Speciation and chromosomal rearrangements in the Australian Morabine Grasshopper Vandiemenella viatica species group

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Speciation and Chromosomal Rearrangements in the Australian Morabine Grasshopper *Vandiemenella viatica* Species Group

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A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

Morabine grasshopper *Vandiemenella viatica* (the *viatica* species group)
(family, Eumastacidae; order Orthoptera)

Originality Statement

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List of Publications and Presentations

Publications by the Candidate Relevant to the Thesis

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Abstract

Recent theoretical developments have led to a renewed interest in the potential role of chromosomal rearrangements in speciation. Australian morabine grasshoppers (genus *Vandiemenella*, *viatica* species group) provide an excellent study system to test this potential role, because they show extensive chromosomal variation: 12 chromosomal races/species with parapatric distributions. The research in this thesis involves the application of molecular genetic analyses to examine patterns of gene introgression among chromosomal races of *Vandiemenella* at three different spatial scales: local-scale hybrid zone analysis, island-scale phylogeography, and continental-scale phylogeography. The aims of these multi-scale analyses are to investigate whether chromosomal races represent genetically distinct taxa with limited gene flow, and to infer the historical biogeography of *Vandiemenella* and evolutionary origins of their parapatric distributions.

Karyotype and 11 nuclear markers revealed a remarkably narrow hybrid zone with substantial linkage disequilibrium and strong deficits of heterozygotes between the chromosome races P24(XY) and *viatica*17 on Kangaroo Island, suggesting that the zone is maintained by a balance between dispersal and selection against hybrids (tension zone). Selection that maintains the stable hybrid zone is unlikely to be operating only on loci linked to rearranged chromosomes. Island-scale and continental-scale phylogeography using multiple nuclear markers indicated that *Vandiemenella* chromosome races/species generally represent genetically distinct taxa with reduced gene flow between them. In contrast, analyses of a mitochondrial gene showed the presence of distinctive and geographically localised phylogroups that do not correspond with the distribution of the *Vandiemenella* taxa. These discordant population genetic patterns are likely to result from introgressive hybridization between the taxa and range expansions and contractions.

Overall, our molecular analyses favour the allopatric mode of diversification for the evolution of *Vandiemenella* and do not support the stasipatric speciation model of

White (1978). Patterns of genetic differentiation between the chromosomal races analysed at three different spatial scales show dynamic responses of the grasshoppers to past climatic fluctuations, leading to opportunities for long-term isolation and allopatric fixation of new chromosome variants and molecular mutations at many loci. Further analyses are necessary to assess potential roles of chromosomal rearrangements in facilitating diversification in *Vandiemenella* by reducing recombination within the rearranged chromosome segments.

Table of Contents

List of Figures

List of Tables

CHAPTER 1

INTRODUCTION

Speciation, the process where ancestral species split into two or more descendant species, has been a major research area in evolutionary biology. Speciation can be viewed as the evolution of barriers to gene flow (*i.e.*, restriction of genetic recombination) between populations or incipient species (Butlin 2005). As organisms may respond to spatially heterogeneous and constantly changing environments, development of barriers to gene flow may also be spatially and temporally dynamic. Such a spatial-temporal dynamism of the speciation process is nicely represented by three major modes of speciation, namely allopatric, parapatric and sympatric speciation. These three modes of speciation differ from each other by the degree and period of geographic isolation by an extrinsic barrier (*i.e.*, complete isolation in allopatry, partial isolation in parapatry, and absence of geographic isolation in sympatry) and, hence, level of gene exchange between incipient species. In turn, to understand mechanisms of barriers to gene flow and evolutionary processes for the initial establishment of these barriers, it is important to analyse spatial and temporal subdivision of populations and the extent of gene flow between them within appropriate spatial and time scales.

Two approaches, namely hybrid zone analysis and phylogeography, provide a powerful theoretical and analytical framework in speciation studies (Avise 2000; Hewitt 2001). Hybrid zones are geographically narrow contact zones where distinct groups of individuals based on some heritable characters (*e.g.*, morphological, genetic, and cytological characters) meet, hybridize, and produce at least some offspring of mixed ancestry (Barton & Hewitt 1989; Harrison 1993b). Hybrid zone researchers aim to investigate the biogeographic history of the zone formation, evolutionary mechanisms for zone maintenance, and underlying genes responsible for population divergence, which may provide potential candidates for 'speciation genes'. Phylogeography deals with geographically structured (or non-structured if null models are accepted) populations with unique gene genealogies to infer

historical biogeographic scenarios that may have resulted in contemporary geographic distributions (Avise *et al.* 1987; Avise 2000). Both approaches concern patterns of gene frequencies in space and time, and explicitly test hypotheses to infer how, when, and where speciation events have occurred. Since advances in molecular and computational technologies have made these two approaches very powerful and popular in speciation studies, I make use of these approaches in this thesis to explore Darwin's 'mystery of mysteries' — the origin of species, and, in particular, potential roles of chromosomal rearrangements in speciation.

The frequent occurrence of chromosome polymorphisms within and between related species is known in diverse groups of organisms (White 1978; Grant 1981; King 1993). These chromosomal taxa often have parapatric distributions and hybridize at narrow contact zones. It has been suggested that natural and laboratory-bred chromosomal heterozygotes have abnormalities of meiosis, which impair the fertility or viability of chromosomal hybrids and, therefore, reduce gene flow between different chromosomal taxa. This has led to the formulation of 'traditional chromosomal speciation models', in which structural chromosomal changes can play a causative role in speciation through reducing fitness in heterokaryotypes (reviewed in Sites & Moritz 1987; Rieseberg 2001). White (1978) particularly emphasized the importance of chromosomal rearrangements as primary isolating mechanisms in speciation, and stated, 'over 90 percent (and perhaps 98 percent) of all speciation events are accompanied by karyotypic changes, and [that] in the majority of these cases the structural chromosomal rearrangements have played a primary role in initiating divergence' (p. 324). However, some of the chromosomal speciation models, including the 'stasipatric speciation model' developed by White (1968), have been criticized from theoretical and empirical viewpoints (Key 1968; Futuyma & Mayer 1980; Sites & Moritz 1987; Coyne & Orr 1998; Spirito 1998; and briefly reviewed in Chapter 2). It is, therefore, apparent that other schools view chromosomal polymorphisms as incidental by-products of speciation processes and present parapatric distributions of chromosomal taxa are a result of fixation of chromosome (potentially neutral) mutations in allopatry, followed by secondary contacts (Key 1968; Futuyma & Mayer 1980; Mayr 1982; Coyne & Orr 1998).

Because there are a number of animals and plants which may have speciated without apparent chromosomal rearrangements, it has been suggested that 'White exaggerated the importance of chromosomal speciation' (Spirito 1998, p. 320).

Nevertheless, certain types of chromosomal rearrangements have the opportunity to be actively involved in speciation processes by reducing fertility and viability of chromosomal heterozygotes (*e.g.*, monobrachial centric fusions, Baker & Bickham 1986; Searle 1993). Moreover, recent studies suggest that chromosomal rearrangements may act as barriers to gene flow not by reducing fitness of chromosomal heterozygotes but by reducing recombination rates within and near rearranged chromosomal segments in heterokaryotypes and thereby accelerate genetic divergence between chromosomal taxa (Noor *et al.* 2001b; Rieseberg 2001; Navarro & Barton 2003a; Kirkpatrick & Barton 2006). These chromosome regions protected from gene flow may spread 'isolation genes' and/or maintain 'coadapted gene complexes' that may be compatible within each of the chromosomal taxa but may cause reproductive isolation between taxa. These non-traditional models, referred to as the 'suppressed-recombination models' (Ayala & Coluzzi 2005), have re-invigorated research interest on the potential role of chromosomal rearrangements in speciation in the last decade (*e.g.*, *Helianthus* sunflowers, Rieseberg *et al.* 1999; human/chimpanzee, Navarro & Barton 2003b; *Anopheles* mosquitos, Ayala & Coluzzi 2005). However, only a handful of empirical studies (*e.g.*, Rieseberg *et al.* 1999; Noor *et al.* 2001b) have tested these new theories, and, therefore, the plausibility and relative frequency of some of the traditional chromosomal speciation models as well as non-traditional suppressed-recombination models are still uncertain (Butlin 2005).

The development of White's stasipatric speciation model (1968; 1978) was largely based on a group of Australian morabine grasshoppers, *Vandiemenella* (formerly *Moraba*) *viatica* species group. The *V. viatica* species group first appeared in the literature in 1964 (White *et al.* 1964). Since then, many empirical studies in ecology, morphology, and cytology of this grasshopper group were carried out by White and his colleagues until the last paper published in 1979 (Mrongovius 1979). These

empirical studies characterize its unique biogeography and chromosomal diversity, which are summarized in several books and journal reviews (White 1969; 1973; 1974; 1978). In addition, their work on *Vandiemenella* has been frequently quoted in various papers and books until today (Coyne & Orr 2004; Barton 2007), implying a large influence and enormous interest in the evolution of *Vandiemenella*. Despite the large amount of work and its importance in the study of chromosomal speciation, however, the *Vandiemenella* study system has been abandoned after White's death in 1983 and no further research has been published on *Vandiemenella* since 1979. Now 30 years have passed since White wrote an influential book, *Modes of Speciation* (1978). These 30 years exactly correspond to the shift from a 'pre-molecular era' to the 'molecular era' in evolutionary biology, represented by the advent of PCR, direct DNA sequencing, and high-throughput genotyping technologies. Thus, it is not surprising that further studies on the *Vandiemenella viatica* species group using modern molecular genetic and new analytical methods have been eagerly awaited.

Since cytological studies have been reviewed elsewhere (White 1973; 1978), Chapter 2 briefly summarizes the cytological characteristics of the species group and focuses more on the taxonomy and ecology of *Vandiemenella* to highlight systematic issues, possible reproductive isolation mechanisms, and the inferred biogeographic history. In addition, Section 2.4 briefly summarizes three key speciation models, which are important to elucidate the origin of parapatric distributions and chromosomal diversification of *Vandiemenella*.

DNA markers have made a significant contribution to hybrid zone and phylogeographic studies (Avise 1994; Avise 2000). Among them, microsatellites are one of the most powerful and widely used markers because of their abundance in the genome, high variability allowing resolution of contemporary population processes, and accompanied high-throughput genotyping technologies (Behura 2006). Chapter 3 describes the isolation and characterization of nine polymorphic microsatellite loci and one insertion/deletion polymorphic locus to investigate population genetic structure and the extent of gene flow between chromosomal races/species of *Vandiemenella*.

The following three chapters examine patterns of gene introgression among chromosomal races of *Vandiemenella* at three different spatial scales (*i.e.*, local-scale hybrid zone analysis, island-scale phylogeography, and continental-scale phylogeography). Chapter 4 uses a hybrid zone between P24(XY) and *viatica*17 on Kangaroo Island (KI) in South Australia to investigate how the barrier to gene flow between these chromosomal races varies for multiple molecular markers (five allozyme loci, five microsatellite loci, the elongation factor-1α [*EF*-1α] gene, and the mitochondrial cytochrome c oxidase subunit I [*COI*] gene). Chapter 5 uses molecular data of three chromosomal races of *Vandiemenella* [*viatica*19, *viatica*17, and P24(XY)] on KI to investigate the extent to which chromosomal variation among these populations may be associated with barriers to gene flow. Chapter 6 describes broad phylogeographic patterns and the origin of parapatric distributions of 11 *Vandiemenella* chromosome races/species across their entire range in southern Australia, using 35 allozyme, two nuclear DNA sequence (*EF*-1α and an anonymous locus), and one mitochondrial (*COI*) markers. In addition, conflicting taxonomic classifications in the *viatica* species group (White *et al.* 1967; Key 1976) are addressed.

Finally, Chapter 7 summarizes results and biological implications obtained from this research. Chapters 4, 5, and 6 critically examine two contrasting biogeographic scenarios: one based on the stasipatric speciation model and the other based on the allopatric speciation model. White *et al*. (1967) argue that the parapatric distributions of *Vandiemenella* taxa originated by primary contact based on the assumption that the distribution of the *viatica* species group was contiguous and never separated by major physical barriers over the entire period of chromosomal diversification. In contrast, Key (1968) and Hewitt (1979) argue that the *Vandiemenella* chromosomal races evolved in allopatry and formed parapatric distribution by secondary contact after climate amelioration. Overall, although the *Vandiemenella viatica* species group forms the basis of the stasipatric speciation model, our molecular analyses do not support this model and favour the allopatric mode of diversification for the evolution of the *Vandiemenella* chromosomal races. Nevertheless, a question remains.

According to the suppressed recombination models, chromosomal rearrangements may play an active role in the diversification of the *Vandiemenella* chromosomal races by reinforcing linkage of many genes in a large block of the genome. Future directions of the *Vandiemenella* study system are discussed to test this prediction.

CHAPTER 2
AUSTRALIAN MORABINE GRASSHOPPER (GENUS *VANDIEMENELLA*): A MODEL FOR SPECIATION RESEARCH

2.1 Taxonomy and systematics

2.1.1 Taxonomy of the morabine grasshopper

The morabine grasshoppers comprise a distinctive group endemic to Australia. First described as the subfamily Morabinae (family, Eumastacidae; superfamily, Eumastacoidea; order Orthoptera) by Rehn (1948), the group was later proposed as a distinct family Morabidae by Descamps (1973). However, this higher-level classification has not been settled, and Key and his colleagues (Key 1976; 1977; 1979; Key & Colless 1982) preferred to use the former classification even after Descamps' proposal. There are several recent molecular systematics studies to investigate basal phylogenetic relationships within Orthoptera (*e.g.*, Flook *et al.* 1999); however, these studies do not include morabine grasshopper specimens, and hence, have not helped to resolve the taxonomic uncertainty at the family/subfamily level. Since the latter classification (family Morabidae) is currently used in public databases, such as National Center for Biotechnology Information (NCBI) taxonomy database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Taxonomy) and the Orthoptera Species File Online (http://osf2x.orthoptera.org/HomePage.aspx) (21 May 2007), this classification has been adopted herein.

Table 2.1 Tribes, genera and the number of species in each genus of the family Morabidae (Orthoptera Species File Online, see text for the web address). The number of species excludes provisional species.

Tribe	Genus	No. of Species
Callitalini	*Bundinja*	3
	Callita	1
	Callitala	3
	Carnarvonella	1
	Furculifera	1
	Malleolopha	1
	Micromeeka	1
	Moritala	3
	Prorifera	5
Capsigerini	*Aliena*	1
	Amangu	2
	Aruntina	1
	Capsigera	1
	Crois	1
	Namatjira	2
	Proscopiomima	1
	Swanea	2
Keyacridini	*Achuraba*	1
	Achurimima	4
	Alatriplica	1
	Baruca	1
	Chinnicka	1
	Flindersella	2
	Heide	6
	Keyacris	3
	Vandiemenella	2
	Whiteacris	1
Morabini	*Drysdalopila*	1
	Filoraba	1
	Moraba	8
	Spectriforma	4
Warramungini	*Callimunga*	1
	Culmacris	4
	Geckomima	12
	Georgina	1
	Hastella	6
	Nanihospita	1
	Stiletta	2
	Warramaba	2
	Warramunga	1
Total*		96

*, The total number of species including provisional species is 245 (Key 1976).

Based on morphological and cytological characters, Key (1976) defined five morabine grasshopper tribes (Table 2.1). With respect to morphology, four of these tribes exhibit a satisfactory degree of homogeneity, whereas members of Capsigerini are much less homogeneous, so their monophyly is less clear (type material stored at the Australian National Insect Collection [ANIC] CSIRO Entomology). Within the Morabidae a large number of 'provisional species' comprising numerous chromosomal races have also been identified, many of which may warrant separate species status (White 1974; 1978). Thus, the full extent of the biodiversity in the family Morabidae is currently uncertain, however, a multi-faceted investigation incorporating molecular, cytological, and morphological techniques should help to assess the biodiversity of this group (Chapter 6).

Vandiemenella, known as the *viatica* species group belongs to the tribe Keyacridini, within which *Flindersella, Keyacris*, and *Whiteacris* may be closely related to *Vandiemenella* (Table 2.1). Similar to other members of the Morabidae, *Vandiemenella* also shows extensive chromosomal variation. This group currently comprises at least two distinct species (*V. pichirichi* and *V. viatica*) and 11 chromosomal races (Table 2.2). White *et al.* (1967) postulated that all five provisional species should be regarded as full species because changes in chromosome numbers and structure have provided strong reproductive barriers between them (see Section 2.3). In contrast, Key (1976) regards at least two of these provisional species as chromosomal races of *V. viatica* on the basis of their morphological characters.

Table 2.2 Taxonomic conflict between White *et al.* (1967) and Key (1976) in *Vandiemenella*. P24, P25, P45b, P45c, and P50 are provisional species (Australian National Insect Collection).

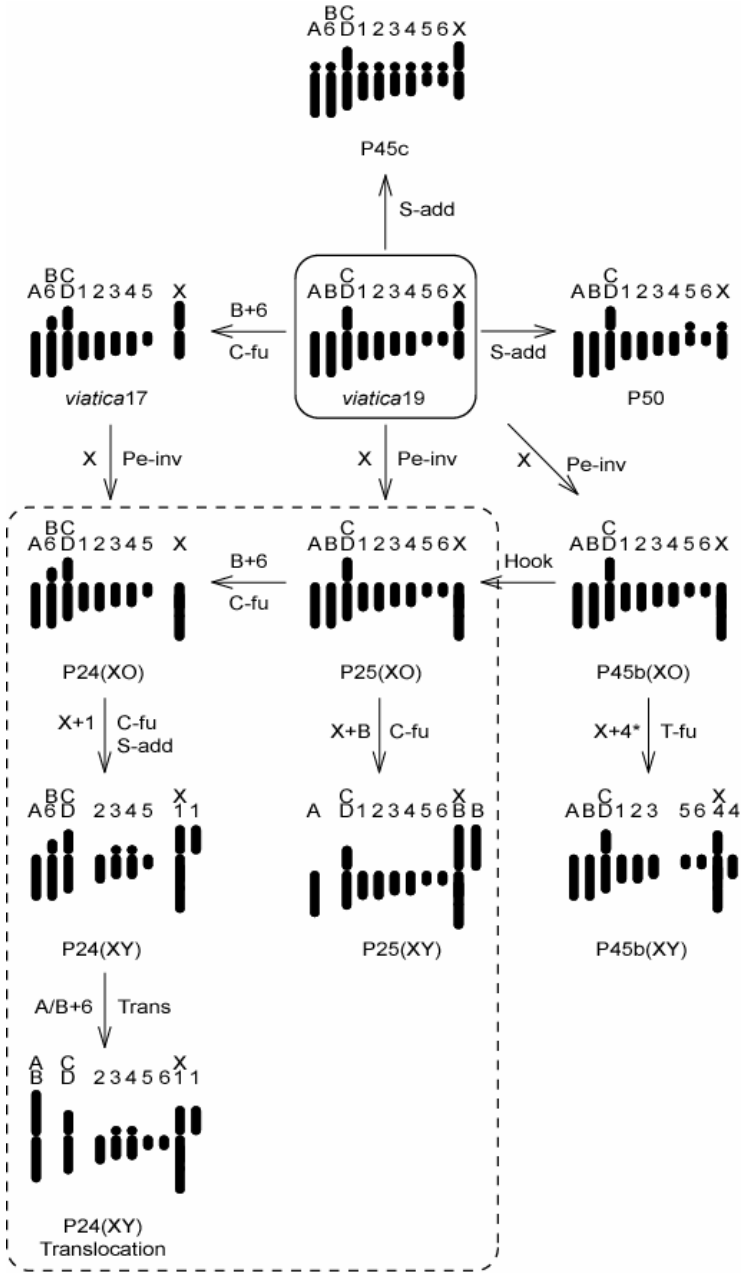
White *et al.* (1967)		Key (1976)	
Species	Races	Species	Races
V. viatica	*viatica*19	*V. viatica*	*viatica*19
	*viatica*17		*viatica*17
P24	XO		P24 XO
	XY		P24(XY)
	Translocation		P24(XY)-Translocation
P25	XO		P25 XO
	XY		P25XY
P45b	XO	P45bXO	- Species/race uncertain
	XY	P45bXY	- Species/race uncertain
P45c		P45c	- Species/race uncertain
P50		P50	
V. pichirichi (P26/142)		*V. pichirichi* (P26/142)	

2.1.2 Chromosomal phylogenetics of Vandiemenella

Karyotypes of each *Vandiemenella* chromosomal race have been described elsewhere (White *et al.* 1967; White 1973; 1978; John 1981) (Fig. 2.1). The number of chromosomes ranges from 2n = 16 to 2n = 19 in males ('XO' or 'XY' sex chromosomes) and from 2n = 16 to 2n = 20 in females ('XX' sex chromosomes). White *et al*. (1967) hypothesized that chromosomal races have arisen from a single ancestral species ('*proto-viatica*') with a karyotype 2n = 19 (XO) in males, which is retained in the present-day *viatica*19. This hypothesis assumes that the *proto-viatica* has experienced two major stages of chromosome mutations and some additional mutations to form the present-day taxa. The first stage involves the B+6 fusion and X-chromosome inversion, forming *viatica*17 and three provisional species lineages (P24, P25, and P45b). However, the order of these two rearrangements is uncertain, and this uncertainty results in a reticulated network in chromosome phylogeny, where the lineage of P24 has been derived either from *viatica*17 (*i.e.*, B+6 fusion first and X chromosome inversion second) or P25/P45b (*i.e.*, X chromosome inversion first and B+6 fusion second) (Fig. 2.1). A close morphological affinity between P24 and P25 based on the presence of a supplementary hook on the male cercus (see Section 2.2.5) implies that these two lineages might share a most recent common

ancestor (MRCA) if this morphological character is a synapomorphy (*i.e.*, character states that are shared due to common ancestry). Nonetheless, White *et al.* (1967) postulated that, because of the geographic contiguity of P24 and *viatica*17, P24 lineage shares a MRCA with *viatica*17 with the B+6 fusion being a synapomorphic mutation. The second stage of chromosome mutations involves fusions between the X chromosome and one of the acrocentric autosomes, creating a neo-XY system in the male in each of the P24, P25, and P45b lineages. The race P24(XY)-Translocation [hereafter, P24(XY)-Trans] has probably been derived from P24(XY) by translocation between the A and B+6 chromosomes, resulting in an AB metacentric and a free No. 6 autosome. Finally, some additional mutations include short arm additions in some of the acrocentric autosomes and X chromosomes presumably due to an increase in the amount of satellite DNA, differentiating P45c and P50 from *viatica*19 (Webb & White 1975; White 1978). In addition, White *et al.* (1964; 1967) also noted that there are measurable variations in the length of short arms of autosomes among populations of *viatica*17, which have resulted from at least five pericentric inversions.

Chromosome rearrangements can be powerful characters for inferring phylogenetic relationships when chromosomal mutations are justified as synapomorphies using various cytogenetic techniques (*e.g.,* chromosome banding, fluorescent *in-situ* hybridisation, and chromosome painting) (Farris 1978; Dobigny *et al.* 2004). However, as manifested by the reticulated network in the chromosomal phylogeny (Fig. 2.1), some of the chromosome mutations in *Vandiemenella* may be homoplasies (character states that are independently derived). In addition, it should also be noted that the chromosomal rearrangements observed in the present *Vandiemenella* taxa do not necessarily represent the total number of rearrangements that has occurred during the entire evolutionary history of the group (*i.e.*, some have arisen but been eliminated by natural selection or genetic drift) (White *et al.* 1967). Therefore, rigorous cytological examinations using conventional and modern cytogenetic analyses coupled with molecular phylogenetic analyses are required to infer phylogenetic relationships of the chromosomal races of *Vandiemenella*.



* X+4 tandem fusion in White *et al.* (1967) but X+6 tandem fusion in White (1978)

Fig. 2.1 Proposed chromosomal evolution of *Vandiemenella* (White 1978; John 1981). *Viatica*19 represents the presumed ancestral karyotype. Arrows indicate the possible mutational direction: C-fu, centric fusion; T-fu, tandem fusion; Pe-inv, pericentric inversion; Trans, translocation; S-add, short arm addition. A broken square indicates taxa with an extra hook on the male cerci (see the Section 2.2.5). *V. pichirichi* was excluded as there is little information about phylogenetic relationships between *V. pichirichi* and other *Vandiemenella* taxa (but see White 1978).

2.2 Biology and Ecology of *Vandiemenella*

2.2.1 Distribution and contact zone

Early cytological and biogeographic studies revealed that the *Vandiemenella* chromosomal races are distributed across south-eastern South Australia, western New South Wales, coastal Victoria and Tasmania (~ 400 000 km^2) (Fig. 2.2) (White *et al.* 1964; White 1978). All the chromosomal races except one pair (*V. pichirichi* and the race P25XO) have parapatric distributions (*i.e.*, each race with a discrete distribution meets other races and forms narrow hybrid zones between them).

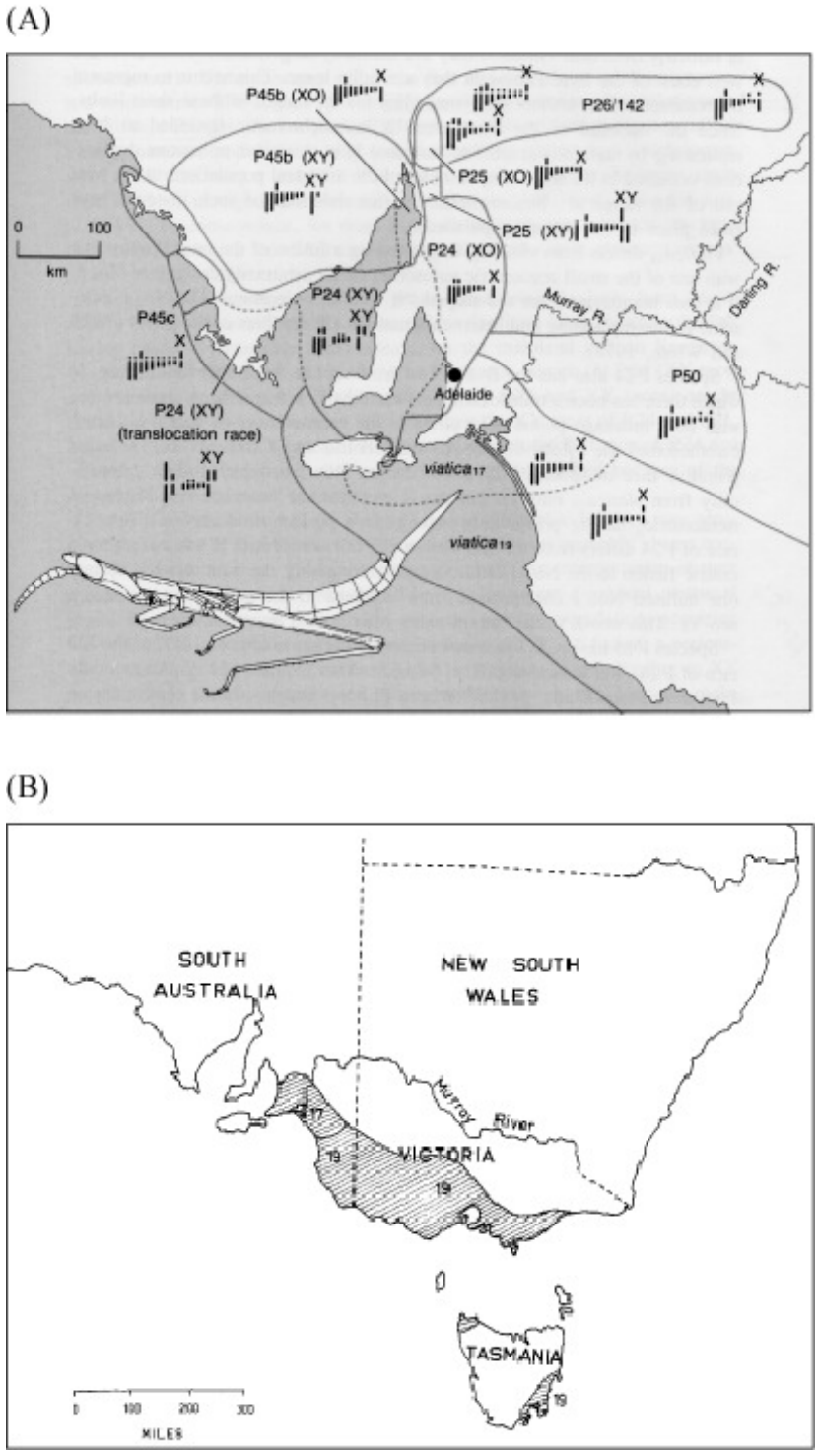


Fig. 2.2 Distribution of *Vandiemenella* chromosomal races in South Australia (A) and *V. viatica* chromosomal races (*viatica*19 and *viatica*17) in Victoria and Tasmania (B) (taken from White *et al.* 1964; White 1978)

Five contact zones have been reported since the early 1960s (Table 2.3). Widths of those contact zones range from 200 m to 800 m, but the contact zone between *viatica*19 and *viatica*17 and the contact zone between *viatica*19 and P24(XY) on Kangaroo Island (KI) may be narrower (White *et al.* 1967; Mrongovius 1975; 1979). Chromosomal heterozygotes (hybrids) were collected by these authors from all contact zones except the P24(XO)/P24(XY) contact zone. Grasshoppers collected from some of these contact zones were analysed cytologically, morphologically, and morphometrically (see Section 2.2.5, 2.2.6, and 2.3.2). It should be noted that the original distribution and contact zones of *Vandiemenella* have probably altered the last 200 years due to landscape changes resulting from agriculture (White *et al.* 1967; Key 1981).

Table 2.3 Reported contact zones of *Vandiemenella*

Chromosomal races		Location	Year	Width*	Ref.
*viatica*19	*viatica*17	Keith, South Australia	1962	800 m	1
*viatica*19	*viatica*17	Kangaroo Is., South Australia	1972	-	4, 5
*viatica*19	P24(XY)	Kangaroo Is., South Australia	1966-1967	-	3, 4, 5
*viatica*17	P24(XY)	Kangaroo Is., South Australia	1966-1967	200-300 m	3, 4, 5
P24(XO)	P24(XY)	Port Gawler, South Australia	-	-	2

* Widths of contact zones are either approximate estimates or areas where two chromosomal races were found

Ref: reference 1, White *et al.* 1964; 2, White *et al.* 1967; 3, White *et al.* 1969; 4, Mrongovius 1975; 5, Mrongovius 1979

2.2.2 Life cycle

The *viatica* group of morabine grasshoppers have been called the 'winter species' (White *et al.* 1967; Blackith & Blackith 1969a). Eggs hatch throughout the summer, and the individuals reach maturity toward the winter months. Blackith and Blackith (1969a) reported the greater part of the P24(XY) population at the southern tip of the Yorke Peninsula to be adult by early June in 1966. However, the timing of maturity may depend on local temperature as laboratory breeding experiments demonstrated development of nymphal instars to be 1.3 - 1.5 times slower at low temperature (18 - 24 °C) than high temperature (30 - 35 °C). Egg hatching, nymphal development, and

longevity of P24, P25, and *V. pichirichi* were studied under laboratory and wild conditions (Blackith & Blackith 1969a) (Table 2.4). Blackith (1967a; 1967b) noted that predation and parasitisation was a major cause of mortality.

Table 2.4 Observations on the biology of *Vandiemenella* P24, P25, and *V. pichirichi* (Blackith & Blackith 1969a)

Fecundity	Number of batches: 9-11/female	
	Mean batch size: 7eggs/batch	
Egg development	Eggs absorb water in the first 6-12 days (30-35 °C), egg weight increases (~1.5 g to ~3.5 g)	
	Dry tolerance: water absorption can be observed after a 49 day dry treatment	
	Eggs hatch after 25-35 days when damp and at high temperature (30-35 °C)	
	Possible to hatch at 20 °C	
	Unfertilized egg hatches (possibly haploid) at low frequency (7/164 eggs), all died within 1 week	
Number of nymphal stages	5 (♂), 6 (♀) with some geographic variation probably due to environmental conditions	
Duration from hatchlings to adults:	18-24 °C	30-35 °C
	128 days (♂, n = 6-9)	87 days (♂, n = 4)
	199+ days (♀, n = 3-6)	155 days (♀, n = 3-4)
Life span	~300 days after hatching	
	16 weeks in adults (wild)	
	30 weeks in adults (laboratory)	

2.2.3 Dispersal ability

Because morabine grasshoppers are wingless, it has been believed that they have avery limited dispersal ability. For example, White (1973, p. 371) stated, 'These (morabine grasshoppers) are insects with quite feeble powers of locomotion, which are usually restricted to a limited range of food plants; individuals are unlikely to travel more than a few metres from their birthplace in the course of an entire life-cycle.' However, mark-recapture studies indicated that individuals of P24(XY) travelled up to 4.0 m in two days (median = 1.0 m in males, n = 8; median = 1.5 m in females, n = 10) and 11.3 m in 12 days (median = 5.2 m in males, n = 6; median = 4.1 m in females, n = 6) (Blackith & Blackith 1969a). This result suggests much larger potential dispersal distances for *Vandiemenella* taxa within a lifetime. It is

important to note that rare long-distance dispersal events, which are not normally measured in mark-recapture studies, may have a significant influence on patterns of gene flow, population structuring, and colonisation following glacial periods (Nichols & Hewitt 1994).

2.2.4 Habitat

Vandiemenella grasshoppers tends to occur in coastal heath habitats, composed of sclerophyllous shrubs, such as Proteaceae, Dilleniaceae, Casuarinaceae, Myrtaceae, Epacridaceae, Xanthorrhoeaceae, Leguminosae, and Restionaceae, while certain chromosomal races occur in areas of saltbush or mallee vegetation (White *et al.* 1964; 1967). They tend to be found at ground level or on low vegetation where the eucalypts do not form a dense cover. Field observations and laboratory feeding experiments indicate that *Vandiemenella* grasshoppers are polyphagous and accept a broad range of plants and plant parts (*e.g.,* hard leaves, growing tips and flowers), although there are slight differences in food preferences between the chromosomal races (Blackith & Blackith 1966b). Interestingly, some plants observed to be closely associated with grasshoppers in the field were not preferably eaten in laboratory feeding experiments, suggesting those plants to serve primarily as shelter and are eaten only when preferred food sources are no longer available (Blackith & Blackith 1966b).

According to White *et al*. (1967), geographic limits to *Vandiemenella* taxa do not appear to correspond to ecological and environmental discontinuities. Nonetheless, there are at least two cases that suggest such a correspondence. First, the XO race of P45b on both sides of Spencer Gulf lives in saltbush country or in areas ecotonal between mallee and saltbush where various Chenopodiaceae species form the main vegetation. In contrast, the XY race of P45b occupies mallee country where the stands of *Eucalyptus* species are thinner and composites are growing (Blackith & Blackith 1966b). Second, on KI, *viatica*19 appears to be associated with relatively dense and continuous sclerophyllous shrub vegetation primarily found at the interior of the island, while *viatica*17 appears to be associated with open eucalyptus mallee with scattered low forbs and grasses along the coastline (White *et al.* 1969).

Moreover, detailed field observations near the contact zone between these two races on KI suggests that the position of the contact zone corresponds to a transition in the soil and vegetation (*i.e.*, *viatica*19 in inland duplex soil country with high frequency of *Grevillea ilicifolia* and *viatica*17 in coastal limestone country with high frequency of *Correa reflexa*) (Mrongovius 1975; 1979). However, although some parapatric boundaries of *Vandiemenella* grasshoppers appear to coincide with environmental gradients, available evidence does not provide strong support for a causative role of environmental variables on the positions of contact zones. First, as mentioned previously, *Vandiemenella* grasshoppers are food generalists, so their distribution is unlikely to be limited by food plants alone. Furthermore, *viatica*19 and *viatica*17 form another contact zone in mainland of Australia, where there is no sharp discontinuity of soil, vegetation, or climate to distinguish habitats (White *et al.* 1964).

2.2.5 Morphological variation

Vandiemenella grasshoppers are sexually dimorphic, but few morphological characters are available to distinguish members of this uniform group. Some differences have been observed in female egg guides (White *et al.* 1967), colour morphs (Blackith & Blackith 1966b), number of female ovarioles, number of moults, and body size (Blackith & Blackith 1969b). However, the taxonomic utility of these characters may be limited due to substantial variations within chromosomal races (Blackith & Blackith 1969b). One notable exception is a characteristic supplementary hook on the male cercus of chromosomal races P24 and P25 (Fig. 2.3) which is absent from chromosomal races of *viatica*, P45b, P45c, and P50 (White *et al.* 1967).

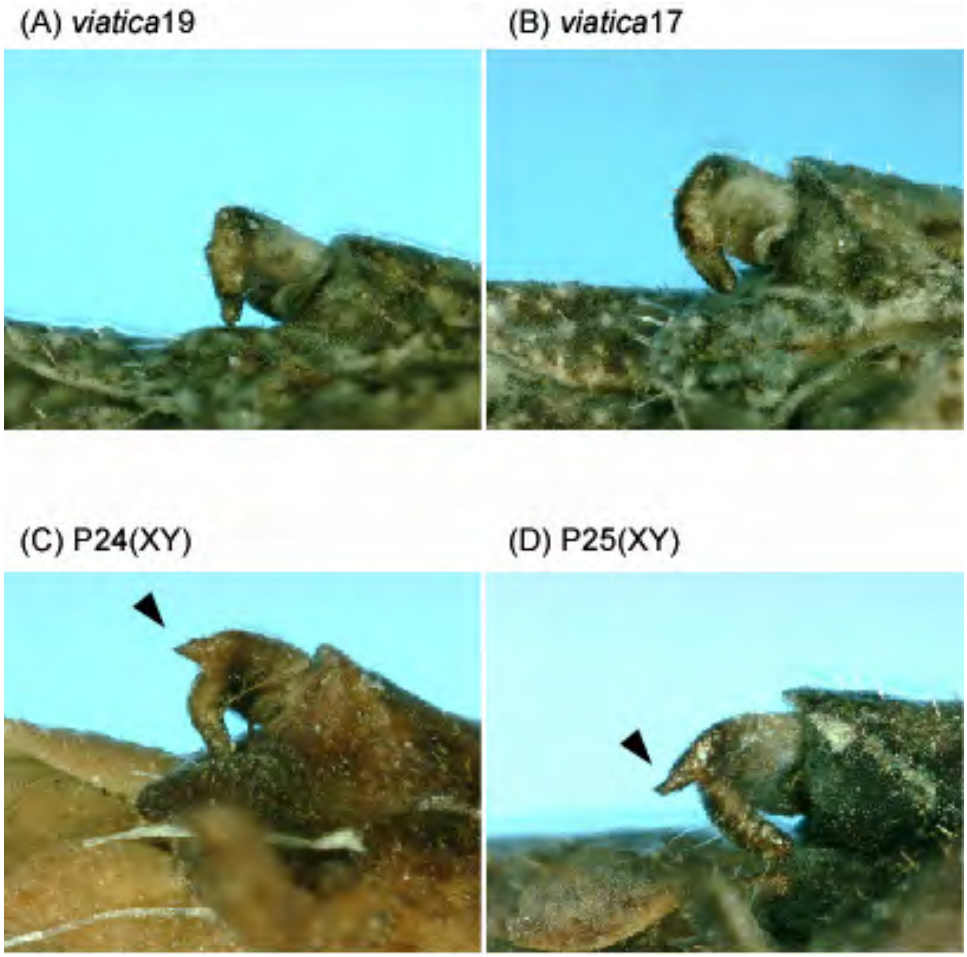


Fig. 2.3 Dorsolateral views of the male cercus of *viatica*19 (A), *viatica*17 (B), P24(XY) (C), and P25(XY) (D). Arrows indicate supplementary hooks.

The size of the supplementary hook on the male cercus was analysed to infer a level of gene flow across the contact zone between *viatica*17 and P24(XY) on KI (White *et al.* 1969). The size of the hook was rated on an arbitrary scale from 0 (no hook) to 3 (extreme development of hook). 'Pure' populations of P24(XY) and *viatica*17, which were far away from the contact zone, showed a typical size of supplementary hook [size rating of P24(XY) = 2.36, n = 14; and size rating of *viatica*17 = 0.00, n = 5], whereas populations near the contact zone possessed an intermediate size of the supplementary hook [size rating = 0.89 ~ 2.00, n = 9 - 24) (White *et al.* 1969). Introgression of this morphological trait and, perhaps, underlying genes controlling this trait appeared to be bidirectional and to extend much further than the width of the chromosomal hybrid zone. This implication may support the concept of the

'tension zone', which acts like 'semi-permeable membranes', holding back some genes and chromosomal rearrangements to varying degrees, but permitting other genes to introgress (Key 1968). Similar introgression of the cercal hook trait, however, was not found near the hybrid zone between *viatica*19 and P24(XY) on KI (White *et al.* 1969).

2.2.6 Morphometric analyses

Blackith and Blackith (1969b) and, later, Atchley and colleagues (Atchley 1974a; Atchley & Cheney 1974; Atchley & Hensleig 1974) carried out morphometric analyses to (i) discriminate *Vandiemenella* taxa and (ii) infer their evolutionary relationships. Multivariate morphometric analyses indicated that there was considerable phenetic divergence with minimal overlap among *Vandiemenella* taxa. For instance, posterior probability classification analyses using 14 characters on male body size and ten characters on female egg guide of *viatica*19 and *viatica*17 showed that about 90 % of males and 72 % of females were unambiguously distinguishable from the non-conspecific chromosomal races (Atchley 1974b; Atchley & Cheney 1974). Similar levels of morphometric divergence were also observed between races within each of P24, P25, and P45b, as well as between these provisional species. In addition to the inter-taxon divergence, small but apparent inter-populational divergence within each taxon was also evident (Atchley 1974a). It is unknown whether such inter-populational divergence would be due to random genetic drift resulting from low population density coupled with the isolated nature of many sub-populations or due to some natural selection operating at the level of the local environment.

Next, these morphometric data were used to test whether morphometric differentiation has paralleled cytogenetic differentiation. Morphometric distance analyses using males of five taxa and females of all *Vandiemenella* taxa indicated a very poor relationship overall, although a weak relationship between morphometric distance and cytological distance was observed (Fig. 2.4). For example, a phenogram based on male morphometric characters (Fig. 2.4A) appears to support the cytological evolutionary sequence (*viatica*19 → *viatica*17 → P24(XO) → P24(XY)

→ P24(XY)-Trans, see Section 2.1.2); however, a phenogram based on female morphometric characters (Fig. 2.4B) does not support this hypothesis. In addition, although the *viatica*19, P45c and P50 are cytologically close to one another (White 1978), they were not morphometrically clustered together (Fig. 2.4B and C). An important difficulty in morphometric phylogenetics is convergence, which results in misleading phylogenetic estimates by clustering morphometrically similar taxa despite their independent evolutionary history (*e.g.*, cichlid fish, Ruber & Adams 2001). To overcome that difficulty and fill in the discrepancy between cytological and morphometric analyses, evolutionary relationships of the chromosomal races will be inferred by a combination of molecular genetic methods.

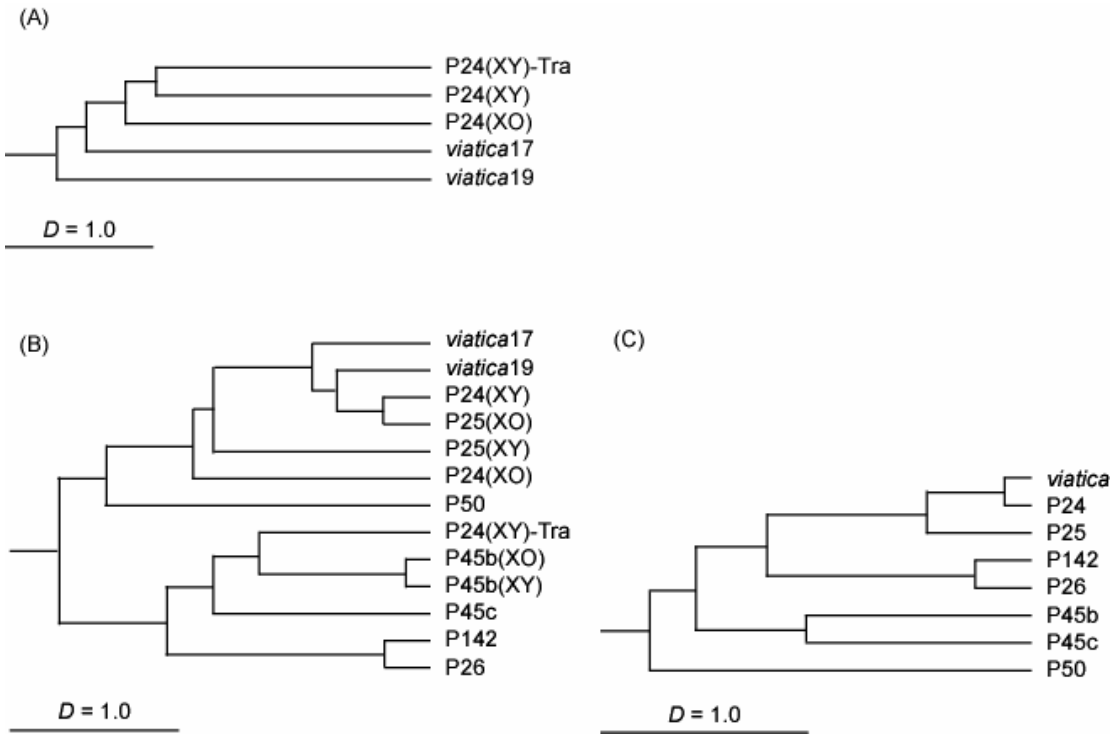


Fig. 2.4 Phenograms from unweighted pair group method analysis (UPGMA) of Mahalanobis *D* values based on morphometric characters of male body size for the five *Vandiemenella* taxa (A), female egg guide for the 13 taxa (B), and eight taxa by pooling the chromosomal races of each species/provisional species (C). The UPGMA phenogram in Fig. 2.4A was generated using pairwise *D* values presented in Atchley (1974b) in PHYLIP version 3.66 (Felsenstein 1995) by M. Adams, and phenograms in Fig. (B) and (C) were modified from Atchley and Cheney (1974). P26 and P142: *V. pichirichi*; P24(XY)-Tra: P24(XY)-Translocation.

2.3 Reproductive isolation of *Vandiemenella*

White (1968; 1978) proposed that chromosomal rearrangements generate powerful post-zygotic isolation mechanisms between *Vandiemenella* taxa as a result of the reduced fitness of chromosomal heterozygotes. Evidence for fitness reduction is primarily based on abnormalities of meiosis (*e.g.,* asynapsis, de-synapsis, malorientation and malsegregation of the chromosomes involved), which may produce chromosomally imbalanced and inviable gametes in F_1 chromosomal hybrids. However, there are very few direct studies to evaluate the extent of fitness reduction of F_1 hybrids (Mrongovius 1975; 1979). Here I review studies on pre-mating, post-mating pre-zygotic, and post-zygotic isolation among *viatica*19, *viatica*17, and P24(XY) to discuss whether any reproductive isolation mechanism is evident in *Vandiemenella* (cytological studies using other pairs of taxa were reported in White *et al*. 1967 and summarised in Hewitt 1979).

2.3.1 Pre-mating isolation

Observations in laboratory crossbreeding experiments suggests no evidence of any pre-mating reproductive barrier between *Vandiemenella* taxa (White *et al.* 1967; Mrongovius 1975). For example, a crossbreeding experiment using 35 pairs of *viatica*19 and *viatica*17 collected from KI showed no apparent differences between inter-racial and intra-racial (control) crosses in mating behaviour (*e.g.,* approaching and mounting) and the time taken from introduction to the start of copulation (Mrongovius 1975). In addition, inter-generic crossbreeding experiments using P24(XY) and sympatric morabine species of *Achurimima asinus* showed that about 17 % of *A. asinus* males and 50 % of P24(XY) males copulated with non-conspecific females when both *A. asinus* and P24(XY) females were presented (White *et al.* 1967). Because any stridulatory organs and auditory tympana are not known in morabine grasshoppers, the courtship behaviour of the morabine grasshoppers does not seem to involve auditory sense (Blackith & Blackith 1966a). The absence of auditory courtship behaviour in addition to matings with non-conspecifics would suggest only weak, if any pre-mating isolating mechanisms among morabine

grasshoppers (White *et al.* 1967). However, little is currently known about other possible (*e.g.,* chemical) mating cues in this group.

2.3.2 Post-mating prezygotic isolation

In contrast to the absence of any evidence of pre-mating isolation mechanisms, Mrongovius (1975) suggested that 'post-mating pre-zygotic' isolating mechanisms might be partially effective. Crossbreeding insemination experiments using *viatica*19 and *viatica*17 showed that female spermatheca tended to be empty more frequently in inter-racial crosses than intra-racial crosses probably due to unsuccessful transfer of sperm to the female spermatheca or later expulsion of sperm from the spermatheca (Fisher's exact test, n = 9 - 13 per cross, P = 0.004) (Mrongovius 1975). Similarly, insemination failure appeared to be more evident in inter-racial crosses between *viatica*19 and P24(XY) than intra-racial crosses, although there was no significant difference between inter- and intra-racial crosses between *viatica*17 and P24(XY) (Mrongovius 1975). Since the sample sizes of these experiments were too small to provide robust statistical support (n = 5 - 13 per crosses), further follow-up study is necessary to rigorously test whether 'post-mating prezygotic' isolation mechanisms are effective in this species group. It would also be interesting to investigate whether there is any sperm competition after multiple copulations as females of the *viatica* species group are known to mate several times (Mrongovius 1975).

2.3.3 Post-zygotic isolation

Reduction of fitness by post-zygotic isolation has been assessed indirectly and directly (White *et al.* 1964; 1969; Mrongovius 1975; 1979). The indirect estimates of fertility reduction in chromosomal heterozygotes were based on the frequency of abnormal meiotic cells and spermatids in natural hybrids (chromosomal heterozygotes caught in the wild) and F_1 hybrids reared by laboratory crossing (Table 2.5). The underlying assumptions of these studies are (i) at metaphase I, a univalent chromosome segregates at random to either pole, resulting in 50 % of aneuploid and inviable gametes and (ii) abnormal spermatids are dysfunctional (Mrongovius 1979). The frequency of abnormal meiotic cells appeared higher in *viatica*19/*viatica*17 and

*viatica*19/P24(XY) crosses than in *viatica*17/P24(XY) crosses. In natural and laboratory-reared hybrids between *viatica*19 and *viatica*17, 7.2 - 10.0 % of spermatids (n = 500) had abnormally large heads (Mrongovius 1975; 1979). However, it is unclear whether the frequency values and reduced fertility estimates were significantly higher for the chromosomal hybrids than the parental karyotype individuals because few comparable data were available in parental forms. Abnormal karyotype frequencies in embryos reared from chromosomal hybrid females or parental form females collected around the contact zone between *viatica*17 and P24(XY) on KI (the bottom row in Table 2.5), suggested no apparent difference in meiotic abnormality between hybrids and parental forms. Thus, White *et al.* (1969, p. 321) concluded that 'there seems no reason to doubt that two forms [*i.e.*, *viatica*17 and P24(XY)] are interbreeding freely… Neither is there any reason to believe that the viability of female hybrids is to any extent subnormal'.

Table 2.5 Frequency of abnormal karyotype in meiosis metaphase I and estimated fertility reduction of natural hybrids (chromosomal heterozygotes caught in the wild) and laboratory-bred F_1 hybrids between *viatica*19, *viatica*17, and P24(XY).

Cross	Hybrid types	Sample sizes [3]	Abnormal karyotype [5]	Estimated fertility reduction	Ref.
V19/V17 [1]	Natural	2 (86 - 100)	12 - 29 %	~10 % aneuploid sperms	1
	Natural [2]	1 (78)	35 %	2 - 35 % fertility reduction	2, 3
	Laboratory-reared	1	No data	45 - 55 % fertility reduction	2, 3
V19/P24 [1]	Laboratory-reared	2 (118 - 156)	21 - 45 %	-	2, 3
V17/P24 [1]	Laboratory-reared	30 (1,171 - 1,185)	0.8 - 2.4 % (Male)	-	2, 3
	Embryos reared from natural hybrid females [4]	9 hybrid females 298 embryos (5)	~4 % (0 - 7.8%) [6]	-	2, 3

[1] V19: *viatica*19, V17: *viatica*17, P24: P24(XY).
[2] Only F_1-like hybrids were presented. The other hybrid-like males caught in the same contact zone were excluded because of their unknown hybrid origin (*i.e.,* non-F_1-like karyotype with an extra chromosome).
[3] Numbers in brackets are the number of cells examined per individual.
[4] Paternal karyotype unknown because the natural hybrid females were used (possibly inseminated in the wild).
[5] Trivalents with incorrect orientation and univalents in metaphase I.
[6] Abnormal karyotype observed in embryos reared from non-hybrid females (V17 or P24)
Ref: reference 1, White *et al.* 1964; 2, Mrongovius 1975; and 3, Mrongovius 1979.

To directly estimate fertility reduction in F_1 chromosomal heterozygotes, a series of backcrossing experiments were conducted using *viatica*17 and P24(XY) (Table 2.6) (Mrongovius 1975). Results showed no significant difference between backcross females and control crosses with respect to the number of eggs laid over time and hatching frequency. In addition, nymphs of hybrid females appeared to have a comparable level of mortality to nymphs of non-hybrid females. Therefore, for *viatica*17 and P24(XY), fertility of F_1 hybrids is not significantly different from the control parental forms, so there is no apparent post-zygotic isolation mechanism between these two chromosomal races.

Table 2.6 Fertility of laboratory-reared F_1 hybrid females (Mrongovius 1975)

Female [1]	Male	n	No. of days [2]	No. of eggs [3]	No. of hatched eggs [4]	Nymphal mortality [5]
F_1 (P24 × V17)	V17	10	25.0	24.8	16.1	
	P24	17	22.4	24.1	13.9	
F_1 (V17 × P24)	V17	16	21.5	23.9	12.3	
	P24	11	23.0	23.3	13.4	
						36-64%
P24	P24	13	25.5	23.5	16.4	
V17	V17	22	24.0	23.2	13.9	
						38-62%

[1] F_1 hybrid females reared from the crosses between (female × male) parents
[2] Number of days between 1st and 4th batch of eggs laid (analysis of variance [ANOVA], $P > 0.05$)
[3] Total number of eggs laid in four batches (ANOVA, $P > 0.05$)
[4] Mean number of eggs hatched in three batches (ANOVA, $P > 0.05$)
[5] Nymphal mortality in the hybrid crosses and control crosses. Breakdown in each cross is unknown.

In addition to fertility reduction, the viability of F_1 hybrids was assessed by examining the frequency of abnormal bases of embryo development and hatching frequency (Table 2.7) (Mrongovius 1975). In the crosses between *viatica*19 and *viatica*17 and between *viatica*19 and P24(XY), abnormal embryo development occurred with greater frequency in hybrids than in the control parental forms, and hatching frequency appeared to be lower in hybrids than in the control parental forms. However, there was no apparent reduction in viability of hybrids in the cross between *viatica*17 and P24(XY). This trend is consistent with field observation, in which natural chromosomal hybrids were rare at the *viatica*19/*viatica*17 contact zone and the *viatica*19/P24(XY) contact zone but were relatively abundant at the *viatica*17/P24(XY) contact zone on KI (four, one, and 23 hybrid individuals were collected over a period of eight years, respectively) (Mrongovius 1975; 1979). Thus, this result suggests that some post-zygotic isolation mechanisms might be partially effective to produce fewer viable F_1 offspring in some pairs of inter-racial crosses than in intra-racial crosses. However, such mechanisms are incomplete as evident by the presence of natural F_1-like hybrids and laboratory hybrids (29 - 82 % of hatching frequency). In addition, it should be noted that even if F_1 chromosomal hybrids are less viable than parental forms, this type of post-zygotic isolating mechanism does not support White's hypothesis (1968; 1978), where reproductive isolation results from meiotic abnormality and fertility reduction in F_1 chromosomal hybrids.

Table 2.7 Frequency of abnormal embryo development and hatching of F_1 chromosomal hybrids obtained by laboratory crosses among *viatica*19, *viatica*17, and P24(XY) (data from Mrongovius 1975).

				Abnormal embryo development			Hatching frequency	
	Crosses F/M [2]	No. of pairs [4]	No. of batches [5]	n_1	Abnormal karyotype [6]	Un-developed	n_2	
V19/V17 [1]	V19/V19 [3]	9	35	154	12%	11%	71	63%
	V17/V17 [3]	9	20	94	10%	14%	45	80%
	V19/V17	7	20	86	9%	23%	41	54%
	V17/V19	5	14	67	36%	22%	35	37%
V19/P24(XY) [1]	V19/V19 [3]	8	31	118	5%	14%	57	86%
	P24/P24 [3]	6	27	103	2%	2%	46	93%
	V19/P24	1	1	1	100%	0%	0	-
	P24/V19	5	21	84	57%	20%	52	29%
V17/P24(XY) [1]	V17/V17 [3]	8	31	141	4%	6%	68	79%
	P24/P24 [3]	6	27	103	2%	2%	46	93%
	V17/P24	7	25	116	2%	4%	56	82%
	P24/V17	6	23	85	0%	12%	40	78%

[1] V19: *viatica*19, V17: *viatica*17, P24: P24(XY)
[2] F/M: crosses presented as female/male
[3] Control crosses were presented in each pair of crosses because of differences in experiment equipments and years (V19/V17 in 1973, and V19/P24(XY) and V17/P24 in 1972)
[4] Number of pairs of males and females used for crossing
[5] Number of eggs batches laid by total number of females
[6] Abnormal karyotype includes haploid/multiploid cells and polysomy
n_1: Number of eggs used for embryo development experiment
n_2: Number of eggs used for hatching frequency experiment

In summary, reproductive isolating mechanisms appear to be absent or very weak among the *Vandiemenella* taxa. F_1 and backcross hybrids are produced without difficulty between some members of *Vandiemenella* and exhibit normal meiosis in most cases. Although some chromosomal hybrids do show some meiotic abnormalities that are likely to produce non-functional gametes, the effect on fertility seems to vary between pairs of patrental taxa. Nonetheless, stable hybrid zones are evident between all pairs of taxa wherever they meet. Therefore, chromosomal rearrangements may play some roles to provide an effective barrier to gene flow, but additional mechanisms may be needed to maintain distinct chromosomal races in the face of gene flow (Barton 1979; Spirito *et al.* 1983; Spirito 1998).

2.4 Models to explain the origin of *Vandiemenella* taxa

2.4.1 Stasipatric speciation model

Detailed description of the stasipatric speciation model can be found elsewhere (White 1968; 1973; 1974; 1978; King 1993). Briefly, characteristics of this model include (i) strong selection against chromosomal heterozygotes due to meiotic abnormalities, (ii) establishment of new chromosome types due to homozygous advantage, meiotic drive, and genetic drift, and (iii) spread of new chromosome types from their point of origin into the distribution of a parental chromosome types without apparent geographic isolation.

The origin of the mosaic papapatric distribution of *Vandiemenella* is explained by the stasipatric speciation model as follows (Fig. 2.5) (White *et al.* 1967):

Stage 0 (not drawn in Fig. 2.5)

- The area occupied by the present-day *viatica* species group was originally occupied by a parental species ('*proto-viatica*'), and its distribution was essentially contiguous over the entire period of evolution of *Vandiemenella* chromosomal races.

Stage 1A and 1B

- Daughter forms carrying new chromosomal rearrangements (B+6 fusion and a pericentric inversion in the X-chromosome) arise at points near the centre of the distribution of *proto-viatica* and separate *viatica*17, P24, P25, and P45b lineages from the ancestral *proto-viatica* lineage with karyotype 2n (♂) = 19.

Stage 2

- After establishment in local populations, the daughter forms (*viatica*17, P24, P25, and P45b) spread out from their origin and displace the parental forms until their expansion is arrested at some geographical point(s) due to selection against the homozygote of daughter forms in the parental distribution.
- P24 and P25 lineages acquired the male cercal hook character

Stage 3

- XY sex chromosome races of P24, P25, and P45b were formed by three independent X-autosome fusions, and later the translocation race was formed in the population of P24(XY)
- Narrow hybrid zones become stabilized between parental and daughter forms. The narrowness of the hybrid zones reflects strong selection against chromosomal hybrids and a low level of gene flow across the hybrid zones.

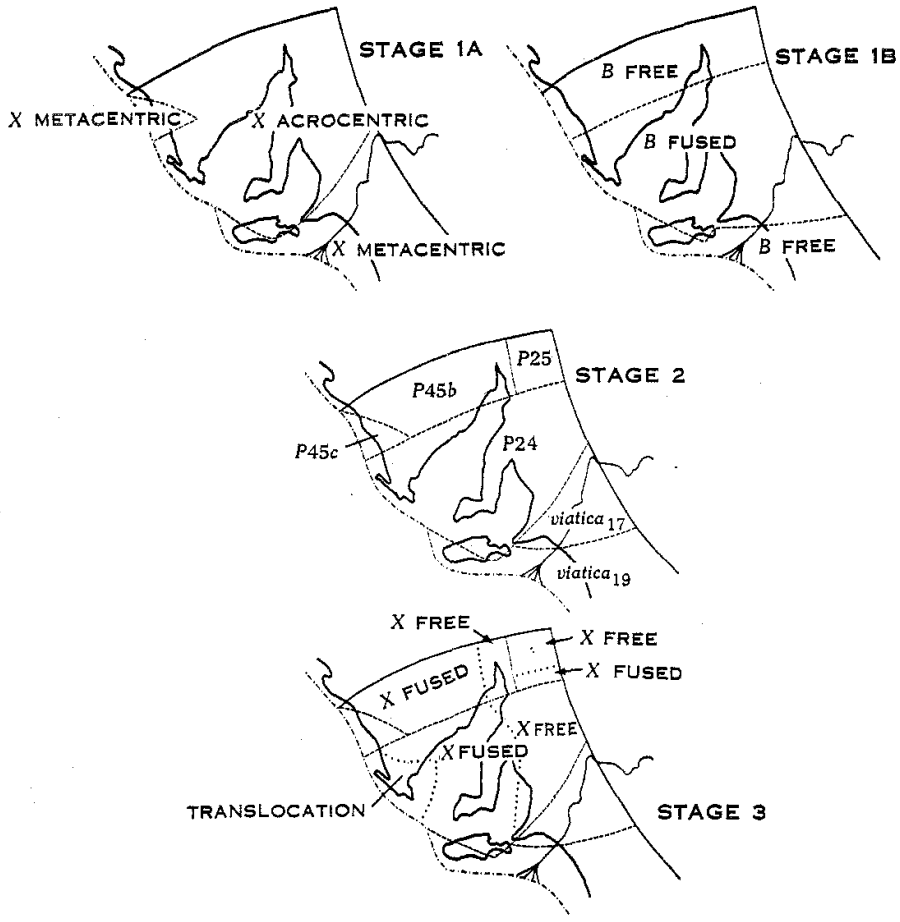


Fig. 2.5 Diagram illustrating chromosomal evolution of *Vandiemenella* based on the stasipatric speciation model (White *et al.* 1967).

The stasipatric speciation model has been criticized because new chromosomal rearrangements with strong underdominance in heterozygotes are unlikely to be established in a population without very special circumstances, such as inbreeding in small isolated populations, strong selective advantages of chromosomal homozygotes, and strong meiotic drive (Walsh 1982; Sites & Moritz 1987; Spirito 1998; Turelli *et al.* 2001). For instance, a number of theoretical studies suggest that chromosomal rearrangements with sufficient meiotic effects to provide an effective barrier to gene flow can only become fixed in very small populations ($n < 10$) with no gene flow (Sites & Moritz 1987 and references therein). Since initial establishment of new chromosomal rearrangements requires small isolated populations in the stasipatric speciation model, Futuyma and Mayer (1980) argued that such a geographic mode of speciation is essentially identical to the classic allopatric speciation model (see also Key 1968; 1974). To avoid such theoretical problems, the model further assumed that the homozygous advantage of new chromosome types and segregational advantage at meiosis (meiotic drive) helped establishment and spread of new chromosome types (White 1968; 1978). However, there is little evidence of such a homozygous advantage and meiotic drive in *Vandiemenella*. Mrongovius (1975; 1979) reported some evidence of meiotic drive, where meiotic segregation slightly favoured the X chromosome of *viatica*17 over the one of P24(XY) in F_1 hybrid females (53 - 55 % of *viatica*17-type X chromosome was inherited to backcrossed offspring), although it was in the reverse direction (*viatica*17-type X chromosome being 'ancestral').

2.4.2 Allopatric speciation model

Even though White *et al.* (1967) argued that the diversification and parapatric distribution of *Vandiemenella* does not seem to be explained by allopatric or sympatric speciation models, Key (1968) and Hewitt (1979) proposed an equally parsimonious evolutionary scenario based on the traditional allopatric mode of speciation. Because there is substantial evidence indicating that glacial-interglacial cycles during the Pleistocene resulted in vegetational and climatic fluctuations in south-eastern Australia (reviewed by Markgraf *et al.* 1995; Hesse *et al.* 2004), the *proto-viatica* is likely to have experienced cyclic expansion-contractions of its

distribution and population fragmentation during the Pleistocene. Key (1968) suggested that fluctuations in population size are likely to be the greatest at the periphery of the *Vandiemenella* distribution, and the surviving new chromosome types will be better adapted to the marginal environment than those in the centre of the range. Hewitt (1979) further predicted that the geographic origin of those new chromosome types may coincide with the Flinders Ranges and the coastal fringes of South Australia, because these areas are likely to provide refuges during the Glacial Maxima (*e.g.*, late Pleistocene wetland within the Flinders Ranges, Williams 2001).

An evolutionary sequence in allopatry is composed of five stages (Fig. 2.6): (A) B+6 fusion, (B) X inversion, (C) cercal hook acquisition, (D) X+1, X+B centric fusions, and (E) X tandem fusion and A/B+6 translocation (Hewitt 1979). The B+6 fusion, X inversion, cercal hook acquisition, X+B centric fusions, and X tandem fusion have primary distributions around the Flinders Ranges, while the X+1 centric fusion and A/B+6 translocation may have their origins along the coastal fringes of Eyre Peninsula. In addition, to explain the distributions of *viatica*19, *viatica*17, and P24(XY), the spread of these mutations must have occurred during the Pleistocene when the sea level was periodically much lower than present, and KI and Tasmania were connected to the mainland of Australia (Bowler 1978; 1982; Lambeck & Chappell 2001). The significance of this hypothesis is not only to demonstrate the plausibility of the allopatric mode of speciation for *Vandiemenella* but also to provide an empirically testable phylogeographic hypothesis using a combination of molecular, cytological, and morphological analyses (Chapter 6).

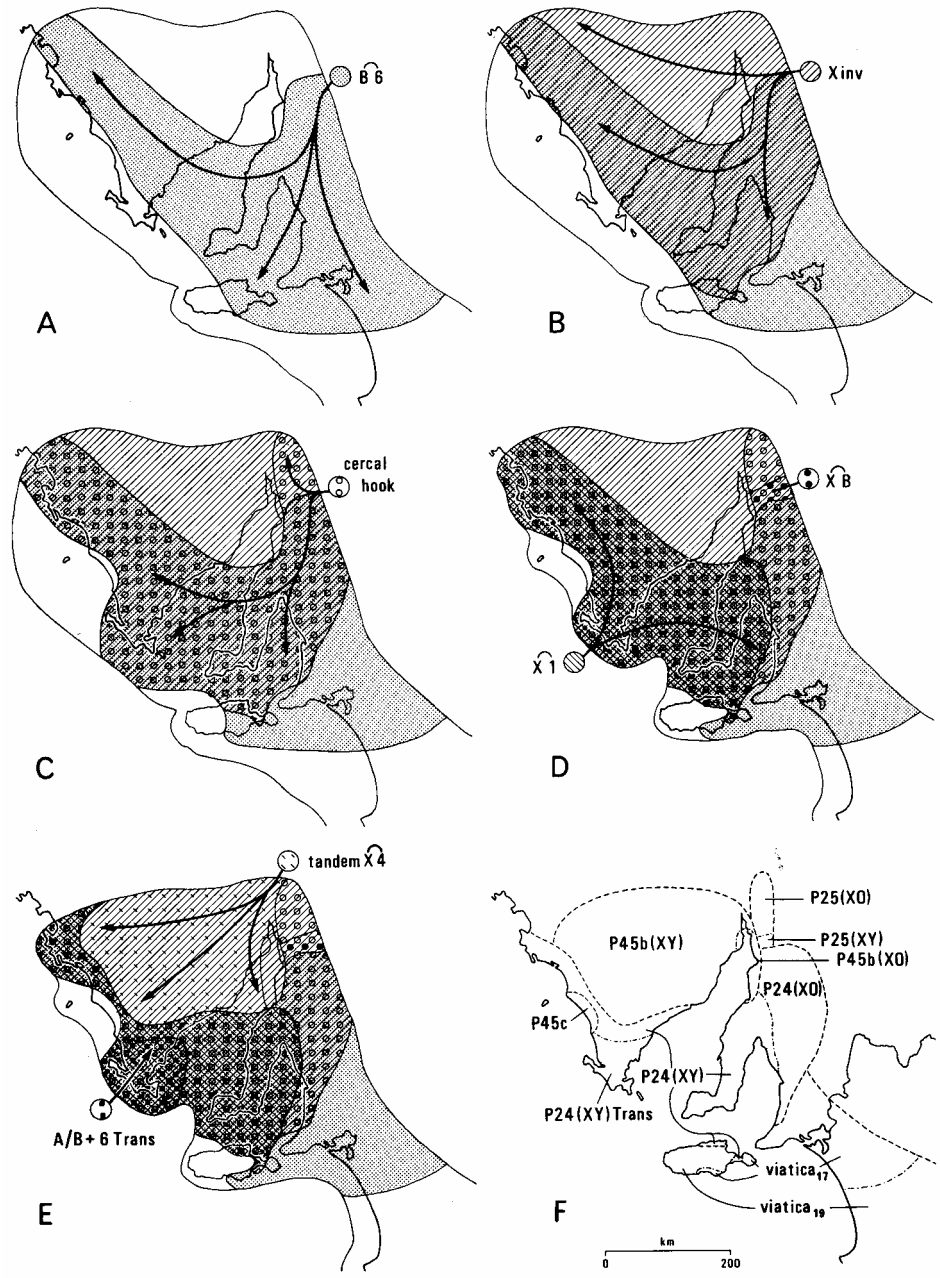


Fig. 2.6 A possible origin of the chromosomal races of *Vandiemenella* based on the allopatric speciation model (Hewitt 1979). Note that in the stage E, X tandem fusion with autosome number 4 is from White *et al*. (1967).

2.4.3 Recombination suppression models

As discussed in Section 2.4.1, a critical theoretical dilemma exists for the stasipatric speciation model. Specifically, if fitness reduction by chromosomal rearrangements is sufficiently high to reduce gene flow and promote speciation, then it may be difficult for these new rearrangements to become fixed in populations. Because of this theoretical dilemma, others view chromosomal rearrangements as incidental by-products of speciation processes (*e.g*., Coyne & Orr 1998). In contrast, recently proposed chromosomal speciation models (Noor *et al.* 2001b; Rieseberg 2001; Navarro & Barton 2003a; Kirkpatrick & Barton 2006), referred to as the 'suppressed recombination model' by Ayala and Coluzzi (2005), suggest that certain types of chromosomal rearrangements, such as inversions, translocations, and tandem fusions, may contribute to speciation by suppressing recombination and reducing gene flow near rearranged regions in chromosomal heterozygotes (reviewed by Ortiz-Barrientos *et al.* 2002; Coyne & Orr 2004; Butlin 2005). Such rearranged chromosomal regions may accumulate sets of genes responsible for local adaptation or reproductive isolation (*e.g.,* assortative mating and post-zygotic isolation) and increase linkage disequilibrium between those genes in the presence of gene flow. In contrast, other genomic regions away from the rearranged regions may maintain recombination and gene flow in chromosomal heterozygotes.

Importantly, suppressed recombination models do not assume fitness reduction as an isolation mechanism to reduce gene flow. This distinction means that establishment of new chromosomal rearrangements in a population is more likely because these models do not rely heavily on genetic drift in small isolated populations. In turn, new chromosomal rearrangements can become fixed not only in strict allopatry but also in parapatry and sympatry. For instance, some of the suppressed recombination models assume initial population isolation followed by secondary contact of populations for establishment of new chromosomal rearrangements and differential accumulation of isolation genes (secondary parapatry) (Noor *et al.* 2001b; Rieseberg 2001). Others suggest that accumulation of reproductive isolating mechanisms is possible in parapatry without an initial allopatric phase (primary parapatry) (Navarro & Barton 2003a; Kirkpatrick & Barton 2006). Whether primary or secondary, all of these

models involve a parapatric phase, and a condition for fixation of new chromosomal rearrangements may be much more relaxed than in the stasipatric speciation model.

Suppressed recombination models appear to be 'somewhat more plausible' than the stasipatric speciation model (Coyne & Orr 2004), although empirical evidence is still scarce and sometimes conflicting (*e.g.*, speciation of humans and chimpanzees in Navarro & Barton 2003b; Szamalek *et al.* 2007). It is still an open question whether chromosomal rearrangements may play a causative role in speciation or are incidental by-products of speciation events. These chromosomal speciation models in combination with the classic speciation models (*e.g.,* allopatric speciation model) provide a theoretical framework with which to study chromosomal speciation. The extent of gene flow between rearranged and collinear chromosomal regions represents a testable hypothesis for the suppressed recombination model. The *Vandiemenella viatica* species group provides a powerful model system to elucidate classic and recent speciation theories and to examine the potential role of chromosomal rearrangements in speciation.

2.5 Conclusion

Information regarding the biology, ecology and genetics of non-model organisms is generally scarce, however, the previous Sections have shown that a fair degree is known about the *Vandiemenella viatica* species group. In particular, numerous chromosomal races, replicated parapatric distributions, and known hybrid zones make the *viatica* species group a unique study system to investigate speciation mechanisms. A combination of molecular, cytological, and morphological analyses will shed light on speciation processes and genetic mechanisms of maintaining species boundaries in *Vandiemenella* and other organisms.

CHAPTER 3

DEVELOPMENT OF POLYMORPHIC MICROSATELLITE MARKERS

(*Accepted for publication in Molecular Ecology Notes under the same title*)

3.1 Introduction

Australian morabine grasshoppers of the genus *Vandiemenella* (*viatica* species group) show extensive chromosomal variation, with the group currently comprising two named species (*V. pichirichi* and *V. viatica*) and 11 chromosomal races (Key 1976). With only two exceptions, the 12 taxa have parapatric distributions, often with narrow contact zones (White 1978). The group formed the basis of the classic 'stasipatric' chromosomal speciation model of White (1978), which suggested that chromosomal rearrangements played a key role in promoting speciation. Four cases of natural hybridisation have been reported at contact zones between different combinations of chromosomal races in South Australia (reviewed in White 1978, and references therein). However, there have been no molecular genetic analyses to investigate the extent of gene flow between chromosomal races. Here, we report the isolation of microsatellite markers, which will be used to investigate the population genetic structure of *Vandiemenella viatica*.

3.2 Methods

Microsatellite loci from *Vandiemenella* were isolated using the protocol of Gardner *et al.* (1999). A total of seven individuals of four chromosomal races of *Vandiemenella* from different localities were used for genomic library construction: *viatica*17 (n = 3), *viatica*19 (n = 1), P24(XO) (n = 1) and P24(XY) (n = 2). Two micrograms of total DNA from each of the seven individuals were combined and digested with *Rsa*I or *Bst*UI (New England Biolabs). Digested DNA samples were size-selected by gel electrophoresis (300 - 1000 bp). Microsatellite-enriched fragments were cloned using the TOPO TA Cloning Kit (Invitrogen®). Positive clones were detected using a colony-based DNA hybridization technique with biotin-

labeled oligonucleotides for $(AAAG)_n$ or $(AC)_n$ as probes and an alkaline phosphatase/streptavidin colourimetric detection system (Roche). Cloned inserts were PCR-amplified using M13 plasmid vector primers and were sequenced on both strands using the ABI Prism™ Big Dye Cycle sequencing kit in ABI 3700 sequencers (Applied Biosystems). Primers were designed in the flanking regions of microsatellite loci using OLIGO version 4.0 (Table 3.1). Sequencing analysis of the *Viat4* microsatellite locus in six individuals revealed 16 bp and 9 bp indels located 70 bp and 40 bp respectively 5' of the $(AAAG)_n$ microsatellite (data not shown). An internal primer was designed to exclude the microsatellite and specifically target the indels in population analyses (referred to as locus *ViaID4*, Table 3.1).

PCR-amplifications of microsatellite loci were performed for one population each of *viatica*17, *viatica*19, and P24(XY) on Kangaroo Island. Populations were composed of 7 - 11 sampling sites per population, which were away from contact zones to avoid potential influences of introgression from adjacent chromosomal races. The forward primer of each microsatellite locus was 5' end-labeled with a fluorescent dye FAM (GeneWorks), NED, PET, or VIC (Applied Biosystems). Multiplex PCR-amplifications were carried out for four sets of loci (set 1-4 in Table 3.1) in 10µl reaction volumes, containing 1 × reaction buffer with 2.5 mM $MgCl_2$ (Eppendorf), 0.2 mM of each dNTP, 0.5 - 2.5 pmol of each primer, 1 unit of HotMaster Taq DNA polymerase (Eppendorf), 4µg of BSA (Ambion), 9.5µmol of betaine (Sigma), and 2µL of genomic DNA (*c*. 20ng). PCR cycling conditions were as follows: 1 cycle of 94°C for 2 min, 8 touchdown cycles (94°C, 30 s; 55 - 47°C, 30 s; and 65°C, 30 s), 25 cycles with constant annealing temperature (94°C, 30 s; 47°C, 30 s; and 65°C, 30 s), followed by a final extension at 65°C for 30 min. Microsatellite alleles were detected using an ABI 3730 DNA Analyzer, and allele size data were analysed by the program GeneMapper® version 3.7 (Applied Biosystems). Standard singleplex PCR for individual loci using 36 samples confirmed that there were no apparent allele dropouts and allele size differences between multiplex and singleplex PCRs.

Table 3.1 Characteristics of nine microsatellite loci and one insertion/deletion polymorphic locus isolated from the *Vandiemenella viatica* species group. Repeat motifs are those found in the cloned allele.

PCR set	Locus	Primer sequence (5' - 3')	Repeat motifs	Allele size (bp)	N_A	*Viatica*$_{19}$ (N = 79)			*Viatica*$_{17}$ (N = 33)			P24(XY) (N = 25)			Accession No.
						H_O	H_E	N_{null}	H_O	H_E	N_{null}	H_O	H_E	N_{null}	
Set-1	*Viat2*	F: GGTGCCTCGAAGTGGTAATA R: GCTATCGGTGTGAAAGAAT	†$(AAAG)_9$	157-236	10	-	-	79	0.08*	0.49	9	0.24*	0.75	0	EF458151
	Viat4	F: TGAGAGCAAAATAGMACCAA R: AGGACACAGAATCAAAGTT	$(AAAG)_{12}$	96-172	11	0.01*	0.04	2	0.49	0.61	0	0.08	0.15	0	EF458152
	Viat8	F: CTCGGGTAAACAGATTCGGC R: AGGAGTCCAAGGTAGAGAGG	†$(AAAG)_9$	181-205	11	-	-	79	0.39*	0.76	0	0.76	0.84	0	EF458154
Set-2	*Viat32*	F: GATGTAATGATTAAGTGAG R: GCATCAAGAAGTACCGTTTT	†$(AAAT)_2$	283-292	2	-	-	65	0.28*	0.50	1	0.00	0.00	0	EF458159
Set-3	*Viat10*	F: GTCTCCACTTTAGTCACATA R: GTTTAATAGCAAGACATCGC	†$(AAAG)_8G(AAAC)_5$	219-373	21	0.50*	0.76	3	0.52*	0.88	2	0.32	0.55	0	EF458155
	Viat27	F: CAGGGCTTAGTGATGATCCA R: CGTTACAACTCAAACCAATGAA	$(AC)_{15}$	169-245	34	0.65	0.80	0	0.79	0.94	0	0.80	0.86	0	EF458157
Set-4	*Viat5*	F: TTATTGTTTTGTCACCTTGTC R: AGGGACGAAACTGATGTAG	†$(ACAT)_9(ACTT)_{23}$ $(CTTT)_{14}$	194-260	11	-	-	75	0.42	0.44	0	0.44	0.50	0	EF458153
	Viat21	F: AGTGTGGTTTTGGCAGATGTC R: CTACTCAGAAGACTAAGGCTC	†$(AAAG)_8$	150-196	16	0.65*	0.89	14	0.30	0.48	2	0.32	0.27	0	EF458156
	Viat31	F: ACCCCATGTCTTGTACGGAT R: ACCCTCTTATTTCACTGCTTAG	†$(TC)_{10}(AC)_{16}$	269-327	23	0.90	0.92	0	0.76	0.85	0	0.70	0.79	0	EF458158
	ViaID4	F: AGACGATATGCCTAAAAGAG R: TTGGTKCTATTTTGCTCTCA	(TGGSTAAATTG AAAAA) (GAAAAAGAG)	90-148	6	0.48	0.45	2	0.52	0.58	0	0.35	0.41	0	EF458152

(Table 3.1 Footnote)

N_A, Number of alleles for three chromosomal races; H_O, Observed heterozygosity; H_E, Expected heterozygosity; N_{null}, Number of putative null homozygous individuals.
H_O and H_E were not calculated for *Viat2*, 5, 8, and 32 for *viatica*19 because of high N_{null} values.
N_A for each chromosomal race is presented in Table 3.2.
†, compound locus; *, significant deviation from Hardy-Weinberg equilibrium with $\alpha = 0.05$ using the sequential Bonferroni correction.
Set-2 originally contained two loci, but one was excluded due to inconsistent amplification.

3.3 Results and discussion

Numbers of alleles, size ranges, observed and expected heterozygosities (H_O and H_E) were calculated using Cervus 3.0 (Marshall *et al.* 1998) (Table 3.1). When all three races are considered, eight microsatellite loci are highly polymorphic (10 - 34 alleles), while one locus (*Viat32*) has only two alleles. The indel locus (*ViaID4*) has four alleles with expected size differences based on combinations of presence/absence of 16 bp and 9 bp indels. Two allele sizes were different from expectation, probably due to unidentified indel polymorphisms in the flanking regions. Tests of deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were conducted using GENEPOP (http://wbiomed.curtin.edu.au/genepop/). Significant heterozygote deficiencies were observed at three loci for *viatica*19 (*Viat4*, 10, and 21), four loci for *viatica*17 (*Viat2*, 8, and 10, and 32), and one locus for P24(XY) (*Viat2*) after sequential Bonferroni correction (Rice 1989). Presence of null alleles was inferred for these loci because other loci in the same populations did not show significant deviation from HWE and several individuals showed no PCR-amplification of alleles (*i.e.*, null homozygotes). However, it is also possible that population processes, such as population subdivision and inbreeding may have contributed to the observed heterozygote deficits. Only one locus pair (*Viat4* and *ViaID4*) showed significant LD after sequential Bonferroni correction, as expected for loci derived from the same flanking sequence. Cross-species/races amplification of all loci was generally successful for eight other *Vandiemenella* taxa (Table 3.2). However, no amplification was observed at *Viat2*, 5, 8, and 32 for 1 - 6 *Vandiemenella* taxa. Cross-genus amplification using *Keyacris sp*., *Prorifera sp*. and

Warramaba sp. was generally unsuccessful, with the exception of two loci (*Viat4* and *Viat31*) in *Keyacris sp*.

In summary, the 10 polymorphic loci reported here will be useful for investigations of the population genetic structure and gene flow among *Vandiemenella* taxa, and to investigate patterns of introgression across hybrid zones to assess the potential role of chromosomal rearrangements in speciation.

Table 3.2 The number of alleles amplified across 11 taxa of *Vandiemenella viatica* species and three other morabine grasshopper genera.

				Locus									
Genus	Species	races	*N*	*Viat2*	*Viat4*	*Viat5*	*Viat8*	*Viat10*	*Viat21*	*Viat27*	*Viat31*	*Viat32*	*ViaID4*
Vandiemenella	*viatica*	19	79	0	3	0	0	12	16	14	20	1	4
		17	33	7	10	6	7	12	2	18	12	2	3
	*P24	(XO)	12	5	5	1	8	11	5	14	14	3	1
		(XY)	25	5	3	5	9	3	2	11	7	1	2
		(XY) Translocation	8	4	2	0	2	1	2	6	6	2	1
	*P25	(XY)	2	2	2	0	1	3	1	3	2	0	2
	*P45b	(XO)	12	1	2	0	5	7	4	11	12	2	2
		(XY)	4	2	2	0	3	3	1	6	6	1	2
	*P45c		6	0	2	0	2	1	1	1	9	1	2
	*P50		5	0	2	4	2	6	2	5	9	3	2
	pichirichi		4	0	5	0	2	1	1	2	4	2	3
Keyacris	*P110a		2	0	2	0	0	0	0	0	2	0	1
Prorifera	*P28		3	0	0	0	0	0	0	0	0	0	0
Warramaba	*P169/P196		2	0	0	0	0	1	0	0	0	0	0
(Total)			197	22	14	13	18	31	19	33	36	9	7

*, provisional species code, Australian National Insect Collection (Key 1976). *N*, number of individuals tested.

CHAPTER 4

GENETIC ANALYSIS OF A CHROMOSOMAL HYBRID ZONE IN THE AUSTRALIAN MORABINE GRASHOPPERS (*VANDIEMENELLA*, *VIATICA* SPECIES GROUP)

4.1 Introduction

Hybrid zones have been found in a wide range of organisms and have been extensively studied as 'natural laboratories' to investigate levels of gene flow and to compare the involvement of different parts of the genome in forming barriers to gene flow (Barton & Hewitt 1985; 1989; Harrison 1990; 1993a). The spatial and temporal dynamics and mechanisms that maintain hybrid zones are highly variable, but the most common form appears to be a 'tension zone' in which a balance between dispersal and selection against individuals of mixed ancestry maintains narrow hybrid zones (Key 1968; Barton & Hewitt 1985; Harrison 1990). In general, tension zones involve changes in a variety of characters, and so consist of a cluster of parallel gradients in allele frequencies (these gradients are termed 'clines') (Barton & Hewitt 1989). The tension zone model predicts that genes under selection or linked to genomic regions under selection experience a barrier to gene flow, while unlinked genomic regions sustain gene flow (Barton & Gale 1993). In turn, it is possible to detect genomic regions that are under strong selection and, hence, may be involved in the speciation process by comparing patterns of variation across tension zones between multiple independent characters.

Chromosomal rearrangements have been considered to alter patterns of gene flow between hybridizing taxa, but whether such changes play a direct role in the process of speciation has been much debated (White 1978; King 1993; Coyne & Orr 1998; Spirito 1998; Butlin 2005). It is traditionally considered that chromosomal changes play a causative role in speciation by providing genetic barriers to gene flow between

populations fixed for different karyotypes (*e.g.*, the 'stasipatric' speciation model, White 1968; White 1978; see Sites & Moritz 1987; Rieseberg 2001 for other traditional chromosomal speciation models). According to the stasipatric model, hybrid individuals with a heterokaryotype are partly sterile due to abnormalities of meiosis, such as asynapsis and malorientation of the rearranged chromosomes. In contrast, others argue that chromosomal variations have been incidentally accumulated after the actual speciation event and, hence, are far less important than genic incompatibility in speciation (Futuyma & Mayer 1980; Charlesworth *et al.* 1982; Coyne & Orr 1998). More recently, it has been suggested that chromosomal rearrangements may facilitate divergence and speciation by suppressing recombination within the rearranged chromosomal regions in heterokaryotypes, hence, extending the effects of linked 'isolation genes' or preserving combinations of locally adapted alleles that evolved in allopatry (Noor *et al.* 2001b; Rieseberg 2001; Navarro & Barton 2003a; Kirkpatrick & Barton 2006). The chromosomal speciation models (both stasipatric and recombination suppression models) predict that gene flow (recombination) will be heterogeneous in different parts of the genome, with regions of low gene flow associated with chromosomal rearrangements. In contrast, the genic view predicts that regions of low gene flow are associated with underlying 'isolation' genes that cause reduced hybrid fitness rather than chromosomal rearrangements. It has been suggested that reproductively isolated species are often separated by many isolation genes of weak effect on their own, but strong effect in combination (Wu & Hollocher 1998; Wu & Ting 2004). Since these isolation genes are likely to be spread throughout the genome, barriers to gene flow would be less localized than is expected for the chromosomal speciation models. Hybrid zones characterized by chromosomal differences, therefore, provide an ideal study system for exploring the potential role of chromosomal rearrangements in speciation.

Australian morabine grasshoppers in the genus *Vandiemenella*, known as the *viatica* species group, is a classic model system for studies of chromosomal speciation and formed the basis of White's stasipatric speciation model (1968; 1978). The group is distributed in south-eastern Australia and currently comprises at least 11 chromosomal races discriminated by various rearrangements (fusions, fissions,

translocations, or inversions). All taxa, with two exceptions, have parapatric distributions and in most cases they show narrow zones of hybridization, with respect to the chromosomal variation. Among several contact zones reported previously (White *et al.* 1967; 1969), we have identified an intact contact zone on Kangaroo Island in South Australia, between the provisional species P24 (2n = 16 XY in males and XX in females), henceforth referred to as the P24(XY) chromosomal race, and the *viatica*17 chromosomal race of *V. viatica* (2n = 17 XO in males, 2n = 18 XX in females) (Fig. 4.1). Cytological studies suggested that P24(XY) evolved from a common ancestor with *viatica*17 through a pericentric inversion on the X-chromosome and a centric fusion (Robertsonian fusion) between the inverted acrocentric X-chromosome and one of the small acrocentric autosomes (Fig. 4.1C) (White *et al.* 1967).

White *et al*. (1969) reported that both chromosomal races and their hybrids were found within a zone of overlap of 200 - 300 m. In contrast to this narrow zone for chromosomes, introgression of a morphological trait appeared to be bidirectional and to extend much further than the width of the chromosomal hybrid zone (White *et al.* 1969). Moreover, little evidence of non-disjunction and malsegregation in meiosis was seen in chromosomal heterozygotes, and laboratory experiments showed little or no evidence for reduced F_1 viability and fertility (Mrongovius 1975; 1979). To date, there are no data to quantitatively evaluate how the barrier to gene flow between P24(XY) and *viatica*17 varies for chromosomal and multiple molecular markers.

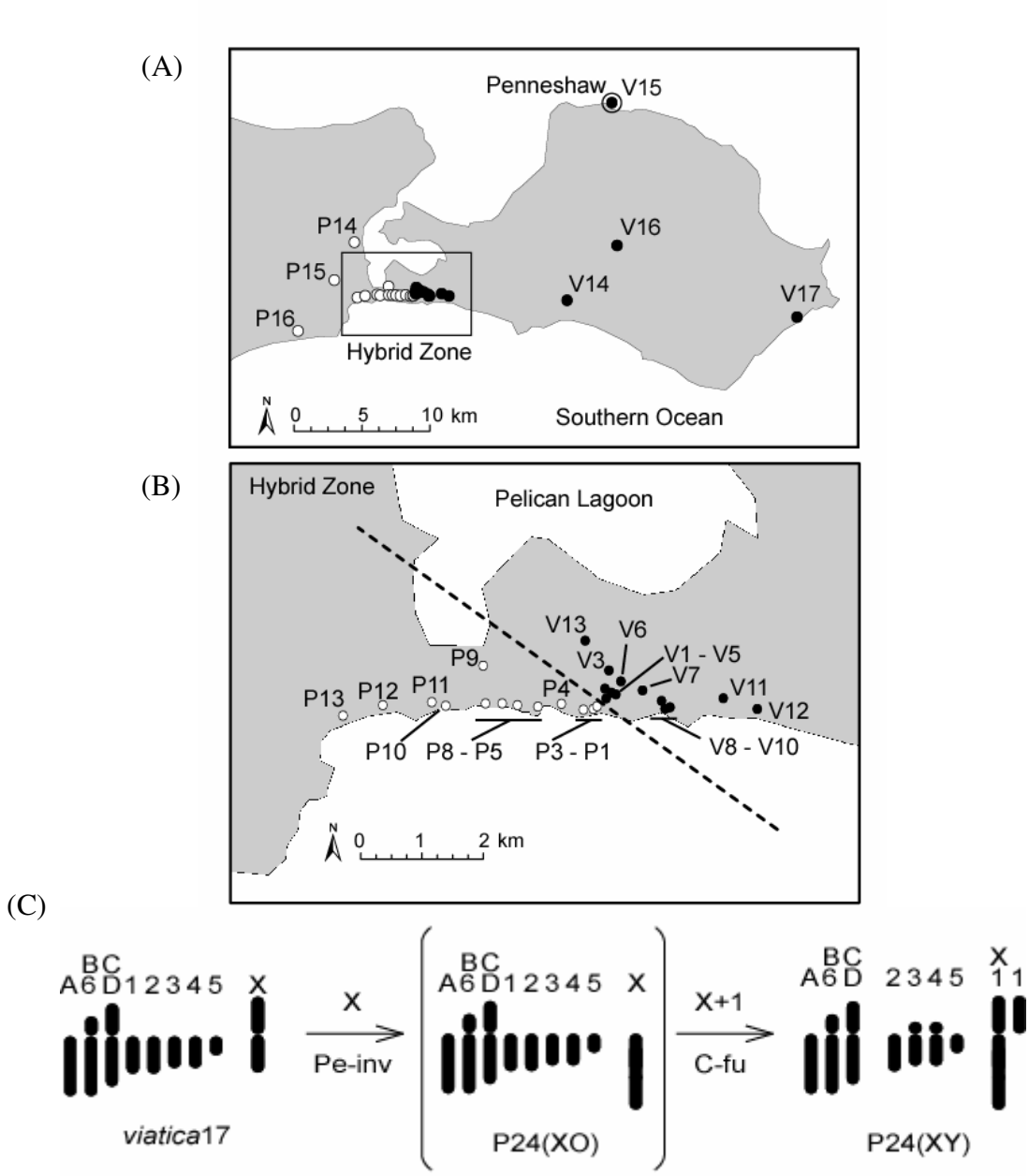


Fig. 4.1 Map of sampling sites across the transect between P24(XY) (open circles) and *viatica*17 (filled circles) chromosomal races of *Vandiemenella* on Kangaroo Island (KI), South Australia (A and B). The dashed line indicates the best fit axis. Cytological evolutionary sequence proposed by White *et al*. (1967) (C): Pe-inv, pericentric inversion on X-chromosome; X +1, centric fusion between the inverted X sex chromosome and No. 1 autosome, resulting in XY sex chromosome system. The 3 and 4 autosomes in P24(XY) are subacrocentric. P24(XO) is a presumed intermediate race, which is distributed only on the mainland of Australia.

White's stasipatric speciation model (1968; 1978) leads to several distinct predictions about the origins and dynamics of the *Vandiemenella* hybrid zone. First, the hybrid zones have arisen by primary contact since the geographic distribution of the species group was 'essentially' contiguous without major allopatric separations through the entire period of chromosomal diversification. Second, the hybrid zones moved uni-directionally from the point of origin of the new chromosome types into the distribution of parental chromosome types owing to homozygous advantage and/or meiotic drive of the new chromosome types (but see Veltsos 2008 for an alternative explanation for the spread of neo-XY chromosome types). Finally, barriers to gene flow are stronger for loci linked with rearranged chromosomes than loci on non-rearranged chromosomes because selection directly acts on meiotic abnormalities of the rearranged chromosomes. Unless selection against heterozygous chromosomes is so extensive that barriers to gene flow affect the whole genome, differences in barriers to gene flow would result in different cline positions and widths between loci (*i.e.*, 'staggered hybrid zone', Barton & Hewitt 1981a; Searle 1993).

In contrast, Key (1968) and Hewitt (1979) argued that chromosomal variants in *Vandiemenella* established in allopatric populations, and contact zones between the chromosomal races formed by secondary contact after climate change. Under an allopatric mode of diversification, genetic mutations may have been randomly accumulated across genomes in each of the geographically separated populations, and some mutations may have resulted in hybrid incompatibility once those allopatric populations met with each other by secondary contact (Barton & Hewitt 1981a). Chromosomal mutations may also have been established during the allopatric phase, although, different from the prediction based on the stasipatric model, these chromosomal mutations may not necessarily have been involved in the formation of barriers to gene flow. Moreover, hybrid zones formed by secondary contact may also move, but their movement does not depend only on the selective advantage of the new homokaryotype over the other, but also on fitness differences between races within local environments, frequency-dependent selection against rare genotypes, and population density gradients (Barton & Hewitt 1985). Such moving

hybrid zones may be trapped by local barriers (either physical or genetic) or population density troughs (Barton 1979; Barton & Hewitt 1981a). Furthermore, coincidence and concordance of clines for multiple loci may be expected as a result of secondary contact. However, such patterns do not provide definitive evidence for an allopatric origin of the chromosomal races because they may also be due to coadaptation of many genes and population density troughs (Barton & Hewitt 1981a). In addition, even if the origin of the zone is secondary, clines can show staggered patterns because selection may act on a variety of characters, and, therefore, alleles at loci associated with those characters may introgress through the hybrid zone to different extents. Such a cline displacement is particularly pronounced in mitochondrial clines because mitochondrial genes are traditionally considered to be neutral and, hence, can introgress though the hybrid zone more easily than nuclear genes (Barton & Jones 1983; Ballard & Whitlock 2004).

To characterize the hybrid zone of the two chromosomal races P24(XY) and *viatica*17 of *Vandiemenella*, we have employed a set of microsatellite loci, allozyme loci, the elongation factor-1α (*EF*-1α) gene, and mitochondrial cytochrome oxidase subunit I (*COI*) gene. Cline shape, patterns of linkage disequilibria, dispersal rate, and selection against hybrids were estimated from the data. Finally, by comparing cline shapes between markers, we discuss the origin and maintenance of chromosomal hybrid zones in *Vandiemenella* (stasipatric vs. allopatric).

4.2 Methods

4.2.1 Taxon sampling

Two chromosomal races of *Vandiemenella* [*viatica*17 and P24(XY)] were collected in 2002, 2004 and 2005 at 26 sites along an east-west transect (~ 6 km) across a hybrid zone and seven sites outside of the zone on Kangaroo Island (Fig. 4.1). Over four years, 206 males and 194 females of *viatica*17 and 233 males and 194 females of P24(XY) were collected in the study area. While samples of P24(XY) were collected in coastal native vegetation evenly distributed over the length (~ 1 km) of the western side of the transect, very few samples of *viatica*17 were collected at the

eastern side of the transect in this coastal vegetation. Instead, *viatica*17 samples were collected from the weed *Acaena* found in cleared vegetation. In addition, very few grasshoppers were collected at sites north of the transect, including the original transect studied by White et al. (1969) and Mongrovius (1975; 1979), probably due to land clearing for agriculture. Latitude and longitude of sampling sites were recorded immediately after a first grasshopper was collected, and subsequent grasshoppers were collected within a 20 m radius of the recorded positions. Testes from males were removed for chromosomal analyses and bodies were frozen in liquid N_2 and then stored at -80 °C, prior to allozyme and DNA analyses. In most cases, the same male specimens used for chromosomal analyses were also used for allozyme and DNA sequence analyses.

4.2.2 Markers

For cytogenetic analysis, fresh testes were removed from 439 males and were placed in 1 % sodium citrate solution for approximately 10 - 15 minutes and squash preparations of the chromosomes were made in 2 % aceto-orcein stain presented in White *et al*. (1967). Given previous cytogenetic studies of the chromosomal races of *Vandiemenella* on Kangaroo Island (White *et al.* 1969), which provided evidence for parapatric distributions and narrow contact zones (~ 200 - 300 metres), it was assumed that males and females in populations more than 300 metres from a contact zone would have a similar karyotype to that of karyotyped males of the same population. In addition, male chromosomal heterozygotes cannot be detected by this method because they appear to be either the P24(XY) or *viatica*17 karyotype, according to the type of X-chromosome they are carrying (White *et al.* 1969).

Following previous allozyme polymorphism screening (Kawakami *et al.* 2007a), five putative allozyme loci, which were observed to be diagnostic between *viatica*17 and P24(XY), were used: *Acyc*, *Ak1*, *Ak2*, *Idh2*, and *PepA*; locus abbreviations follow Richardson *et al.* (1986). A locus was considered diagnostic if one chromosome race shared no or few alleles with the alternative race (< 5 %), based on a comparison of 126 individuals [n = 56, *viatica*17; and n = 70, P24(XY)] representing seven sites well removed from the contact zone to avoid potential influences of gene

introgression from adjacent chromosomal races. The same criterion of diagnosability was used for microsatellite, *COI* and *EF*-1α loci. Allozyme electrophoresis was undertaken on cellulose acetate gels (Cellogel©, MALTA, Milan) following methods in Kawakami *et al*. (2007a) and general protocols described in Richardson *et al.* (1986).

Total DNA was extracted from single hind legs using the GENTRA DNA extraction kit and using the protocol specified by the manufacturer. Sampling sites along the transect across the contact zone and outside the zone were screened at five microsatellite loci (*Viat2*, *Viat4*, *Viat5*, *Viat8*, and *Viat10*; Kawakami *et al.* 2007b). These were considered diagnostic based on a comparison of 121 individuals [n = 56, *viatica*17; and n = 65, P24(XY)] from seven sites outside the hybrid zone. A diagnostic restriction endonuclease (RE) assay for the two chromosomal races was designed using the *COI* and *EF*-1α sequence data in Kawakami *et al*. (2007a). A 637 bp region of the *COI* gene and an 825 bp region of the *EF*-1α gene were PCR-amplified using the protocol described in Kawakami *et al*. (2007a). Amplified products of *COI* were digested separately with *Bgl*II (Promega) at 37 °C for 8 h and with *Fau*I (New England Biolabs) at 55 °C for 8 h, and amplified products of *EF*-1α were digested with *Bsr*GI (New England Biolabs) at 37 °C for 8 h under buffer conditions specified by the manufacturer. RE fragments were separated by electrophoresis on 1.5 % agarose gels and stained with Ethidium Bromide. Fragment sizes were estimated by comparison with a 100 bp ladder. RE digestion for heterozygote individuals for *EF*-1α was repeated to ensure that fragment patterns were not due to incomplete digestion. In addition, representative heterozygote individuals [five P24(XY) and five *viatica*17 individuals] were sequenced.

To pool samples collected over four years, we assumed that the position and width of the contact zone had not significantly changed from year to year. To test this assumption, temporal homogeneity of allele frequencies at five sites with sufficient sample sizes were tested: P15 (2002: n = 19; 2004: n = 8), V1 (2002: n = 10; 2004: n = 18), P3 (2002: n = 10; 2004: n = 10), P2 (2002: n = 9; 2004: n = 4; 2005: n = 17), and V5 (2002: n = 10; 2004: n = 10). None of the five sites showed significant

differences in allele frequencies for five microsatellite, *COI* and *EF*-1α loci after sequential Bonferroni correction (Rice 1989). Thus, changes in allele frequencies at each locus are likely to be small on this short time-scale.

4.2.3 Marker variability

Allele frequencies, numbers of alleles, observed and expected heterozygosities (H_O and H_E), inbreeding coefficients (F_{IS}), tests of deviation from Hardy-Weinberg equilibrium (HWE), and linkage disequilibrium were calculated for five microsatellite and five allozyme loci in each sampling site using GENEPOP (http://wbiomed.curtin.edu.au/genepop/) and FSTAT version 2.9.3 (Goudet 1995). Males and females were analysed separately for a putative X-linked locus, *Acyc*, where all males appeared homozygous (*i.e.,* putatively hemizygous). A permutation test (5000 runs of multilocus genotype randomization) was used to determine whether observed F_{IS} values were significantly different from zero for each locus and over all loci using FSTAT. The statistical significance was adjusted using a sequential Bonferroni correction for multiple comparisons (Rice 1989).

4.2.4 Cline fitting

Microsatellite and allozyme loci were collapsed to two allele systems [*i.e*., P24(XY) alleles and *viatica*17 alleles]. Allele frequencies for the *Acyc* locus was calculated by treating males as haploid [XO in *viatica* 17 or XY in P24(XY)] and females as diploid (XX) in both races. Maximum-likelihood (ML) clines were fitted independently at each locus to population allele frequency data of allozyme loci, microsatellite loci, *EF*-1α, *COI* genes, and a cytological character, across the transect using the compound hyperbolic tangent function (tanh) and exponential model of Szymura and Barton (1986). Because the transect used in the present study lies parallel to the narrow strip of coastal habitat along a coastline, a one dimensional (1D) cline was used in the program ANALYSE version 1.30 (Barton & Baird 2002). Sample sites were collapsed onto a 1D transect along a best-fit axis oriented 49° anti-clockwise from the north-south direction (Fig. 4.1B). The best-fit axis was estimated by a 2-dimensional (2D) cline fitting with a single straight line. Although we also

used angled lines made up by two or more segments for estimating the best-fit axis (Bridle *et al.* 2001), these segmented lines did not provide significantly better fits (likelihood ratio tests, $p > 0.05$). Perpendicular distances from each sampling site to the centre of the cline were measured. As perpendicular lines from the distant sites (sites P13, P12, V8, V9, V10, V11, and V12) were intercepted by sea, we adjusted the distance as follows; we followed the perpendicular line until it met the coast at which point the angle of measurement changed and followed a straight line along the coast to the centre of the cline. The distance of the centre of the best-fit average cline over all ten autosomal loci (allozyme loci excluding *Acyc*, microsatellite loci, and *EF*-1α locus) was set to zero, and all other distances were recalculated [*i.e.*, distances of sampling sites in the P24(XY) territory were negative and on the *viatica*17 territory they were positive]. Error associated with the 1D collapse and its orientation may result in an overestimate of the width, but may not significantly affect the centre of the cline and its relative widths between loci.

Because allele frequencies between sites may fluctuate randomly due to small sample sizes, allele frequencies at each site were weighted in proportion to the effective sample size (N_e). N_e estimates, taking into account maximum likelihood estimates (MLEs) of F_{IS}, were calculated for each sampling site at each locus from the equation (Phillips *et al.* 2004; Raufaste *et al.* 2005):

$$N_e = 2N/(2N \times F_{ST} + 1 + F_{IS}) \quad (1)$$

where N is the number of diploid individuals sampled, and F_{ST} represents the fluctuations of allele frequencies between loci that are not accounted by differences in their cline shapes. This correction was for a single locus, and N_e was taken as the sum of N_e for each locus when ten autosomal loci were pooled. To obtain MLEs of F_{IS} within the contact zone, HWE was assessed at each site for each marker using ANALYSE. In addition, standardized pairwise linkage disequilibrium [$R_{ij} = D_{ij}/(p_i q_i\ p_j q_j)^{1/2}$] through the cline was assessed for autsomal loci and averaged across these

loci (Barton & Gale 1993). Only sampling sites with six or more individuals were used for estimating R_{ij} (Hatfield *et al*. 1992).

Three cline models have been proposed: sigmoid (Sig) model, symmetrical stepped (Sstep) model, and asymmetrical stepped (Astep) model (Szymura & Barton 1986; 1991). Sig model is the simplest cline model, defined by a hyperbolic tangent function of its width and centre:

$$p_i = (1 + \tanh[2(x_i - c)/w])/2 \qquad (2)$$

where p_i is the allele frequencies at each locus, $(x_i - c)$ is the distance from the centre of the cline (c), and w is the cline width, defined as l/(maximum slope of the curve). The Sig model cline is a general product of gene flow with and without intrinsic and extrinsic selection (Barton & Gale 1993; Kruuk *et al.* 1999). The Sstep model cline is composed of three segments, namely a central segment where selection acting on different characters combines synergistically, and left and right side segments outside the central segment where selection independently acts on introgressed foreign alleles, because linkage disequilibria at the central segment are broken down by recombination (Szymura & Barton 1986). The allele frequencies at the central segment are defined by the Sig model, while the allele frequencies of the left and right side segments decline exponentially at a rate:

$$p \propto \exp(-4x\theta^{1/2}/w) \qquad (3)$$

where θ is the rate of exponential decay. θ is proportional to the level of selection acting on a character outside the central segment. The strength of the barrier to gene flow outside the central segment can be measured by:

$$B = \Delta p/(\mathrm{d}p/\mathrm{d}x) \qquad (4)$$

where Δp is the difference in allele frequency, and $\mathrm{d}p/\mathrm{d}x$ is the initial gradient in gene frequency along distance x at the edges outside the central segment. In the Sstep model, θ and B are the same in both the left and right side of the central segment (symmetrical), but are different in the Astep model (asymmetrical). In sum, these three models can be described by two, four and six parameters, respectively (Sig: c and w; Sstep: c, w, θ and B; Astep: c, w, θ_0 and B_0, θ_1 and B_1). The maximum and minimum gene frequency values at the tail ends of a cline ($p_{\max}$ and $p_{\min}$) were allowed to vary. We used all three models, and the most likely model was determined by a Likelihood Ratio Test (LRT), where the test statistic (2 × differences in log likelihood: 2ΔML) approximately follows the chi-square distribution with Δd.f. (degrees of freedom: differences in the number of free parameters between more complex models and simpler models). It should be noted that using the Sstep and Astep models seem valid when there is extensive sampling both within and outside the cline (Barton & Gale 1993).

4.2.5 Cline coincidence and concordance

Coincidence of cline centre (c) between loci was evaluated by two methods, namely the polynomial fitting method described by Szymura and Barton (1986) and the composite likelihood method described by Phillips *et al*. (2004). In the polynomial fitting method, allele frequencies at each of the 12 molecular markers and one chromosome marker (p_i) were plotted against the average frequency ($\bar{p}$). If the clines coincided, all the points would lie on the diagonal, whereas if not, the points would be scattered around the diagonal. This scatter was evaluated by fitting a polynomial:

$$p_i = \bar{p} + 2\,\bar{p}\,\bar{q}\,(\alpha + (\bar{p} - \bar{q})\beta) \qquad (5)$$

where α describes an increase of *viatica*17 alleles above the average [*i.e.*, a shift of the cline towards P24(XY) territory] and β describes a narrowing of the cline below the average. In the composite likelihood method, a log-likelihood profile for each locus was constructed stepwise (1m) along the transect axes for *c* with the other parameters free to vary at each point. Then, a composite log-likelihood profile was constructed by summing log-likelihood profiles for all individual loci. The ML value of this composite log-likelihood profile (ML_{comp}) was compared with the sum of all MLEs for individual loci (ML_{sum}) using a LRT. If the clines of individual loci coincide and have the same *c* values, ML_{comp} is not significantly different from ML_{sum} (*i.e.*, $\Delta ML = ML_{sum} - ML_{comp} \approx 0$). Conversely, if the clines do not coincide, ML_{comp} is significantly smaller than ML_{sum} (*i.e.*, $\Delta ML > 0$). The significance of any difference of ML_{comp} and ML_{sum} was determined using a chi-squared test with $n - 1$ degrees of freedom where *n* is the number of loci ($\alpha = 0.05$). In the same way, concordance of cline width (*w*) was evaluated by constructing log-likelihood profiles for the cline width and comparing ML_{comp} and ML_{sum} of *w* values. These LRTs were performed for all three cline models separately.

A genetic hybrid index was calculated from ten autosomal loci, ranging from 0 [theoretical 'pure' P24(XY)] to 1 [theoretical 'pure' *viatica*17]. Cytonuclear disequilibria levels were evaluated for five sites where both mitochondrial and nuclear markers were segregating (P11, P6, P5, P4, P3, and P2), using a programme CND (Asmussen & Basten 1994). Two types of cytonuclear disequilibria, namely allelic disequilibria (*D*-AM) and genotypic disequilibria (*D*-AAM, *D*-AaM, and *D*-aaM), were assessed [A and M denote P24(XY) nuclear alleles and *COI* haplotypes, respectively]. Allelic and genotypic disequilibria respectively measure the extent of non-random associations between the mitochondrial *COI* haplotypes and the nuclear alleles and genotypes at ten diploid loci. The program also estimates minimum sample sizes to detect the observed levels of disequilibria with 50 % and 90 % probabilities. Sequential Bonferroni corrections (Rice 1989) were applied for multiple tests as adjacent sites were not independent.

4.2.6 Estimate of dispersal and selection paprameters

The dispersal parameter σ, defined as the standard deviation of the distance between parent and offspring along a linear axis, was estimated directly and indirectly. For the direct estimates of σ, results of mark-recapture and laboratory breeding studies using the P24(XY) race on the mainland (Blackith & Blackith 1969a) were used to estimate the standard deviation of distance travelled over 2 - 12 days and expected longevity. Indirect estimates of σ can be obtained using the R_{ij} estimate at the centre of the zone by:

$$R_{ij} = 4\sigma^2/w^2 r \qquad (6)$$

where r is the recombination rate and approximately 0.5 for unlinked loci (Szymura & Barton 1986). Underlying assumptions include that linkage disequilibrium primarily result from the dispersal of parental allele combinations into the hybrid zone, and other effects in producing linkage disequilibrium, such as epistasis and selection, are weak. If these assumptions are valid, linkage disequilibrium will be greatest in the centre and decline towards the edge (forming a quadratic curve along some axis). The R_{ij} value in the zone centre was estimated by a linear regression line of average R_{ij} across all pairs of autosomal loci plotted against average allele frequencies ($\bar{p}\ \bar{q}$) over ten autosomal loci. The regression line gave the R_{ij} value in the zone centre at $\bar{p}\ \bar{q} = 0.25$ ($\bar{p} = \bar{q} = 0.50$ at a theoretical zone centre). Effective selection pressure on a locus at the zone centre due to association with other loci, which is defined as the difference in mean fitness between populations at the centre and the edge, is given by Barton and Gale (1993):

$$s^* = 8(\sigma/w)^2 \qquad (7)$$

4.3 Results

4.3.1 Identification of diagnostic markers

Cytological examination of 292 male grasshoppers revealed that samples from 13 western sites (P1 - P13) had the P24(XY) karyotype, and samples from 13 eastern sites (V1 - V13) had the *viatica*17 karyotype (Appendix Table 4.2). There was no site where both P24(XY) and *viatica*17 samples were found. All samples had balanced chromosome complements without apparent meiotic abnormalities. Cytological examination of an additional 147 males from seven sites outside of the hybrid zone confirmed that samples collected from both ends of the hybrid zones had identical karyotypes with 'pure' P24(XY) and *viatica*17 karyotypes.

Numbers of alleles, observed and expected heterozygosities (H_O and H_E), and inbreeding coefficients (F_{IS}) of five microsatellite and five allozyme loci are presented in Appendix Table 4.1. The exact test for Hardy-Weinberg equilibrium (HWE) for each microsatellite and allozyme locus for each of seven sampling sites outside the contact zone detected significant deviations from HWE in site V16 for *Viat2* (P = 0.002). The permutation test for F_{IS} also detected significantly positive F_{IS} values for this sampling site for the same locus (P = 0.001). Presence of null alleles was inferred for this locus because other loci in the same sites did not show significant deviation from HWE and several individuals showed no PCR-amplification of alleles (*i.e.*, null homozygotes). However, it is possible that population processes, such as inbreeding and mixing of very localized demes within samples, also may have contributed to the observed heterozygote deficits. The low F_{IS} in the other six sites outside the contact zone and sites near the contact zone makes it unlikely that null alleles are common in these localities.

Restriction endonuclease (RE) analyses confirmed that the *COI* and *EF*-1α loci showed a fixed difference between P24(XY) and *viatica*17 populations (Appendix Table 4.2). Overall, individuals collected within the zone appeared to have complex hybrid ancestry (*i.e.*, F_2 or later generations), and no putative F_1 hybrids, those heterozygous at all nuclear loci, were found. In addition, no individual homozygous at all nuclear loci was found on the opposite side of the contact zone [i.e., no 'pure'

*viatica*17 individual on P24 (XY) side and vice versa], suggesting an absence of recurrent occasional long-distance migration.

4.3.2 Heterozygote deficit and linkage disequilibrium

After collapsing multiple alleles into two allele systems at the five microsatellite and five allozyme loci, F_{IS} was estimated using ML in ANALYSE. Significant heterozygote deficit (*i.e.*, $F_{IS} > 0$) was found at four out of ten nuclear autosomal markers (*Viat2*, *Viat5*, *Viat8*, and *Viat10*), while an overall estimate of F_{IS} averaged across loci across sampling sites was significantly positive ($F_{IS} = 0.16$, 2u = 0.10 - 0.23). No significant heterogeneity among ten autosomal loci ($\Delta ML = 14.37$, d.f. = 9, P = 0.11) and among 26 sites ($\Delta ML = 16.69$, d.f. = 25, P = 0.89) was detected. However, consensus estimates of F_{IS} against distance along the transect suggested high F_{IS} values near the zone centre but relatively low F_{IS} values at both ends of the transect (Fig. 4.2A). When F_{IS} values were plotted against the product of average allele frequencies $\bar{p}\,\bar{q}$, there was a significant relationship between F_{IS} and $\bar{p}\,\bar{q}$ (the regression line: $y = 1.549x - 0.012$, $R^2 = 0.255$, p = 0.013) (Fig. 4.2B). High F_{IS} values at the zone centre are consistent with expectation, where selection, differential environmental preferences, and/or assortative mating are likely to be effective at the centre of the hybrid zone (Szymura & Barton 1986; 1991; MacCallum *et al.* 1998; Kruuk *et al.* 1999). Although no significant difference in F_{IS} was detected across sampling sites, there was an outlier (site P4, HI = 0.09), which had a high F_{IS} value relative to $\bar{p}\,\bar{q}$. This may be due to mixing of highly localized demes within the sampling site, recent migrants from the opposite side of the hybrid zone, and/or presence of null alleles. A regression correlation between F_{IS} and $\bar{p}\,\bar{q}$ became stronger after removing this outlier site ($R^2 = 0.396$, P = 0.002).

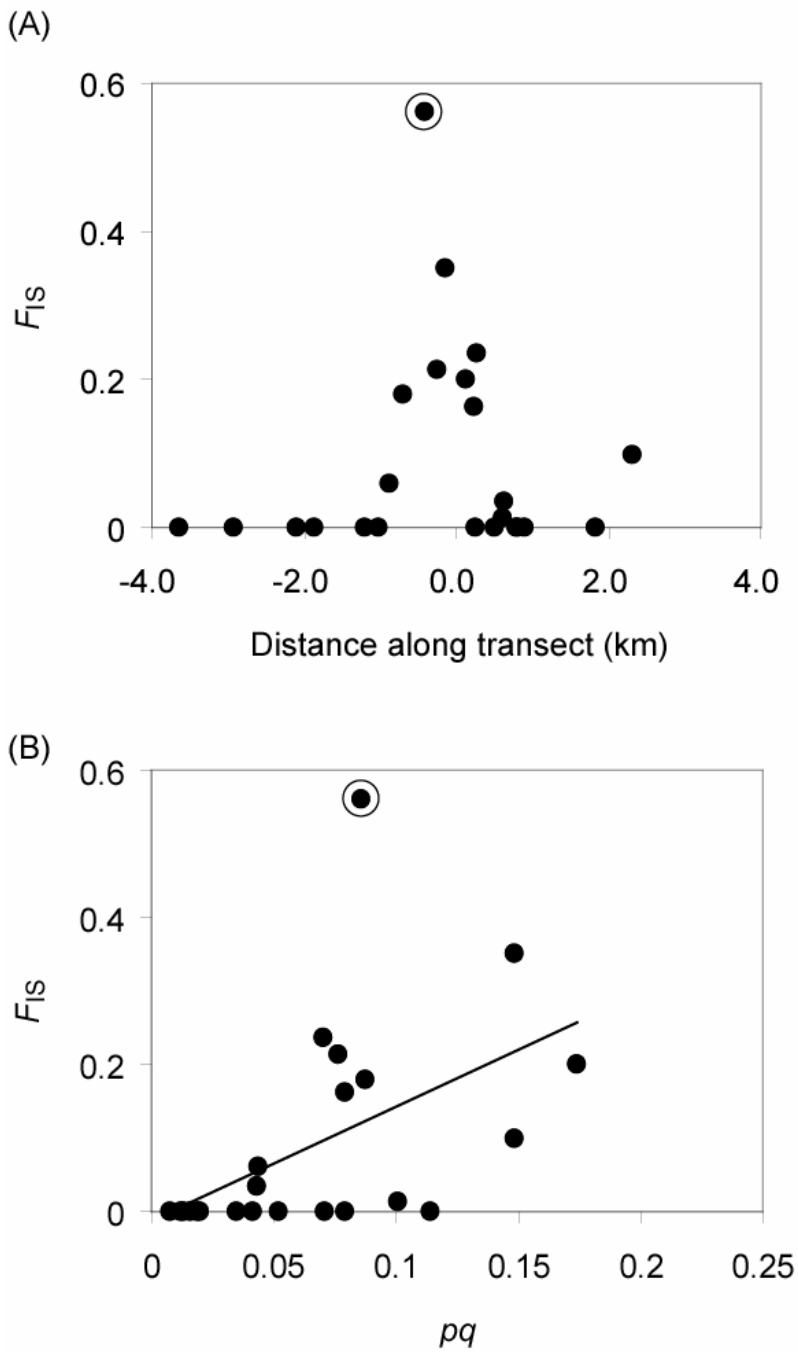


Fig. 4.2 Consensus F_{IS} estimates for ten autosomal loci plotted along the transect (A) and the product of average allele frequencies (*pq*) (B). An outlier site (P4) is marked with circle.

Maximum-likelihood estimates of standardized average pairwise linkage disequilibrium (R_{ij}) between pairs of ten autosomal loci are presented in Table 4.1. Overall R_{ij} averaged across sampling sites was significantly positive (R_{ij} = 0.051, 2u = 0.023 - 0.081). No heterogeneity among sites (ΔML = 12.01, d.f. = 25, P = 0.99) and among pairs of loci (ΔML = 43.23, d.f. = 44, P = 0.51) were detected. Similar to F_{IS}, high R_{ij} values were observed in the central part of the zone, while relatively low R_{ij} values were observed at sites away from the zone centre (Fig. 4.3A). An exception included the site V12, where P24(XY) alleles were common in the *viatica*17 side. This high R_{ij} value at this site was primarily generated by the presence of individuals with extreme hybrid index values (HI = 0.556 - 0.950, mean HI = 0.794). A possible cause of these individuals with extreme HI may include rare long distance dispersal of individuals from the P24(XY) side, which carries a 'pure' set of P24(XY) alleles across the hybrid zone. Another possibility is gene introgression from unknown (either extant or extinct) P24(XY) populations on the *viatica*17 side. Although there is no evidence for the existence of these populations, we cannot rule out this possibility due to the patchy distribution of this grasshopper and/or likelihood of local population extinction by agriculture. When the R_{ij} values were plotted against the product of average allele frequencies, $\bar{p}\,\bar{q}$ (Szymura & Barton 1991; Barton & Gale 1993), there was a significant relationship between R_{ij} and $\bar{p}\,\bar{q}$ (the regression line: $y = 0.871x - 0.021$, $R^2 = 0.408$, P = 0. 001) (Fig. 4.3B). The regression correlation was not improved when this outlier site was excluded from the analysis ($R^2 = 0.326$, P = 0. 006). The R_{ij} value in the zone centre ($\bar{p}\,\bar{q} = 0.25$) was 0.197 (0.078 - 0.315) using all data and 0.174 (0.046 - 0.301) after excluding the site V12.

Table 4.1 Average standardized linkage disequilibrium between each pair of loci (R_{ij})

Locus	*Viat2*	*Viat4*	*Viat5*	*Viat8*	*Viat10*	*EF*-1α	*Ak1*	*Ak2*	*Idh2*	*PepA*
Viat2	-									
Viat4	0.109	-								
Viat5	0.221	0.000	-							
Viat8	0.123	0.196	0.152	-						
Viat10	0.165	0.000	0.143	0.043	-					
EF-1α	0.020	0.107	0.018	0.000	0.000	-				
Ak1	0.031	0.196	0.104	0.650	0.000	0.042	-			
Ak2	0.000	0.000	0.019	0.017	0.036	0.039	0.000	-		
Idh2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.056	-	
PepA	0.216	0.000	0.017	0.000	0.125	0.000	0.013	0.290	0.000	-
All	0.051									

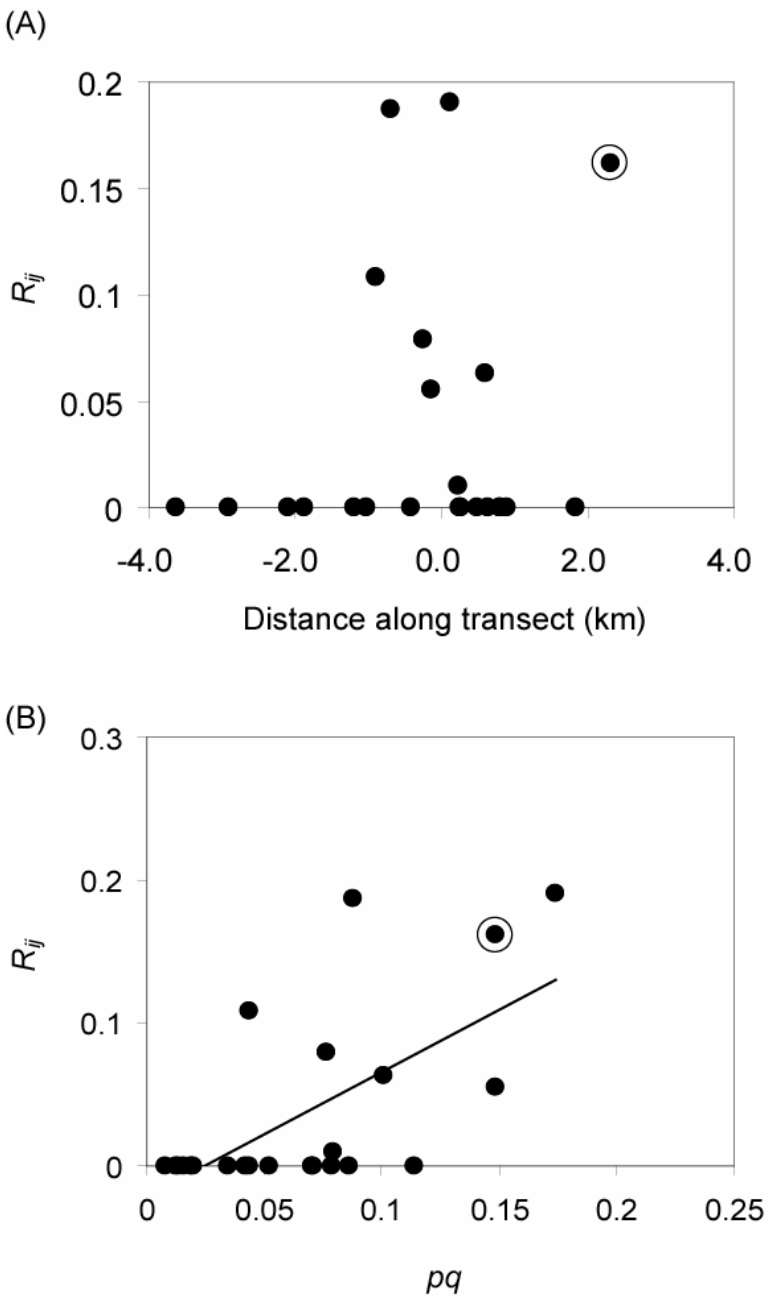


Fig. 4.3 Consensus R_{ij} estimates for ten autosomal loci plotted along the transect (A) and the product of average allele frequencies (*pq*) (B). An outlier site (V12) is marked with circle.

4.3.3 Cline shape

The ML cline fitting analyses revealed that the Sig model was best fitted for seven loci (chromosome marker, *Viat*2, *Viat*8, *Viat*10, *EF*-1α, *PepA*, and *COI*), the Sstep model was best fitted for three loci (*Ak*1, *Ak*2, and *Acyc*), and the Astep model was best fitted for three loci (*Viat*4, *Viat*5, and *Idh*2) (Fig. 4.4, Table 4.2). Asymmetry of the cline shape was consistent across the three loci fitted with the Astep model, and the barrier was stronger toward the *viatica*17 territory than vice versa. In addition, although the Astep model did not provide significant improvement over the Sstep model for *Ak*1, *Ak*2, and *Acyc*, these clines appeared to have the same asymmetrical shape with stronger barriers toward the *viatica*17 side than toward the P24(XY) side. The estimates of cline width (*w*) per nuclear locus range from 93.1 m (*Idh*2) to 347.7 m (*Ak*1), with an overall average 310.4 m. All clines based on nuclear markers (allozyme loci, microsatellite loci and *EF*-1α gene locus) were centred within approximately 200 m of one another. The single locus cline generally had large support limits within loci and variations between loci in the cline parameter estimates, particularly *B*/*w* and θ for loci fitted with the stepped cline models. The average of the ten autosomal loci, however, had more reliable parameter estimates with relatively narrow support limits except *B*/*w* and θ on the *viatica*17 side. The average cline was best-fitted with the Astep model, and, consistent with the single locus analyses, the barrier to introgression appeared stronger toward the *viatica*17 side.

Table 4.2 Cline parameter estimates for 11 nuclear loci (five microsatellite loci: *Viat2*, *Viat4*, *Viat5*, *Viat8*, and *Viat10*; five allozyme loci: *Ak1*, *Ak2*, *Idh2*, *PepA*, and *Acyc*; *EF*-1α gene locus), average cline over 11 nuclear loci, mitochondrial *COI* locus, and karyotype. Sigmoid model (Sig), symmetrical stepped model (Sstep) and asymmetrical stepped (Astep) models were used separately. Parameters are cline centre (*c*), cline width (*w*), barrier strength standardized by *w* (*B*/*w*) and, rate of exponential decay (θ). Subscript numbers indicate parameters for the western P24(XY) territory (B_0/w and θ_0) and eastern *viatica*17 territory (B_1/w and θ_1). Note that B_0/w and θ_0 incidate parameters for both sides for the Sstep model. Numbers in brackets are two-unit support limits (ca. 95 % confidence intervals) for each parameters.

Locus	Model	LnL	*c* (m)	*w* (m)	B_0/w	θ_0	B_1/w	θ_1
Viat2	Sig*	-6.70	-76.8	300.3	-	-	-	-
			(-117.1, -33.7)	(203.7, 406.4)	-	-	-	-
	Sstep	-6.70	-76.8	300.2	5.45×10^9	0.02	-	-
			(-117.2, -35.6)	(203.4, 405.5)	(0.00, inf.)	(0.00, 1.00)	-	-
	Astep	-6.70	-76.8	300.3	0.00	0.35	0.00	0.82
			(-112.2, -34.9)	(203.4, 406.2)	(0.00, inf.)	(0.00, 1.00)	(0.00, inf.)	(0.00, 1.00)
Viat4	Sig	-22.78	64.9	337.7	-	-	-	-
			(10.2, 139.9)	(165.3, 456.9)	-	-	-	-
	Sstep	-20.84	69.4	309.6	2.98	0.40	-	-
			(-46.2, 150.8)	(201.6, 410.2)	(0.00, inf.)	(0.00, 1.00)	-	-
	Astep*	-17.94	110.1	220.5	9.35	0.05	6.41×10^9	0.99
			(13.9, 165.0)	(0.0, 408.5)	(0.90, 304.77)	$(3.9\times10^{-6}, 0.61)$	$(7.5\times10^{-5}$, inf.)	(0.00, 1.00)
Viat5	Sig	-18.84	-50.3	391.3	-	-	-	-
			(-113.8, 8.6)	(283.6, 542.3)	-	-	-	-
	Sstep	-18.84	-50.3	391.2	5.60×10^9	0.00	-	-
			(-113.8, 8.6)	(0.0, 560.9)	(0.00, inf.)	(0.00, 1.00)	-	-
	Astep*	-12.63	99.2	160.5	2.19	0.04	6.32×10^9	0.75
			(-69.7, 293.2)	(0.0, 413.0)	(0.00, 16.20)	$(1.2\times10^{-6}, 0.32)$	(0.00, inf.)	(0.00, 1.00)
Viat8	Sig*	-13.83	52.5	326.9	-	-	-	-
			(-10.4, 121.8)	(216, 451.1)	-	-	-	-
	Sstep	-13.83	52.6	326.8	9.88×10^9	0.38	-	-
			(-63.1, 121.8)	(0.0, 454.0)	(0.00, inf.)	(0.00, 1.00)	-	-
	Astep	-13.83	52.5	326.9	8.44×10^9	0.94	7.38×10^8	0.07
			(-9.8, 121.3)	(218.0, 452.5)	(0.00, inf.)	(0.00, 1.00)	(0.00, inf.)	(0.00, 1.00)

(Table 4.2 Continued)

Viat10	Sig*	-17.87	126.2	93.4	-	-	-	-
			(-44.2, 166.7)	(0.0, 304.6)	-	-	-	-
	Sstep	-16.09	112.7	211.7	109.91	0.01		
			(28.3, 169.6)	(0.0, 350.4)	(0.00, inf)	(0.00, 1.00)	-	-
	Astep	-16.10	121.2	166.4	124.25	0.00	148.13	1.07×10^{-3}
			(11.6, 195.6)	(0.0, 408.9)	(0.00, inf)	(0.00, 1.00)	(0.00, inf)	(0.00, 1.00)
Ak1	Sig	-15.21	26.5	354.9	-	-	-	-
			(-27.5, 78.8)	(227.8, 494.4)	-	-	-	-
	Sstep*	-10.45	22.7	347.7	8.50	0.08		
			(-120.2, 81.9)	(0.0, 510.5)	(1.18, 77.17)	(0.00, 0.44)	-	-
	Astep	-9.22	17.3	335.7	8.05	0.07	1.26×10^{9}	0.88
			(-120.2, 77.3)	(335.7, 335.7)	(1.54, 45.84)	(0.00, 0.43)	(3.3×10^{-6}, inf)	(3.30×10^{-6}, 1.00)
Ak2	Sig	-17.61	-23.4	213.1	-	-	-	-
			(-80.2, 44.2)	(101.4, 364.1)	-	-	-	-
	Sstep*	-14.08	-13.7	272.9	29.95	0.03		
			(-74, 56.2)	(131.9, 444.6)	(3.53, 642.86)	(0.00, 0.24)	-	-
	Astep	-13.62	11.3	315.7	20.03	0.05	52.45	2.11×10^{-03}
			(-70, 141.1)	(0.0, 502.4)	(2.85, 607.88)	(4.5×10-6, 0.39)	(10.06, inf)	(0.00, 1.00)
Idh2	Sig	-36.37	-22.9	247.9	-	-	-	-
			(-75.8, 32.9)	(141.1, 354.3)	-	-	-	-
	Sstep	-34.03	-8.1	325.0	64.77	0.01		
			(-73.6, 36.9)	(180.4, 438.3)	(6.59, 3.3×10^{4})	(0.00, 0.45)	-	-
	Astep*	-28.52	93.4	93.1	12.97	0.01	4.18×10^{9}	0.72
			(-53.2, 141.2)	(0.0, 282.0)	(2.29, 168.74)	(0.00, 0.25)	(0.66, inf)	(0.00, 1.00)
PepA	Sig*	-15.06	-41.3	156.2	-	-	-	-
			(-76.2, 5.3)	(91.1, 242.2)	-	-	-	-
	Sstep	-15.06	-41.3	156.2	9.55×10^{9}	0.14	-	-
			(-76.2, 5.3)	(0.0, 242.2)	(0.00, inf)	(0.00, 1.00)	-	-
	Astep	-14.61	-73.1	102.3	0.03	0.99	7.36×10^{9}	0.08
			(-117.9, -0.6)	(0.0, 234.9)	(0.00, inf)	(0.00, 1.00)	(0.00, inf)	(0.00, 1.00)

(Table 4.2 Continued)

Acyc	Sig	-23.91	10.5	244.3	-	-	-	-
			(-55.4, 75.0)	(105.3, 376.0)	-	-	-	-
	Sstep*	-18.35	7.7	104.9	34.52	0.01	-	-
			(-44.7, 140.7)	(0.0, 295.1)	(4.62, 969.35)	(6.6×10-7, 0.12)	-	-
	Astep	-18.20	52.8	120.6	24.27	0.01	48.58	0.01
			(-46.8, 141.4)	(0.0, 290.3)	(4.68, 342.65)	(7.2×10-7, 0.13)	(5.50, inf)	(7.0×10^{-4}, 1.00)
EF-1α	Sig*	-14.08	34.1	314.6	-	-	-	-
			(-37.8, 95.6)	(179.5, 446.6)	-	-	-	-
	Sstep	-13.14	40.2	304.0	6.20	0.24		
			(-68.2, 142.9)	(0.0, 444.9)	(0.00, inf)	(0.00, 1.00)	-	-
	Astep	-13.04	36.3	294.4	4.30	0.31	2.39×10^{9}	0.15
			(-58.3, 248.8)	(0.0, 474.2)	(0.00, inf)	(0.00, 1.00)	(0.00, inf)	(0.00, 1.00)
10 nuc	Sig	-47.59	5.1	320.9	-	-	-	-
			(-13.0, 23.2)	(281.1, 361.7)	-	-	-	-
	Sstep	-38.65	11.4	338.1	13.73	0.10	-	-
			(-9.6, 30.0)	(285.2, 382.0)	(3.9, 70.9)	(0.03, 0.38)	-	-
	Astep*	-28.92	0.0	310.4	11.10	0.07	3.24×10^{9}	0.79
			(-18.1, 45.4)	(268.7, 351.8)	(3.4, 30.3)	(0.02, 0.20)	(0.00, inf)	(0.00, 1.00)
COI	Sig*	-24.93	-408.7	911.0	-	-	-	-
			(-535.6, -297.6)	(689.4, 1219.3)	-	-	-	-
	Sstep	-24.93	-408.7	911.1	5.89×10^{9}	0.95	-	-
			(-538, -293.4)	(689.9, 1221.9)	(0.30, inf)	(0.00, 1.00)	-	-
	Astep	-23.63	-308.3	661.6	0.98	0.33	8.20×10^{9}	0.99
			(-480.9, 316.7)	(0.0, 1120.8)	(0.00, inf)	(0.00, 1.00)	(0.00, inf)	(0.00, 1.00)
Chromosome	Sig*	-0.03	15.9	38.0	-	-	-	-
			(-119.2, 142)	(0.0, 185.3)	-	-	-	-
	Sstep	-0.03	-8.2	5.4	9.90×10^{9}	0.01		
			(-119.2, 142)	(0.0, 185.3)	(0.00, inf)	(0.00, 1.00)	-	-
	Astep	-0.03	-7.1	5.8	9.90×10^{9}	0.01	0.24	1.00
			(-123.8, 142)	(0.0, 186.4)	(0.00, inf)	(0.00, 1.00)	(0.00, inf)	(0.00, 1.00)

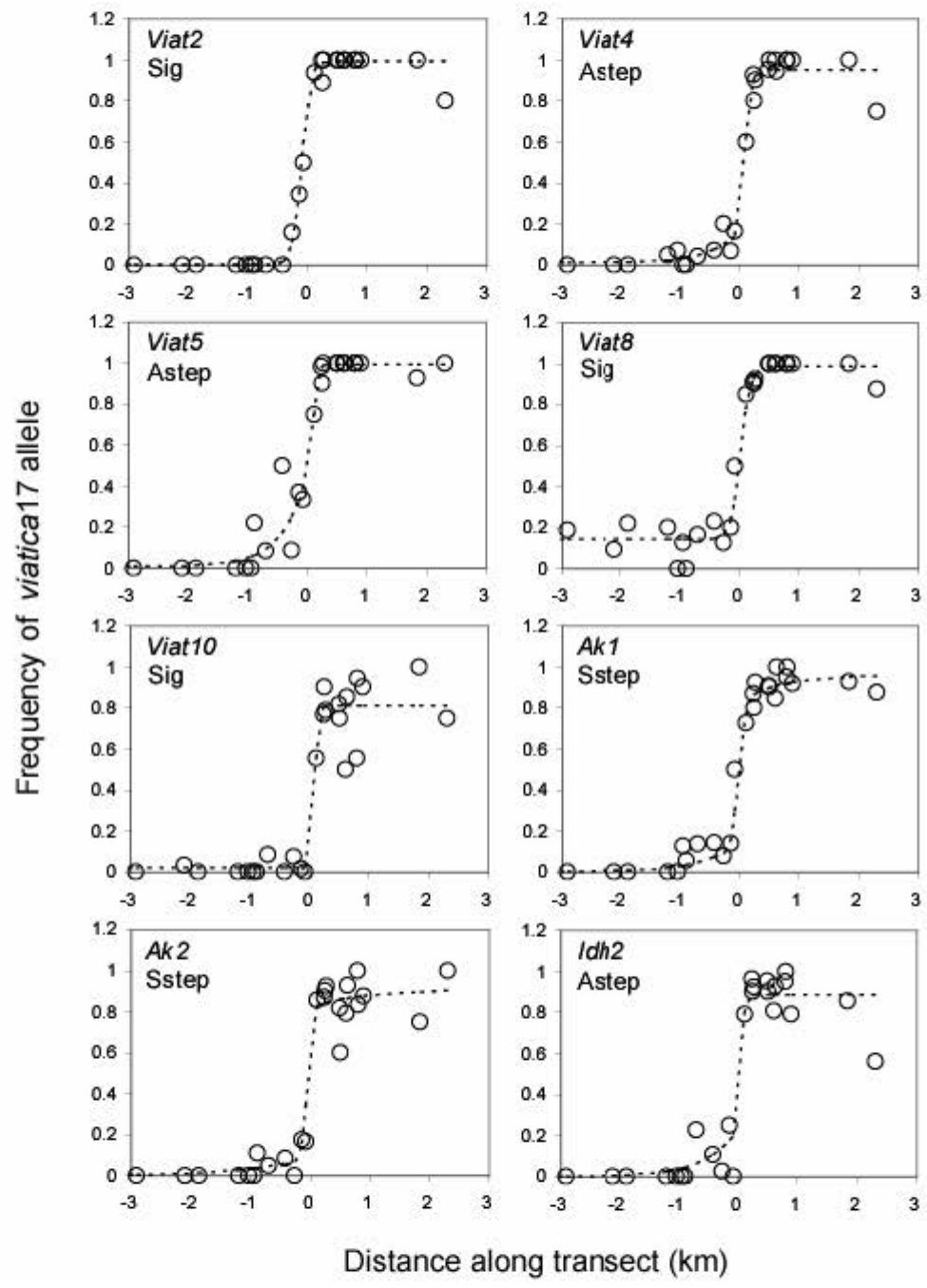
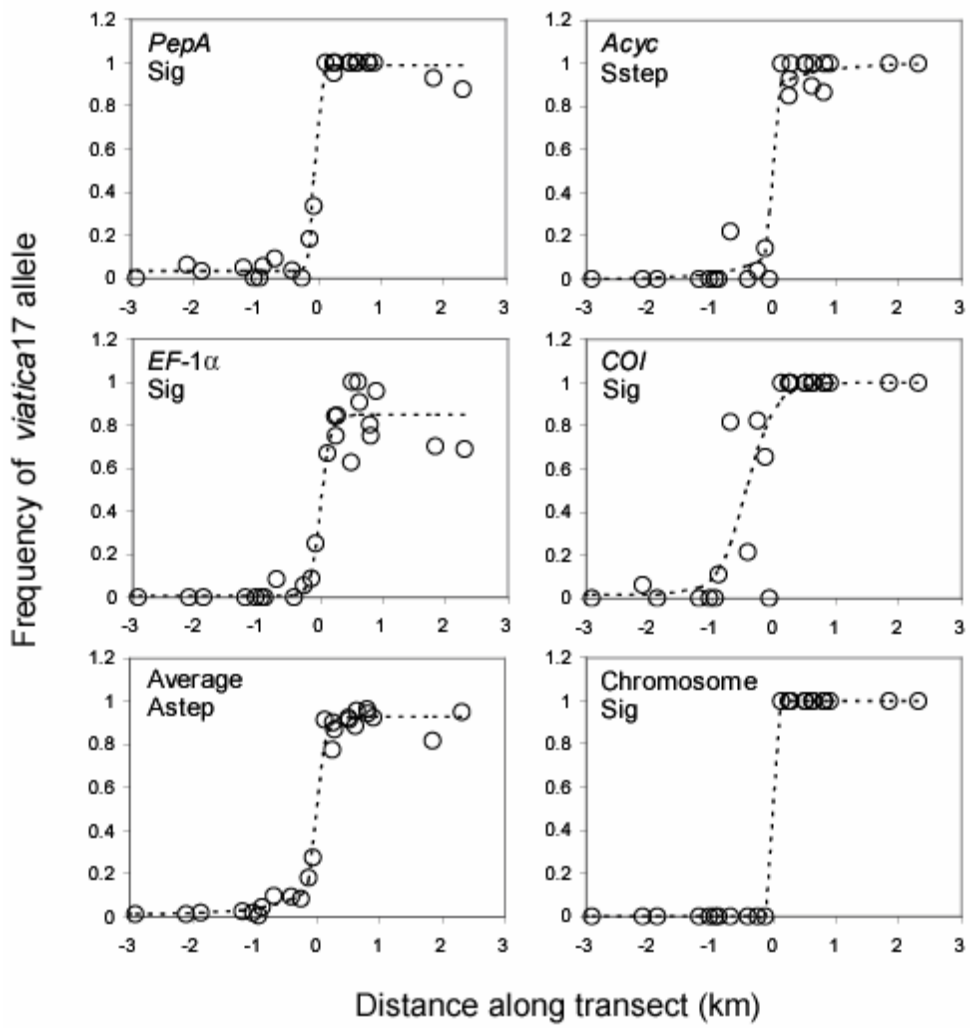


Fig. 4.4 Frequencies of *viatica*17 alleles along the transect across the hybrid zone for ten autosomal loci, their average, putative X-linked locus (*Acyc*), *COI*, and chromosome marker. Circles and dashed lines represent observed data and the best fit clines based on one of the three cline models, respectively. The cline centre (= 0 km) is based on that of the average cline. The best fit models are indicated under the locus names.



(Fig. 4.4 Continued)

When cline coincidence and concordance were visually inspected, the ten autosomal clines and putative X-linked *Acyc* cline appeared very similar in position and width, and these nuclear clines appeared coincident with the chromosomal cline. In contrast, the centre of the mitochondrial cline based on the *COI* gene appeared to have a very different position and shape compared to the nuclear clines. Polynomial fitting of each marker against the average frequency of P24 (XY) alleles ($\bar{p}$) revealed that the *COI* cline was shifted toward the P24(XY) territory ($\alpha = 1.508$) and wider than the average ($\beta = -0.623$).

Coincidence and concordance of chromosomal, nuclear and mitochondrial clines was tested using a Likelihood-Ratio Test (LRT). LRTs for each of the three cline models suggested that the *COI* cline did not have coincident estimates of *c* and concordant estimates of *w* with other nuclear and chromosomal clines when the Sig model and Sstep model were used (Table 4.3). When the Astep model was used, all nuclear, mitochondrial and chromosome clines were coincident and concordant probably because of the higher flexibility of the Astep model composed of six parameters. When LRTs were repeated without the *COI* cline, all 11 nuclear clines and chromosomal cline had a similar position and width for all three cline models (*i.e.*, coincident and concordant).

Table 4.3 Likelihood Ratio Tests (LRTs) for coincidence of centre (A) and concordance of width (B) of 11 nuclear loci (five microsatellite, five allozyme, and *EF*-1α), one mitochondrial locus (*COI*), and chromosome marker. LRTs were repeated with and without *COI*. ΔML, test statistics; d.f., degree of freedom.

A) Centre

	With *COI*			Without *COI*		
	ΔML	d.f.	P	ΔML	d.f.	P
Sig	36.29	12	<0.001	16.20	11	0.134
Sstep	24.87	12	0.015	7.35	11	0.770
Astep	2.08	12	0.999	2.93	11	0.992

B) Width

	With *COI*			Without *COI*		
	ΔML	d.f.	P	ΔML	d.f.	P
Sig	29.73	12	0.003	15.86	11	0.146
Sstep	29.22	12	0.004	15.18	11	0.174
Astep	7.84	12	0.797	11.09	11	0.435

4.3.4 Cytonuclear disequilibria

The shift of the mitochondrial *COI* cline centre toward the west [*i.e.*, P24(XY) territory] was further tested by assessing cytonuclear disequilibria. A total of six sites had both P24(XY) and *viatica*17 *COI* haplotypes (frequency of the *viatica*17 haplotype = 0.063 - 0.824) with a P24(XY)-like nuclear background (HI = 0.019 - 0.184), while no site was found with both *COI* haplotypes in the *viatica*17 territory (Appendix Table 4.2). Cytonuclear allelic disequilibria (*D*-AM) and genotypic disequilibria (*D*-AAM and *D*-AaM) were found only at the site P3. *D*-AM strongly deviated in a negative direction from a no-association hypothesis for *Viat8* (*D*-AM = -0.038, normalised *D*-AM = -39 %, P « 0.008) and *Ak*1 (*D*-AM = -0.043, normalised *D*-AM = -60 %, p « 0.008), suggesting significant association between P24(XY) nuclear alleles and the *viatica*17 mitochondrial haplotype. The genotypic homozygote disequilibria for P24(XY) nuclear alleles (*D*-AAM) were also negative for *Viat8* (*D*-AAM = -0.087, normalised *D* -AAM = -60 %, p = 0.049) and *Ak*1 (*D*-AAM = -0.087, normalised *D*-AAM = -60 %, p = 0.049) but not statistically significant after sequential Bonferroni corrections. Furthermore, the genotypic heterozygote disequilibria (*D*-AaM) were positive for *Viat8* (*D*-AaM = 0.097, normalised *D* -AaM = 100 %, p = 0.005) and *Ak*1 (*D*-AaM = 0.087, normalised *D*-AaM = 60 %, p = 0.049), and *D*-AaM for *Viat8* remained significant after sequential Bonferroni corrections. Allelic and genotypic disequilibria were not detected for other loci. It should be noted that sample sizes were larger than that necessary for detection of these genotypic disequilibria with 50% probability, but smaller than that necessary for detection with 90% probability.

4.3.5 Estimate of dispersal and selection parameters

A mark-recapture study using 30 individuals of P24(XY) in the mainland of Australia indicate the standard deviation of distance travelled was 0.51 m per day (Blackith & Blackith 1969a). These *Vandiemenella* grasshoppers mature in 87 days and can live up to 300 days in a laboratory condition (Blackith & Blackith 1969a). These values gave $\sigma = 4.7 - 8.8$ m/gen$^{1/2}$. Substituting this direct estimate of σ and

average cline width w = 310.4 m in the equation 7 returned the effective selection pressure on an average enzyme locus at the zone centre, s^* = 0.2 - 0.6 %. In addition, given R_{ij} = 0.197 (0.078 - 0.315) at the zone centre and w = 310.4 m gave indirect estimates of σ = 48.7 m/gen$^{1/2}$ (2u support limits 26.5 - 69.8 m/gen$^{1/2}$) (eqn. 6). The effective selection pressure on an average enzyme locus at the zone centre was s^* = 19.7 % (2u support limits 5.8 - 40.5 %) (eqn. 7).

4.4 Discussion

The identification of an intact contact zone between P24(XY) and *viatica*17 on Kangaroo Island allowed investigation of how the barrier to gene flow between these chromosomal races varied for chromosomal and multiple molecular markers using formal statistical analysis of clines based on hybrid zone theory. The hybrid zone is remarkably narrow (< 350 m) relative to the average dispersal distance of individual grasshoppers (σ = 48.7 m/gen$^{1/2}$) (cf. *Podisma pedestris*, chromosomal cline width = 580 - 880 m, σ = 21 m/gen$^{1/2}$, Barton & Hewitt 1981b). The width of the chromosomal cline appears to be narrower (38 m) than nuclear clines (310 m, average autosomal), although the difference is not statistically significant. Centre positions of these chromosomal and nuclear markers are coincident. Coincidence and concordance of nuclear clines for randomly sampled loci suggest that selection is unlikely to be concentrated on a few chromosomes, but may act across the genome. In addition, strong linkage disequilibria among independent nuclear loci and deficits of heterozygotes at the centre of the hybrid zone suggest assortative mating (pre-zygotic selection), selection favouring certain gene combinations (post-zygotic selection), continuous influx of parental genotypes into the zone by dispersal, and/or genomically widespread incompatibilities inconsistent with selection concentrated on the two rearranged chromosomes (Arnold & Bennett 1993; Barton & Gale 1993).

4.4.1 Cline shapes

Changes of allele frequency were best fitted with the stepped cline models (Sstep and Astep) for six nuclear loci (*Viat*4, *Viat*5, *Ak*1, *Ak*2, *Idh*2, and *Acyc*) and with the Sig model for five nuclear loci (*Viat*2, *Viat*8, *Viat*10, *EF*-1α, *PepA*). If the difference in

the best-fit models among loci reflects the true nature of the clines at each locus without stochastic fluctuations of allele frequency due to small sample sizes, there are significant barriers to gene flow at the former six loci but non-significant barriers to gene flow at the other loci. Phillips *et al.* (2004) suggest two possible models for the heterogeneous detection of barriers between loci, namely a mixed model and consistent barrier model. The mixed model assumes that the barrier affects only the chromosome segments marked by loci fitted with stepped cline models, but do not affect other chromosome segments, where changes of allele frequency are best-fitted with the Sig model. The consistent barrier model assumes that the barrier affecting loci best-fitted with the stepped models also affects other loci, but their increased likelihood under this model is not significant due to insufficient power to detect weak effects. Although there is limited statistical power to distinguish these two models due to the small number of loci and sampling sites, we can at least conclude that selection is operating on several loci and creating a tension zone.

Physical obstacles to gene flow, epistatic interactions, and dispersal of parental genotypes into the zone are three possible causes to form stepped clines (Barton & Gale 1993). First, tension zone theory predicts that zone centres tend to coincide with physical obstacles (*e.g.*, river streams, two chromosomal races of the alpine grasshopper, *Podisma pedestris*, Barton & Gale 1993) and/or environmental gradients (*e.g.*, soil type in *Gryllus firmus/pennsylvanicus*, Rand & Harrison 1989). These environmental factors may impede dispersal, and, as a result, a sharp step of allele frequency change may build up at the centre of the cline. However, the position of the hybrid zone between P24(XY) and *viatica*17 does not seem to correspond to any physical barrier along the transect (White 1973; T. Kawakami, personal observation). The only environmental difference across the zone includes soil profile (Fig. 4.5). However, an association between soil profile and grasshopper distribution is not found at other regions on Kangaroo Island and the mainland (White *et al.* 1967; White *et al.* 1969). Thus, even if the difference in soil type affects habitat preferences of the two chromosomal races and reduces dispersal at the zone centre, it is unlikely that the soil type *per se* can explain the stepped cline and the strength of the barrier to gene flow. As suggested for *Podisma pedestris* (Barton &

Gale 1993), additional factors, such as selection against hybrids, may be necessary to form the stepped clines and strong barriers to gene flow.

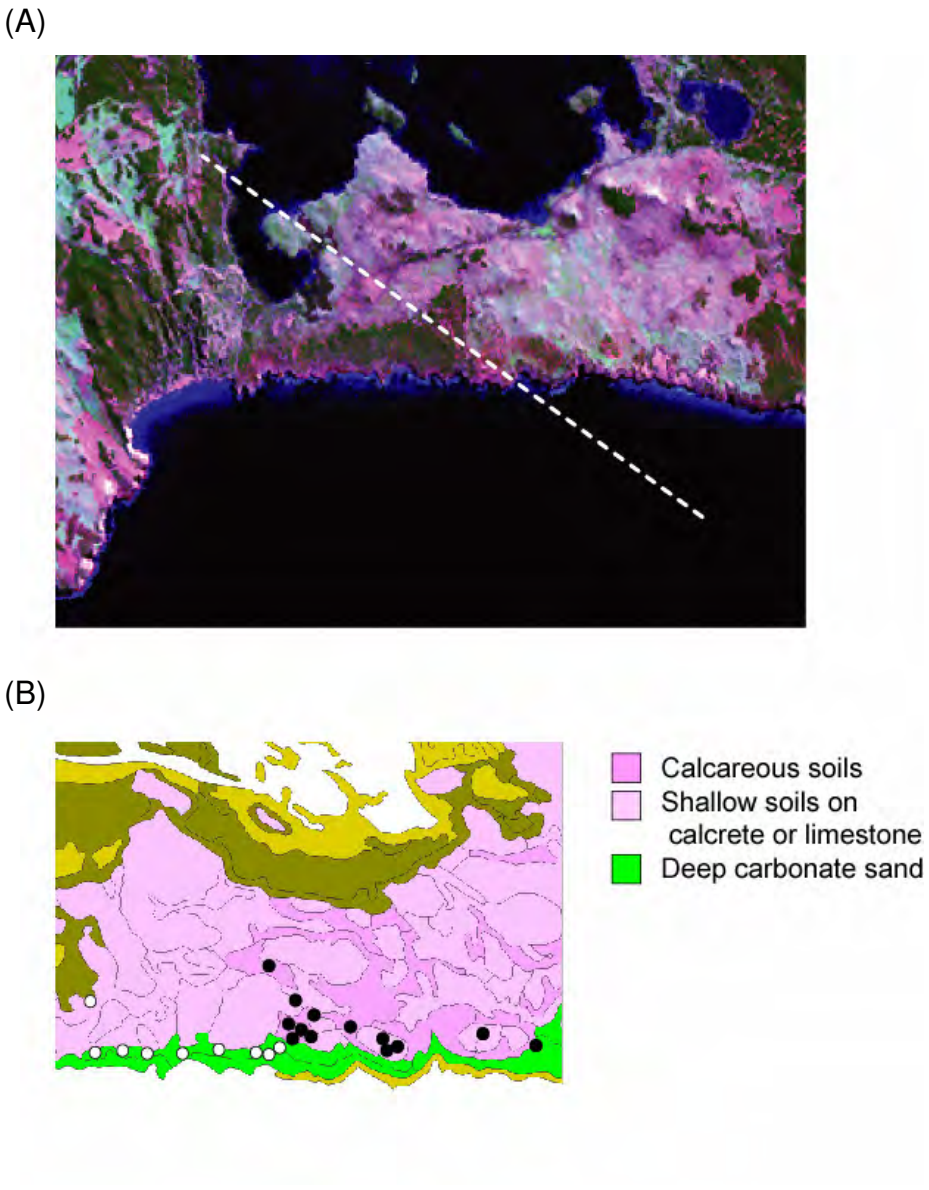


Fig. 4.5 Sampling sites superimposed on a satellite image (A) and soil profile (B). The dashed line indicates the best fit axis of the hybrid zone (Fig. 4.1A). P24(XY) (open circles) and *viatica*17 (filled circles) chromosomal races of *Vandiemenella.*

The second possible cause for the stepped clines is epistatic interactions, in which particular combinations of alleles at different loci are favoured (Barton & Gale 1993; Barton & Shpak 2000). Barton and Gale (1993) introduced the epistatis parameter (β). When β is large (β » 1), individuals with a genotype close to F_1 hybrids (*i.e.*, HI ≈ 0.5) suffer strong selection at the zone centre, while backcrosses are almost as fit as individuals with parental genotypes. Consequently, a cline becomes shallow at the edges and steep at the centre. Epistatic interactions, however, can produce such strong distortions in cline shape only if interactions are extreme, and a moderate to large number of loci interact in their effects on fitness (Barton & Gale 1993). Although it is possible to assume that a large number of loci may be under selection (see *Section 4.4.3*), it is difficult to evaluate the strength of the epistatic interactions among these loci and estimate β without knowing the nature of selection.

Finally, a synergistic effect of linkage disequilibria generated by dispersal and selection acting on individual loci produces a sharp step at the zone centre with shallow introgression tails outside the zone (*i.e.*, broadly introgressed foreign alleles with low frequency outside the central segment of a hybrid zone) (Barton 1983; Barton & Gale 1993). Dispersal from 'pure' parental populations into a hybrid zone increases associations between allele combinations and, hence, results in stronger linkage disequilibria at the zone centre than outside. When linkage disequilibria are generated, selection on one locus also acts on all the loci in linkage disequilibria with it, and thus, the effective selection experienced by each locus (s^*) is increased. As a result of increased effective selection on loci in linkage disequilibria, clines become steeper at the zone centre. This pattern has been found in many hybrid zones (Nichols & Hewitt 1986; Szymura & Barton 1986; Hatfield *et al.* 1992; Virdee & Hewitt 1994). In sum, since these three mechanisms are not mutually exclusive and may work together, it is difficult to know which mechanism is the primary one responsible for the stepped cline in the *viatica* hybrid zone. Regardless of the predominant mechanism, however, it seems reasonable to assume that the *Vandiemenella* hybrid zone between P24(XY) and *viatica*17 is a tension zone, which is maintained by a balance between selection and migration.

4.4.2 Moving hybrid zone

Our cline analyses showed that three single autosomal clines and the average cline over the ten autosomal loci were best fitted with the Astep model, and asymmetry of these clines is in the same direction (Fig. 4.4, Table 4.2). The consistent asymmetry suggests differences in fitness or effective gene flow rate with greater introgression from the *viatcia*17 to P24(XY) than vice versa. Such asymmetric barriers to gene flow are common in a number of organisms (reviewed in Buggs 2007). Asymmetry may be caused by various mechanisms, such as asymmetrical hybridization (*e.g.*, *Chorthippus parallellus parallelus* and *C. parallellus erythropus,* Bella *et al.* 1992), differences in fitness (*e.g.*, butterflies *Anartia fatima* and *A. amathea*, Dasmahapatra *et al.* 2002), differences in female mate preference and/or male aggression (*e.g.*, katydids *Orchelimum nigripes* and *O. pulchellum*, Shapiro 2000; warblers *Dendroica occidentalis* and *D. townsendi*, Rohwer *et al.* 2001), and hybrid zone movement due to climate and/or habitat changes (*e.g.*, gophers, *Thomomys bottae actuosus* and *T. bottae ruidosae*, Ruedi *et al.* 1997; lizards *Sceloporus cowlesi* and *S. tristichus*, Leache & Cole 2007). In the *Vandiemenella* hybrid zone, it seems unlikely that ecological and behavioural differences cause the asymmetrical cline shape because P24(XY) and *viatica*17 are food generalists and require very similar habitat (Blackith & Blackith 1966b; White *et al.* 1969). Although mate choice experiments have not been conducted, cross-breeding experiments showed that F_1 hybrids could be reared for these two chromosomal races with reciprocal crosses without apparent differences in mate choice behaviour, insemination rate, embryonic development, and hatchling rate (Mrongovius 1975). Moreover, although the selective advantage for *viatica*17 alleles may result in the long shallow introgression tails on the P24(XY) side and abrupt allele frequency changes on the *viatica*17 side, it is unlikely that multiple independent loci have coincident cline centres and concordant cline widths because selection may act on a variety of characters.

Although it is difficult to rule out these ecological and behavioural mechanisms, as they are not mutually exclusive with other mechanisms and may be involved simultaneously, the asymmetry of the multiple independent clines with the same directionality suggests that hybrid zone movement is the most likely explanation for

the asymmetry of the *viatica* hybrid zone. The movement of the zone may be eastward with P24(XY) pushing *viatica*17. As selection is strongest at the centre of the tension zone, loci tightly linked to loci under selection may follow the moving front, leaving few alleles behind. In contrast, loci not too tightly linked to selected loci may not follow the front and leave a long tail of introgression behind. The chromosomal cline may represent the former pattern because of its narrow width with no introgression tail, suggesting selection acting directly on chromosomes or some underlying genetic characters tightly linked with the chromosome character. In contrast, the loci fitted with the stepped cline models may represent the latter case because they show steep clines at the central segments and long introgression tails for the *viatica*17 alleles on the P24(XY) side of the zone. Furthermore, the mitochondrial cline is much wider than the chromosomal and nuclear clines, and significantly shifted toward the P24(XY) side. Since mitochondrial genes are generally less closely linked with nuclear genes than nuclear genes are with each other, and since mtDNA polymorphisms are traditionally considered to be selectively neutral or nearly so (Barton & Jones 1983; Ballard & Whitlock 2004), the observed pattern in the mitochondrial cline is consistent with the hypothesis of an eastward moving hybrid zone with mtDNA introgression, where selectively neutral mitochondrial haplotypes have a wider cline and are left in the wake of the moving hybrid zone. This extensive introgression of mtDNA across chromosomal races due to past hybrid zone movement is suggested in other contact zones in the *Vandiemenella* grasshoppers (Kawakami *et al.* 2007a) and other organisms (*e.g.*, grasshopper, *Caledia captiva*, Marchant *et al.* 1988). Moreover, consistent unidirectional asymmetry across multiple markers and a substantial offset of a mitochondrial cline in the Danish house mouse hybrid zone may be due partially to past movement of the zone (Ferris *et al.* 1983; Raufaste *et al.* 2005).

The lack of apparent behavioural differences in female mate preference and/or male aggression for the P24(XY) and *viatica*17 chromosomal races (Mrongovius 1975) and concordant and coincident clines across multiple loci in this study suggest that a direct effect of environmental change is likely to be responsible for the movement of the zone. It has been suggested that climatic fluctuations during the Pleistocene

caused changes in habitat distribution and corresponding parapatric species distributions (*e.g.*, Marchant *et al.* 1988; Ruedi *et al.* 1997). In addition, recent habitat modification induced by agriculture may also have caused hybrid zone movement (*e.g.*, Leache & Cole 2007). A satellite image of the study area shows that the *viatica*17 territory appears to be heavily cultivated for agriculture (pink areas in Fig. 4.5A). Moreover, our field observation suggests that *viatica*17 were patchily distributed in cleared land and often associated with *Acaena* weed. It is, therefore, possible that recent habitat modification may have created regions of low grasshopper density with frequent meta-population extinction and recolonisation on the *viatica*17 side. Tension zones may move down a density gradient and be trapped in regions of low density (Barton 1979; Barton & Hewitt 1981a). It is difficult to disentangle these two possibilities (historical climate change vs. more recent habitat changes) without historical records of population density changes; however, in either case, hybrid zone movement may have resulted from a low population density on the *viatica*17 side created by environmental changes.

4.4.3 Estimate of dispersal and selection

The direct estimate of the dispersal parameter σ based on the mark-recapture study (Blackith & Blackith 1969a) was 5 - 10 times smaller (σ = 4.7 - 8.8 m/gen$^{1/2}$) than the indirect estimate of σ based on the cline analysis (σ = 48.7 m/gen$^{1/2}$). This is not surprising because direct estimates tend to underestimate σ due to juvenile dispersal, rare long-distance migration and historical extinction-recolonisation events, which are not normally measured in mark-recapture studies (Barton & Hewitt 1985; Szymura & Barton 1986; Barton & Gale 1993). Missing these rare dispersal events is likely in the previous mark-recapture study because the simplistic nature of this study does not incorporate the recapture probability of these rare events (Blackith & Blackith 1969a). Therefore, despite some uncertainty in the linkage disequilibria and recombination rate estimates, the indirect estimate of σ seems more realistic than the direct estimate.

The estimates of average effective selection on a locus in this study, $s^* = 19.7$ % (2u support limits 5.8 - 40.5 %), seems comparable to those in other studies (*Heliconius* butterflies, $s^* = 23$ - 25 %, Mallet *et al.* 1990; *Bombina* toads, $s^* = 17$ - 22 %, Szymura & Barton 1991; *Sceloporus* lizards, $s^* = 30$ %, Sites *et al.* 1995; *Pontia* butterflies, $s^* = 47$ - 64 %, Porter *et al.* 1997; *Carlia* skinks, $s^* = 22$ - 70 %, Phillips *et al.* 2004; *Ensatina* salamander, $s^* = 46$ - 75 %, Alexandrino *et al.* 2005; *Mus* house mice, $s^* = 6$ - 9 % for autosomal loci and $s^* = 25$ % for X-linked loci, Macholan *et al.* 2007). Raufaste *et al*. (2005) suggest a method to estimate the number of selected loci (n) and the total selection (S) based on cline shape parameters; however, we did not estimate n and S due to considerable uncertainty in assumptions and the estimated cline shape parameters, particularly barrier strength (B). Nonetheless, given that the s^* estimate is similar to other studies, and more than a half of the randomly chosen loci were best-fitted with the stepped cline models, it seems reasonable to consider that a large number of loci are under selection to maintain the stable hybrid zone (*e.g., Bambina* toads, $n = 55$ - 300; *Mus* house mice, $n = 56$ - 378). Furthermore, if this inference is valid, then it is likely that these selected loci are scattered across the genome, presumably both inside and outside the rearranged chromosomal segments. (see *section 4.4.4*)

4.4.4 Mode of speciation

Our cline shape analyses and dispersal/selection parameter estimates have important implications for the origin of parapatry and mode of speciation in the *Vandiemenella viatica* species group. White's stasipatric speciation model (1968; 1978) predicts that (i) hybrid zones of the *Vandiemenella* have arisen by primary contact as the geographic distribution of the species group was 'essentially' contiguous through the entire period of chromosomal diversification, (ii) hybrid zones have moved from the distribution of a derived chromosome type into the that of a parental chromosome type due to homozygous advantage and/or meiotic drive, and (iii) reduction of gene flow may be much stronger on rearranged chromosomes than collinear chromosomes because of strong selection against chromosomal heterozygotes due to meiotic abnormalities. Evidence from our cline analyses, however, does not support these predictions. First, the narrowness of the clines relative to the dispersal rate and age of

the two chromosomal race lineages strongly support a secondary origin of the contact zone. Separation of these two chromosomal races based on *COI*, *EF*-1α and allozyme is estimated as about 1.0 - 3.7 million years (Ma) (Kawakami *et al.* 2007a). Assuming the zones arose by primary contact about 1.0 - 3.7 Ma ago, cline widths for these markers, in particular the presumably neutral *COI*, should have been much wider, given the dispersal rate ($\sigma = 48.7$ m/gen$^{1/2}$). Thus, it is much more plausible that the contact zone between P24(XY) and *viatica*17 formed by secondary contact much more recently, possibly after the last Glacial Maximum (ca. 18 000 years ago).

Most importantly, the coincidence and concordance of the clines for nuclear and chromosome markers suggest that the reduction of gene flow is unlikely to be restricted to the rearranged chromosomes, and loci under selection may not be concentrated only on those chromosomes. P24(XY) and *viatica*17 differ by an X-chromosome inversion and a fusion between the inverted X-chromosome and an acrocentric autosome (chromosome No. 1) (Fig. 4.1C). Meiotic abnormalities were reported to be associated with these chromosomes in all F_1 hybrids (Mrongovius 1979). Since the primary isolating mechanism in the stasipatric model is meiotic abnormalities, selection should act predominantly on the X and No. 1 chromosomes. This assumption predicts that loci linked with these chromosomes are under selection with reduced gene flow and should show stepped clines, while loci on the other non-rearranged chromosomes should retain normal gene flow without strong selection and show Sig clines. In addition, because of the difference in the selection strength between loci on the rearranged and non-rearranged chromosomes, widths of clines for the latter loci fitted with the Sig model should be wider than those for the former loci fitted with the stepped models. Although genomic locations of the nuclear markers used in this study are unknown, it is unlikely that all six markers with a stepped clinal pattern are located on these rearranged chromosomes. Moreover, the cline widths of loci fitted with the Sig model and the putative X-linked *Acyc* are not significantly different from those of other nuclear loci. Taken all together, the stasipatric model of speciation does not explain the observed clinal patterns in the *Vandiemenella* hybrid zone, and an alternative model is needed.

Our analyses support Key (1968) and Hewitt's (1979) proposal, in which the origin of the chromosomal races of *Vandiemenella* and their hybrid zones occurred in allopatry with subsequent secondary contact. Thus, it is reasonable to hypothesize that new chromosome types have been established in allopatry by drift, and genetic differences have accumulated during an allopatric phase. The contact zone between the chromosomal races may have formed possibly after the Last Glacial Maximum. An unanswered question is whether karyotypic differences acted as a reproductive barrier after the secondary contact. The narrowness of the chromosomal cline implies some association between the karyoptypic differences and barriers to gene flow between the two chromosomal races. However, the similarity in the stepped cline patterns at multiple presumably unlinked loci argues that selection is not concentrated on a few chromosomes, but is scattered across the genome. Furthermore, the recombination suppression models (Ayala & Coluzzi 2005; Butlin 2005) predict that genetic structure is higher with more restricted gene flow for the rearranged chromosome segments than outside the rearranged regions and other non-rearranged chromosomes. In order to test these predictions and discriminate between reproductive barriers caused only by genetic factors or in combination with chromosomal differences, further analyses are required to investigate differential gene flow using a large number of genetic markers within and outside the rearranged chromosome regions.

CHAPTER 5

DIFFERENTIAL GENE FLOW OF MITOCHONDRIAL AND NUCLEAR DNA AMONG HYBRIDISING CHROMOSOMAL RACES OF AUSTRALIAN MORABINE GRASSHOPPERS (*VANDIEMENELLA*, *VIATICA* SPECIES GROUP) ON KANGAROO ISLAND

(*Accepted for publication in Molecular Ecology under the same title*)

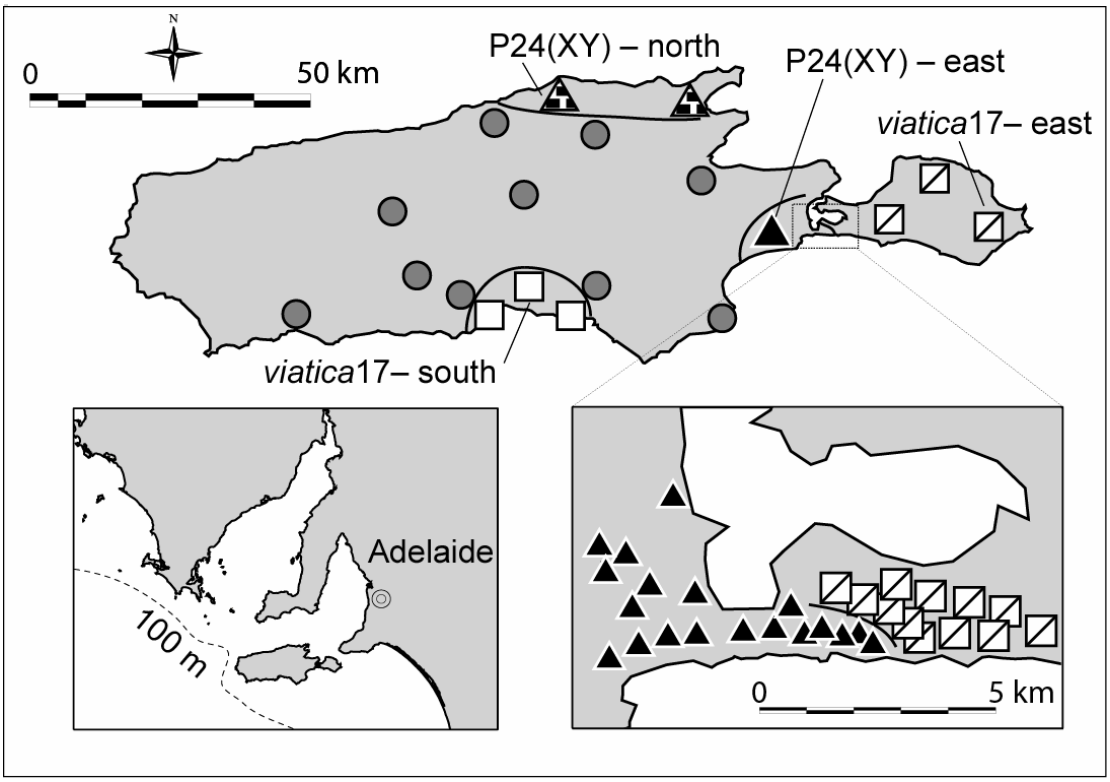
5.1 Introduction

The existence of chromosomal variation both within and between closely related species is known from a wide range of taxa, but whether chromosomal rearrangements play a direct role in the process of speciation has been much debated (White 1978; John 1981; King 1993). White (1968; 1978) proposed that chromosomal changes play a causative role in speciation by leading to hybrid dysfunction or underdominance of heterokaryotypic individuals (the 'stasipatric' speciation model). Other characteristics of this model include (i) establishment of new chromosome types due to homozygous advantage, meiotic drive, and genetic drift, and (ii) spread of new chromosome types from their point of origin into the distribution of a parental chromosome type without apparent geographic isolation, leading to parapatric distributions of chromosomal races. In contrast, others view chromosomal rearrangements as incidental by-products of speciation processes (Coyne & Orr 1998) with parapatric distributions resulting from secondary contact. More recently, however, it has been suggested that suppressed recombination resulting from chromosomal rearrangements may favour speciation and accelerate divergence between populations by promoting the spread of 'isolation genes' or locally adapted alleles, or by preserving combinations of alleles that evolved in allopatry (Noor *et al.* 2001b; Rieseberg 2001; Navarro & Barton 2003a; Kirkpatrick & Barton 2006). These theories, referred to as the 'suppressed-recombination models' by Ayala and Coluzzi (2005), have led to a renewed interest in the potential role of chromosomal rearrangements in speciation and environmental adaptation.

However, there is only a limited number of empirical studies to test these theories and, hence, the frequency and importance of the role of chromosomal rearrangements in speciation is uncertain (Butlin 2005).

The development of White's stasipatric speciation model (1968; 1978) was largely based on a group of Australian morabine grasshoppers in the genus *Vandiemenella*, known as the *viatica* species group. *Vandiemenella* are wingless grasshoppers that show extensive chromosomal variation, with chromosomal variants having restricted distributions in south-eastern Australia. The group currently comprises two formally designated species (*V. pichirichi* and *V. viatica*) and 11 chromosomal forms that are considered to be either races within these species or distinct species awaiting formal description (Key 1976). These taxa have been discriminated by chromosomal rearrangements (fusions, fissions, translocations, or inversions), characters of the male cercus and genitalia, and morphometrics (White 1978 and references therein). All taxa, with two exceptions, have parapatric distributions and in most cases they show narrow zones of hybridization, usually less than a few hundred metres wide (White *et al.* 1967; 1969). Although these hybrid zones have been studied using cytological and morphological analyses, there are no data on the extent to which karyotypic differences are associated with barriers to gene flow. Therefore, it is currently unknown whether the chromosomal differentiation between races generates partial or complete reproductive isolation or whether there has been extensive gene flow between races, despite the fixation of different chromosomal variants in different geographic regions.

(A)



(B)

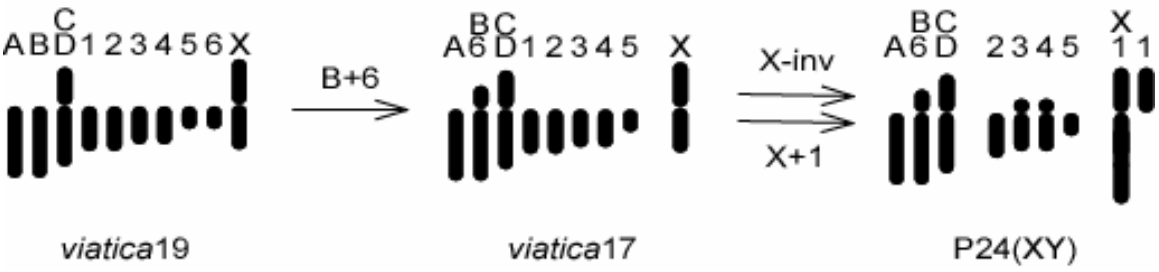


Fig. 5.1 Geographic distribution of three chromosomal races of *Vandiemenella* on Kangaroo Island (KI), South Australia (A). Five populations are indicated as follows: *viatica*19 (shaded circle), *viatica*17-south (open square), *viatica*17-east (hatched square), P24(XY)-east (filled triangle), and P24(XY)-north (hatched triangle). Lines indicate provisional contact zones (White et al. 1969). Right inset: sampling sites near the P24(XY)/*viatica*17 contact zone. Left inset: a map of South Australia indicating 100 m isobath. Note that only representative sampling sites are shown. Cytological evolutionary sequence (B) proposed by White *et al.* (1967): B+6, centric fusion between B and 6 autosomes; X-inv, X sex chromosome pericentric inversion; X+1, centric fusion between the inverted X sex chromosome and 1 autosome, resulting in XY sex chromosome system. The 3 and 4 autosomes in P24(XY) are subacrocentric (White *et al.* 1967)

Kangaroo Island (KI) in South Australia is inhabited by three chromosomal races of *Vandiemenella*: one widely distributed population of the *viatica*19 chromosomal race of *V. viatica* (2n = 19 XO in males, 2n = 20 XX in females); two isolated populations (east and south, KI) of the *viatica*17 chromosomal race of *V. viatica* (2n = 17 XO in males, 2n = 18 XX in females); and two isolated populations (north and east, KI) of the provisional species P24 (2n = 16 XY in males and XX in females), henceforth referred to as the P24(XY) chromosomal race (Fig. 5.1) (White *et al.* 1969). White *et al*. (1967) hypothesize that the cytological evolutionary sequence in these three chromosomal races was *viatica*19 → *viatica*17 → P24(XY) (Fig. 5.1B). It is possible that the now isolated *viatica*17 populations were once connected by exposed continental shelf at the south shore of KI, and that the P24(XY) populations were similarly connected to the north of KI, and that all three races were connected to populations on the mainland that have the same karyotypes, prior to a rise in sea level after the last glaciation (White *et al.* 1967). It is expected that these wingless grasshoppers, with relatively feeble dispersal abilities, will show strong population genetic structuring, unless their distributions were influenced by historical changes to landscape and climate, resulting in range expansions/contractions (Hewitt 1999). Furthermore, each population of *Vandiemenella* on KI forms a parapatric distribution with a neighbouring chromosomal race. Importantly, every pairwise combination is evident [*i.e.*, *viatica*17/*viatica*19 in south KI, *viatica*19/P24(XY) in east KI and *viatica*17/P24(XY) in east KI], allowing an assessment of gene flow between each pair of chromosomal races without the potentially confounding effects of historical migrations from mainland populations, which are bordered by a variety of different chromosomal races. Therefore, the diverse distribution patterns of *Vandiemenella* on KI, coupled with multiple contact zones comprising different pairs of chromosomal races, provide an excellent system to investigate barriers to gene flow and phylogeography using multiple independent molecular markers.

The mitochondrial cytochrome c oxidase subunit I (*COI*) gene has been extensively used to elucidate phylogenetic relationships and phylogeographical variation within species (Avise 2000). However, a number of studies suggest that mitochondrial DNA (mtDNA) gene trees are often incongruent with species trees based on morphological,

cytological or nuclear markers due to introgression among species, the maternal mode of inheritance of mtDNA and a different rate of lineage sorting (reviewed in Funk & Omland 2003; Ballard & Whitlock 2004). For this reason, it is generally recommended to include multiple nuclear markers for investigating the evolutionary history of a recently diverged species complex. Therefore, we have employed a series of independent molecular markers comprising DNA sequence data from the *COI* gene and the nuclear elongation factor-1α (*EF*-1α) gene, and allozyme electrophoretic data to (i) test whether the three chromosomal races of *Vandiemenella* on KI represent genetically distinct taxa, (ii) examine population structure and patterns of gene flow of various markers among chromosomal races to establish whether chromosomal variants are associated with barriers to gene flow, and (iii) explore whether historical demographic changes may have affected the population genetic patterns of each molecular marker.

5.2 Methods

5.2.1 Taxon sampling

Grasshoppers of *Vandiemenella* chromosomal races were collected on KI between 2002 and 2005 from 95 sites within the five populations (5 - 34 sites/population), defined according to karyotype and geographic location: *viatica*19 population; *viatica*17-east and *viatica*17-south populations; and P24(XY)-east and P24(XY)-north populations (Fig. 5.1). Of the three previously reported contact zones (White *et al.* 1969; Mrongovius 1975; 1979), we identified only one contact zone that has survived habitat changes due to agriculture in the last 20 - 30 years, composed of *viatica*17 and P24(XY). However, no sites were identified showing both *viatica*17 and P24(XY) karyotypes in the area of this contact zone (65 males collected < 200 m from the putative contact zone were analysed) (details of sampling sites in Appendix Table 5.1). Testes from males were removed for chromosomal analyses and bodies were frozen in liquid N_2 and stored at -80°C, prior to allozyme and DNA analyses. In most cases, the same male specimens used for chromosomal analyses were also used for allozyme and DNA sequence analyses. *Prorifera sp.* P28 and *Keyacris sp.* P110a ('P' for provisional species code, Australian National Insect Collection) were used as

outgroup taxa. Fresh testes were removed from 1066 males and were placed in 1 % sodium citrate solution for approximately 10 - 15 minutes and squash preparations of the chromosomes were made in 2 % aceto-orcein stain presented in White *et al.* (1967). At least two meiotic preparations were counted per male.

5.2.2 Allozyme analyses

Allozyme electrophoresis of leg homogenates was undertaken on cellulose acetate gels (Cellogel©, MALTA, Milan) according to the principles and procedures of Richardson *et al.* (1986). A preliminary screen of 27 individuals [n = 7 *viatica*19; n = 10 *viatica*17; and n = 10, P24(XY); two sites for each of the three races], chosen from sites well removed from any contact zone, resolved 40 putative allozyme loci. Of these, 15 loci were selected for the allozyme study based on (i) being either apparently diagnostic for at least one taxon and/or commonly polymorphic (q > 30%) at one or more sites, and (ii) the ease of assigning genotypes (*i.e.*, how active, well-resolved, and well-separated were putative allozymes). Locus abbreviations, electrophoretic conditions, and staining protocols follow Richardson *et al.* (1986).

The allozyme data were analysed using two approaches. First, UPGMA dendrograms were constructed from pairwise matrices of both Nei's *D* and Rogers' *R* among the 56 sampling sites represented. As Nei's *D* and Rogers' *R* produced UPGMA dendrograms with similar topologies, a single UPGMA dendrogram is presented herein (Fig. 5.2). An estimate of the robustness of major nodes was obtained through bootstrapping (100 pseudoreplicates). In addition, we constructed Neighbour-Joining trees. Since they had identical topologies, we present only a UPGMA dendrogram. Second, Principal Co-ordinates Analysis (PCoA) on a pairwise matrix of Rogers' *R* was used to assess the genetic relationships among individuals, without requiring the *a priori* assignment of individuals to any taxon. All loci, including sex-linked loci (*Acyc* and *Pgm*), were used in these analyses. Details of all procedures used to analyse the allozyme data are presented in Hammer *et al*. (2007) or Maryan *et al*. (2007). Given the small sample sizes per site (n = 1 - 17), site-based statistical assessments of Hardy Weinberg expectations (HWE) and linkage disequilibrium (LD) were not undertaken.

5.2.3 Polymerase Chain Reaction (PCR) amplification and sequencing

DNA was extracted from single hind legs using the PUREGENE® DNA Isolation Kit (GENTRA). An approximately 650 bp region of the *COI* gene was PCR-amplified and sequenced using the primers LCO1490 (forward, 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (reverse, 5'-AAC TTC AGG GTG ACC AAA AAA TCA-3') (Folmer *et al.* 1994). Mitochondrial enrichment procedures (Donnellan *et al.* 1999) were used for one individual of each chromosome race to verify the mitochondrial origins of sequences. Primers for the *EF*-1α region were: G605 (forward, 5'-GGG YAA AGG WTC CTT CAA RTA TGC-3', designed by B. Danforth personal communication) and G1001 (reverse, 5'- TGC GTC GTG GTT AYG TWG CTG-3', designed by T. Kawakami). This primer pair usually amplified sufficiently strong PCR products (~ 880 bp), but for individuals with poor amplifications, a two-step nested PCR was used with a nested forward primer G508 (5'-AGT ACG CCT GGG TGT TGG AC-3', designed by S. Tierney). Standard PCR amplifications included 1x reaction buffer (Perkin Elmer), 0.2 mM of each dNTP, 5 pmol of each primer, 1 unit of Amplitaq Gold (Perkin Elmer), 2 mM $MgCl_2$ and 2.5 µL of template DNA (*c*. 50 ng) in a 25 µl reaction volume. PCR-amplifications were carried out for 1 cycle of 94 °C for 9 min and 35 cycles (94 °C, 45 s; 55 °C, 45 s; and 72 °C, 60 s), followed by a final incubation step at 72 °C for 6 min. PCR products were purified using the UltraClean™ PCR Clean-up Kit (MoBio), and sequenced on both strands using the ABI Prism™ Big Dye Terminator Cycle in ABI 3700 automated sequencers (Applied Biosystems). Sequences have been submitted to GenBank (Accession numbers: EU121420 - EU121509).

5.2.4 DNA sequence analysis

Multiple sequence alignments of *COI* were performed using Clustal W (Thompson *et al.* 1994). Direct sequencing of *EF*-1α resulted in several sites that were heterozygous for single nucleotide polymorphisms (SNPs) or insertions/deletions (indels). Heterozygous SNP positions were identified by the presence of double peaks in sequence chromatograms (Brumfield *et al.* 2003). Heterozygous indel

positions could be identified from sequence chromatograms, as the indel results in a frame-shift of the sequence of one allele relative to the other, leading to double peaks in the chromatogram after the position of the indel (Bhangale *et al.* 2005). Sizes of identified indels varied from 2 bp to 3 bp and these multiple site indels were scored as a single mutation event and used as a fifth character for phylogenetic analyses. Alignments of *EF*-1α were performed using Clustal W and then adjusted by eye in the locality of these indels. Exon/intron regions were identified with reference to the conserved splice sites for insects (Mount *et al.* 1992; Danforth & Ji 1998). The haplotypic phases of heterozygotes for *EF*-1α were inferred from the genotype data of all individuals using the Bayesian statistical methods implemented in the program Phase 2.1.1 (Stephens *et al.* 2001). Five independent runs were performed, each with 1000 iterations and a burn-in value of 100 to reduce the error due to incorrect haplotype assignments. As the haplotype frequency estimates were consistent, and the goodness-of-fit values were very similar across the five runs, the lengths of the runs were considered sufficient to obtain reliable results. Seven individuals with more than one SNP position were found in 99 individuals analysed. The haplotypic phase of these seven was determined with a posterior probability of > 95% in all five runs.

5.2.5 Tests for recombination

To test whether there is evidence for recombination in the *EF*-1α fragment, estimates of the minimum number of recombination events (R_m) (Hudson & Kaplan 1985) were obtained in the program DnaSP 4.10.9 (Rozas *et al.* 2003), and the population recombination parameter ($\rho = 4N_e r$) was estimated by the modified version of the composite-likelihood method (Hudson 2001) in the program LDhat 2.0 (McVean *et al.* 2002). The estimate of R_m was 4 per locus (825 bp) with a 95% confidence Interval (CI) ranging from 1 to 6, and the estimate of ρ was 0.69 per locus. Although a nonparametric permutation test indicated that ρ was not significantly different from 0 (P = 0.89), the CI of R_m did not include 0, suggesting potential recombination events. The haplotype network analysis, some of the neutrality and demographic analyses, and mismatch distribution analysis are inappropriate for recombining DNA

regions because recombination increases the number of haplotypes and generates Poisson-like mismatch distributions by shuffling nucleotide variation among DNA sequences (Ramos-Onsins & Rozas 2002). Therefore, although we have conducted these analyses for both *COI* and *EF*-1α datasets, results of these analyses for *EF*-1α are not presented.

5.2.6 Phylogenetic analysis

Phylogenetic analyses were conducted for *COI* and *EF*-1α separately using maximum parsimony (MP) in PAUP* version 4b10 (Swofford 2000) and Bayesian inference (BI) in MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003). For MP analyses, an unweighted heuristic search, with TBR branch swapping, character state optimisation using the MINF option and random addition of taxa with 10 replications, was performed. The reliability of tree topologies from these analyses was estimated using the bootstrap with 1000 pseudoreplicates. For BI analyses, the sequence matrix of *EF*-1α was partitioned into three regions, corresponding to exons, introns and indels, with separate models of evolution applied to each partition. The best-fit models of molecular evolution for BI were assessed using the Akaike Information Criterion in Modeltest version 3.7 (Posada & Crandall 1998). The best-fit models were a TVM+G (six substitution types [Rmat] = 2.58, 16.08, 1.62, 0.36, 16.08, and1.00; G = 0.126) for *COI*, K81uf+I (Rmat = 1.00, 1.92, 0.18, 0.18, 1.92, 1.00; I = 0.922) for the exon region of *EF*-1α, and TIM+I (Rmat = 1.00, 2.45, 0.40, 0.40, 1.07, 1.00; I = 0.500) for the intron region. Indels were coded as binary characters with ascertainment bias being variable (see MrBayes documentation). BI analyses were conducted on the same data sets with two simultaneous runs of Metropolis-coupled Markov Chain Monte Carlo analysis and four chains for 5 000 000 generations, starting with a random tree and saving one tree every 100 generations. Run length was sufficient to obtain reliable results as the average standard deviation of split frequencies was ~0.007 in *COI* and ~0.002 in *EF*-1α datasets at the completion of the runs, suggestive of convergence of the two runs on a stationary distribution. The log likelihood reached stable values by 14 000 generations for *COI* and 283 000 generations for the *EF*-1α, but 25 % of the sampled trees (1 250 000 generations)

were excluded as a burn-in to ensure stationarity. Mean pairwise genetic distances were calculated using a maximum likelihood (ML) distance implemented in PAUP* with the best-fit molecular evolution models (HKY+I+G for *COI* [parameters above] and K81uf+I (Rmat = 1.00, 1.89, 0.41, 0.41, 1.89, 1.00; I = 0.814 for *EF*-1α without partitions).

Initially, phylogenetic analyses were conducted including *Prorifera* P28 and *Keyacris* P110a to evaluate whether *V. pichirichi* is a sister taxon to the three chromosome races of *Vandiemenella*. Taxonomic classification of these genera based on morphology suggests that *Prorifera* is most distantly related to *Vandiemenella* (Key 1976). Our MP and BI analyses of *COI* and the exon regions of *EF*-1α with *Prorifera sp.* as an outgroup confirmed that *V. pichirichi* is a sister taxon to other members of *Vandiemenella* with high bootstrap values and posterior probabilities (100 % for both *COI* and *EF*-1α in MP and BI) (data not shown). Large genetic distances between *Prorifera sp.* and *Vandiemenella* in this study support a distant relationship. To avoid possible phylogenetic errors that may arise from long-branch attraction (Felsenstein 1978) when using *Prorifera* P28 as an outgroup, for subsequent analyses *V. pichirichi* was used instead.

5.2.7 Neutrality and demographic analyses

To test whether genetic populations, or 'phylogroups' (see results for a definition of phylogroups), of *COI* and *EF*-1α conform to neutral expectations and demographic equilibria, the Tajima's (1989) *D* and Fu's F_s (1997) were independently calculated for each marker using Arlequin 3.11 (Excoffier *et al.* 2005), and Ramos-Onsins & Rozas's R_2 (2002) were calculated using DnaSP 4.10.9 (Rozas *et al.* 2003). The statistical significance was tested by generating random samples under the hypothesis of selective neutrality and demographic equilibrium, using coalescent simulations with 10 000 permutations. Mismatch distributions was computed only for the *COI* dataset using Arlequin, and the empirical distributions were compared with the null distributions based on a demographic expansion model (DEM) and a

spatial expansion model (SEM) by the generalized least-squares approach (Schneider & Excoffier 1999).

In addition, coalescent-based analyses were used to estimate θ ($2N_e\mu$ for *COI* or $4N_e\mu$ for *EF*-1α) and *g* (population growth parameter) for phylogroups of *COI* using FLUCTUATE version 1.4 (Kuhner *et al.* 1998) and for *EF*-1α using LAMARC version 2.0.2 (Kuhner 2006). The parameter θ was estimated jointly with *g* (initial value $g = 1$) and with *g* held at zero (θ_G and θ_{NG}, respectively). Search settings in FLUCTUATE were: 50 short chains (10 000 steps) and 5 long chains (100 000 steps) sampling every 20th step, random starting trees, transition/transversion ratio 2.0, empirical base frequencies, and starting θ from Watterson's (1975) estimate. Search settings in LAMARC were: Bayesian analysis with the best-fit models of molecular evolution selected in Modeltest, empirical base frequencies, initial values of θ from Watterson's (1975) estimate, initial values of *M* (migration parameter) = 20, *r* (recombination rate) = 0.001, 1 short chains (10 000 steps, sampling every 20th step, burn-in of 1000 trees) and 1 long chains (1 000 000 steps, sampling every 100th step, burn-in of 2000 trees). Runs were repeated five times, and mean and standard deviation (SD) of θ_G, θ_{NG}, and *g* were calculated. We considered *g* to indicate population growth when $g - 3\,SD(g) > 0$) (Lessa *et al.* 2003)

5.2.8 Population genetic differentiation

To assess the degree of population genetic differentiation within and among the chromosomal races based on *COI* and *EF*-1α sequences, analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) was undertaken using Arlequin. Genetic distance matrices were calculated based on the ML-distance with the best-fit molecular evolution models. Hierarchy levels were defined *a priori* based on chromosomal race and geographic population, *i.e.*, among the three chromosomal races, within races between populations and within populations. Significance of Φ_{ST} was evaluated by 10 000 permutations.

5.3 Results

5.3.1 Allozyme analyses

In total, 233 individuals from 56 sampling sites were screened for 15 variable allozyme loci (allele frequencies in Appendix Table 5.2). Seven loci were found to be fully diagnostic for at least one chromosomal race (*Acyc*, *Ak2*, *Fum*, *Hex*, *Idh2*, *Mdh2*, and *Me*), five loci were partially diagnostic (*Ak1*, *Argk*, *Mpi*, *PepA*, and *PepC*), and three loci were nearly invariant (*Got*-1, *PepD1*, and *Pgm*). A locus was considered diagnostic if one or more chromosome races shared no alleles based on a comparison of 61 individuals [n = 17, *viatica*19; n = 12, *viatica*17; and n = 27, P24(XY)] representing 15 sites well removed from contact zones to avoid potential influences of gene introgression from adjacent chromosomal races.

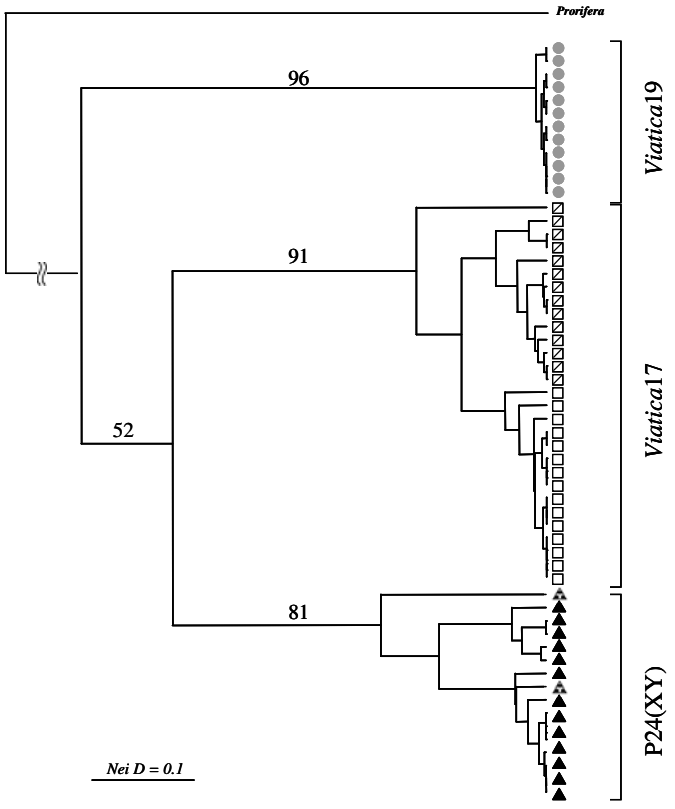


Fig. 5.2 An UPGMA dendrogram showing the relationships among the three chromosomal races of *Vandiemenella* on Kangaroo Island based on the unbiased genetic distances of Nei (1978) using 15 allozyme loci. Numbers indicate bootstap support (> 50 %). Each symbol represents a sampling site (see Fig. 5.1).

The UPGMA dendrogram based on the genetic distances between sampling sites (Fig. 5.2) and Principal Co-ordinate Analysis (PCoA) based on the genetic distances between individuals (Fig. 5.3) indicated that the three chromosomal races were clearly distinguishable from one another, forming three monophyletic groups in the dendogram with strong bootstrap support. Moreover, the *viatica*17-east and *viatica*17-south populations were genetically distinct within the *viatica*17 cluster. In contrast, the cluster of P24(XY) did not indicate such clear population subdivisions due partly to the small sample size of P24(XY)-north (n = 6). Importantly, individuals of *viatica*17 and P24(XY) collected near the contact zone in east KI were observed in intermediate positions between *viatica*17 and P24(XY) clusters in the PCoA, suggesting that these individuals may be of hybrid origin.

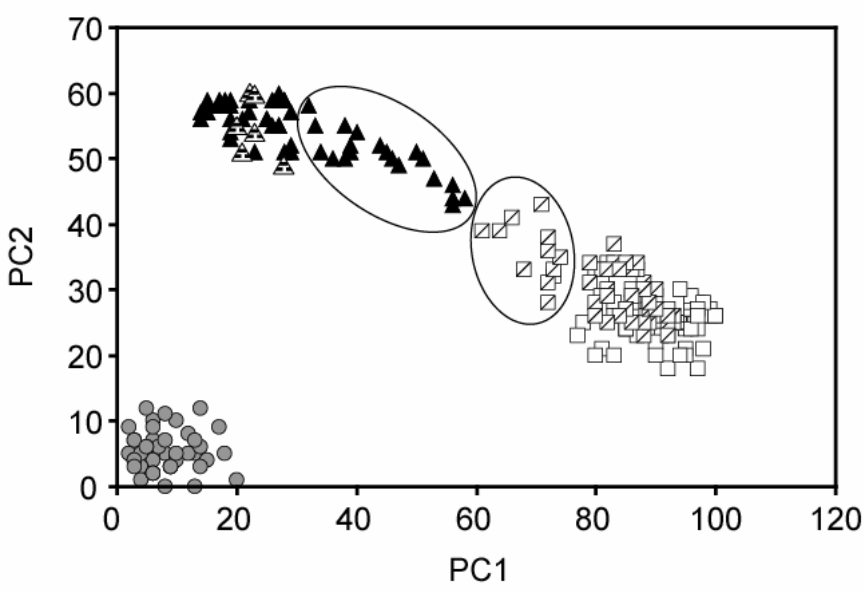


Fig. 5.3 Principal Co-ordinate Analysis of the allozyme data from the population study with 15 loci. The relative PCoA values were plotted for the first and second PC axis, which individually explained 32 % and 24 %, respectively, of the total multivariate variation. The five populations are indicated as follows: *viatica*19 (shaded circle), *viatica*17-south (open square), *viatica*17-east (hatched square), P24(XY)-east (filled triangle), and P24(XY)-north (hatched triangle). Ellipses denote individuals collected from sampling sites < 1 km from the approximate centre of the contact zone between P24(XY) and *viatica*17 in east KI.

The preferential selection of diagnostic loci over-estimates the Nei's *D* values underlying Figure 5.2. When all 40 putative allozyme loci were analysed in the preliminary screen of 27 individuals, pairwise Nei's *D* between the three chromosomal races ranged from 0.2 [P24(XY) and *viatica*17] to 0.4 [*viatica*19 and *viatica*17], suggesting divergence 1 - 2 million years (Ma) based on a conventional allozyme clock calibration [divergence time (Ma) = Nei's *D* ×5] (Nei 1987).

5.3.2 Sequence variability

Seventy-four nucleotide sites of the 637 bp *COI* fragment showed variation, and 61 haplotypes were identified from the 145 *Vandiemenella* individuals (Appendix Fig. 5.1). Nucleotide diversity for the total sample was 0.01602 (SD = 0.00069). We deem this amplified region to be derived from functional mtDNA because (i) all fragments from *Vandiemenella* and two outgroup genera (*Keyacris* and *Prorifera*) had an open reading frame without indels and stop codons, (ii) a large proportion of substitutions were synonymous (78 %), and (iii) *COI* sequence data from dilutions of the mtDNA enrichments were identical to that from total genomic DNA, suggesting an absence of nuclear copies.

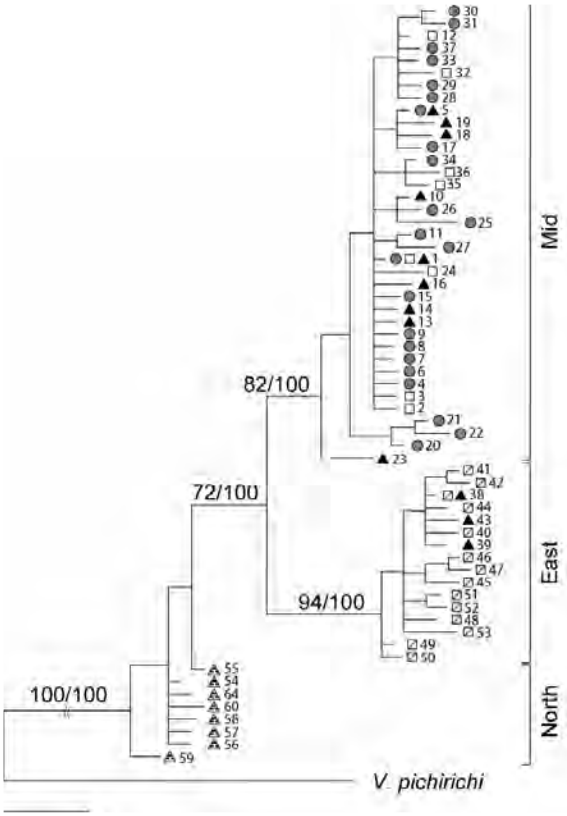
The 825 bp *EF*-1α fragment included two complete exons and two partial exons, separated by three introns. Twenty-nine nucleotide polymorphic sites (10 sites in the exon and 20 in the intron regions) and five indel polymorphic sites in intron regions were identified from the 99 *Vandiemenella* individuals, representing 22 haplotypes (Appendix Fig. 5.2, Supplementary material). Nucleotide diversity was 0.00761 (SD = 0.00019). Our results suggest that this amplified region is derived from a single orthologous functional copy of an *EF*-1α gene because (i) all substitutions in exon regions from *Vandiemenella* and two outgroup genera had an open reading frame without indels and stop codons, (ii) there was no evidence of more than two SNPs at any site within a single individual (*i.e.*, only homozygotes or heterozygotes with two alleles were identified), and (iii) exon regions had high homology with published *EF*-1α sequences of other insects as determined using BLAST analyses (*e.g.*, Hymenoptera, Danforth & Ji 1998).

5.3.3 Phylogenetic analyses

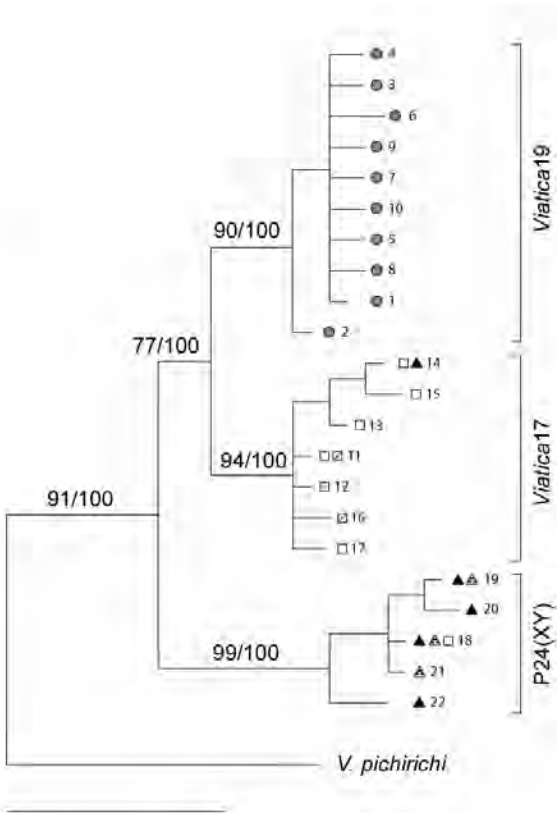
Phylogenetic and haplotype network analyses of *COI* data resolved three geographically localised phylogroups (hereafter referred to as mid, east, and north phylogroups); however, the geographic distribution of these phylogroups did not correspond to the distribution of the three chromosomal races (Figs. 5.4A, 5.5). Among the three phylogroups, the mid phylogroup was found in all chromosomal races, including the geographically wide-spread *viatica*19 and two relatively isolated populations containing the *viatica*17-south population and P24(XY)-east population. The east phylogroup, on the other hand, was predominantly found in the *viatica*17-east population, with the exception of four P24(XY) individuals from sites near the presumed contact zone between *viatica*17 and P24(XY). The north phylogroup was found only in the P24(XY)-north population.

In contrast, phylogenetic analyses of *EF*-1α resolved individuals from *viatica*19, *viatica*17 and P24(XY) into three distinct monophyletic groups, regardless of their geographic distribution (Fig. 5.4B). Analyses using only exon regions (567 bp) confirmed these three groups with moderate to high bootstrap values and posterior probabilities (data not shown). Exceptions included three *viatica*17 and one P24(XY) individuals collected near the contact zone between P24(XY) and *viatica*17 in east KI. These *viatica*17 individuals had two alleles belonging to the P24(XY) phylogroup (allele 15), and the P24(XY) individual had two alleles belonging to the *viatica*17 phylogroup (allele 19).

(A)



(B)



(C)

	North	East	Mid
North	0.44		
East	3.58	0.65	
Mid	2.44	3.42	0.69

(D)

	P24(XY)	*viatica*17	*viatica*19
P24(XY)	0.32		
	(0.11)		
*viatica*17	1.35	0.23	
	(0.49)	(0.15)	
*viatica*19	1.61	1.07	0.24
	(0.45)	(0.48)	(0.22)

Fig. 5.4 Majority rule consensus phylogram of *COI* (A) and *EF*-1α (B) haplotypes based on the last 37 500 trees from Bayesian analyses. Numbers on branches indicate bootstrap support in MP and posterior probability in BI analyses, respectively. The mid, east, and north phylogroups of *COI* and the *viatica*19, *viatica*17, and P24(XY) phylogroups of *EF*-1α are indicated. The scale bar is 0.01 substitutions/site. Symbols are as given in Fig. 5.1. Numbers next to the symbols indicate haplotype codes and the frequency of each haplotype is given in Appendix Figs. 5.1 and 5.2. Tables below phylograms show mean pairwise genetic distances (maximum likelihood distance) within and among three phylogroups in *COI* (C) and *EF*-1α (D). Genetic distances for *EF*-1α. were calculated using exon and intron (top row) and exon only (bottom row in bracket).

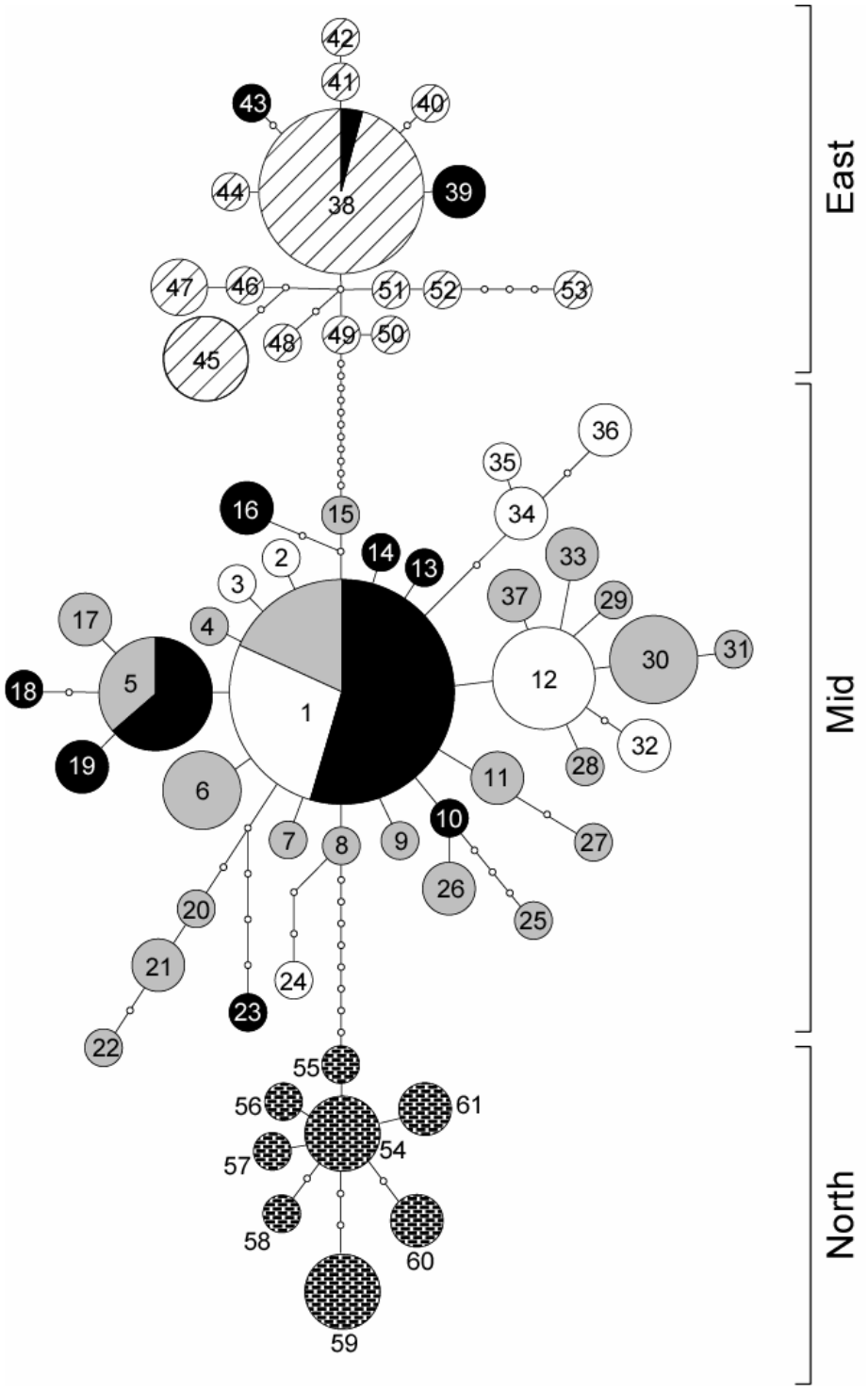


Fig. 5.5 Statistical parsimony network for *COI* for the three chromosomal races of *Vandiemenella*: *viatica*19 (gray shaded), *viatica*17 in the south (open), *viatica*17 in the east (diagonal line hatched), P24(XY) in the east (filled), and P24(XY) in the north (dashed line hatched). Haplotype node area is proportional to the number of individuals contained. Line lengths do not reflect actual branch lengths between haplotypes.

5.3.4 Neutrality and demographic analyses

Evidence of population growth was detected for all three *COI* phylogroups and two of the three *EF*-1α phylogroups (Table 5.1). In the *COI* dataset, of the three neutrality/demography tests (Tajima's *D*, Fu's F_s, and R_2), all three tests detected population growth for the mid and east phylogroups, while only R_2 detected population growth for the north phylogroup. Observed mismatch distributions of all three *COI* phylogroups were unimodal and fit both demographic (DEM) and spatial (SEM) expansion models. Significantly positive *g* in coalescent simulations suggested population growth for all three *COI* phylogroups. For the *EF*-1α dataset, at least two of the three neutrality/demography tests detected population growth for the *viatica*19 and P24(XY) phylogroups, whereas none of the three tests detected population growth for the *viatica*17 phylogroup. It is suggested that recombination, by shuffling nucleotide variation among DNA sequences and increasing the number of haplotypes, reduces the statistical power of the F_s test, which is based on the haplotype distribution (Ramos-Onsins & Rozas 2002). Thus, non-significant results for *EF*-1α should be taken with caution. Estimated *g* was positive for all three *EF*-1α phylogroups in all five runs, but was not significantly different from zero.

5.3.5 Population differentiation

Results of AMOVA indicate that variations between chromosomal races for the *EF*-1α datasets account for a large proportion of the total genetic variation (83 %, $p < 0.005$), whereas variation among populations within chromosomal races accounted for only a small proportion (2 %, $p < 0.005$). In contrast, molecular variation between chromosomal races in *COI* was zero, and genetic variation among populations within chromosomal races accounted for 75 % of the total variation ($p < 0.005$), indicating strong genetic structuring at the level of population.

Table 5.1 Tests of neutrality/demography tests (Tajima's *D* , Fu's F_s, and Ramos-Onsins & Rozas's R_2), mismatch distribution analyses based on demographic (DEM) and spatial (SEM) expansion models, and coalescent simulation analyses for phylogroups of *COI* and *EF-1α* loci. Values in bold are significant: significantly negative in *D* and F_s, significantly small in R_2, and significantly positive in *g*. Parameters: *N*, number of gene copies sampled; *h*, number of unique haplotypes/alleles in the sample; NS, not significant at $P < 0.05$; DEM and SEM, statistical tests based on the generalized least-squares approach; τ, relative time since expansion; -, not estimated; θ_{NG}, θ estimated with *g* held at zero (no growth); θ_G, θ estimated with *g* at maximum likelihood (growth). Numbers in brackets are 95% Confidence Intervals for τ or Standard Deviations for θ_{NG}, θ_G, and *g*.

				Neutrality/demography tests			Mismatch distribution				Coalescent simulation		
Locus	Phylogroup	*N*	*h*	*D*	F_s	R_2	DEM	τ	SEM	τ	θ_{NG}	θ_G	*g*
COI	Mid	87	37	**-2.268**	**-26.611**	**0.028**	NS	2.9	NS	1.6	0.0311	0.1249	**1407.9**
				P < 0.01	**P < 0.01**	**P < 0.01**		(1.1-4.4)		(0.8-3.9)	(0.0009)	(0.0177)	(255.5)
	East	44	16	**-1.864**	**-7.879**	**0.048**	NS	4.7	NS	3.2	0.0123	0.0248	**1118.4**
				P = 0.01	**P < 0.01**	**P < 0.01**		(0.1-9.3)		(0.4-6.8)	(0.0003)	(0.0010)	(38.0)
	North	14	8	-0.986	-2.413	**0.102**	NS	3.1	NS	3.0	0.0074	0.0254	**730.8**
				NS	NS	**P = 0.03**		(1.0-4.9)		(0.9-4.7)	(0.0001)	(0.0024)	(79.8)
EF-1α	*Viatica*19	68	10	**-1.933**	**-7.893**	**0.034**	-		-		0.0024	0.0055	1203.8
				P < 0.01	**P < 0.01**	**P = 0.03**					(0.0002)	(0.0009)	(579.5)
	*Viatica*17	78	7	-0.312	-1.732	0.088	-		-		0.0012	0.0034	730.0
				NS	NS	NS					(0.0001)	(0.0003)	(425.5)
	P24(XY)	52	5	**-1.533**	-0.934	**0.043**	-		-		0.0012	0.0026	287.6
				P = 0.04	NS	**P = 0.04**					(0.0001)	(0.0005)	(158.8)

N, number of gene copies; *h*, number of haplotypes

5.4 Discussion

5.4.1 Restricted nuclear gene flow

Since White (1968) proposed the stasipatric speciation model based on studies of the *Vandiemenella viatica* species group, the question of whether chromosomal rearrangements have played a causative role in diversification within this group has remained open. The first question to address is whether chromosomal races of *Vandiemenella* represent genetically distinct taxa with reduced gene flow associated with chromosomal variants. Our molecular analyses using nuclear markers (allozymes and *EF*-1α) demonstrate strong genetic structure among the three chromosomal races on KI, regardless of their geographic distribution. Three chromosomal races were completely or nearly fixed with race-specific alleles at 12 independent allozyme and *EF*-1α loci. Such strong genetic structure in nuclear markers implies that these three chromosomal races have diverged over a considerable time period and show highly restricted gene flow for substantial portions of the nuclear genome.

Although estimating the time since divergence between the chromosomal races is difficult without reliable fossil records to calibrate the rate of evolution of each marker, estimates of the divergence time between populations can be given by allozyme Nei's *D* and *EF*-1α genetic distances. A conventional allozyme clock calibration (Nei 1987) would give estimated divergence times of the three chromosomal races as 1.0 - 2.0 Ma. An inferred substitution rate of the *EF*-1α exon region in *Papilio* butterflies of 1.3 - 2.0 × 10^{-9} per site per year (Zakharov *et al.* 2004) would give estimated divergence times between 2.3 - 3.7 Ma. Because substitution rates may be significantly different between distantly related organisms (Pulquerio & Nichols 2007) and molecular rates may be time-dependent due to purifying selection (Ho *et al.* 2005), these estimated divergence times should be treated with caution. However, it seems reasonable to conclude that these three chromosomal races show long-term isolation that predates the last ice age and possibly the Pleistocene. Furthermore, the nuclear genetic architecture of each chromosomal race has been

maintained despite the fragmentation of the populations that most likely occurred following the rise of sea levels after the last Glacial Maximum.

It should be highlighted that a substantial but incomplete barrier to nuclear gene exchange exists at chromosomal contact zones, and, importantly, this genetic barrier is probably not restricted to loci on the rearranged chromosomes but spread across the genome. The suppressed-recombination models predict that gene flow will be restricted in rearranged chromosomal segments in heterozygotes, but is likely to be maintained in collinear chromosomal segments (Ayala & Coluzzi 2005). The number of chromosomal rearrangements differentiating these three races is from one to three (Fig. 5.1B), so it seems unlikely that the 12 independent allozyme and *EF*-1α loci, which differentiate the three races, are all on rearranged chromosomal segments. However, the genetic barrier appears to be incomplete, at least between P24(XY) and *viatica*17, as a number of individuals with allozyme and *EF*-1α alleles typical of the alternative chromosomal races were found near the contact zone of the two races, suggesting that there is on-going gene introgression across the zone that extends further than the introgression of rearranged chromosomes. To establish the strength of the barrier to gene flow and to test for genomic localization of the barrier, further sampling and more nuclear markers of known chromosomal location will be needed.

5.4.2 Incomplete lineage sorting or introgressive hybridization of mtDNA

In contrast to the concordance of genetic structure for nuclear gene markers and chromosomes, the population genetic structure based on mitochondrial *COI* was discordant with the distribution of chromosomal races. Haplotypes were extensively shared among populations of *viatica*19, *viatica*17-south, and P24(XY)-east. However, some population substructuring associated with chromosomal races was still evident for mtDNA, with *viatica*17-east and P24(XY)-north, each comprising a distinctive monophyletic group of *CO1* haplotypes (Figs. 5.4A, 5.5). These differential patterns of population structure for the nuclear and mtDNA genomes are likely to have resulted from either stochastic lineage sorting of ancestral haplotypes or

introgression of mtDNA between chromosome races through hybridization (Funk & Omland 2003; Ballard & Whitlock 2004).

Incomplete lineage sorting alone is insufficient to explain the observed population genetic pattern of mtDNA given the geographic distribution of the haplotypes and theoretical expectations for the coalescent time of mitochondrial and nuclear genes. If lineage sorting were a purely stochastic process, it would be unlikely that three adjacent parapatric populations [*viatica*19, *viatica*17-south and P24(XY)-east] would retain an identical set of haplotypes, when *viatica*17-east and P24(XY)-north had evolved a different set. More importantly, retention of ancestral polymorphisms through stochastic lineage sorting is more likely to be observed in nuclear genes than mitochondrial genes because the evolution of monophyly is expected to be four times faster for mtDNA than nuclear DNA, owing to the smaller effective population size and uniparental inheritance of mitochondrial genes (Palumbi *et al.* 2001). This theoretical prediction contradicts our present dataset, where each of the three chromosomal races was in reciprocal monophyly with respect to allozyme and nuclear *EF*-1α alleles, but not with respect to mitochondrial *COI* haplotypes. Given the estimated divergence time of the three chromosomal races of >1.0 Ma (see above), it would also seem likely that *viatica*17-south, P24(XY)-east and *viatica*19 populations would show considerable divergence in mtDNA, as this molecule generally has a faster rate of evolution than genic regions of the nuclear genome (Vawter & Brown 1986). Therefore, introgressive hybridization better explains the monophyly of the three chromosomal races with fixed nuclear gene alleles and extensive sharing of *COI* haplotypes in the mid phylogroup among the three populations of *viatica*19, *viatica*17-south, and P24(XY)-east.

5.4.3 Mechanisms of introgressive hybridization

There are at least three mechanisms that might result in introgressive hybridization of mtDNA, namely (i) reproductive asymmetry, (ii) differential selection, and (iii) chance fixation by genetic drift in small populations. First, reproductive asymmetry due to behavioural and ecological differentiation can skew gene flow of maternally inherited mitochondria (*e.g.,* Townsend's and hermit warblers, Rohwer *et al.* 2001).

However, a preliminary study suggested little or no behavioural or ecological evidence for strong mating asymmetry between any pairs of *Vandiemenella* chromosomal races (Mrongovius 1975). Cross-breeding experiments showed that F_1 hybrid individuals could be reared for all combinations of the three chromosomal races on KI, with reciprocal crosses under laboratory conditions (*i.e.*, males and females of each race crossed with males and females of a different race) without apparent differences in mate choice behaviour, insemination rate, embryonic development, and hatchling rate (Mrongovius 1975). In addition, fertility of F_1 hybrid individuals reared from P24(XY) × *viatica*17 crosses and the viability of backcrossed individuals reared from these F_1 hybrids × parental forms appeared to be comparable with the fertility of non-hybrid individuals (Mrongovius 1975). However, reproductive asymmetry and cross-specific fitness reduction of F_2 or later generations (hybrid breakdown) may not have been detected due to experimental limitations, such as small sample sizes. Moreover, it is still unknown whether reproductive asymmetry is also absent in wild *Vandiemenella* populations.

A second mechanism that might result in rapid introgression of genes across hybrid zones is positive selection (Barton & Hewitt 1985; 1989). Although mitochondrial genes are traditionally considered to be selectively neutral or nearly so, it has been suggested that they are often under indirect selection (*e.g.*, by differential fitness reductions between male and female hybrids, known as Haldane's rule, and *Wolbachia*-induced cytoplasmic incompatibility) or direct selection (*e.g.*, cyto-nuclear incompatibility, and selective advantage of mtDNA genes in a local environment) (Gerber *et al.* 2001). Significant results in Tajima's D, Fu's F_s, and R_2 tests suggest either selective sweeps of mtDNA genes or demographic expansion of the *Vandiemenella* chromosomal races; however, mismatch distributions and coalescent simulation analyses suggest demographic expansion for all three *COI* phylogroups (Table 5.1). In addition, similar results were obtained for analyses of an independent nuclear marker (*EF*-1α), suggesting that demographic expansion may be a more likely explanation for the observed disequilibrium patterns.

Finally, owing to the small effective population size of mtDNA, introgression and fixation of mtDNA into foreign taxa may occur via founder effects or drift following hybridization in small populations with little or no gene flow at nuclear loci (Avise *et al.* 1984). Empirical examples for this 'founder hypothesis' include the *Mus musculus/domesticus* species complex (reviewed in Boursot *et al.* 1993) and the Australian *Caledia captiva* grasshopper species complex (reviewed in Arnold *et al.* 1999). Although other mechanisms cannot be ruled out, consistent population expansion patterns inferred through multiple independent analyses in our study support the founder hypothesis as the most plausible explanation for the introgression of the mid *COI* phylogroup in the populations of *viatica*17-south and P24(XY)-east. In the following section, a biogeographic scenario is offered to explain how mtDNA introgression may have occurred.

5.4.4 Historical demography

White (1968; 1978) hypothesized that the distribution of *Vandiemenella* was 'essentially continuous' through the entire process of chromosomal diversification. However, in contrast to this prediction, our molecular studies indicate significant population expansion in neutrality and demographic analyses. Glacial/inter-glacial cycles during the Pleistocene may have lead to a series of population contractions and expansions of the three *Vandiemenella* chromosomal races (Hewitt 1979), resulting in introgression by hybridization of the mid *COI* phylogroup, which subsequently became fixed in small isolated populations with different nuclear backgrounds. The most parsimonious direction of introgression is from *viatica*19 into *viatica*17-south and P24(XY)-east. Less plausible explanations require multiple introgression/replacement events (*e.g.*, introgression from *viatica*17-south to *viatica*19, then to P24(XY)-east and replacement of the mtDNA genome in *viatica*17-east).

It has been well documented that climatic oscillations due to glacial cycles can have significant impacts on population distributions of many species (Hewitt 2004). Glacial cycles would also have affected climate and landscape in southern Australia, where it was generally cooler, drier and much less vegetated during Glacial Maxima

(Hesse *et al.* 2004). The continental shelf around KI is likely to have been exposed repeatedly during Glacial Maxima due to sea level fluctuations, regressing the shoreline approximately 70 km south of KI (near the present 100 m isobath; see Fig. 5.1) (Bowler 1978). At such times, *i.e.* when KI was joined to mainland Australia, dry northwesterly winds from the continental interior are thought to have resulted in a more arid-continental climate on KI (Bowler 1978).

Anecdotal evidence presented in White *et al*. (1969) indicated that *viatica*19 is associated with dense and continuous sclerophyllous (hard-leafed) shrub vegetation primarily found at the interior of KI, while *viatica*17 and P24(XY) are associated with open eucalyptus mallee with scattered low forbs (broad-leafed herbaceous plants) and grasses along the coastline. If these habitat associations are valid and have been reinforced over time by local environmental adaptation, then it is possible that the three chromosomal races altered their distributions in accordance with vegetational shifts during the Pleistocene. For example, during sea-level lows, coastal vegetation would have followed the retreating shoreline, possibly accompanied by the *viatica*17-south population (Fig. 5.1). Similarly, inland sclerophyllous shrub vegetation and the associated *viatica*19 population would have extended southward. These two races would then have formed an historical contact zone south of the present position following the shift of the ecotonal boundary. If hybridizing populations were small, haplotypes of the mid *COI* phylogroup could have been fixed by chance in the *viatica*17 population. Natural processes, such as periodic bushfires, might have facilitated the fixation of foreign mtDNA by creating micro-scale population extinctions and recolonisations. After climate amelioration and sea-level rise, the *viatica*19/*viatica*17 contact zone may have moved northwards, with the expanding front of the *viatica*17 population fixed for haplotypes of the mid *COI* phylogroup. Similar contact zone movement may have resulted in mtDNA introgression into the P24(XY)-east population, although the possible direction of its movement is less clear.

In conclusion, our molecular analyses indicate that there has been differential gene flow of mitochondrial and nuclear gene markers among the three chromosomal races

of *Vandiemenella,* with strong barriers to gene flow among races evident for multiple independent nuclear markers. An important question is whether genetic differentiation of the nuclear markers used in this study is maintained as a direct consequence of restricted gene flow by chromosomal rearrangements or a consequence of multiple genic incompatibilities, which reduce gene flow generally across the nuclear genome, regardless of chromosomal rearrangements. Although initial evidence presented herein seems to favour the latter possibility, additional analyses are needed to test these hypotheses. The development of a series of polymorphic microsatellite markers (Kawakami *et al.* 2007b) and identification of a contact zone between the P24(XY)-east and *viatica*17-east populations offers considerable potential for further investigation of the molecular mechanisms that maintain genetic boundaries among the chromosomal races of *Vandiemenella*.

CHAPTER 6

ALLOPATRY OR STASIPATRY? THE EVOLUTIONARY ORIGIN OF CHROMOSOMAL RACES OF THE AUSTRALIAN MORABINE GRASSHOPPERS (*VANDIEMENELLA*, *VIATICA* SPECIES GROUP)

6.1 Introduction

Whether chromosomal rearrangements promote speciation has been much debated over several decades (White 1978; Sites & Moritz 1987; King 1993; Butlin 2005). White (1968; 1978) argued that chromosomal changes play a causative role in speciation and proposed the 'stasipatric speciation model'. Characteristics of this model include (i) strong selection against chromosomal heterozygotes due to meiotic abnormalities, resulting in a barrier to gene flow between parental and daughter chromosome types, (ii) establishment of new chromosome types due to homozygous advantage, meiotic drive, and genetic drift, and (iii) spread of new chromosome types from their point of origin into the distribution of a parental chromosome type without apparent geographic isolation, leading to parapatric distributions of chromosomal races. In contrast, others view chromosomal rearrangements as incidental by-products of speciation processes (Coyne & Orr 1998) with parapatric distributions resulting from secondary contact. Recent theoretical developments and several experimental studies have suggested that chromosomal rearrangements substantially reduce gene flow and accelerate divergence between populations by suppressing recombination and extending the effects of linked 'isolation genes' or locally adapted alleles (Noor *et al.* 2001b; Rieseberg 2001; Navarro & Barton 2003a; Kirkpatrick & Barton 2006). These theories, referred to as the 'suppressed-recombination models' by Ayala and Coluzzi (2005), have led to a renewed interest in the potential role of chromosomal rearrangements in speciation and environmental adaptation. Although there are a number of chromosomally characterized species complex with parapatric distributions (*e.g.*, insects, Shaw 1981; mammals, Searle 1993; lizards, Sites *et al.* 1995; plants, Rieseberg *et al.* 1999), the frequency and importance of the role of

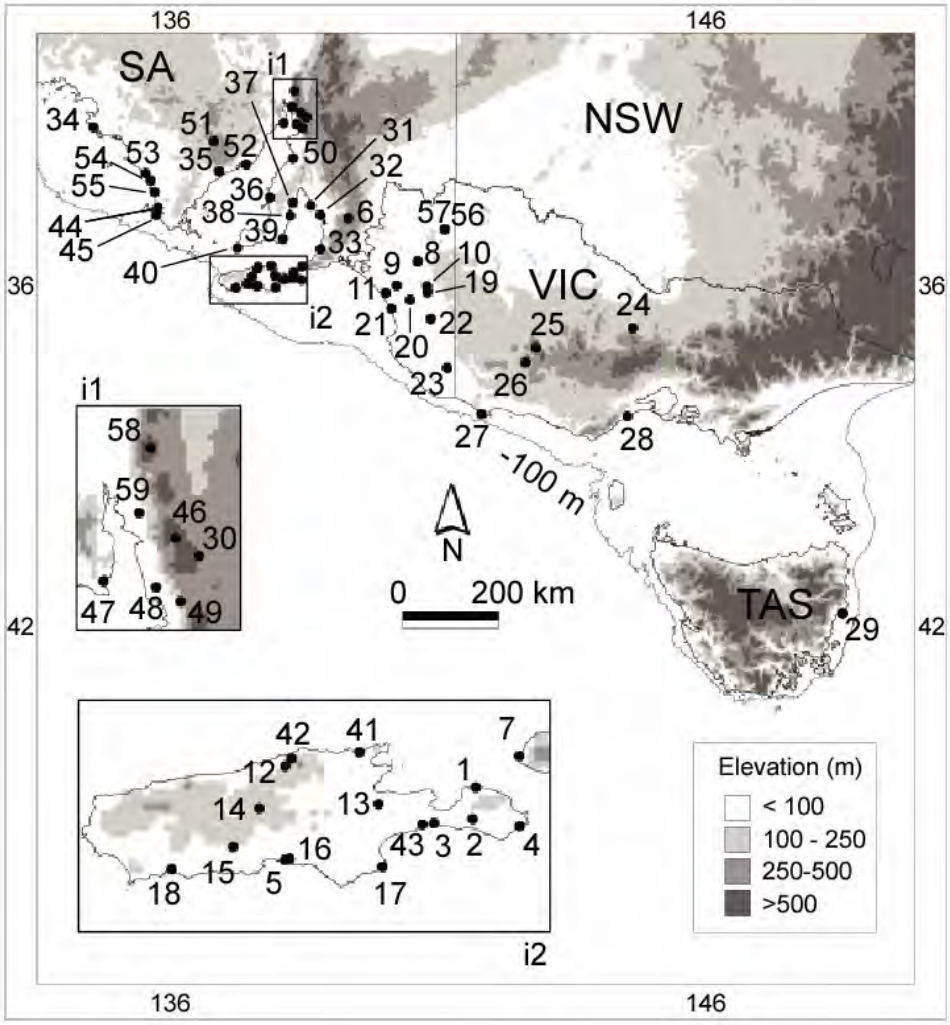
chromosomal rearrangements in speciation and distribution patterns is uncertain (Butlin 2005).

Australian morabine grasshoppers in the genus *Vandiemenella*, known as the *viatica* species group, formed the basis of White's stasipatric speciation model (1968; 1978). The group is distributed across south-eastern South Australia, western New South Wales, coastal Victoria and Tasmania (Fig. 6.1). The group currently comprises two nominal species (*V. pichirichi* and *V. viatica*) and five provisional species (P24, P25, P45b, P45c, and P50; Australian National Insect Collection [ANIC], CSIRO Entomology) (Key 1976). Within many of these species/provisional species, there are also numerous chromosomal races differentiated by sex chromosomes (hereafter *Vandiemenella* species, provisional species, and chromosomal races are called 'taxa'). White *et al*. (1967) hypothesize that *viatica*19 (2n = 19 XO in males) is regarded as the direct descendant of an ancestral species ('*proto-viatica*'), and subsequent sequential chromosomal rearrangement formed the present taxa and their parapatric distributions (Figs. 6.1B and 6.2). Although few morphological characters are available to distinguish the taxa, one notable exception is a characteristic supplementary hook on the male cercus of P24 and P25, which is absent from the remaining taxa (White *et al.* 1967).

White *et al*. (1967) argued that the evolutionary history and origins of parapatric distributions of *Vandiemenella* taxa can be better explained by the stasipatric speciation model than the traditional allopatric speciation model. They postulated that the geographic distribution of the *proto-viatica* was 'essentially' contiguous over the entire period of evolution of the different chromosomal races. A series of new mutant chromosome types associated with hybrid infertility established themselves within the range of the *proto-viatica* and then spread from their point of origin. Such spreads were eventually arrested, forming a hybrid zone between the parental and daughter chromosome forms and ultimately leading to a mosaic pattern in the distribution of chromosomal taxa, each separated by narrow hybrid zones. In contrast, Key (1968) and Hewitt (1979) argue that the *Vandiemenella* taxa evolved in allopatry, with populations isolated following climatic changes during the

Pleistocene. Due to the glacial-interglacial cycles during the Pleistocene, the *proto-viatica* is likely to have experienced cyclic expansions and contractions of its distribution and population fragmentation corresponding to fluctuations of available habitat. New chromosome types may have arisen and become fixed in such fragmented peripheral isolates and spread through a part of the whole range following climate amelioration.

(A)



(B)

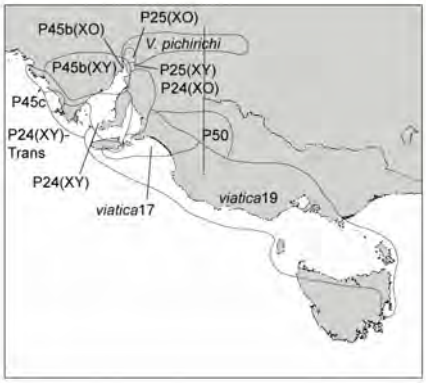
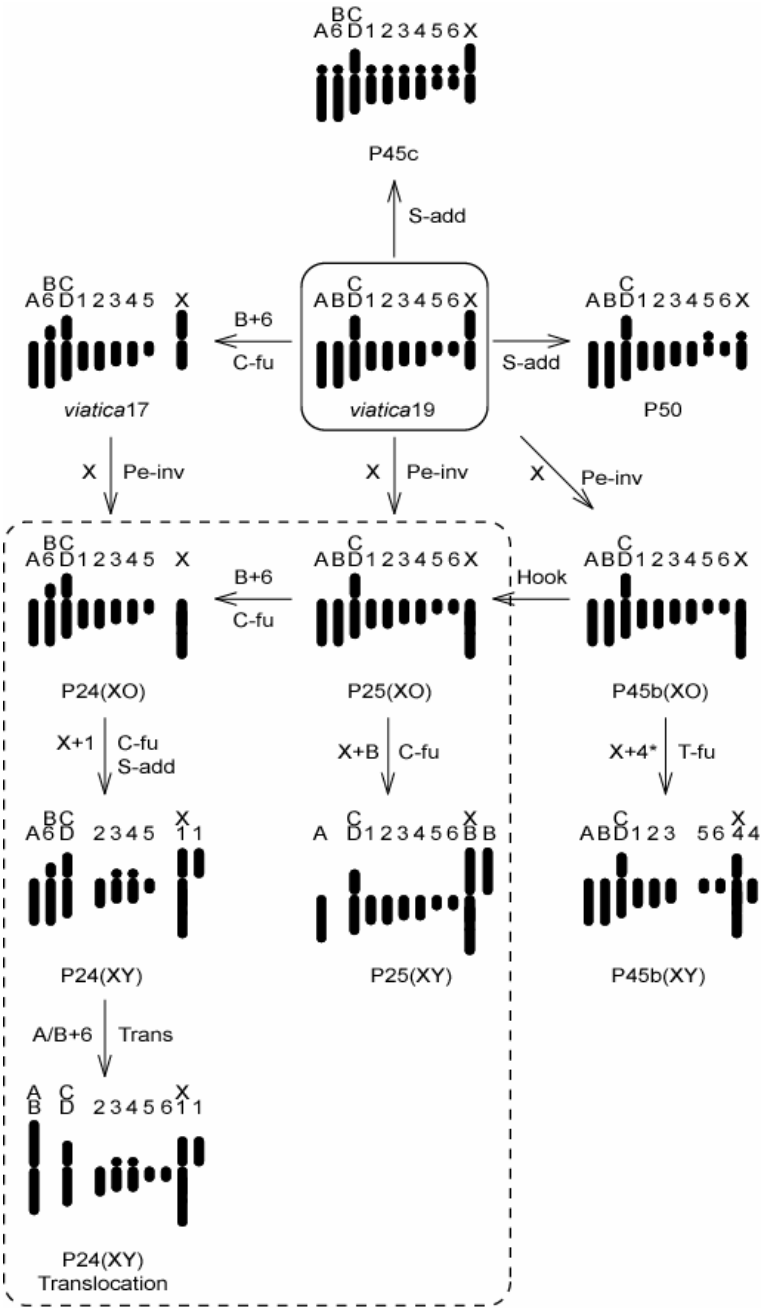


Fig. 6.1 Localities sampled for populations of the *viatica* species group in southeastern Australia. Place names are given in Appendix Table 6.1 for each number (A). Insets are Flinders Ranges (i1) and Kangaroo Island (i2). State abbreviations are NSW (New South Wales), SA (South Australia), VIC (Victoria), and TAS (Tasmania). Parapatric distribution of chromosomal races of the group proposed by White *et al.* (1964; 1967) (B). P24(XY)-Translocation is abbreviated as P24(XY)-Trans.



* X+4 tandem fusion in White *et al.* (1967) but X+6 tandem fusion in White (1978)

Fig. 6.2 Proposed chromosomal evolution of *Vandiemenella* (White 1978). *Viatica*19 represents the presumed ancestral karyotype. Arrows indicate the possible mutational direction: C-fu, centric fusion; T-fu, tandem fusion; Pe-inv, pericentric inversion; Trans, translocation; S-add, short arm addition. A broken square indicates taxa with an extra hook on the male cerci (see the Section 2.2.5). *V. pichirichi* was excluded as there is little information about cytological relationships between *V. pichirichi* and other *Vandiemenella* taxa (but see White 1978).

These two models make contrasting predictions for the biogeographic history and genetic structure of the *V. viatica* species group. Since the stasipatric model assumes a contiguous distribution of the chromosomal taxa, one would expect no evidence for fragmentation of populations within chromosomal taxa. In addition, the stasipatric model predicts that gene flow between chromosomal taxa is significantly reduced at loci closely linked to chromosomal rearrangements but is maintained at other loci (*i.e.*, no genetic differentiation among taxa except for loci closely linked to rearrangements). Moreover, if establishment and spread of new chromosome variants is due to homozygous advantage and meiotic drive, alleles may have spread with the new chromosome variants, showing evidence of selective sweeps. In contrast, if allopatry is the dominant mode of diversification in the *V. viatica* species group, population genetic patterns may indicate past fragmentation events associated with climate change. It has been proposed that the geographic origin of new chromosomal taxa may coincide with the Flinders Ranges and the coastal fringes of South Australia because these areas are likely to have provided refuges during the last glacial maximum (ca. 18 000 years ago) (Hewitt 1979). Physical barriers to gene flow, such as the Murray River and Lake Bungunnia (*e.g.*, Cooper *et al.* 2000), may coincide with abrupt population genetic changes. Moreover, the allopatric model predicts genome-wide divergence associated with the period of separation. Incomplete reproductive isolation may allow break down of such genome-wide divergence by introgression near contact zones after secondary contact. Furthermore, introgression might be restricted in rearranged chromosomal segments in heterozygotes but be maintained outside the rearranged segments if patterns of gene flow after secondary contact follow the suppressed-recombination models.

Putatively neutral markers, such as mitochondrial DNA (mtDNA) sequences, are useful tools to elucidate phylogenetic relationships and phylogeographical variation within species (Avise 2000). However, over-reliance on a single locus mtDNA sequence in phylogenetic and phylogeographic analyses has been problematic because of violations of a basic assumption, in which evolutionary patterns inferred by those single loci are suitable proxies for the evolutionary history of the organisms (Ballard & Whitlock 2004; Sites & Marshall 2004). Population genetic and

phylogeographic analyses using 15 variable allozyme loci, the elongation factor-1α (*EF*-1α) and cytochrome oxidase subunit I (*COI*) gene revealed significant discordance in population genetic structure of the three *Vandiemenella* taxa on Kangaroo Island between mitochondrial and nuclear markers (Kawakami *et al.* 2007a). The analyses of allozyme and *EF*-1α indicate that the three taxa represent genetically distinct groups, whereas the analyses of *COI* show the presence of three geographically localized groups that do not correspond with the distribution of the chromosomal races. Here we broaden these analyses to explore the phylogeography of *Vandiemenella* chromosome races across their range in southern Australia. We have employed a series of independent molecular markers comprising allozyme electrophoretic data, DNA sequence data from the *COI* gene, the nuclear *EF*-1α gene and an anonymous nuclear DNA locus (*Mvia11*) to (i) examine population structure and patterns of gene flow among the *Vandiemenella* taxa to establish whether the chromosomally defined taxa represent genetically distinct units with marked barriers to gene flow, (ii) explore the origin of the parapatric distribution of the *Vandiemenella viatica* species group in relation to vegetation and climatic fluctuations in south-eastern Australia, and (iii) evaluate whether previously proposed species/provisional species represent evolutionarily and taxonomically meaningful units.

6.2 Methods

6.2.1 Taxon sampling

Eleven of the known 12 chromosomal races/species of *Vandiemenella* were collected between 2002 and 2005 from 305 sites in southeastern Australia. A total of 385 sub-samples were selected from 58 sites to cover the entire distributional range and maximise the underlying genetic variation within each taxon (Appendix Table 6.1). Samples of the chromosomal race P25(XO) were not collected despite intensive searching around the area where it had previously been collected in the 1960s (ANIC Specimen Database; http://anic.ento.csiro.au/database/biota_details.aspx?BiotaID=25284). There were five previously reported contact zones in South Australia (three on Kangaroo Island

[pairs of *viatica*19 and *viatica*17, *viatica*19 and P24(XY), and *viatica*17 and P24(XY)], one near Gawler between P24(XO) and P24(XY) and one near Keith between *viatica*19 and *viatica*17) (White *et al.* 1964; 1967; 1969; Mrongovius 1975; 1979). We re-identified one of the contact zones, composed of *viatica*17 and P24(XY) on Kangaroo Island (Kawakami *et al.* 2007a), but were unable to collect any specimens in the remaining contact zones. The majority of grasshoppers were chosen from sites well removed from any known contact zones, while 23 grasshoppers were collected from sites within 1 km of those contact zones (Appendix Table 6.1). In most cases, the specimens were used for cytological, allozyme and DNA sequence analyses. Only males were karyotyped because karypotyping a large number of females was laborious and impractical. We assumed that chromosomal races of females were the same as males collected within a 500 m radius because of the low dispersal ability of this grasshopper (White 1973). Fresh testes were removed from all 147 males and placed in 1 % sodium citrate solution for approximately 10-15 minutes prior to making squash preparations in 2 % aceto-orcein stain. Following the previous study (Kawakami *et al.* 2007a), *Keyacris* sp. P110a was used as an outgroup taxon.

6.2.2 Molecular analyses

Allozyme electrophoresis was undertaken on cellulose acetate gels (Cellogel©, MALTA, Milan) according to the principles and procedures of Richardson *et al.* (1986). A full suite of enzymes was examined on both leg and abdomen homogenates, prepared separately for each individual, to identify suitable genetic markers. Of these, 35 enzymes produced zymograms of sufficient activity and clarity for allozymic interpretation to occur (Appendix Table 6.2). Electrophoretic conditions and staining protocols are detailed in Richardson *et al.* (1986).

DNA was extracted from single hind legs using the PUREGENE® DNA Isolation Kit (GENTRA). The primers used for PCR and direct sequencing of mitochondrial *COI* and nuclear *EF*-1α are described in (Kawakami *et al.* 2007a). Mitochondrial enrichment procedures (Donnellan *et al.* 1999) were used for one individual of each chromosome race to verify the mitochondrial origins of sequences. In addition, 11

individuals of three chromosomal races [*viatica*19, *viatica*17 and P24(XY)] were sequenced at the 3'-end of *COI* using an independent primer pair, C1-J-2183 (forward, 5'-CAA CAT TAA TTT TGA TTT TTT GG-3') and TL2-N3014 (reverse, 5'-TCC AAT GCA CTA ATC TGC CAT ATT A-3') (Simon *et al.* 1994) to check that topologies were identical for the 3' and 5'-ends of *COI*. Primers for an anonymous locus (*Mvia11*) were: G837 (forward, 5'- GGA GCA TTT TGT GTA TTG GAG -3') and G1003 (reverse, 5'- TCT ATT AAA TCA TGG CAG GTG -3'), which were designed from the sequence of a cloned DNA fragment obtained during microsatellite development (Kawakami *et al.* 2007a). Standard PCR amplifications included 1 × reaction buffer (Perkin Elmer), 0.2 mM of each dNTP, 5 pmol of each primer, 1 unit of Amplitaq Gold (Perkin Elmer), 2 mM $MgCl_2$ and 2.5 µL of template DNA (*c*. 50 ng) in a 25 µl reaction volume. PCR-amplifications were carried out for 1 cycle of 94 °C for 9 min and 35 cycles (94 °C, 45 s; 55 °C, 45 s; and 72 °C, 60 s), followed by a final incubation step at 72 °C for 6 min. PCR products were purified using the UltraClean™ PCR Clean-up Kit (MoBio), and sequenced on both strands using the ABI Prism™ Big Dye Terminator Cycle kit in ABI 3700 automated sequencers (Applied Biosystems).

Multiple sequence alignments were performed using Clustal W (Thompson *et al.* 1994). Direct sequencing of *EF*-1α and *Mvia11* resulted in several sites that were heterozygous for single nucleotide polymorphisms (SNPs) or insertions/deletions (indels), which were identified according to Kawakami *et al.* (2007a). Sequence alignments of *EF*-1α and *Mvia11* were then adjusted by eye in the locality of indels. The haplotypic phases of heterozygotes for *EF*-1α and *Mvia11* were inferred from the genotype data of all individuals using the Bayesian statistical methods implemented in the program Phase version 2.1.1 (Stephens *et al.* 2001). Five independent runs were performed, each with 1000 iterations and a burn-in value of 100 to reduce the error of incorrect haplotypic phasing. As the haplotype frequency estimates were consistent, and the goodness-of-fit values were very similar across the five runs, the lengths of the runs were sufficient to obtain reliable results. Only individuals whose haplotypic phase was determined with a posterior probability of ≥ 95% in all five runs were used for subsequent analyses.

To test whether there is evidence for recombination in the *EF*-1α and *Mvia11* fragments, estimates of the minimum number of recombination events (R_m) (Hudson & Kaplan 1985) were obtained in the program DnaSP 4.10.9 (Rozas *et al.* 2003), and the population recombination parameter ($\rho = 4N_e r$) was estimated by the modified version of the composite-likelihood method (Hudson 2001) in the program LDhat 2.0 (McVean *et al.* 2002).

6.2.3 Population genetic analyses

The number of haplotypes/alleles, nucleotide diversity (π), and the number of synomymous/non-synonymous mutations were calculated for *COI*, *EF*-1α and *Mvia11* sequence datasets using DnaSP version 4.10.9 (Rozas *et al.* 2003). To test whether there is significant variation among chromosomally defined taxa relative to variation among sampling sites within them, analysis of molecular variance (AMOVA) was undertaken for allozyme, *COI*, *EF*-1α and *Mvia11* datasets using ARLEQUIN version 3.11 (Excoffier *et al.* 2005). Genetic distance matrices for *COI*, *EF*-1α and *Mvia11* were calculated based on the ML-distance with the best-fit molecular evolution models implemented by Modeltest version 3.7 (Posada & Crandall 1998). Hierarchy levels were defined *a priori* based on karyotype and geographic location, *i.e.*, among the *Vandiemenella* taxa, within taxa between sampling sites and within sites. Significance of Φ_{ST} was evaluated by 10,000 permutations. In addition, AMOVA was repeated for all four datasets using another population hierarchy defined by allozyme clusters (see Section *6.2.*).

To test whether character-based 'chromosomal distances' and geographical distances influenced population structure in *Vandiemenella*, we performed partial Mantel tests using IBDWS version 3.15 (Jensen *et al.* 2005). The chromosomal distance matrix was calculated by pairwise comparison of the presence/absence of a (i) B+6 fusion, (ii) X chromosome inversion, (iii) X+B fusion, (iv) X+1 fusion, (v) X+4 tandem fusion, and (vi) A/B+6 translocation (Fig. 6.2. sequence of the chromosome mutation). The second matrix consisted of log-transformed Euclidean geographic

distances between sampling sites (geographic distance matrix). The third matrix was comprised of pairwise genetic distances between sites (log-transformed): Rogers' genetic distances for allozyme, and Rousset's distance measure Φ_{ST} /(1-Φ_{ST}) based on Kimura-2-Parameter genetic distances for *COI*, *EF*-1α, and *Mvia11* (log-transformed). Rogers' genetic distance was used for allozymes because F_{ST}-based distance measures, which are commonly employed in this sort of analysis (Weir 1990), are not accurately estimated by the small sample sizes per sites. The partial Mantel correlations (*r*) estimate the correlation between the chromosome distance matrix and genetic distance matrix while controlling for the effect of the geographic distance matrix. This procedure allows separation of the effect of chromosomal mutation on genetic isolation from the effect of isolation by geographic distance. Significance of the partial correlation was evaluated by 10 000 permutations.

6.2.4 Tree-based analysis

In addition to population genetic analysis, we used tree-based analyses to characterise whether chromosomally defined *Vandiemenella* taxa consist of genetically congruent groups based on allozyme, *COI*, *EF*-1α and *Mvia11* markers. The allozyme data were analysed using Neighbour-Joining (NJ) and UPGMA analysis. Since these analyses had identical topologies we present only the NJ tree. Rogers' genetic distances among individuals were calculated using all 35 loci. Alternative approaches, such as the continuous maximum-likelihood method were not implemented because of violations of the assumptions (*i.e.*, no fixation or loss of alleles nor any introduction of new alleles through mutation; Felsenstein 1981), and because of the fact that not all allozyme loci were scored for all individuals.

Phylogenetic analyses were conducted for *COI*, *EF*-1α and *Mvia11* separately using maximum parsimony (MP) in PAUP* version 4b10 (Swofford 2000) and Bayesian inference (BI) in MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003). For MP analyses, an unweighted heuristic search, with TBR branch swapping, character state optimisation using the MINF option and random addition of taxa with 10 replications, was performed. The reliability of tree topologies from these analyses was estimated

using the bootstrap with 1000 pseudoreplicates. For BI analyses, the sequence matrix of *EF*-1α was partitioned into two regions, corresponding to exons and introns, for which separate models of evolution were applied to each partition. The best-fit models of molecular evolution for BI were assessed using the Akaike Information Criterion in Modeltest version 3.7 (Posada & Crandall 1998). The best-fit models were a TVM+I+G (six substitution types [Rmat] = 1.59, 13.24 0.78, 0.14 13.24, and1.00; I = 0.601; G = 0.680) for *COI*, TIM+I+G (Rmat = 1.00 1.20 0.35, 0.35, 3.43, and 1.00; I = 0.734; G = 0.858) for the exon region of *EF*-1α, TVM (Rmat = 3.30, 3.93, 0.85, 2.51, 3.93, and 1.00) for the intron region, and TVM+G (Rmat = 0.57, 1.23, 0.32 0.52, 1.23, and 1.00; G = 0.980) for *Mvia11*. BI analyses were conducted on the same data sets with two simultaneous runs of Metropolis-coupled Markov Chain Monte Carlo analysis and four chains for 5 000 000 generations, starting with a random tree and saving one tree every 100 generations. Run length was sufficient to obtain reliable results as the average standard deviation of split frequencies was ~0.0069 in *COI*, ~0.0021 in *EF*-1α and ~0.004 in *Mvia11* datasets at the completion of the runs, suggestive of convergence of the two runs on a stationary distribution. The log likelihood reached stable values by 11 000 generations for *COI*, 283 000 generations for the *EF*-1α, and ~20 000 generations for the *Mvia11* but 25 % of the sampled trees (1 250 000 generations) were excluded as a burn-in to ensure stationarity. Moreover, to examine whether mtDNA haplotypes form geographically coherent clusters, we constructed haplotype networks of *COI* based on the statistical parsimony method using the software TCS version 1.21 (Clement *et al.* 2000).

6.2.5 Clustering analysis

The allozyme data were analysed using distance-based Principal Co-ordinates Analysis (PCoA) and Bayesian model-based clustering analysis to identify genetically homogeneous clusters of individuals without *a priori* knowledge of population units based on morphology or karyotype. First, PCoA was performed in a stepwise manner to increase resolution for detecting major patterns within a multi-locus dataset (Horner & Adams 2007). The stepwise PCoA consisted of a series of PCoAs, starting with the entire dataset (in this case, all 177 individuals of the *Vandiemenella* taxa) and thereafter sequentially removing those individuals that can

be assigned to discrete genetic groups based on the initial PCoA. This cycle of genetic group identification followed by further PCoAs on the reduced subset of individuals was repeated until all subgroups had been recognized and became genetically 'homogeneous' (*i.e.*, no discrete PCoA clusters are apparent within a subgroup, or those present were not diagnosable by any 'fixed' allozyme differences). The operational definition of a 'fixed' difference was relaxed slightly to allow taxa/groups to cumulatively share up to 10 % of their alleles in common (as compared to the 5 % tolerance advocated by Richardson *et al*. (1986). To perform the stepwise PCoA, pairwise matrices of Rogers' genetic distances among individuals were calculated.

In addition to the stepwise PCoA, we used model-based methods implemented in the software STRUCTURE version 2.2 (Pritchard *et al.* 2000), BAPS 5 (Corander *et al.* 2008), and GENELAND (Guillot *et al.* 2005), which cluster individuals on the basis of their genotypes using a Bayesian approach. It has been shown that different methods often infer different numbers of clusters for the same dataset (*e.g*., Frantz *et al.* 2006), so using more than one program is recommended to investigate the spatial genetic structure (Pearse & Crandall 2004). STRUCTURE attempts to find population groupings to minimise deviations from Hardy-Weinberg equilibria or linkage disequilibria between loci within groups. We assumed that the admixture model with independent allele frequencies between clusters is the most appropriate model because (i) *Vandiemenella* taxa were likely to have had mixed ancestry during the course of chromosomal race evolution and formation of parapatric distributions, and (ii) allele frequencies in different parapatric taxa are likely to be reasonably different from each other due to limited hybridization within contact zones. The numbers of clusters (K) were changed from two to 16, and for each K, ten independent replicates of the Markov Chain Monte Carlo (MCMC) of 1 000 000 iterations after a burn-in of 1 000 000 were conducted. To detect the most likely K, we used the method proposed by Evanno *et al.* (2005). Convergence of the MCMC runs were checked by visual inspection of time series plots of log-posterior probability of K (*i.e*., reaching equilibrium) and the consistency of individual assignments between runs. As individual assignments into the best inferred clusters

(K = 13, see results) were consistent across eight replicate runs (*i.e.*, two out of the 10 replicate runs had different individual assignments), we performed 90 extra runs for K = 13 with the same MCMC and burn-in. The mean log-posterior probabilities of K for each of the 100 runs were calculated. The ten runs with the highest log-posterior probability were selected and were aligned using CLUMPP version 1.1 (Jakobsson & Rosenberg 2007) with the LargeKGreedy option and 10 000 repeats to confirm consistency of the individual assignments and calculate the mean of the estimated cluster membership coefficient matrices (Q-matrices). The mean Q-matrices were plotted using DISTRUCT (Rosenberg 2004).

BAPS 5 uses stochastic optimisation to infer the posterior mode of genetic structure, incorporating individual genotype data with geographical information (the locations in which individuals were sampled). Both individual and group analysis modes were used. The program was run 10 times for each of K = 2 - 16 in each mode. Bayesian posterior mean estimates of the proportion of the genome represented by given clusters (posterior allele frequencies) were computed by the admixture analysis option (10 000 iterations, 200 reference individuals). The clustering pattern of individual and group analysis without using geographical information was identical to the group mode with geographical information, so only results with geographical information were shown.

Finally, we used the program GENELAND version 2.0.8 (Guillot *et al.* 2005) to infer K using both genotypic and geographical information. Ten independent runs with 1 000 000 MCMC iterations (with results saved every 100 iterations) consistently inferred K = 8; however, assignment of individuals into clusters varied across replicate runs despite 'good' posterior density distributions in each run, and varying numbers of 'ghost populations' (*i.e.*, clusters with no individuals assigned; Guillot *et al.* 2005) were observed. Since GENELAND did not offer biologically meaningful clustering solutions, we did not include this result.

6.3 Results

6.3.1 Marker variability

A total of 35 putative allozyme loci were successfully scored for the 177 individuals of *Vandiemenella* taxa and three individuals of *Keyacris* P110a from 53 sampling sites screened in this study (allele frequencies in Appendix Table 6.3). The number of haplotypes/alleles, segregating sites (*S*), and nucleotide diversity (π) of *COI*, *EF*-1α, and *Mvia11* are shown in Appendix Table 6.4. Within the *Vandiemenella* taxa, 159 nucleotide sites of the 637 bp *COI* fragment showed variation, and 134 haplotypes were identified from the 254 individuals (Appendix Fig. 6.1). It is most likely that this amplified region is derived from functional mtDNA because (i) all fragments from the *Vandiemenella* taxa and *Keyacris* P110a had an open reading frame without indels and stop codons, (ii) a large proportion of substitutions were synonymous (91 %), (iii) *COI* sequence data from dilutions of the mtDNA enrichments were identical to that from total genomic DNA, and (iv) phylogenetic analyses using 11 sequences of the 3'-end of *COI* showed identical topologies with the 5'-end of *COI*, suggesting an absence of nuclear copies. In this study, all individuals were unambiguously sequenced for *COI* except individuals of P24(XY)-Translocation [hereafter, P24(XY)-Trans] (n = 8), which showed high background noise and several double peaks in sequence chromatograms. Thus, these individuals were excluded from the analyses.

The *EF*-1α fragment included two complete exons and two partial exons, separated by three introns (Kawakami *et al.* 2007a). The fragment length ranges from 805 bp to 837 bp due to indel polymorphisms in the intron regions. Twenty-five individuals with more than one SNP position were found in 227 individuals of the 11 *Vandiemenella* taxa analysed in *EF*-1α. Of these 25 individuals, the haplotypic phase of ten individuals was not determined with a posterior probability of $\geq$ 95 % in the program PHASE, and these individuals were removed from the following analyses. Within the *Vandiemenella* taxa, 105 nucleotide polymorphic sites (45 and 60 sites in the exon and intron regions, respectively) were identified from the remaining 216 individuals, representing 85 alleles (Appendix Fig. 6.2). The estimates of R_m and ρ

were eight per locus [95 % Confidence Interval (CI): 3 - 11] and 1.59 per locus (nonparametric permutation test, $\rho > 0$, P = 0.94). Although a nonparametric permutation test indicated that ρ was not significantly different from 0, the CI of R_m did not include 0, suggesting potential recombination events in the *EF*-1α loci. Consistent with the previous study (Kawakami *et al.* 2007a), our results suggest that this amplified region is derived from a single orthologous functional copy of an *EF*-1α gene because (i) all substitutions in exon regions from the *Vandiemenella* taxa and *Keyacris* P110a had an open reading frame without indels and stop codons, and (ii) there was no evidence of more than two SNPs at any site within a single individual (*i.e.,* only homozygotes or heterozygotes with two alleles were identified).

In *Mvia11*, 15 individuals with more than one SNP position were found in 111 individuals of the 11 *Vandiemenella* taxa analysed. Of these 15 individuals, the haplotypic phase of 11 individuals was not determined with a posterior probability of $\geq$ 95 % in the program. Within the remaining 100 individuals of the *Vandiemenella* taxa, 107 nucleotide sites of the anonymous *Mvia11* fragment showed variation, and 76 alleles were identified (Appendix Fig. 6.3). The fragment length ranges from 511 bp to 643 bp due to indel polymorphisms. The estimates of R_m and ρ were five per locus [95 % Confidence Interval (CI): 1 - 7] and 0.24 per locus (nonparametric permutation test, $\rho > 0$, P = 0.40). Thus, a possibility of recombination events was not completely ruled out. We deem this amplified region to be derived from a single, orthologous, non-protein-coding nuclear DNA region because (i) sequences from *Vandiemenella* and *Keyacris* had several indels and stop codons, with no evidence of any long open reading frames, (ii) there was no evidence of more than two SNPs at any site within a single individual (*i.e.,* only homozygotes or heterozygotes with two alleles were identified), and (iii) a BLAST search did not find any known homologous protein-coding sequences.

6.3.2 Population genetic structure

The Analyses of Molecular Variance (AMOVA) of the *Vandiemenella* populations indicated that a significant but relatively low proportion of the total genetic variation

was distributed among *Vandiemenella* taxa defined by chromosome type for *COI* data (15 %), whereas a moderate proportion of the total genetic variation was distributed among *Vandiemenella* taxa in allozyme, *EF*-1α and *Mvia11* (40 - 65 %) (permutation tests, $P < 0.001$) (Table 6.1). Genetic and geographic distances were found to be significantly correlated for all four types of markers (Table 6.2). This pattern of isolation by distance (IBD) was also significant even after the effect of chromosomal variation was controlled (partial Mantel tests in Table 6.2). For allozyme, *COI*, and *EF*-1α, the general pattern of IBD was clearer when correlation was compared within each of the 11 *Vandiemenella* taxa, whereas it was very weak when correlation was compared between taxa (Fig. 6.3). In addition, there was a significant correlation between the character-based chromosomal distance and genetic distance for all four types of markers, even after the effect of geographic distance was removed (Table 6.2).

Table 6.1 Results of two analyses of molecular variance (AMOVAs) of the *Vandiemenella* populations nested within chromosomal taxa (Chromosome) or allozyme clusters defined by the clustering analyses (Cluster) (Fig. 6.12, Table 6.3). d.f., degrees of freedom; Φ-statistics; and Var (percentage of molecular variance). All the variance components were highly significant (P < 0.001)

		Among taxa/clusters				Within taxa/clusters between populations				Within populations			
Markers		d.f.	Variance	Φ_{CT}	Var	d.f.	Variance	Φ_{SC}	Var	d.f.	Variance	Φ_{ST}	Var
Allozyme	Chromosome	10	2.34	0.396	39.7	41	1.72	0.482	29.1	302	1.84	0.687	31.3
	Cluster	12	2.90	0.558	55.8	38	0.60	0.261	11.6	297	1.70	0.673	32.7
COI	Chromosome	9	1.71	0.153	15.3	43	7.44	0.784	66.4	197	2.05	0.817	18.3
	Cluster	11	2.91	0.255	25.5	34	6.28	0.737	54.9	152	2.24	0.804	19.6
EF-1a	Chromosome	10	4.93	0.650	65.0	46	1.99	0.752	26.3	365	0.66	0.913	8.7
	Cluster	12	6.23	0.803	80.3	37	0.85	0.556	11.0	296	0.68	0.913	8.7
Vv11a	Chromosome	10	13.82	0.508	50.8	34	8.16	0.608	30.0	153	5.25	0.807	19.3
	Cluster	12	21.74	0.762	76.2	28	1.45	0.214	5.1	145	5.32	0.813	18.7

Table 6.2 Results of Mantel and partial Mantel tests of the *Vandiemenella* populations for allozyme, *COI*, *EF*-1α, and *Mvia11*.

		Allozyme		*COI*		*EF* -1α		*Mvia11*	
Distance		*r*	P	*r*	P	*r*	P	*r*	P
Geographic	Mantel	0.314	<0.001	0.274	<0.001	0.257	<0.001	0.274	<0.001
	Partial Mantel[1]	0.296	<0.001	0.271	<0.001	0.251	<0.001	0.249	<0.001
Chromosomal	Mantel	0.444	<0.001	0.136	0.005	0.277	<0.001	0.234	<0.001
	Partial Mantel[2]	0.433	<0.001	0.128	0.010	0.272	<0.001	0.204	<0.001

1: The effect of chromosomal variation was controlled

2: The effect of geographic variation was controlled

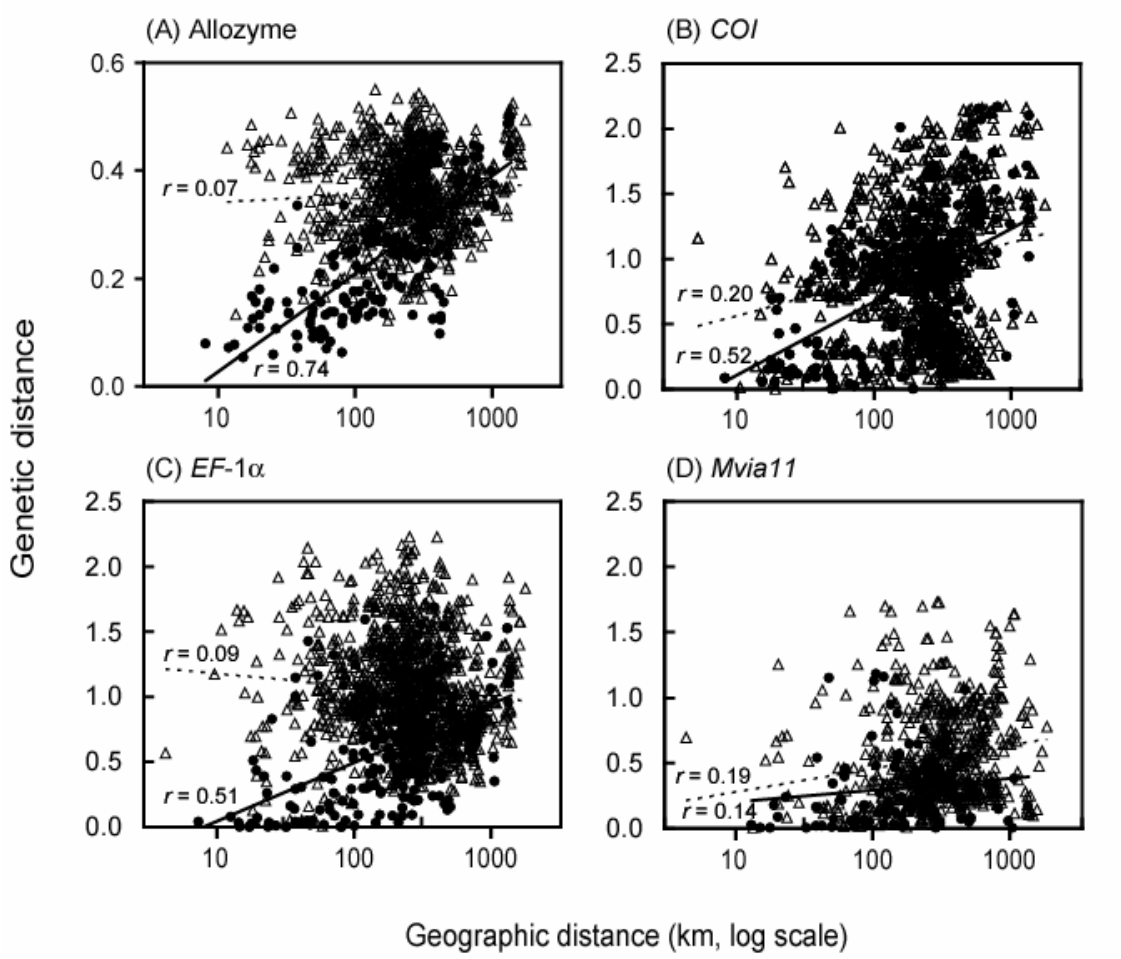


Fig. 6.3 Relationship between genetic distance and geographical distance for allozyme (A), *COI* (B), *EF*-1α (C), and *Mvia11* (D). Genetic distances: Rogers' genetic differentiation (A) and log-transformed Rousset's distance measure F_{ST} /(1-F_{ST}) (B-D) between sampling sites. Geographic distances (km) are log-transformed (A-D). Pairwise comparisions between sites within each of 11 taxa (filled circle) between sites between taxa (open triangle). Corresponding regression lines are indicated: within taxa (solid line) and between taxa (dashed line).

6.3.3 Phylogenetic relationships

The NJ tree based on Rogers' genetic distances between individuals using 35 allozyme loci revealed seven geographically structured clades (Fig. 6.4). (i) P24 and P25 taxa formed a large monophyletic clade. Geographically restricted P24(XY)-Trans and P25(XY) respectively formed monophyletic clades within a large clade, whereas widespread P24(XO) and P24(XY) comprised polyphyletic clades. One individual grouped together with the P24/P25 clade despite the fact that it was collected from the P45c distribution range (site 55). (ii) *Viatica*17 individuals from Kangaroo Island and the adjacent mainland, northwest of the Murray River, formed a second clade (sites 1 - 7). (iii) The third clade comprised individuals of three taxa, namely *viatica*17 (sites 8 - 10), *viatica*19 (sites 19 - 29), and P50 (sites 56 - 57), and all of these individuals were collected from southeast of the Murray River. (iv) *Viatica*19 formed another distinct clade, which was exclusively composed of individuals from Kangaroo Island (sites 12 - 18). (v) A monophyletic clade comprised P45b individuals, within which P45b(XY) was nested within P45b(XO). (vi) P45c formed a distinct monophyletic clade. (vii) *Vandiemenella pichirichi* was resolved as sister taxon of the *viatica*19 population on Kangaroo Island.

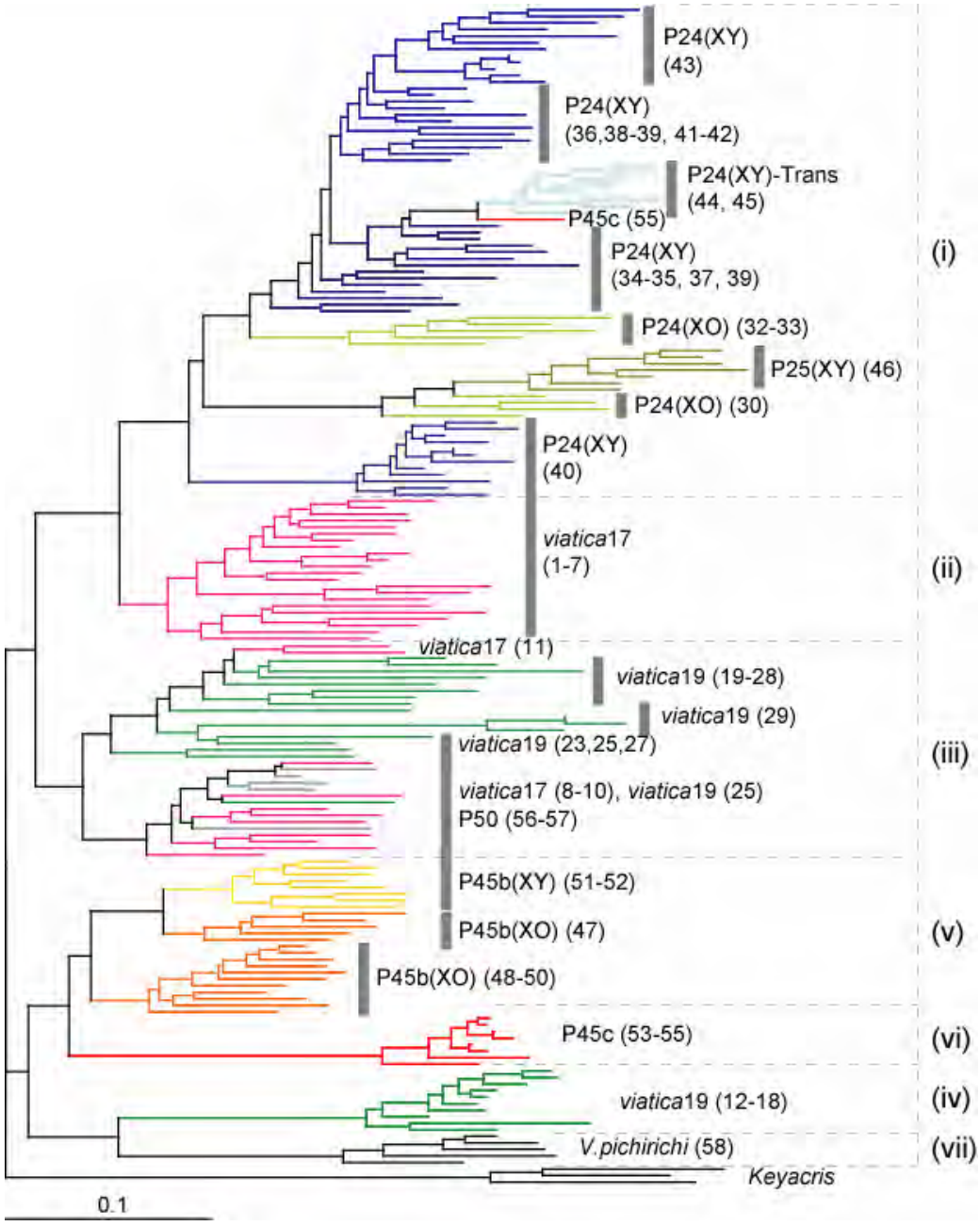


Fig. 6.4 NJ tree based on Rogers' genetic distances using 35 allozyme loci. Branches are coloured for each taxon, and taxon names are indicated with vertical bars. Sample locality numbers are indicated with as in Appendix Table 6.1 and Figure 6.1. Roman numerals in brackets and dotted lines indicate geographically structured clades described in the text.

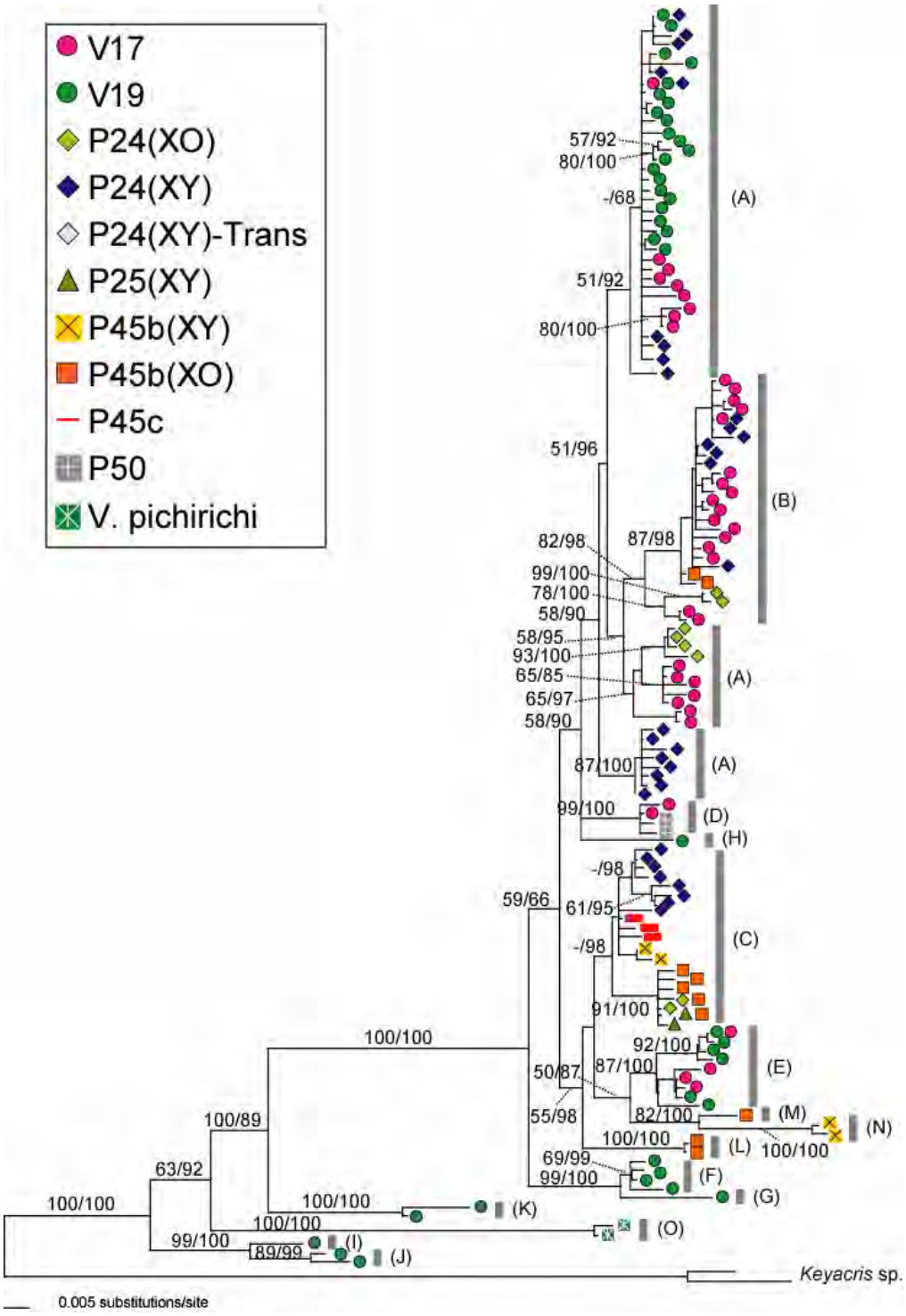


Fig. 6.5 Majority rule consensus phylogram of *COI* based on the last 37 500 trees from Bayesian analyses. Numbers on branches indicate bootstrap support in MP and posterior probability in BI analyses, respectively. (A)-(O) indicate haplotype networks in Fig. 6.6.

It is evident that the *Vandiemenella* chromosomal taxa are incongruent with phylogenetic groupings inferred by MP and BI analyses of *CO1* sequence data (Fig. 6.5). Individuals of *viatica*19 from south-eastern Australia (sites 23-29) had haplotypes that were distantly related to the remaining haplotypes (maximum pairwise genetic distance = 21% between haplotypes found at site 26 and 51). Statistical parsimony analyses of *COI* data revealed geographically coherent groups to some extent, representing 15 disconnected haplotype networks with 95 % confidence (Figs. 6.6 and 6.7). As shown in the bifurcating trees, distantly related haplotypes of *viatica*19 collected from south-eastern Australia comprised networks with from one to two haplotypes (networks G - K). The remaining haplotypes found in *viatica*19 on the mainland Australia formed two haplotype groups (network E and F). One of them (network E) had a haplotype shared with *viatica*17, and these *viatica*19 and *viatica*17 individuals were collected from sites 10 and 19, where these two chromosomal races form a hybrid zone (White *et al.* 1964). Network D was composed of haplotypes found in *viatica*17 and P50 collected from eastern South Australia. Three extended networks (A - C) were composed of haplotypes found in more than two *Vandiemenella* taxa. Network A was primarily composed of populations of *viatica*19, *viatica*17 and P24(XY) on Kangaroo Island [sites 12 - 18 for *viatica*19, site 5 for *viatica*17, and sites 41 - 43 for P24(XY)]. Haplotypes connected by several mutation steps were from adjacent mainland populations of *viatica*17 (sites 9 - 11) and P24(XO) (sites 31 - 32). The network B was largely composed of *viatica*17 individuals collected from eastern Kangaroo Island (sites 1 - 4) and adjacent mainland (sites 6 - 7), but several individuals of P24(XO), P24(XY), and P45b(XO), which were from populations along St. Vincent Gulf and east of Spencer Gulf, had haplotypes belonging to this network. Finally, the network C was composed of haplotypes from Eyre Peninsula [sites 34 - 35 for P24(XY), site 52 for P45b(XY), and sites 53 - 55 for P45c], southern tip of Yorke Peninsula [P24(XY), site 40], and north of Spencer Gulf [P24(XO), site 30; P25(XY), site 46; P45b(XO), site 48 - 49].

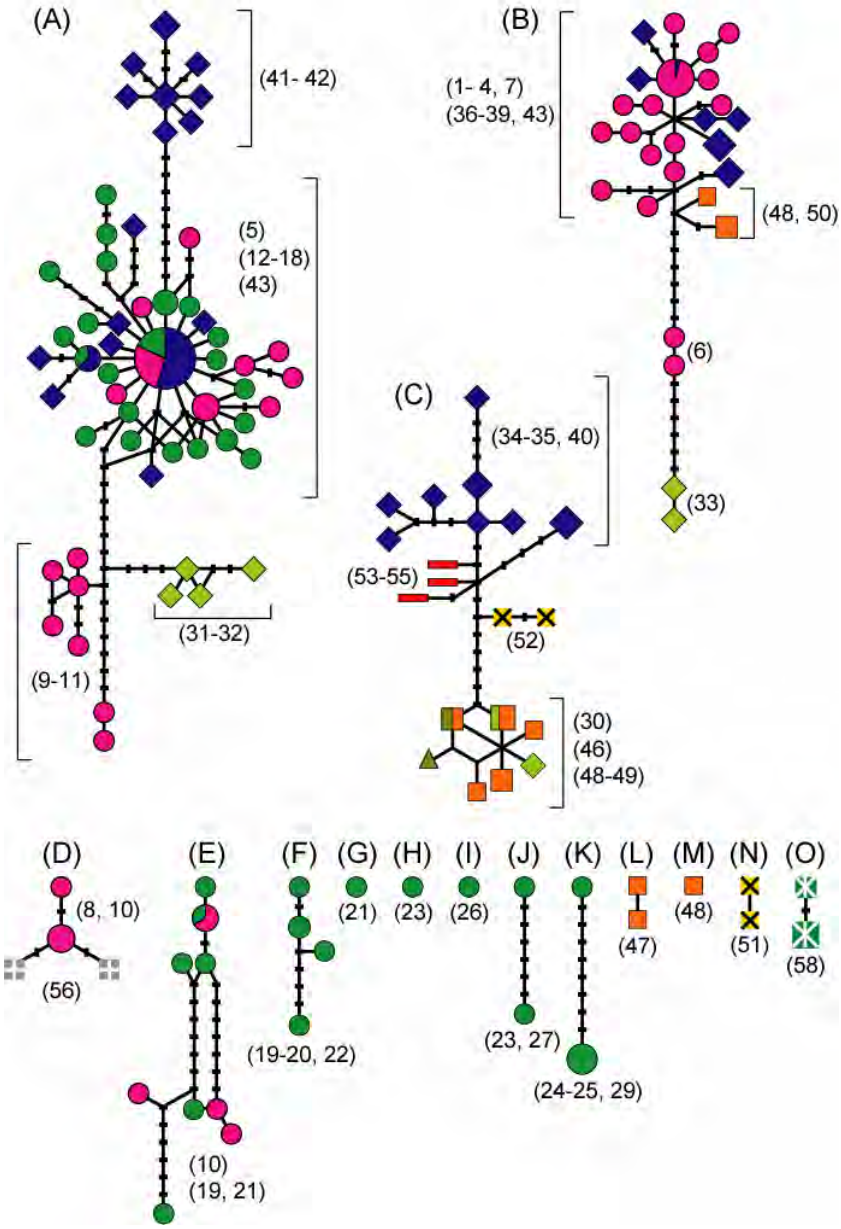


Fig. 6.6 Statistical parsimony network for *COI* for the 11 *Vandiemenella* taxa. Haplotype node area is approximately proportional to the number of individuals contained. Points in the lines represent missing haplotypes. Taxon symbols are the same with Fig. 6.5.

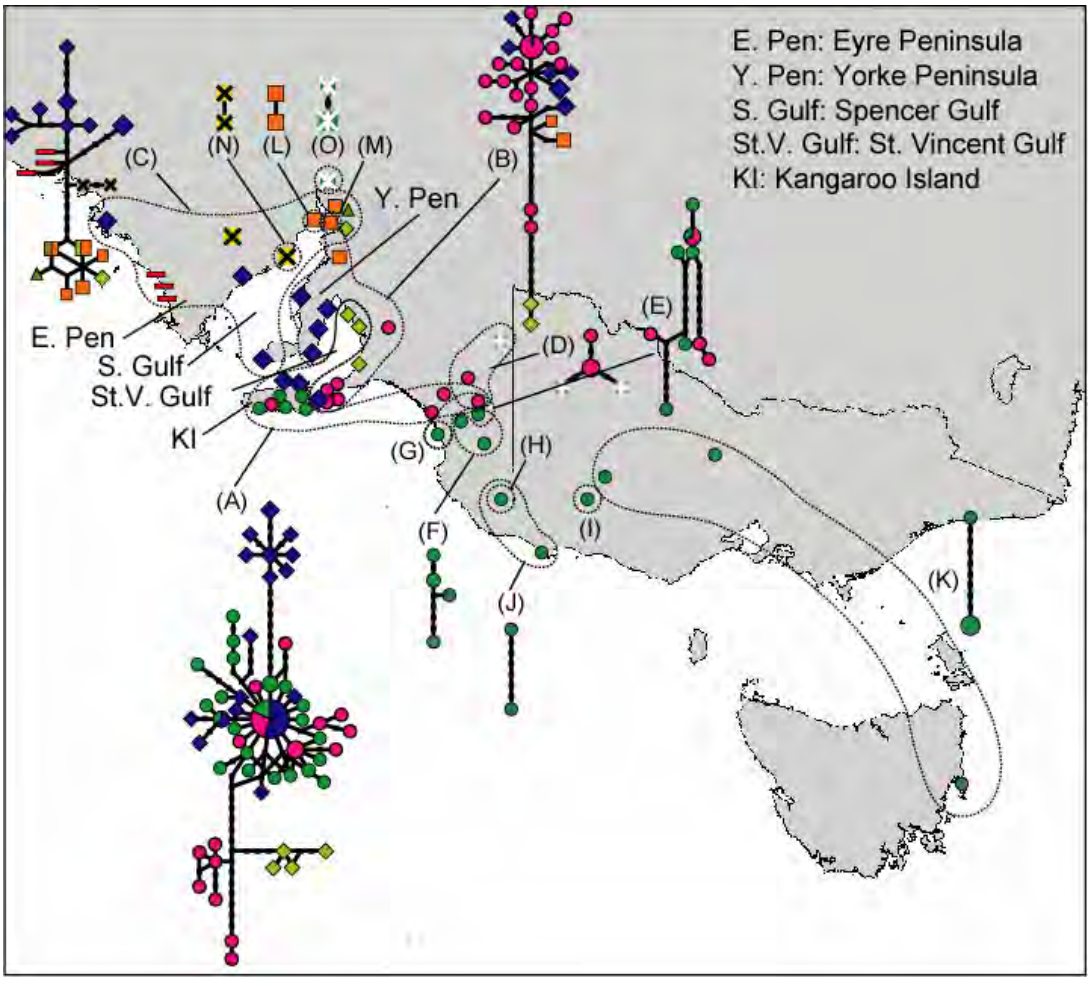


Fig. 6.7 Statistical parsimony network for *COI* for the 11 *Vandiemenella* taxa superimposed on a map. Dotted lines indicate approximate distributions of haplotype groups. Sampling localities are indicated by taxon symbols (see legends in Fig. 6.5).

Phylogenetic analyses of the *EF*-1α sequence data showed that the alleles of several *Vandiemenella* taxa were monophyletic or nearly so (Fig. 6.8). P24(XY)-Trans (sites 44 - 45) and P45b(XY) (sites 51 - 52) formed monophyletic clades without sharing alleles with other taxa, although they were nested within other clades. Nine individuals of P45c (sites 53 - 55) had an identical allele, and five individuals of *V. pichirichi* (site 58) had an identical allele, representing exclusive clades. P24(XY) comprised a nearly monophyletic clade except one haplotype shared with five *viatica*17 individuals, all of which were collected within 1 km from the centre of the contact zone of these two races on Kangaroo Island. Unlike the allozyme NJ tree, P24/P25 taxa did not form a monophyletic clade. P24(XO) and P25(XY) interdigitated among P45b(XO) individuals. Alleles of *viatica*19 and *viatica*17 did not show monophyly, although there were three geographically localised *viatica*19 clades: Kangaroo Island (sites 12 - 18), near the South Australia-Victoria border (sites 23, 27), and Tasmania (site 29).

Phylogenetic analyses of the anonymous *Mvia11* fragment revealed only the monophyly of P45c (sites 53 - 55) (Fig. 6.9). Although monophyly of each taxon of *Vandiemenella* was poorly supported, alleles at *Mvia11* may be broadly grouped into two: one in northwest of the Murray River, composed of *viatica*17 on Kangaroo Island and northeast of Murray River (sites 1 - 6), *viatica*19 on Kangaroo Island (site 17), P24(XO) (sites 32 - 34), P24(XY) (sites 35 - 44), P24(XY)-Trans (sites 45 - 46), P45b(XO) (sites 48 - 50), P45b(XY) (51), and P45c; and one southeast of the Murray River, composed of *viatica*17 (sites 7 - 11), *viatica*19 (sites 19 - 29), and P50 (56 - 57).

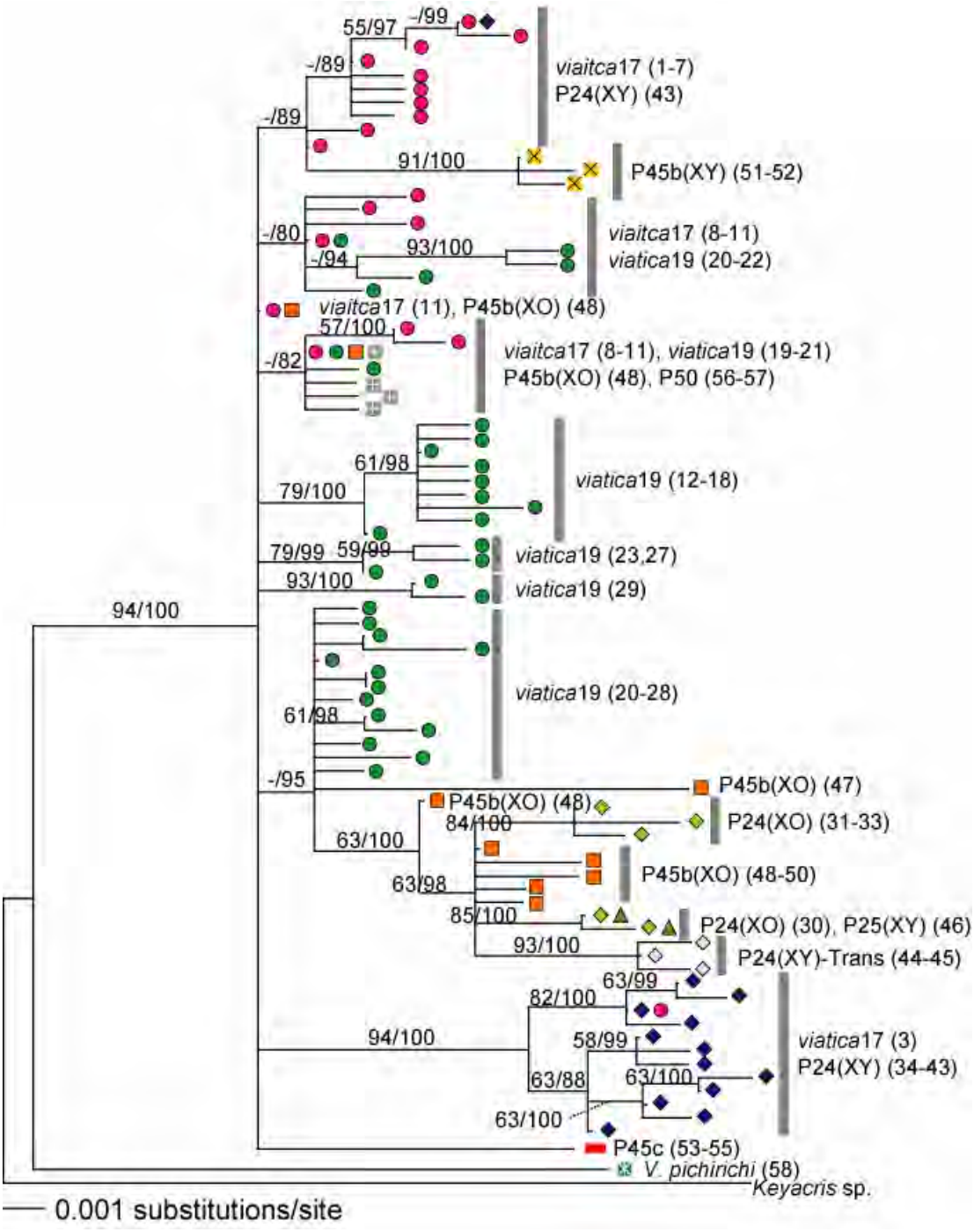


Fig. 6.8 Majority rule consensus phylogram of *EF*-1α based on the last 37 500 trees from Bayesian analyses. Numbers on branches indicate bootstrap support in MP and posterior probability in BI analyses, respectively. Taxon symbols are the same with Fig. 6.5.

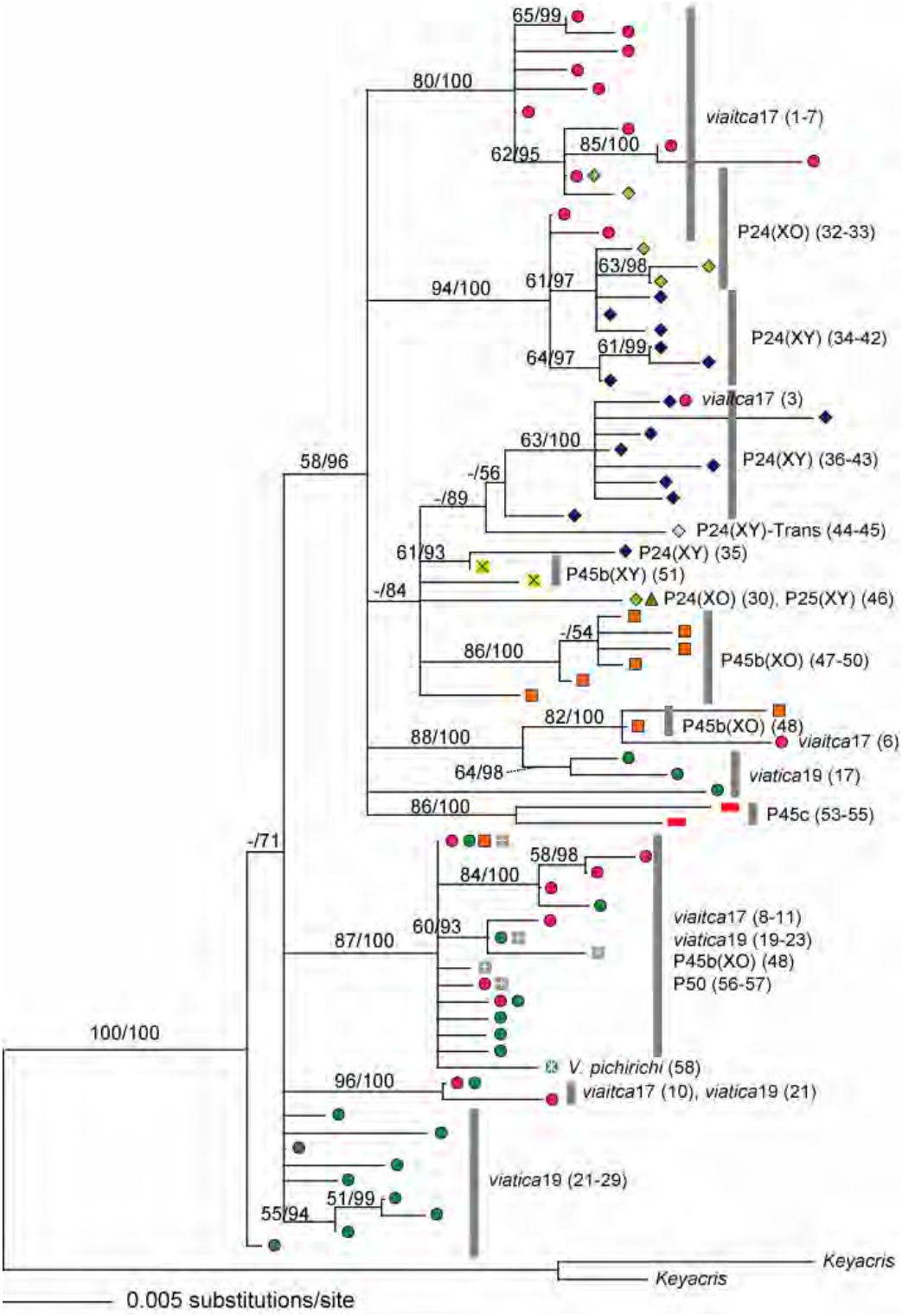


Fig. 6.9 Majority rule consensus phylogram of *Mvia11* based on the last 37 500 trees from Bayesian analyses. Numbers on branches indicate bootstrap support in MP and posterior probability in BI analyses, respectively. Taxon symbols are the same with Fig. 6.5.

6.3.4 Allozyme population clusters

Stepwise Principal Co-ordinate Analysis (PCoAs) comprising three steps resolved the *viatica* species group into 12 clusters (Fig. 6.10). The first-step with all individuals of the *Vandiemenella* taxa resolved the Cluster 1-1 (P24 and P25) and 1-2 (*viatica*, P45b, P45c, P50, and *V. pichirichi*). The second and third steps resolved a total of 12 clusters with each cluster representing distinct taxa with two exceptions (Cluster 3 - 10 and 3 - 11).

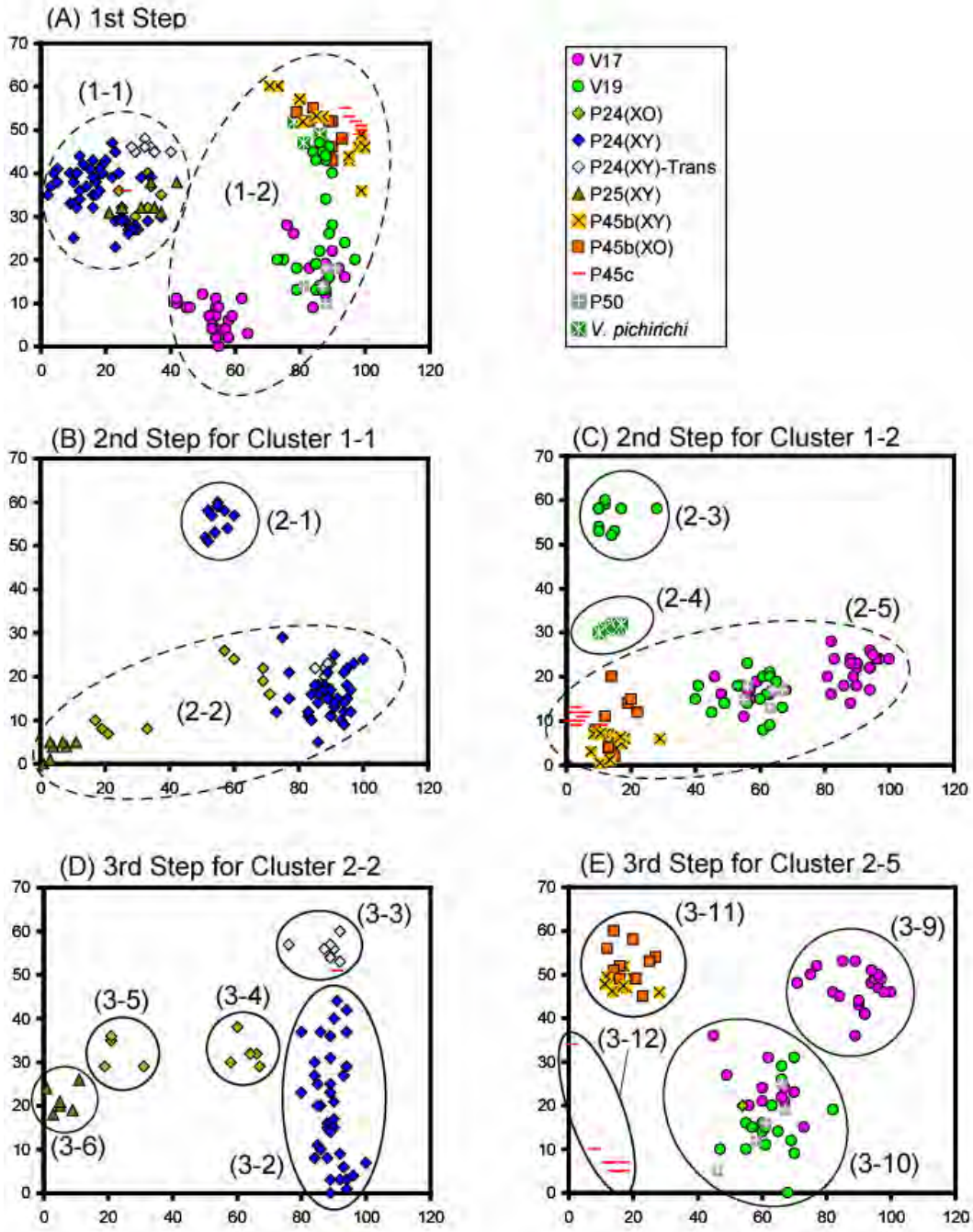


Fig. 6.10 Stepwise Principal Component Analyses (PCoA) based on Rogers' genetic distances using 35 allozyme loci for (A) first-step PCoA with all 11 taxa of the *viatica* species group, (B) second-step PCoA for the cluster 1-1, (C) second-step PCoA for cluster 1-2, (D) third-step PCoA for cluster 2-2, and (E) third-step PCoA for the cluster2-5. X-axis and Y-axis are the first and second principal components. Ellipses denote genetic clusters of individuals. Dashed ellipses denote clusters that are shown in the next step PCoA.

The Bayesian clustering analysis implemented by STRUCTURE resolved 13 clusters within the *Vandiemenella* taxa according to the method proposed by Evanno *et al.* (2005) (Fig. 6.11). These clusters were generally consistent with the clusters inferred by the stepwise PCoA (Fig. 6.12, Table 6.3). However, there were three exceptions; (i) the Cluster 3-5 and 3-6 in the stepwise PCoA were not separated in the STRUCTURE analysis (Cluster 5), (ii) the Cluster 3-10 in the stepwise PCoA was subdivided into two clusters in the STRUCTURE analysis by separating Tasmanian *viatica*19 individuals (Cluster 10) from the mainland sites (Cluster 9), and (iii) the Cluster 3-11 in the stepwise PCoA was subdivided into two clusters in the STRUCTURE analysis (Cluster 11 and 12), corresponding to east and west of Spencer Gulf in South Australia (Fig. 6.13). Posterior probabilities of membership coefficients were moderate to high, but there were several individuals with low posterior probabilities of membership (arrows in Fig. 6.12). Two individuals of *viatica*19 collected from Penola (site 23) were not assigned to any of the 13 clusters with > 50 % posterior probabilities of membership. Notably, an individual from Mt Hope (site 55), which is within the P45c distribution range, was assigned to cluster 3. Cluster 3 was exclusively composed of P24(XY)-Trans individuals, from a population adjacent to the P45c population. As the karyotype of this individual is unknown (females were not karyotyped), it is unclear whether it had a hybrid origin or was a long-distance migrant from the P24(XY)-Trans territory.

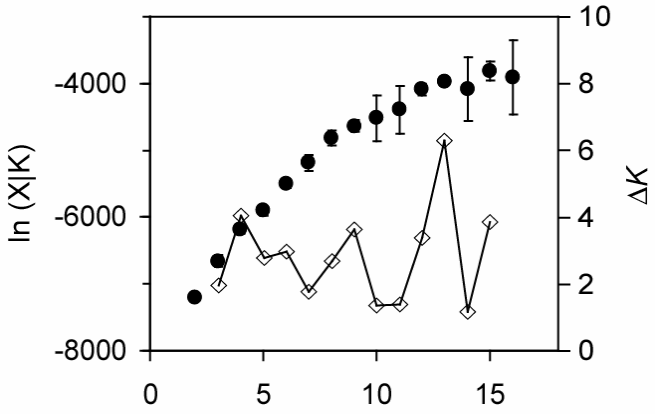


Fig. 6.11 Inference of the number of genetic clusters (*K*) of *Vandiemenella* data based on STRUCTURE. Mean log posterior probability of K, ln(X|*K*) (+-SD), filled circle; Δ*K* according to Evanno *et al.* (2005) (open diamond).

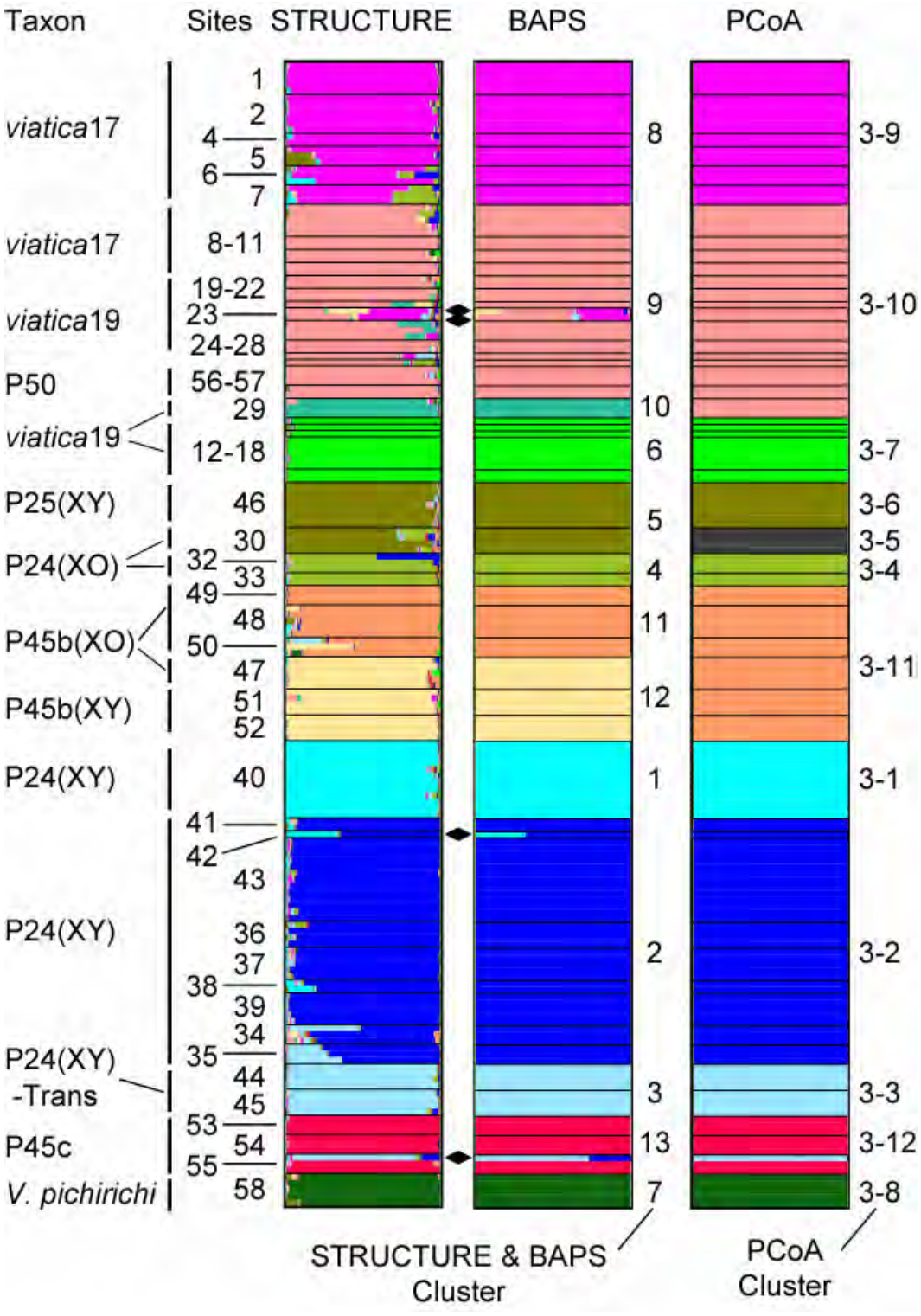


Fig. 6.12 Posterior probabilities of assignment of 177 individuals of *Vandiemenella* taxa using STRUCTURE and BAPS. Taxon names and sampling sites are indicated. Clusters are indicated with different colour, and cluster numbers are indicated. Diamond arrows indicate individuals with low posterior probabilities described in the text. Twelve clusters resolved by the stepwise PCoA (Fig. 6.10) are presented with the same colour codes.

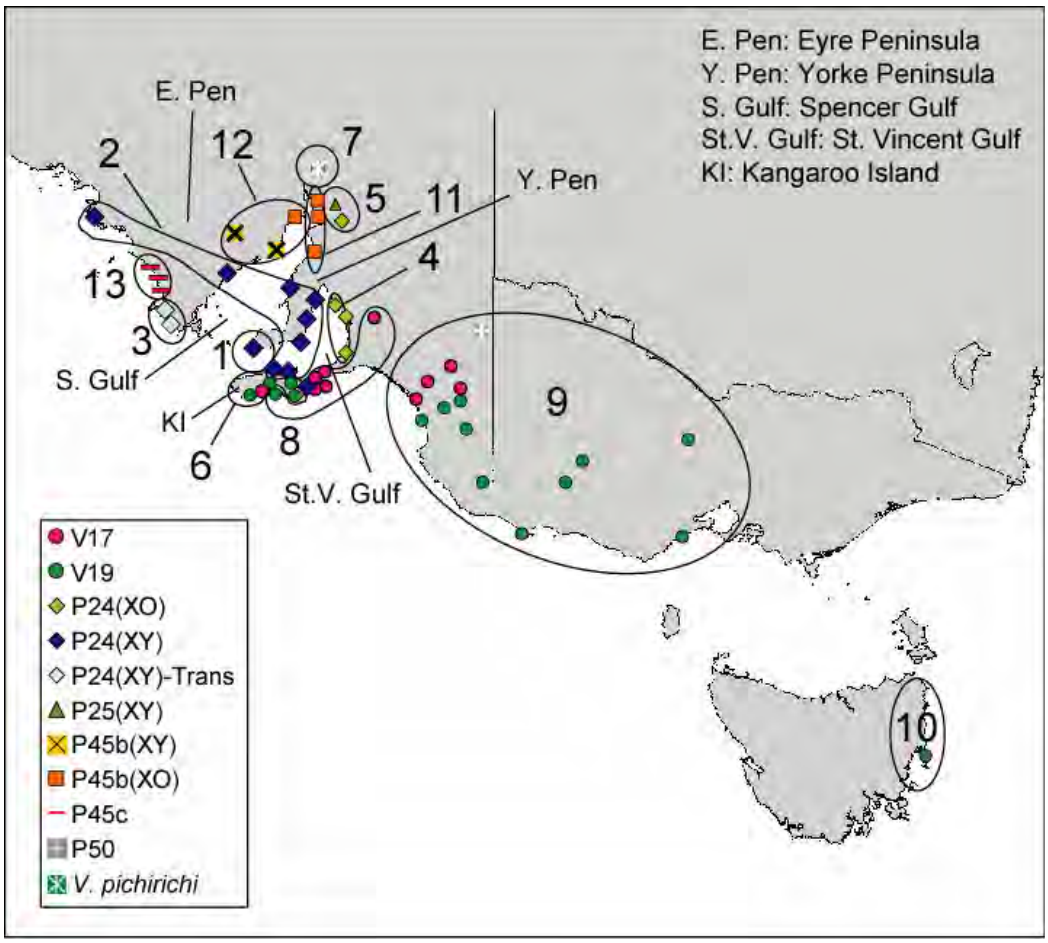


Fig. 6.13 Geographic distribution of 13 allozyme clusters resolved by STRUCTURE and BAPS (Fig. 6.12, Table 6.3). Sample localities are marked with chromosomal taxa.

Table 6.3 Allozyme clusters resolved by stepwise Principal Coordinate Analysis (PCoA), STRUCTURE, and BAPS. Posterior probabilities of membership for each cluster (median and highest and lowest values) are indicated in STRUCTURE and BAPS analyses.

PCoA						STRUCTURE						BAPS					
Cluster (steps)										Probability						Probability	
1st	2nd	3rd	Taxon	Site	*N*	Cluster	Taxa	Site	*N*	Median	(range)	Cluster	Taxa	Site	*N*	Median	(range)
1-1	2-1	3-1	P24(XY)	40	12	1	P24(XY)	40	12	98%	(90, 99%)	1	P24(XY)	40	12	100%	(100%)
	2-2	3-2	P24(XY)	34 - 39	38	2	P24(XY)	34 - 39	38	94%	(51, 98%)	2	P24(XY)	34 - 39	37	100%	(100%)
				41 - 43				41 - 43						41 - 43			
		3-3	P24(XY)-Trans	44 - 45	8	3	P24(XY)-Trans	44 - 45	8	96%	(89, 98%)	3	P24(XY)-Trans	44 - 45	8	100%	(100%)
			P45c	55	1		P45c	55	1	80%	-		P45c	55	1	73%	-
		3-4	P24(XO)	32-33	5	4	P24(XO)	32 - 33	5	94%	(57, 99%)	4	P24(XO)	32 - 33	5	100%	(100%)
		3-5	P24(XO)	30	4	5	P24(XO)	30	4	79%	(72, 95%)	5	P24(XO)	30	4	100%	(100%)
		3-6	P25(XY)	46	7		P25(XY)	46	7	98%	(91, 99%)		P25(XY)	46	7	100%	(100%)
1-2	2-3	3-7	*viatica*19	12 - 18	10	6	*viatica*19	12 - 18	10	98%	(93, 99%)	6	*viatica*19	12 - 18	10	100%	(100%)
	2-4	3-8	*V. pichirichi*	58	5	7	*V. pichirichi*	58	5	97%	(90, 98%)	7	*V. pichirichi*	58	5	100%	(100%)
	2-5	3-9	*viatica*17	1 - 2	22	8	*viatica*17	1 - 2	22	92%	(61, 98%)	8	*viatica*17	1 - 2	22	100%	(100%)
				4 - 7				4 - 7						4 - 7			
		3-10	*viatica*17	8-11	11	9	*viatica*17	8 - 11	11	94%	(82, 98%)	9	*viatica*17	8 - 11	11	100%	(100%)
			*viatica*19	19 - 29	17		*viatica*19	19 - 28	12	88%	(67, 98%)		*viatica*19	19 - 28	12	100%	(100%)
			P50	56 - 57	5		P50	56 - 57	5	97%	(89, 98%)		P50	56 - 57	5	100%	(100%)
						10	*viatica*19	29	3	97%	(84, 98%)	10	*viatica*19	29	3	100%	(100%)
		3-11	P45b(XO)	48 - 50	16	11	P45b(XO)	48 - 50	11	98%	(98, 99%)	11	P45b(XO)	48 - 50	11	100%	(100%)
			P45b(XY)	51 - 52	8	12	P45b(XO)	47	5	90%	(89, 97%)	12	P45b(XO)	47	5	100%	(100%)
							P45b(XY)	51 - 52	8	90%	(84, 98%)		P45b(XY)	51 - 52	8	100%	(100%)
		3-12	P45c	53 - 55	8	13	P45c	53 - 55	8	99%	(91, 99%)	13	P45c	53 - 55	8	100%	(100%)
						N/A	*viatica*19	23	2	40%	(38, 41%)	N/A	*viatica*19	23	2	54%	(47, 61%)
													P24(XY)	43	1	68%	-

N: number of samples

N/A: not assigned to any of the clusters

The BAPS 5 analyses (spatial model with group analysis mode) suggested an identical clustering pattern as the STRUCTURE analysis with *K*=13 (Fig. 6.12, Table 6.3). Posterior allele frequencies of individuals were 100% except four individuals, namely the P24(XY)-Trans-like individual in site 55, two individuals of *viatica*19 in site 23, and one P24(XY) individual collected from site 42 on northern Kangaroo Island. The last is interesting as 68 % of its posterior allele frequencies were from the Cluster 2, while the remaining 32 % were from the Cluster 1, which is exclusively composed of P24(XY) individuals from site 40, an adjacent mainland location. The spatial model with individual analysis mode suggested slightly different clustering solutions with $K = 11$ by putting together Cluster 2 and 4, and Cluster 11 and 12.

Assuming that the most likely number of population clusters is 13, AMOVA was repeated for allozyme, *COI*, *EF*-1α, and *Mvia11* using these 13 clusters as a first population hierarchy level (allozyme clusters). The analyses indicated that significant proportions of the total genetic variations were distributed among the allozyme groups in all four markers (permutation tests, $P < 0.001$) (Table 6.1). Importantly, the proportions of the total genetic variations among the groups increased compared with those when *a priori* defined chromosomal taxa were used. Nei's *D* genetic distances between the allozyme groups range from 0.10 (Cluster 2 and 3) to 0.62 (Cluster 5 and 10), suggesting divergence 0.5 - 3.1 million years (Ma) based on a conventional allozyme clock calibration [divergence time (Ma) = Nei's $D \times 5$] (Nei 1987).

6.4 Discussion

The *Vandiemenella viatica* species group is an unusual species complex as it is composed of numerous distinct chromosomal taxa in parapatry with narrow hybrid zones between them. Since the time White (1968) proposed that chromosomal rearrangements played a causative role in diversification and speciation within this group, the evolutionary origin of the chromosomal taxa and whether they are genetically distinct have not been explored. Our molecular analyses show that the chromosomal taxa of the V. *viatica* species group generally represent genetically distinct units with reduced gene flow at nuclear loci. However, a substantial portion of the total genetic variation was not explained by the chromosomal variation, with a

number of genetically distinct populations being composed of multiple chromosomal taxa (*e.g.*, Cluster 9 in south-east South Australia) and distinct chromosomal taxa (*e.g.*, *viatica*19) being composed of at least two genetically distinct populations (Fig. 6.12, Table 6.3). AMOVA revealed that chromosomal variants explain 40 % - 65 % of the total genetic variation of the nuclear markers, but that the clusters defined by allozyme analyses explained between 56 % and 80 % of the total genetic variation (Table 6.1). Partial Mantel tests showed that populations are highly geographically structured within each of the *Vandiemenella* taxa, whereas this pattern of isolation by distance (IBD) is very weak between the taxa (Fig. 6.3). This genetic structure suggests that the *Vandiemenella* taxa have remained fairly isolated from each other, although they have maintained gene flow at a local scale within each of the taxa. In addition, the significant association detected between chromosomal character distance and genetic distance, which remained significant after removing the effect of geographic distance, indicates that genetic differences are not simply due to IBD. This pattern of isolation by 'chromosomal rearrangements' suggests that factors other than restricted gene flow by IBD, such as chromosomal variations or associated genetic variation, may have contributed to the observed differentiation.

6.4.1 Patterns of differentiation of the Vandiemenella taxa

Phylogenetic analyses based on one mitochondrial and two nuclear loci show some differentiation among the *Vandiemenella* chromosomal taxa, but extensive non-monophyly with shared alleles between the taxa. Including taxa represented by single alleles, only five and one taxa formed monophyletic or nearly monophyletic groups for *EF-1*α and *Mvia11*, respectively. This discordant genetic structure may reflect incomplete lineage sorting of ancestral polymorphisms as well as historical and contemporary gene introgression. Retention of ancestral polymorphisms in nuclear markers is common in recently radiating taxa (*e.g.*, *Drosophila pseudoobscula/persimilis*, Machado & Hey 2003). However, phylogeographic analyses using the three chromosomal taxa on Kangaroo Island demonstrated that there were shared alleles among the taxa mainly in the region of contact zones, suggesting that there is on-going gene introgression across these zones (Kawakami *et al.* 2007a). Gene introgression appears to be particularly extensive for mtDNA in the

V. viatica species group (Kawakami *et al.* 2007a) and other recently radiating species (*e.g.*, Hawaiian crickets, Shaw 2002). To distinguish these two possibilities (*i.e.*, incomplete lineage sorting and gene introgression), fine-scale analyses of contact zones between the chromosomal taxa are necessary. However, the distribution of the *V. viatica* species group is so extensive with a number of taxon pairs that such fine-scale analyses were beyond the scope of the current project.

Individual-level clustering analyses using the allozyme data show that there may be 13 major genetic subdivisions in the *V. viatica* species group, and differentiation attributable to the chromosomal variants is limited to only a few taxa. It should be noted that the inferred number of population clusters may not be accurate because the underlying model in the Bayesian clustering analyses is not well suited to data from populations with IBD (Pritchard *et al.* 2000). However, distance-based PCoA inferred a similar population clustering solution, so the inferred number of population clusters may be close to the 'correct' number. Only four taxa [P24(XY), P24(XY)-Trans, P45c, and *V. pichirichi*] comprise exclusive clusters, whereas the other seven taxa share clusters with another taxon. These patterns were consistent across loci and were unlikely to have resulted from a major effect of a single locus. This notion is further supported by AMOVAs for independent nuclear DNA markers using these 13 allozyme clusters as the first population hierarchy (Table 6.1). The allozyme clusters better explained the total genetic variations of *EF*-1α and *Mvia11* among the groups than *a priori* defined chromosomal taxa, indicating that genetic subdivision suggested by allozymes is also evident for the nuclear DNA sequence markers. Importantly, these 13 clusters are clearly geographically structured (Fig. 6.13). There were four allozyme clusters containing individuals of more than one chromosomal taxon (Cluster 5, 9, 12, and 13), and among these, the distribution ranges of the Cluster 9 and 12 overlapped with contact zones that were previously reported [*viatica*19/*viatica*17 in Cluster 9 and P45b(XO)/P45b(XY) in Cluster 12] (White *et al.* 1964; White *et al.* 1967). Although contact zones have not been reported, distribution of the other two clusters appear to contain the presumed parapatric boundaries [P24(XO)/P25(XY) in Cluster 5 and P45c/P24(XY)-Trans in Cluster 13]. Given incomplete reproductive isolation in some taxon pairs, extensive

gene flow may partly be responsible for the genetic admixture between parapatric taxa.

6.4.2 Ancestral taxon of Vandiemenella

According to White's hypothesis using the stasipatric speciation model, the geographic distribution of the *V. viatica* species group was 'essentially' contiguous through the entire period of chromosomal diversification. On the contrary, Key (1968) and Hewitt (1979) proposed that a series of allopatric fragmentation events may be responsible for fixation of the different chromosomal variants in peripheral isolates. To examine these two conflicting biogeographic scenarios and explore the evolutionary history of the *V. viatica* species group, we start by considering whether *viatica*19 is a direct descendant of an ancestral species ('*proto-viatica*').

Based on comparative cytological studies within the morabine grasshopper subfamily (Morabinae), White (1969; 1973) suggested that the karyotype (2n = 19 XO in males) possessed by *viatica*19 is an ancestral karyotype (Fig. 6.2). Our molecular analyses partially support this hypothesis. First, the allozyme NJ-tree and individual clustering analyses resolved three distinct, geographically separated groups, which are more divergent from one another than any other groups within each of the taxa. Nei's *D* distance between the *viatica*19 population on Kangaroo Island and the mainland population (Clusters 6 and 9, respectively in Table 6.3) is 0.33, which gives estimated divergence times of approximately 1.7 Ma, using a conventional allozyme clock calibration (Nei 1987). The *viatica*19 population in Tasmania (Cluster 10) may have diverged from the mainland population about 1.3 Ma (Nei's $D = 0.26$), although this estimate should be taken with caution due to the small sample size. In contrast, Nei's *D* distances between the two clusters containing *viatica*17 (Clusters 8 and 9) is 0.14 (divergence time = 0.7 Ma), and those between the two clusters containing P24(XY) (Clusters 1 and 2) is 0.20 (divergence time = 1.0 Ma). Second, genetic diversity is the highest in *viatica*19 in allozymes (the number of alleles and gene diversity), *COI* (the number of haplotypes and nucleotide diversity), and *EF*-1α (the number of haplotypes and nucleotide diversity) (Appendix Tables 6.3 and 6.4). Finally, although topologies are polyphyletic, alleles of *viatica*19 tend to be resolved

as basal in the *COI* and *Mvia11* gene trees relative to alleles from the other chromosomal taxa. Taken overall, there is good evidence that *viatica*19 is an ancestral lineage and represents the ancestral karyotype in support of the hypothesis of White.

6.4.3 Allopatric fragmentation

An obvious difference in biogeographic configuration between the stasipatric and allopatric mode of diversification of the *V. viatica* species group is whether the distribution has been contiguous or fragmented. Assuming that *viatica*19 represents the direct descendant of an ancestral *proto-viatica*, the existence of highly divergent populations and the wide distribution of *viatica*19 suggests repeated population fragmentation of the ancestral population. As discussed above, the approximate divergence time based on Nei's *D* distance and the allozyme clock calibration has shown that the *viatica*19 populations on Kangaroo Island and Tasmania have been isolated from the mainland population of *viatica*19 for a prolonged period (1.4 - 1.7 Ma). In addition, an inferred substitution rate of the *EF*-1α exon region in *Papilio* butterflies of 1.3 - 2.0 × 10^{-9} per site per year (Zakharov *et al.* 2004) would give estimated divergence times 1.8 - 2.7 Ma between the mainland and Tasmanian populations, and 2.1 - 3.2 Ma between the mainland and Kangaroo Island populations. Moreover, a conventional molecular clock calibration for *COI* in insects (2.0 × 10^{-8} per site per year) would give estimated divergence times of the three *viatica*19 populations as 3.6 - 6.0 Ma. These estimated divergence times should be treated with caution because substitution rates may be significantly different between distantly related organisms (Pulquerio & Nichols 2007), and the coalescent time for the haplotypes will be greater than the divergence time for the populations. However, it seems reasonable to conclude that the pronounced genetic footprint of this isolation at allozyme, nuclear and mtDNA sequence loci reflect historical allopatric fragmentations of the ancestral *viatica*19 poulations that might date back to the late Pliocene or early Pleistocene. White *et al.* (1967, p. 292) suggest that *viatica*19 colonized Tasmania after the Last Glacial Maximum because 'at the height of the Pleistocene glaciation Tasmania was too cold and wet to support populations of any species of morabine grasshopper'; however, our molecular analyses imply a long-

term persistence of *viatica*19 in potential glacial refugia in Tasmania. Such glacial refugia in Tasmania are suggested for several organisms (*e.g.*, Eucalypts, McKinnon *et al.* 2004). Therefore, presumed ancestral *viatica*19 taxon seems to have had a wide distribution including presently isolated islands but has experienced a series of population fragmentations, which resulted in distinct genetic differences among those island populations and the mainland population.

Similar to the *viatica*19 populations, two distinct clusters, one northwest of the Murray River including Kangaroo Island and the other southeast of the Murray River, were resolved within *viatica*17 for the allozyme dataset (and less clearly in two nuclear sequence datasets). Allozyme and the *EF*-1α datasets suggest a long term isolation of these two populations (0.7 Ma in allozyme and 2.4 - 3.6 Ma in *EF*-1α). Furthermore, White *et al.* (1967) reported that these two populations can be clearly distinguishable by the length of short arms in five acrocentric autosomes, in which pericentric inversions are probably involved, providing additional support. Therefore, our molecular analyses and the cytological differences support the hypothesis that these two populations have evolved independently for a long time. A number of other species show such separation (*e.g.*, dunnarts, *Sminthopsis crassicaudata*, Cooper *et al.* 2000; lizard, *Tiliqua rugosa*, Cooper, unpublished data). These studies suggest that an ancient mega-lake, called Lake Bungunnia, occupying the Western Murray Basin about 3.5 - 0.7 Ma, and the periodic flooding of the Murray River due to increased discharge from the Great Divide during glacial maxima in the Pleistocene (Bowler *et al.* 2006), may have acted as a physical barrier to gene flow. It has been suggested that the contact zone between *viatica*19 and *viatica*17 on the mainland (near Keith, South Australia) was once contiguous to the one on Kangaroo Island during sea-level lows (White *et al.* 1969); however, significant population genetic differentiation within each of the taxa suggest that these two contact zones have an independent origin.

6.4.4 Historical biogeography

Repeated climatic oscillations due to glacial cycles during the Pleistocene would have affected landscape and population distributions of many species (Hewitt 2004). In southern Australia, it is suggested that climate was generally cooler and drier and the land was much less vegetated during glacial maxima (Bowler 1982; Hesse *et al.* 2004). It is likely that the gulfs and straits have become dry land, and the continental shelf has been exposed repeatedly during glacial maxima due to sea level fluctuations (near the present 100 m isobath; see Fig. 6.1). Drastic fluctuations in temperature (particularly the impact of severe frosts) and aridity during this period are likely to have significantly altered the distribution of many species (*e.g.*, skink, *Egernia whitii*, Chapple *et al.* 2004; Australian magpie, *Gymnorhina tibicen*, Toon *et al.* 2007). At such times during glacial maxima, dry northwesterly winds from the continental interior are thought to have resulted in a more arid-continental climate in southern Australia (Bowler 1978). However, substantial regional climatic variations are evident particularly at the southern Flinders Ranges, where geological evidence suggests the presence of wetlands during the Last Glacial Maximum (Williams *et al.* 2001), which might have provided refugia for wetland and surrounding grassland dependent organisms.

The *V. viatica* species group tends to occur in coastal heath habitats, composed of sclerophyllous (hard-leafed) shrubs, open *Eucalyptus* mallee with scattered low forbs (broad-leafed herbaceous plants), and sand dunes (White *et al.* 1964; 1967). If these habitat associations are valid, it is possible that the species altered its distribution and populations were fragmented in accordance with vegetational shifts and marine incursions during the Pleistocene. These distribution shifts and population fragmentation events might have facilitated fixation of different cytological traits in isolated populations and promoted genetic divergence between populations.

Several *viatica*19 populations characterized by allozyme clusters (Clusters 6, 9, and 10) and cytologically similar taxa, such as P45c and *V. pichirichi* (Clusters 13 and 7, respectively), appear to be relics of the early allopatric fragmentation of the ancestral *proto-viatica*. Since P50 has substantially the same karyotype as *viatica*19, and both

taxa are adjacent to each other on the mainland, P50 seems to be a local chromosomal variant of *viatica*19. *Viatica*17 may have arisen from the *proto-viatica* in allopatry with subsequent gene introgression across the hybrid zone in the mainland following secondary contact. However, the northwestern population of *viatica*17, separated by the Murray River, has not been exposed to such gene introgression possibly from *viatica*19. P24/P25 and P45b may each be associated with two separate putative refugia at an early stage of the diversification of the *Vandiemenella* group, and the subsequent isolation may have resulted in acquisition of further chromosomal polymorphisms (*i.e.*, XY sex chromosome systems and A/B+6 translocation). Given their present distribution, the geographic origin of these daughter taxa may coincide with the Flinders Ranges, which is likely to have provided refugia during glacial maxima (Hewitt 1979). Although a detailed chronological order and geographic locations of these isolating events are unclear, our documentation of a possible biogeographic barrier and presumed location of refugia provides testable hypotheses in a phylogeographic framework by fine-scale sampling of *Vandiemenella* grasshoppers in southern Victoria and Tasmania, and near the Flinders Ranges.

In summary, our molecular analyses do not support the stasipatric speciation model and favour the allopatric mode of diversification for the evolution of *Vandiemenella*. The population genetic pattern indicates past fragmentation events associated with climate change and landscape evolution, such as the formation of Lake Bungunnia. Divergence of the 13 genetic clusters inferred by our allozyme dataset does not appear to be restricted to loci closely linked to chromosomal rearrangements but is probably genome-wide. Such genome-wide divergence is not predicted by the stasipatric model but may be better explained by the allopatric model. New chromosomal variants may have fixed in allopatry, but gene exchange may have been maintained at narrow contact zones after secondary contact. The inferred biogeographic history of the *V. viatica* species group is still inconclusive, but, at least, the present study demonstrates the inappropriateness of the stasipatric speciation model in the group.

6.4.5 Taxonomic implications

Allozyme clustering analyses supported by independent sequence markers indicate 13 genetic subdivisions in the *V. viatica* species group, but only three of these subdivisions are exclusive to each of the chromosomal taxa [P24(XY)-Trans, P45c, and *V. pichirichi*]. As eight other chromosomal taxa comprise either more than one allozyme cluster or clusters shared with other races, chromosomal variation alone is not sufficient to define species or subspecies. There may be long-term barriers to gene exchange between the 13 allozyme clusters that appear to have been maintained to accumulate genetic differentiation at multiple loci despite some evidence for introgression near contact zones. The 13 clusters represent diagnosable units based on genetics and in some cases chromosomes [P24(XY)-Trans, P45c, and *V. pichirichi*] and morphology (*V. pichirichi*) (Key 1976). Some ecological differences are also likely to occur. For example, White *et al*. (1969) suggest that *viatica*19 is associated with dense and continuous sclerophyllous shrub vegetation, while *viatica*17 and P24(XY) are associated with open eucalyptus with scattered low forbs and grasses on Kangaroo Island. Therefore, all of these attributes provides a basis for recognising them as 13 evolutionarily significant units (ESUs) under several different criteria (Ryder 1986; Avise 1989; Moritz 1994; Crandall *et al.* 2000). Among these putative ESUs, the only one that can be considered a distinct species is *V. pichirichi,* because it could clearly be distinguished from the other *V. viatica* species group based on chromosomes, morphology, and molecular markers. Nonetheless, phylogenetic analyses of the *COI* and *Mvia11* datasets suggested that *V. pichirichi* was paraphyletic within the *V. viatica* species group, while *EF*-1α showed *V. pichrichi* to be a sister lineage to the *V. viatica* species group. Overall, our genetic analyses support the distinct species status of *V. pichirichi*; however, it is possible that the other provisional species of *Vandiemenella* chromosomal taxa (*e.g*., P24 and P25) may maintain gene flow with other taxa and/or populations, and, therefore, most of them are unlikely to represent distinct species under a biological species concept.

CHAPTER 7
Discussion and Conclusions

7.1 Conclusions

The research in this thesis was motivated by the long-standing debate of whether chromosomal rearrangements play a causative role in speciation. Morabine grasshoppers of the *Vandiemenella viatica* species group formed the basis of the development of one of the traditional chromosomal speciation models (the stasipatric speciation model, White *et al.* 1967; White 1968); however, a potential role of chromosomal rearrangement in the diversification of the *viatica* species group has not been explicitly tested. In addition, a number of questions have remained open since the *viatica* species group was discovered in 1960s: Do chromosomal races represent genetically distinct taxa? Do chromosomal variants provide taxonomically meaningful characters? Are hybrid zones primary or secondary contacts? Do hybrid zones move? How much does gene flow occur between the races? Are barriers to gene flow, if any, associated with the rearranged chromosomes? Moreover, previous cytological and morphological studies during the 1960s and 1970s have generated several historical biogeographic scenarios, but these have not been tested using modern molecular genetic and new analytical methods. This thesis explored a number of these questions in Chapter 4, 5 and 6.

Chapter 4 investigated patterns of gene flow between P24(XY) and *viatica*17 on Kangaroo Island (KI) using multiple molecular markers. Karyotypes and 11 nuclear markers revealed a remarkably narrow (width < 350 m) hybrid zone with substantial linkage disequilibrium and strong deficits of heterozygotes. Widths and centre positions of these nuclear markers and chromosome marker are concordant and coincident respectively, suggesting that selection is unlikely to be concentrated on a few chromosomes. In contrast, mitochondrial *COI* markers showed a significantly wider (911 m) cline width and offset centre [about 400 m offset toward the P24(XY) side]. We argue that the discordance between the mitochondrial cline and the nuclear

and chromosomal clines suggests a secondary origin of the contact zone and potential movement of the zone after the contact.

Chapter 5 used molecular data of three chromosomal races of *Vandiemenella* [*viatica*19, *viatica*17, and P24(XY)] on KI to investigate the extent to which chromosomal variation among these populations may be associated with barriers to gene flow. Population genetic and phylogeographic analyses using 15 allozyme loci and the *EF*-1α gene indicated that the three races represent genetically distinct taxa. In contrast, analyses of the mitochondrial *COI* show the presence of three distinctive and geographically localised groups that do not correspond with the distribution of the chromosomal races. These discordant population genetic patterns are likely to result from introgressive hybridisation between the chromosomal races and range expansions/contractions. Overall, these results suggest that reduction of nuclear gene flow may be associated with chromosomal variation or underlying genetic variation linked with chromosomal variation, whereas mitochondrial gene flow appears to be independent of those variations in these morabine grasshoppers.

Chapter 6 described broad phylogeographic patterns and the origin of parapatric distributions of 11 *Vandiemenella* chromosome races/species across their entire range in southern Australia, using 35 allozyme, two nuclear DNA sequence (*EF-1*α and an anonymous locus), and one mitochondrial (*COI*) markers. These molecular analyses show that the *Vandiemenella* chromosome races/species generally represent genetically distinct units with reduced gene flow at nuclear loci. However, a substantial portion of the total genetic variation was not explained by the chromosomal variation, with a total of 13 genetically distinct populations being composed of multiple chromosome races. Evolutionary history of these genetic populations may be better explained by the allopatric speciation model than the stasipatric speciation model. In addition, our molecular analyses indicate that most of the previously proposed species and provisional species defined primarily by chromosomal variation do not represent evolutionarily and taxonomically meaningful units.

Overall, although the *Vandiemenella viatica* species group forms the basis of the stasipatric speciation model, our molecular analyses do not support the stasipatric speciation model and favour the allopatric mode of diversification for the evolution of the *Vandiemenella*. Patterns of genetic differentiation between the *Vandiemenella* chromosomal taxa analysed at three different spatial scales show dynamic responses of the grasshoppers to climatic fluctuations, leading to opportunities for long-term isolation and allopatric fixation of new chromosome variants and molecular mutations in many loci. They may have repeatedly experienced population expansions and contractions in accord with long-term climatic fluctuations and, consequently, have formed parapatric distributions by secondary contact. Some chromosomal races represent genetically and cytologically distinct taxa, while others are genetically indistinguishable from adjacent chromosomal races based on allozyme, mtDNA and nucDNA markers, suggesting that incomplete reproductive isolation in some taxon pairs allowed gene exchanges across their parapatric boundaries. The level of gene exchanges is highly variable between pairs of chromosomal taxa and even between different locations of the same pairs of contact zones (*e.g.*, *viatica*19/*viatica*17 contact zones on Kangaroo Island and mainland). Such heterogeneous gene introgression suggests that chromosomal variations alone cannot explain the underlying population genetic patterns within *Vandiemenella*.

7.2 Future directions

7.2.1 Recombination suppression models

A critical unresolved question remains concerning a potential role of chromosomal rearrangements in speciation. Even if new chromosomal variants have emerged and established by drift in isolated allopatric populations, some of the suppressed recombination models (Noor *et al.* 2001b; Rieseberg 2001) suggest that chromosomal rearrangements may facilitate diversification of the *Vandiemenella* chromosomal races after secondary contact by reducing recombination and reinforcing linkage between genes in a large block of the genome. Such large blocks of the genome protected from recombination, or 'genomic islands' (Turner *et al.* 2005), would provide a strong genetic barrier if these genomic islands have

accumulated incompatible alleles in a foreign genetic background or allele combinations favourable for the local environment during an allopatric phase. In contrast, non-rearranged homologous chromosomes and chromosomal regions outside the rearranged segments (collinear genomic regions) may sustain normal recombination rates and, hence, retain gene flow between chromosomally differentiated populations after secondary contact. Such heterogeneous gene introgression across genomes has been reported in several taxa (*e.g., Helianthus* sunflowers, Rieseberg *et al.* 1999; *Drosophila*, Noor *et al.* 2001a; Mus house mouse, Panithanarak et al. 2004; *Rhagoletis* apple maggot fly, Feder et al. 2005; *Anopheles* mosquitos, Stump et al. 2005; *Solex* shrew, Basset *et al.* 2006). Because these genomic islands tend to remain differentiated in the face of gene introgression, identification of such genomic islands is expected to be useful for tracking down 'speciation genes' that are responsible for barriers to gene flow and/or differential environmental adaptation (Butlin & Roper 2005). One of the exciting features of the *viatica* species group is that various types and numbers of chromosomal rearrangements are involved, and a number of hybrid zones composed of different combinations of chromosomal races are available. It is expected that different pairs of chromosomal races, different levels of genetic divergence through different degrees of isolation in allopatry, and different ages of contact zones (*e.g.*, possibly *viatica*19 and *viatica*17 contact zones on Kangaroo Island and the mainland Australia), may show different levels of genetic admixture between pairs of chromosomal races and, hence, have different sizes/numbers of genomic islands.

In order to investigate whether there are genomic islands where genes do not easily introgress between chromosomal races, and whether these genomic islands are predominantly found within the rearranged chromosomal segments, we need to identify many genetic markers with known genomic locations. A first step is to generate genetic linkage maps based on the recombination frequency for each of the chromosomal taxa. Amplified fragment length polymorphism (AFLP) markers (Vos *et al.* 1995) for intermediate-scale linkage mapping, and single nucleotide polymorphism (SNP) markers for large-scale linkage mapping have been commonly used for whole genome mappings in insects (Behura 2006). By comparing these

linkage maps, it is possible to investigate in more detail chromosomal structural relationships among the chromosomal taxa of the *Vandiemenella* group and differences in the degree of introgression between rearranged and collinear genomic regions using natural hybrid zones (*e.g.*, Rieseberg *et al.* 1995; Rieseberg *et al.* 1999). In addition, all microsatellite and nuclear sequence loci used in this research can be placed on the obtained linkage maps to further investigate associations between levels of genetic differentiation and chromosomal rearrangements, although more loci will be needed for good coverage of the genome. Comparative genetic mapping is a powerful approach to delineate chromosome structures with precise localization of known orthologous sequences (Livingstone & Rieseberg 2004); however, it is often difficult to obtain accurate linkage maps without errors due to genotyping errors, limited number of informative meioses, incorrect identification of marker positions, and unknown cryptic chromosomal rearrangements (Semagn *et al.* 2006). To overcome this difficulty, DNA markers with known positions on linkage maps are needed to be physically mapped on chromosomes. A second step, therefore, is to generate physically anchored genetic maps using fluorescent *in situ* hybridization (FISH) localization of grasshopper genomic clones. Bacterial artificial chromosome (BAC) and cosmid genomic libraries have been used for developing physical maps for a number of insect species using FISH technology (Brown *et al.* 1995; Brown & Knudson 1997; Behura *et al.* 2004). Once genomic libraries are constructed from one of the *Vandiemenella* taxa, genomic regions of 80-200 kb in length (BAC clones) can be used for FISH as well as conventional library screening analyses. It is possible to obtain a number of loci with known genomic regions from BAC libraries for sequencing and genotyping analyses, which fulfill the current shortage of DNA markers for genome-wide analyses of introgression.

7.2.2 Molecular cytogenetics

At present, description of karyotypic differences in the *viatica* species group is based on traditional cytological examinations, which detect mainly macro chromosomal mutations; however, there may be much more small and cryptic chromosomal rearrangements. For instance, it is unclear whether the short arms of sub-metacentric chromosomes in P24(XY) (chromosome 3 and 4) are derived from corresponding acrocentrics in P24(XO) by pericentric inversions (White *et al.* 1967) (Fig. 2.1). If

this is the case, the number of chromosomal rearrangements involved between P24(XY) and *viatica*17 would become double (*i.e*., a pericentric inversion on X-chromosome, a centric fusion between X+1 chromosomes, and two pericentric inversions on chromosome 3 and 4). Since pericentric inversions may have a strong effect on suppressing recombination in heterokaryotypes (Hartl & Jones 2005), it would become more likely that gene introgression is reduced by the structural change in chromosomes across the hybrid zone on Kangaroo Island, and/or rearranged chromosomal segments capture some 'speciation genes.' Moreover, White et al. (1967) noted that two populations of *viatica*17, one in the southeast and the other in the northwest of Murray River, can be clearly distinguishable by the length of short arms in five acrocentric autosomes, in which pericentric inversions are probably involved. It is possible that other chromosomal races that are composed of multiple genetic populations, such as *viatica*19 and P45b(XO) (Chapter 6), may have similar cryptic chromosome differences between the populations. Therefore, we need to have a clearer picture of the amount of both macro and micro chromosome mutations and karyotypic evolution of the *viatica* species group. In addition to traditional cytological techniques, molecular cytogenetic techniques, such as FISH and chromosome painting, allow detection of cryptic chromosomal rearrangements and chromosome arm homology between the chromosomal races. It is suggested that FISH generally works well for a number of organisms but sometime does not work for certain arthropods without apparent reasons, regardless of proves used (F. Grützner, personal communication). To test if FISH works for *Vandiemenella*, we carried out a preliminary FISH analysis using *viatica*17 with 18s and 28s ribosomal DNA fragments as probes, which clearly detected genomic locations of these markers (Fig. 7.1). In addition to these markers, satellite DNA repeats $(TTAGG)_n$, which are often conserved in telomeres of chromosomes in grasshoppers and many other insects (Frydrychova *et al.* 2004; Lopez-Fernandez *et al.* 2004) will provide useful markers for detection of pericentric inversions.

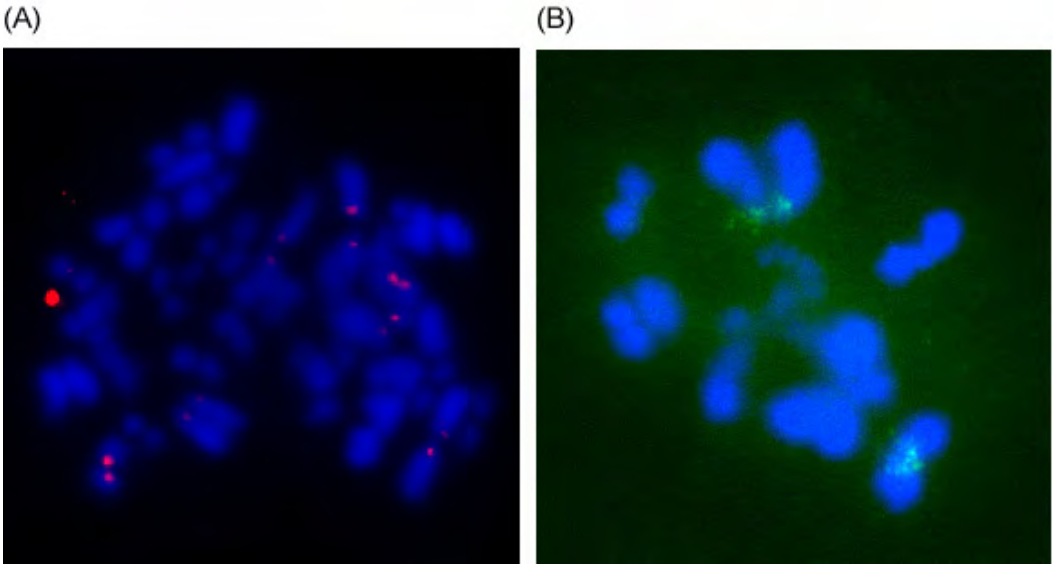


Fig. 7.1. Fluorescence *in situ* hybridization (FISH) of mitotic metaphase chromosomes labeled with 18s rDNA (red, Cy3) (A) and miotic metaphase chromosomes (B) of *viatica*17 chromosomal race of *Vandiemenella* labeled with 28s rDNA (green, FITC).

7.2.3 Statistical phylogeography

Chapter 6 identified several genetically distinct population clusters within each of the chromosomal taxa, which may have diverged over a considerable time period; however, the timing and geographic locations of diversification events are largely unknown. Identification of these genetic populations generates testable phylogeographic hypotheses. Four chromosomal taxa are highlighted here: (i) three *viatica*19 populations on Kangaroo Island, mainland Australia, and Tasmania, (ii) two *viatica*17 populations separated by the Murray River, (iii) two populations of P24(XY), one widely distributed across two Peninsulas and Kangaroo Island and the other restricted to the tip of Yorke Peninsula, and (iv) two populations of P45b(XO) separated by Spencer Gulf (Fig. 6.13). Separation of these populations appears to correspond mostly with geographic barriers, such as gulfs, rivers and oceanic straits, and, therefore, these genetically distinct populations within races may represent

separate glacial refugia during the Pleistocene. Statistical phylogeography, in particular model-based coalescent simulation approaches, will provide a powerful framework to explore biogeographic history and processes of diversification with various population demographic parameters, such as divergence times, population sizes, and gene flow between populations (Knowles & Maddison 2002; Hey & Nielsen 2004). Since historical biogeography inferred by single locus approaches based on mtDNA can potentially lead to misleading conclusions as pointed out in Chapter 5 and 6, joint analysis of multiple independent nuclear markers is necessary. In addition, because patterns of gene flow may significantly vary across the genome between hybridizing taxa for various reasons, such as variations in the fitness of introgressed alleles in different genetic background or environments (Bull *et al.* 2006) and heterogeneous recombination rates due to chromosomal rearrangements (Rieseberg *et al.* 1999), coalescent simulation approaches require a number of markers for sufficient representation of the genome. Therefore, in addition to the nuclear loci isolated in this research, it is necessary to isolate additional single-copy loci from BAC libraries for complete coverage of both rearranged and collinear genomic regions (Yatabe *et al.* 2007). Moreover, as we pointed out in Chapter 5, it is important to distinguish incomplete lineage sorting of ancestral polymorphisms and introgressive hybridization for inferring historical biogeography. These two population genetic scenarios can be better distinguished by comparing levels of genetic differentiation at many of these nuclear loci between currently parapatric populations and historically allopatric populations (*e.g.*, parapatric *viatica*19 populations adjacent to *viatica*17 populations and allopatric *viatica*19 populations in Tasmania). In contrast to the Australian wet tropics (Schneider *et al.* 1998), historical biogeography of various organisms in southeastern Australia has not been well studied with a statistically rigorous framework. Therefore, insects with a poor dispersal ability, such as morabine grasshoppers, will provide ideal model systems to investigate population divergence history and fine-scale phylogeography.

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APPENDICES

Appendix Table 4.1 The numbers of alleles (A), observed and expected heterozygocities (H_O and H_E) and fixation index (F_{IS}) of five microsatellite and five allozyme loci at 33 sites before collapsing alleles into two allele system. Males and females for *Acyc* locus were analysed separately because it is a putative X-linked locus (*i.e.*, all homozygotes in males). Numbers in bold indicate statistical significance after the sequential Bonferroni corrections.

Site	N		*Viat2*	*Viat4*	*Viat5*	*Viat8*	*Viat10*	*Ak1*	*Ak2*	*Idh2*	*PepA*	*Acyc* (♂)	*Acyc* (♀)
P16	24	A	3	1	2	7	3	1	1	1	2	1	1
		H_O	0.417	0.000	0.000	0.708	0.292	0.000	0.000	0.000	0.043	0.000	0.000
		H_E	0.588	0.000	0.245	0.709	0.484	0.000	0.000	0.000	0.043	0.000	0.000
		F_{IS}	0.291	-	1.000	0.001	0.397	-	-	-	-0.022	-	-
P15	32	A	8	4	7	10	3	1	1	1	1	1	1
		H_O	0.538	0.231	0.577	0.778	0.556	0.000	0.000	0.000	0.000	0.000	0.000
		H_E	0.701	0.334	0.597	0.816	0.501	0.000	0.000	0.000	0.000	0.000	0.000
		F_{IS}	0.232	0.308	0.033	0.046	-0.108	-	-	-	-	-	-
P14	14	A	5	2	2	6	3	1	1	1	1	1	1
		H_O	0.538	0.071	0.357	0.786	0.571	0.000	0.000	0.000	0.000	0.000	0.000
		H_E	0.586	0.191	0.375	0.765	0.503	0.000	0.000	0.000	0.000	0.000	0.000
		F_{IS}	0.081	0.627	0.048	-0.027	-0.137	-	-	-	-	-	-
P13	17	A	6	4	5	7	4	1	1	1	1	1	1
		H_O	0.529	0.176	**0.353**	0.765	0.529	0.000	0.000	0.000	0.000	0.000	0.000
		H_E	0.702	0.166	0.697	0.813	0.580	0.000	0.000	0.000	0.000	0.000	0.000
		F_{IS}	0.246	-0.062	0.494	0.060	0.087	-	-	-	-	-	-
P12	16	A	5	2	6	8	3	1	1	1	2	1	1
		H_O	0.688	0.063	0.667	0.750	0.500	0.000	0.000	0.000	0.067	0.000	0.000
		H_E	0.713	0.061	0.576	0.787	0.482	0.000	0.000	0.000	0.064	0.000	0.000
		F_{IS}	0.036	-0.032	-0.157	0.047	-0.037	-	-	-	-0.034	-	-
P11	16	A	5	3	6	5	3	1	1	1	2	1	1
		H_O	0.625	**0.125**	0.500	0.500	0.313	0.000	0.000	0.000	0.125	0.000	0.000
		H_E	0.691	0.447	0.543	0.736	0.389	0.000	0.000	0.000	0.117	0.000	0.000
		F_{IS}	0.096	0.721	0.079	0.321	0.196	-	-	-	-0.067	-	-
P10	16	A	4	3	5	7	2	1	1	1	2	1	1
		H_O	**0.200**	0.313	0.462	0.938	0.250	0.000	0.000	0.000	0.063	0.000	0.000
		H_E	0.542	0.365	0.571	0.797	0.305	0.000	0.000	0.000	0.061	0.000	0.000
		F_{IS}	**0.631**	0.144	0.192	-0.176	0.179	-	-	-	-0.032	-	-
P9	4	A	6	1	3	3	1	2	1	1	1	1	1
		H_O	0.500	0.000	0.500	0.500	0.000	0.250	0.000	0.000	0.000	0.000	0.000
		H_E	0.813	0.000	0.594	0.594	0.000	0.219	0.000	0.000	0.000	0.000	0.000
		F_{IS}	0.385	-	0.158	0.158	-	-0.143	-	-	-	-	-
P8	10	A	4	4	5	4	2	1	1	1	2	1	1
		H_O	0.333	0.600	0.400	0.500	0.200	0.000	0.000	0.000	0.100	0.000	0.000
		H_E	0.636	0.615	0.745	0.575	0.180	0.000	0.000	0.000	0.095	0.000	0.000
		F_{IS}	0.476	0.024	0.463	0.130	-0.111	-	-	-	-0.053	-	-
P7	7	A	5	3	4	3	1	1	1	1	1	1	1
		H_O	0.600	0.143	0.400	0.714	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		H_E	0.780	0.357	0.740	0.582	0.000	0.000	0.000	0.000	0.000	0.000	0.000
		F_{IS}	0.231	0.600	0.459	-0.228	-	-	-	-	-	-	-

Site	N		*Viat2*	*Viat4*	*Viat5*	*Viat8*	*Viat10*	*Ak1*	*Ak2*	*Idh2*	*PepA*	*Acyc* (♂)	*Acyc* (♀)
P6	9	*A*	4	2	6	4	2	2	2	2	3	1	2
		H_O	0.167	0.556	0.333	0.556	0.000	0.111	0.222	0.111	0.556	0.000	0.125
		H_E	0.597	0.475	0.809	0.512	0.198	0.105	0.198	0.278	0.512	0.000	0.117
		F_{IS}	0.721	-0.169	**0.588**	-0.084	1.000	-0.059	-0.125	0.600	-0.084	-	-0.067
P5	12	*A*	3	4	5	3	3	2	2	3	2	2	3
		H_O	0.273	0.583	**0.167**	0.167	0.417	0.091	0.100	0.545	0.182	0.000	0.375
		H_E	0.649	0.503	0.681	0.344	0.351	0.236	0.095	0.417	0.165	0.500	0.508
		F_{IS}	0.580	-0.159	**0.755**	0.515	-0.188	0.614	-0.053	-0.307	-0.100	1.000	0.262
P4	14	*A*	3	3	4	3	2	2	2	3	3	1	1
		H_O	**0.000**	**0.143**	0.167	0.154	0.071	0.143	0.167	0.429	0.357	0.000	0.000
		H_E	0.595	0.561	0.681	0.370	0.069	0.245	0.153	0.446	0.390	0.000	0.000
		F_{IS}	**1.000**	**0.745**	0.755	0.584	-0.037	0.417	-0.091	0.040	0.085	-	-
P3	20	*A*	3	3	4	3	3	3	1	3	2	2	2
		H_O	0.158	0.400	0.118	0.150	0.150	0.211	0.000	0.263	0.263	0.000	0.167
		H_E	0.309	0.640	0.265	0.224	0.141	0.193	0.000	0.309	0.301	0.142	0.153
		F_{IS}	0.489	0.375	0.556	0.330	-0.062	-0.094	-	0.148	0.124	1.000	-0.091
P2	30	*A*	3	3	4	4	2	3	2	3	3	2	3
		H_O	**0.241**	0.567	**0.148**	0.267	0.033	0.345	0.261	0.300	**0.167**	0.000	0.250
		H_E	0.514	0.559	0.545	0.332	0.033	0.295	0.287	0.466	0.352	0.346	0.344
		F_{IS}	**0.531**	-0.013	**0.728**	0.196	-0.017	-0.169	0.092	0.356	**0.526**	1.000	0.273
P1	3	*A*	3	3	3	2	1	3	2	1	2	0	2
		H_O	1.000	0.667	0.333	0.333	0.000	1.000	0.333	0.000	0.667	-	0.667
		H_E	0.611	0.500	0.611	0.500	0.000	0.611	0.278	0.000	0.444	-	0.444
		F_{IS}	-0.636	-0.333	0.455	0.333	-	-0.636	-0.200	-	-0.500	-	-0.500
V1	28	*A*	2	5	5	6	9	3	2	2	2	2	3
		H_O	0.042	0.536	0.500	**0.393**	**0.429**	0.259	0.259	0.071	0.179	0.000	0.333
		H_E	0.187	0.517	0.552	0.737	0.821	0.230	0.226	0.069	0.163	0.305	0.497
		F_{IS}	0.777	-0.036	0.095	**0.467**	**0.478**	-0.128	-0.149	-0.037	-0.098	1.000	0.329
V2	12	*A*	2	4	4	6	7	2	2	2	1	1	2
		H_O	0.125	0.400	0.400	0.500	0.556	0.364	0.286	0.417	0.000	0.000	0.200
		H_E	0.117	0.615	0.610	0.760	0.796	0.397	0.245	0.330	0.000	0.000	0.180
		F_{IS}	-0.067	0.350	0.344	0.342	0.302	0.083	-0.167	-0.263	-	-	-0.111
V3	5	*A*	2	2	2	3	5	2	2	2	1	1	1
		H_O	0.000	0.500	0.250	0.750	1.000	0.200	0.400	0.200	0.000	0.000	0.000
		H_E	0.444	0.500	0.219	0.531	0.750	0.180	0.480	0.180	0.000	0.000	0.000
		F_{IS}	1.000	0.000	-0.143	-0.412	-0.333	-0.111	0.167	-0.111	-	-	-
V4	10	*A*	2	5	4	5	8	3	2	2	3	2	2
		H_O	0.000	0.500	0.500	0.400	0.700	0.200	0.000	0.200	0.200	0.000	0.250
		H_E	0.198	0.635	0.535	0.745	0.830	0.335	0.180	0.180	0.185	0.278	0.219
		F_{IS}	1.000	0.213	0.065	0.463	0.157	0.403	1.000	-0.111	-0.081	1.000	-0.143
V5	20	*A*	2	4	5	6	7	2	2	2	2	1	2
		H_O	0.105	0.450	0.400	0.600	0.526	0.150	0.050	0.150	0.150	0.000	0.111
		H_E	0.100	0.580	0.384	0.753	0.819	0.139	0.139	0.139	0.139	0.000	0.105
		F_{IS}	-0.056	0.224	-0.042	0.203	**0.357**	-0.081	0.640	-0.081	-0.081	-	-0.059
V6	11	*A*	4	6	4	4	7	2	2	2	1	2	2
		H_O	**0.000**	0.364	0.455	0.455	0.545	0.182	0.364	0.091	0.000	0.000	0.600
		H_E	0.656	0.512	0.446	0.657	0.764	0.165	0.298	0.087	0.000	0.444	0.420
		F_{IS}	**1.000**	0.290	-0.019	0.308	0.286	-0.100	-0.222	-0.048	-	1.000	-0.429

Site	N		*Viat2*	*Viat4*	*Viat5*	*Viat8*	*Viat10*	*Ak1*	*Ak2*	*Idh2*	*PepA*	*Acyc* (♂)	*Acyc* (♀)
V7	13	*A*	2	2	2	3	6	2	2	2	2	1	2
		H_O	0.200	0.333	0.222	0.444	0.667	0.308	0.250	0.385	0.154	0.000	0.000
		H_E	0.500	0.278	0.346	0.623	0.778	0.260	0.330	0.311	0.142	0.000	0.278
		F_{IS}	0.600	-0.200	0.357	0.287	0.143	-0.182	0.242	-0.238	-0.083	-	1.000
V8	12	*A*	2	3	4	3	6	1	2	1	2	1	2
		H_O	0.300	0.600	0.333	0.200	0.444	0.000	0.333	0.000	0.083	0.000	0.167
		H_E	0.455	0.540	0.562	0.515	0.741	0.000	0.278	0.000	0.080	0.000	0.153
		F_{IS}	0.341	-0.111	0.407	0.612	0.400	-	-0.200	-	-0.043	-	-0.091
V9	10	*A*	3	4	3	3	5	2	1	2	1	2	2
		H_O	0.222	0.400	0.300	0.400	**0.000**	0.100	0.000	0.100	0.000	0.000	0.200
		H_E	0.370	0.640	0.265	0.460	0.716	0.095	0.000	0.095	0.000	0.320	0.180
		F_{IS}	0.400	0.375	-0.132	0.130	**1.000**	-0.053	-	-0.053	-	1.000	-0.111
V10	12	*A*	3	4	5	4	7	2	2	2	2	2	2
		H_O	0.167	0.167	0.333	0.667	0.600	0.167	0.250	0.417	0.083	0.000	0.200
		H_E	0.344	0.354	0.299	0.545	0.800	0.153	0.219	0.330	0.080	0.408	0.180
		F_{IS}	0.515	0.529	-0.116	-0.223	0.250	-0.091	-0.143	-0.263	-0.043	1.000	-0.111
V11	7	*A*	2	4	4	3	4	2	2	2	2	1	2
		H_O	0.143	0.429	0.429	0.429	0.600	0.143	0.167	0.286	0.143	0.000	0.833
		H_E	0.133	0.531	0.367	0.500	0.700	0.133	0.375	0.245	0.133	0.000	0.486
		F_{IS}	-0.077	0.192	-0.167	0.143	0.143	-0.077	0.556	-0.167	-0.077	-	-0.714
V12	8	*A*	3	6	4	7	8	2	1	2	2	1	2
		H_O	0.200	0.500	0.375	0.875	**0.250**	0.250	0.000	0.625	0.250	0.000	0.500
		H_E	0.460	0.773	0.484	0.820	0.852	0.219	0.000	0.492	0.219	0.000	0.375
		F_{IS}	0.565	0.354	0.226	-0.067	**0.706**	-0.143	-	-0.270	-0.143	-	-0.333
V13	7	*A*	3	4	5	4	8	1	2	2	2	2	1
		H_O	0.267	0.667	0.278	0.389	0.471	0.000	0.143	0.143	0.143	0.000	0.000
		H_E	0.340	0.542	0.253	0.637	0.777	0.000	0.133	0.133	0.133	0.320	0.000
		F_{IS}	0.216	-0.231	-0.098	0.390	0.394	-	-0.077	-0.077	-0.077	1.000	-
V14	13	*A*	4	6	4	6	7	2	1	2	3	2	2
		H_O	0.100	0.615	0.308	0.846	0.846	0.083	0.000	0.167	0.167	0.000	0.000
		H_E	0.415	0.660	0.388	0.754	0.802	0.080	0.000	0.153	0.156	0.320	0.219
		F_{IS}	0.759	0.067	0.206	-0.122	-0.055	-0.043	-	-0.091	-0.067	1.000	1.000
V15	19	*A*	4	4	4	5	4	1	2	1	1	2	2
		H_O	0.167	0.316	0.368	0.579	0.684	0.000	0.111	0.000	0.000	0.000	0.300
		H_E	0.229	0.411	0.316	0.719	0.688	0.000	0.105	0.000	0.000	0.469	0.455
		F_{IS}	0.273	0.232	-0.167	0.195	0.006	-	-0.059	-	-	1.000	0.341
V16	20	*A*	5	7	4	6	6	2	2	1	2	2	2
		H_O	**0.350**	0.300	0.550	0.650	0.750	0.056	0.000	0.000	0.200	0.000	0.400
		H_E	0.644	0.314	0.486	0.740	0.805	0.054	0.117	0.000	0.180	0.180	0.420
		F_{IS}	**0.456**	0.044	-0.131	0.122	0.068	-0.029	1.000	-	-0.111	1.000	0.048
V17	4	*A*	1	4	3	2	5	1	1	2	1	2	0
		H_O	0.000	0.750	0.750	0.250	1.000	0.000	0.000	0.500	0.000	0.000	-
		H_E	0.000	0.563	0.531	0.469	0.750	0.000	0.000	0.375	0.000	0.500	-
		F_{IS}	-	-0.333	-0.412	0.467	-0.333	-	-	-0.333	-	1.000	-

Appendix Table 4.2 Frequencies of *viatica*17 karyotype, microsatellite alleles, allozyme electromorphs, and mitochondrial *COI* haplotype at 33 sampling sites. Frequencies for 'pure' P24(XY) and 'pure' *viatica*17 are average frequencies of P16-P14 and V14-V17, respectively.

Site	*Distance* (km)†	*Karyotype*	*Viat2*	*Viat4*	*Viat5*	*Viat8*	*Viat10*	*Ak1*	*Ak2*	*Idh2*	*PepA*	*Acyc*	*EF-1a*	*COI*	HI*
Pure' P24(XY)		0.000	0.008	0.000	0.011	0.031	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.005
P16	-8.63	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
P15	-5.73	0.000	0.019	0.000	0.019	0.074	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.010
P14	-6.77	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
P13	-3.63	0.000	0.000	0.029	0.029	0.147	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.023
P12	-2.92	0.000	0.000	0.000	0.000	0.188	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021
P11	-2.09	0.000	0.000	0.000	0.000	0.094	0.031	0.000	0.000	0.000	0.063	0.000	0.000	0.063	0.019
P10	-1.87	0.000	0.000	0.000	0.000	0.219	0.000	0.000	0.000	0.000	0.031	0.000	0.000	0.000	0.025
P9	-0.94	0.000	0.000	0.000	0.000	0.125	0.000	0.125	0.000	0.000	0.000	0.000	0.000	0.000	0.025
P8	-1.20	0.000	0.000	0.050	0.000	0.200	0.000	0.000	0.000	0.000	0.050	0.000	0.000	0.000	0.030
P7	-1.03	0.000	0.000	0.071	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.008
P6	-0.89	0.000	0.000	0.000	0.222	0.000	0.000	0.056	0.111	0.000	0.056	0.000	0.000	0.111	0.046
P5	-0.69	0.000	0.000	0.042	0.083	0.167	0.083	0.136	0.050	0.227	0.091	0.222	0.083	0.818	0.095
P4	-0.41	0.000	0.000	0.071	0.500	0.231	0.000	0.143	0.083	0.107	0.036	0.000	0.000	0.214	0.094
P3	-0.26	0.000	0.158	0.200	0.088	0.125	0.075	0.079	0.000	0.026	0.000	0.040	0.056	0.824	0.098
P2	-0.14	0.000	0.345	0.067	0.370	0.200	0.017	0.138	0.174	0.250	0.183	0.143	0.086	0.654	0.184
P1	-0.07	-	0.500	0.167	0.333	0.500	0.000	0.500	0.167	0.000	0.333	0.000	0.250	0.000	0.274

Site	*Distance* (km)†	*Karyotype*	*Viat2*	*Viat4*	*Viat5*	*Viat8*	*Viat10*	*Ak1*	*Ak2*	*Idh2*	*PepA*	*Acyc*	*EF-1a*	*COI*	HI*
V1	0.24	1.000	1.000	0.929	0.982	0.911	0.768	0.870	0.870	0.964	0.911	0.850	0.840	1.000	0.912
V2	0.12	1.000	0.938	0.600	0.750	0.850	0.556	0.727	0.857	0.792	1.000	1.000	0.667	1.000	0.777
V3	0.50	1.000	1.000	1.000	1.000	1.000	0.750	0.900	0.600	0.900	1.000	1.000	1.000	1.000	0.908
V4	0.25	1.000	0.889	0.800	0.900	0.900	0.900	0.800	0.900	0.900	0.900	0.929	0.750	1.000	0.869
V5	0.28	1.000	1.000	0.900	1.000	0.925	0.789	0.925	0.925	0.925	0.925	1.000	0.842	1.000	0.923
V6	0.49	1.000	1.000	0.955	1.000	1.000	0.818	0.909	0.818	0.955	1.000	1.000	0.625	1.000	0.913
V7	0.61	1.000	1.000	1.000	1.000	1.000	0.500	0.846	0.792	0.808	0.923	0.895	1.000	1.000	0.901
V8	0.80	1.000	1.000	1.000	1.000	1.000	0.944	1.000	0.833	1.000	0.958	1.000	0.750	1.000	0.954
V9	0.80	1.000	1.000	1.000	1.000	1.000	0.556	0.950	1.000	0.950	1.000	0.867	0.800	1.000	0.929
V10	0.89	1.000	1.000	1.000	1.000	1.000	0.900	0.917	0.875	0.792	0.958	1.000	0.958	1.000	0.945
V11	1.84	1.000	1.000	1.000	0.929	1.000	1.000	0.929	0.750	0.857	0.929	1.000	0.700	1.000	0.919
V12	2.31	1.000	0.800	0.750	1.000	0.875	0.750	0.875	1.000	0.563	0.875	1.000	0.688	1.000	0.794
V13	0.63	1.000	1.000	0.944	1.000	1.000	0.853	1.000	0.929	0.929	1.000	1.000	0.906	1.000	0.946
V14	11.80	1.000	0.900	0.885	1.000	1.000	0.885	0.958	1.000	0.917	0.958	1.000	1.000	1.000	0.990
V15	19.97	1.000	1.000	0.974	1.000	1.000	1.000	1.000	0.944	1.000	1.000	1.000	1.000	1.000	0.989
V16	15.87	1.000	1.000	0.975	1.000	1.000	1.000	0.972	0.938	1.000	1.000	1.000	1.000	1.000	0.974
V17	28.80	1.000	1.000	0.875	1.000	1.000	1.000	1.000	1.000	0.750	1.000	1.000	1.000	1.000	0.945
Pure' *viatica*17		1.000	0.983	0.950	1.000	1.000	0.952	0.980	0.957	0.971	0.990	1.000	1.000	1.000	0.978

* Hybrid Index caluculated using all nuclear loci except putative X-linked *Acyc*.
† Distance along the transect. The best-fit average cline over all 10 nuclear loci is set to zero.

Appendix Table 5.1 Locality data and the number of samples analysed for allozyme (N_A), *COI* (N_C), and *EF*-1α (N_E)

Population	Site	Locality Name	Latitude	Longitude	N_A	N_C	N_E
P24(XY)-east	AE	0.9 km NW of Prospect Hill	-35.83489	137.73539	5	0	0
	DD	1.0 km NW of Prospect Hill	-35.83866	137.73471	0	2	0
	AA	1.1 km NW of Prospect Hill	-35.83617	137.73086	4	0	0
	DT	1.4 km E of Prospect Hill (1)	-35.84625	137.76333	0	2	0
	DS	1.4 km E of Prospect Hill (2)	-35.84542	137.76371	0	2	2
	AB	4.8 km S of American River	-35.82397	137.74503	5	2	2
	DJ	8.0 km W of Prospect Hill (1)	-35.84147	137.73976	0	1	0
	U	8.0 km W of Prospect Hill (2)	-35.84147	137.74042	5	0	0
	S	Flour Cask Bay (1)	-35.87664	137.69117	2	0	0
	T	Flour Cask Bay (2)	-35.87742	137.69117	5	1	1
	DG	Flour Cask Bay (3)	-35.87729	137.69327	0	2	1
	DO	Pennington Bay (1)	-35.85495	137.73582	0	1	0
	DK	Pennington Bay (2)	-35.85187	137.74200	0	3	0
	B	Pennington Bay (3)	-35.85197	137.74506	7	4	3
	DC	Pennington Bay (4)	-35.85165	137.74582	0	2	0
	G	Pennington Bay (5)	-35.85242	137.75267	2	1	0
	I	Pennington Bay (6)	-35.85119	137.77283	1	1	0
	J	Pennington Bay (7)	-35.85122	137.77381	5	3	2
	K	Pennington Bay (8)	-35.85072	137.77803	4	0	0
	L	Pennington Bay (9)	-35.85147	137.78200	6	2	0
	M	Pennington Bay (10)	-35.85133	137.78381	8	3	2
P24(XY)-north	EV	Cape Rouge	-35.59208	137.60422	0	1	0
	ET	Emu Bay (1)	-35.59500	137.50948	0	5	5
	C	Emu Bay (2)	-35.59925	137.54086	5	2	2
	BK	Storks Bay (1)	-35.62478	137.20639	1	1	1
	ES	Storks Bay (2)	-35.62299	137.20644	0	5	5
*viatica*17-east	JG	15 km S of Penneshaw	-35.85113	137.90961	0	4	4
	JD	4.5 km E of Prospect Hill	-35.85064	137.81339	0	2	0
	E	Dudley Peninsula	-35.81383	137.94894	6	2	3
	JF	Lesueur Conservation Park	-35.85763	138.09715	0	4	4
	AN	Pelican Lagoon (1)	-35.84222	137.77922	2	0	0
	EE	Pelican Lagoon (2)	-35.83852	137.78041	0	1	0
	F	Pelican Lagoon (3)	-35.84131	137.78200	2	0	0
	AM	Pelican Lagoon (4)	-35.84014	137.78392	3	0	0
	DQ	Pelican Lagoon (5)	-35.83498	137.78902	0	3	2
	D	Penneshaw	-35.71922	137.94125	6	4	4
	EA	Pennington Bay (11)	-35.84807	137.78583	0	2	0
	AF	Pennington Bay (12)	-35.84828	137.78583	3	1	1
	AL	Pennington Bay (13)	-35.84975	137.78606	9	2	2
	AI	Pennington Bay (14)	-35.84628	137.78631	1	0	0
	AH	Pennington Bay (15)	-35.84561	137.78642	2	1	0
	DW	Pennington Bay (16)	-35.84887	137.78699	0	1	0
	DY	Pennington Bay (17)	-35.84756	137.78757	0	2	0
	R	Pennington Bay (18)	-35.84911	137.78783	6	1	0
	DX	Pennington Bay (19)	-35.84877	137.78796	0	2	0
	AG	Pennington Bay (20)	-35.84714	137.78864	3	1	0
	DZ	Pennington Bay (21)	-35.84733	137.78899	0	2	0
	Y	Pennington Bay (22)	-35.84839	137.79261	3	1	0

	X	Pennington Bay (23)	-35.84986	137.79608	3	0	0
	Q	Pennington Bay (24)	-35.85100	137.79675	3	2	2
	W	Pennington Bay (25)	-35.84922	137.80722	3	2	2
*viatica*17-south	EP	0.4 km N of Little Sahara	-35.94473	137.24271	0	2	1
	EM	Seal Bay Conservation Park (1)	-35.98994	137.34764	0	2	0
	EN	Seal Bay Conservation Park (2)	-35.99055	137.34845	0	1	1
	BF	Seal Bay Conservation Park (3)	-35.99197	137.34714	1	0	0
	EL	Seal Bay Conservation Park (4)	-35.99241	137.34955	0	2	2
	IU	Bales Bay	-35.99793	137.36194	0	2	2
	AO	Little Sahara (1)	-35.94750	137.24239	9	1	1
	BC	Little Sahara (2)	-35.94500	137.24258	7	0	0
	BD	Little Sahara (3)	-35.94461	137.24264	7	1	1
	EK	Little Sahara (4)	-35.94822	137.24267	0	2	2
	BR	Little Sahara (5)	-35.94436	137.24275	1	0	0
	AY	Little Sahara (6)	-35.94581	137.24278	7	0	0
	AZ	Little Sahara (7)	-35.94653	137.24283	1	0	0
	AQ	Little Sahara (8)	-35.94431	137.24289	1	0	0
	BA	Little Sahara (9)	-35.94700	137.24292	2	0	0
	AT	Little Sahara (10)	-35.94283	137.24294	1	0	0
	BN	Little Sahara (11)	-35.94744	137.24333	17	0	0
	BO	Little Sahara (12)	-35.94878	137.24381	2	0	0
	BV	Point Ellen (1)	-35.99775	137.18553	1	0	0
	AU	Point Ellen (2)	-35.99753	137.18633	8	2	2
	EJ	Point Ellen (3)	-35.99769	137.18690	0	2	0
	EO	Vivonne Bay (1)	-35.98152	137.18234	0	2	2
	EI	Vivonne Bay (2)	-35.98011	137.18452	0	2	1
*viatica*19	AV	1.0 km SW of Vivonne Bay	-35.98831	137.17503	4	2	2
	AW	1.0 km W of Vivonne Bay	-35.98036	137.17381	4	2	1
	BS	1.1 km SW of Vivonne Bay	-35.99231	137.17642	6	0	0
	IH	12 km NW of Vivonne Bay	-35.91593	137.06073	0	2	2
	IC	14 km E of Storkes Bay, KI	-35.64802	137.36588	0	1	2
	BH	2.7 km NW of Bales Bay	-35.97753	137.32933	7	2	2
	BJ	3.5 W of Parndana	-35.78789	137.25475	5	2	2
	BL	4.0 km SW of Storks Bay	-35.65131	137.17406	3	1	1
	BM	5.0 km E of Burajige	-35.81158	137.02636	2	2	2
	AD	6.0 km S of American River	-35.83383	137.73011	1	0	0
	BE	6.5 km NW of Bales Bay	-35.94200	137.31972	6	1	1
	II	8.9 km WNW of Vivonne Bay	-35.94738	137.08424	0	2	2
	IL	Seal Bay Conservation Park (4)	-35.96196	137.31980	0	2	2
	IJ	Seal Bay Conservation Park (5)	-35.97180	137.32542	0	1	1
	IE	Seal Bay Conservation Park (6)	-35.98153	137.33552	0	2	2
	EU	Beyeria CP	-35.78015	137.59241	0	4	3
	AX	Hanson Bay	-36.01228	136.85339	2	4	3
	IB	Lathami CP (1)	-35.62842	137.21302	0	1	1
	ER	Lathami CP (2)	-35.64399	137.21678	0	2	1
	BI	Parndana	-35.78753	137.21736	2	2	1
	Z	Point Tinline	-36.00233	137.60647	6	3	3

Appendix Table 5.2 Allele frequencies of 15 allozyme loci at 56 sampling sites

Population	Site Code	N	Allele	*Acyc*	*Ak1*	*Ak2*	*Argk*	*Fum*	*Got1*	*Hex*	*Idh2*	*Mdh2*	*Me*	*Mpi*	*PepA*	*PepC*	*PepD1*	*Pgm*
P24(XY) - east	AA	4	1	0.000	0.000	0.000	0.000	1.000	0.000	0.500	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.000	1.000	1.000	0.500	0.000	1.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	0.000	0.000
			3	1.000	0.000	0.000	0.500	0.000	0.000	0.500	1.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
			4	0.000			0.000			0.000	0.000	0.875	0.000	1.000	0.000	0.000	0.750	0.000
			5	0.000						0.000	0.000	0.000	1.000	0.000	0.000		0.000	
			6								0.000	0.125	0.000	0.000			0.000	
			7									0.000					0.250	
			8														0.000	
	AB	5	1	0.000	0.000	0.000	0.000	1.000	0.000	0.500	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.000	1.000	1.000	0.500	0.000	1.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	0.000	0.000
			3	1.000	0.000	0.000	0.500	0.000	0.000	0.500	1.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
			4	0.000			0.000			0.000	0.000	0.600	0.000	1.000	0.000	0.000	0.600	0.000
			5	0.000						0.000	0.000	0.000	1.000	0.000	0.000		0.000	
			6								0.000	0.400	0.000	0.000			0.000	
			7									0.000					0.400	
			8														0.000	
	AE	5	1	0.000	0.000	0.000	0.000	1.000	0.000	0.300	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.000	1.000	1.000	0.500	0.000	1.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	0.000	0.000
			3	1.000	0.000	0.000	0.500	0.000	0.000	0.700	1.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
			4	0.000			0.000			0.000	0.000	0.500	0.000	1.000	0.000	0.000	1.000	0.000
			5	0.000						0.000	0.000	0.000	1.000	0.000	0.000		0.000	
			6								0.000	0.500	0.000	0.000			0.000	
			7									0.000					0.000	
			8														0.000	

Population	Site Code	N	Allele	*Acyc*	*Ak1*	*Ak2*	*Argk*	*Fum*	*Got1*	*Hex*	*Idh2*	*Mdh2*	*Me*	*Mpi*	*PepA*	*PepC*	*PepD1*	*Pgm*
P24(XY) - east	B	7	1	0.000	0.000	0.000	0.000	1.000	0.000	0.286	0.000	0.000	0.000	0.000	0.000	0.143	0.000	0.000
			2	0.000	1.000	1.000	0.357	0.000	1.000	0.000	0.000	0.000	0.000	0.000	1.000	0.857	0.000	0.000
			3	1.000	0.000	0.000	0.643	0.000	0.000	0.714	1.000	0.000	0.214	0.000	0.000	0.000	0.000	1.000
			4	0.000			0.000			0.000	0.000	0.857	0.071	1.000	0.000	0.000	0.714	0.000
			5	0.000						0.000	0.000	0.000	0.643	0.000	0.000		0.000	
			6								0.000	0.143	0.071	0.000			0.214	
			7									0.000					0.071	
			8														0.000	
	G	2	1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.000	1.000	1.000	0.250	0.000	1.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	0.000	0.000
			3	1.000	0.000	0.000	0.750	0.000	0.000	1.000	1.000	0.000	0.250	0.000	0.000	0.000	0.000	1.000
			4	0.000			0.000			0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.500	0.000
			5	0.000						0.000	0.000	0.000	0.750	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.250	
			7									0.000					0.250	
			8														0.000	
	I	1	1	0.000	0.000	0.000	0.000	0.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	0.000	0.000
			3	0.500	0.000	0.000	1.000	0.000	0.000	0.000	0.500	0.000	1.000	0.000	0.000	0.000	0.000	1.000
			4	0.500			0.000			0.000	0.500	1.000	0.000	0.000	0.000	0.000	0.500	0.000
			5	0.000						0.000	0.000	0.000	0.000	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.500	
			8														0.000	

Population	Site Code	N	Allele	*Acyc*	*Ak1*	*Ak2*	*Argk*	*Fum*	*Got1*	*Hex*	*Idh2*	*Mdh2*	*Me*	*Mpi*	*PepA*	*PepC*	*PepD1*	*Pgm*
P24(XY) - east	J	5	1	0.000	0.000	0.000	0.000	1.000	0.500	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.000	0.900	0.900	0.300	0.000	0.500	0.000	0.000	0.000	0.000	0.000	0.900	1.000	0.000	0.000
			3	0.600	0.100	0.100	0.700	0.000	0.000	0.000	0.600	0.000	0.600	1.000	0.100	0.000	0.000	1.000
			4	0.400			0.000			0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.700	0.000
			5	0.000						0.000	0.400	0.000	0.400	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.300	
			8														0.000	
	K	4	1	0.000	0.000	0.000	0.000	1.000	0.000	0.875	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.000	1.000	1.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	0.000	0.000
			3	1.000	0.000	0.000	1.000	0.000	0.000	0.125	0.500	0.000	1.000	1.000	0.000	0.000	0.000	1.000
			4	0.000			0.000			0.000	0.125	1.000	0.000	0.000	0.000	0.000	0.875	0.000
			5	0.000						0.000	0.375	0.000	0.000	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.125	
			8														0.000	
	L	6	1	0.000	0.000	0.000	0.000	1.000	0.000	0.917	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.000	0.833	1.000	0.000	0.000	0.917	0.000	0.000	0.000	0.000	0.000	0.917	0.917	0.000	0.000
			3	1.000	0.167	0.000	1.000	0.000	0.083	0.083	0.917	0.000	1.000	1.000	0.083	0.083	0.000	1.000
			4	0.000			0.000			0.000	0.083	1.000	0.000	0.000	0.000	0.000	0.667	0.000
			5	0.000						0.000	0.000	0.000	0.000	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.333	
			8														0.000	

Population	Site Code	N	Allele	*Acyc*	*Ak1*	*Ak2*	*Argk*	*Fum*	*Got1*	*Hex*	*Idh2*	*Mdh2*	*Me*	*Mpi*	*PepA*	*PepC*	*PepD1*	*Pgm*
P24(XY) - east	M	8	1	0.000	0.000	0.000	0.000	1.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.000	0.813	0.333	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.750	1.000	0.000	0.000
			3	0.750	0.188	0.667	1.000	0.000	0.000	0.000	0.313	0.000	0.875	1.000	0.250	0.000	0.000	1.000
			4	0.250			0.000			0.000	0.188	1.000	0.000	0.000	0.000	0.000	0.875	0.000
			5	0.000						0.000	0.500	0.000	0.125	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.125	
			8														0.000	
	S	2	1	0.000	0.000	0.000	0.000	1.000	0.000	0.500	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.000	1.000	1.000	0.250	0.000	1.000	0.500	0.000	0.000	0.000	0.000	0.750	1.000	0.000	0.000
			3	1.000	0.000	0.000	0.750	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.250	0.000	0.000	1.000
			4	0.000			0.000			0.000	0.000	1.000	0.000	1.000	0.000	0.000	1.000	0.000
			5	0.000						0.000	0.000	0.000	1.000	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.000	
			8														0.000	
	T	5	1	0.000	0.000	0.000	0.000	1.000	0.000	0.900	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.000	1.000	1.000	0.300	0.000	1.000	0.100	0.000	0.000	0.000	0.000	1.000	1.000	0.000	0.000
			3	1.000	0.000	0.000	0.700	0.000	0.000	0.000	1.000	0.000	0.700	0.000	0.000	0.000	0.000	1.000
			4	0.000			0.000			0.000	0.000	1.000	0.000	1.000	0.000	0.000	0.800	0.000
			5	0.000						0.000	0.000	0.000	0.300	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.200	
			8														0.000	

Population	Site Code	N	Allele	*Acyc*	*Ak1*	*Ak2*	*Argk*	*Fum*	*Got1*	*Hex*	*Idh2*	*Mdh2*	*Me*	*Mpi*	*PepA*	*PepC*	*PepD1*	*Pgm*
P24(XY) - east	U	5	1	0.000	0.000	0.000	0.000	1.000	0.000	0.300	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.000	1.000	1.000	0.800	0.000	1.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	0.000	0.000
			3	1.000	0.000	0.000	0.200	0.000	0.000	0.700	1.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
			4	0.000			0.000			0.000	0.000	0.500	0.000	1.000	0.000	0.000	0.700	0.000
			5	0.000						0.000	0.000	0.000	1.000	0.000	0.000		0.000	
			6								0.000	0.500	0.000	0.000			0.100	
			7									0.000					0.200	
			8														0.000	
P24(XY) - north	BK	1	1	0.000	0.000	0.000	0.000	1.000	0.500	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000
			2	0.000	1.000	1.000	0.500	0.000	0.500	1.000	0.000	0.000	0.000	0.000	0.500	0.000	0.500	1.000
			3	1.000	0.000	0.000	0.500	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.500	0.000	0.000	0.000
			4	0.000			0.000			0.000	0.000	0.500	0.000	1.000	0.000	0.000	0.500	0.000
			5	0.000						0.000	0.000	0.000	1.000	0.000	0.000		0.000	
			6								0.000	0.500	0.000	0.000			0.000	
			7									0.000					0.000	
			8														0.000	
	C	5	1	0.200	0.000	0.000	0.100	1.000	0.000	0.300	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.100	1.000	1.000	0.800	0.000	0.700	0.000	0.000	0.000	0.000	0.000	0.900	1.000	0.000	0.000
			3	0.700	0.000	0.000	0.100	0.000	0.300	0.700	1.000	0.000	0.700	0.000	0.100	0.000	0.000	1.000
			4	0.000			0.000			0.000	0.000	0.800	0.000	0.800	0.000	0.000	1.000	0.000
			5	0.000						0.000	0.000	0.000	0.300	0.000	0.000		0.000	
			6								0.000	0.200	0.000	0.200			0.000	
			7									0.000					0.000	
			8														0.000	

Population	Site Code	N	Allele	*Acyc*	*Ak1*	*Ak2*	*Argk*	*Fum*	*Got1*	*Hex*	*Idh2*	*Mdh2*	*Me*	*Mpi*	*PepA*	*PepC*	*PepD1*	*Pgm*
*viatica*17 - east	AF	3	1	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.167	0.500	0.000	0.000
			2	0.000	0.250	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.167	0.000	0.000
			3	0.000	0.750	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.667	0.000	0.833	0.333	0.000	1.000
			4	1.000			0.000			0.000	0.000	1.000	0.167	0.000	0.000	0.000	0.833	0.000
			5	0.000						0.000	1.000	0.000	0.167	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.167	
			8														0.000	
	AG	3	1	0.000	0.000	0.000	0.000	0.000	0.333	1.000	0.000	0.000	0.000	0.000	0.000	0.667	0.000	0.000
			2	0.000	0.167	0.167	0.000	0.000	0.667	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			3	0.000	0.833	0.833	1.000	0.000	0.000	0.000	0.000	0.000	0.667	0.000	1.000	0.333	0.167	1.000
			4	0.667			0.000			0.000	0.000	1.000	0.333	0.000	0.000	0.000	0.333	0.000
			5	0.333						0.000	1.000	0.000	0.000	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.500	
			8														0.000	
	AH	2	1	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.000	0.250	0.250	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.250
			3	0.000	0.750	0.750	1.000	0.000	0.000	0.000	0.000	0.000	0.250	0.000	1.000	1.000	0.000	0.750
			4	1.000			0.000			0.000	0.000	1.000	0.500	0.000	0.000	0.000	0.250	0.000
			5	0.000						0.000	1.000	0.000	0.250	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.500	
			8														0.250	

Population	Site Code	N	Allele	*Acyc*	*Ak1*	*Ak2*	*Argk*	*Fum*	*Got1*	*Hex*	*Idh2*	*Mdh2*	*Me*	*Mpi*	*PepA*	*PepC*	*PepD1*	*Pgm*
*viatica*17 - east	AI	1	1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.500	0.000	0.000
			3	0.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.500	0.500	1.000
			4	1.000			0.000			0.000	0.000	1.000	0.500	0.000	0.000	0.000	0.500	0.000
			5	0.000						0.000	1.000	0.000	0.500	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.000	
			8														0.000	
	AL	9	1	0.000	0.000	0.000	0.000	1.000	0.111	0.722	0.000	0.000	0.000	0.000	0.000	0.167	0.000	0.000
			2	0.000	0.313	0.125	0.056	0.000	0.889	0.167	0.000	0.000	0.000	0.000	0.556	0.056	0.000	0.000
			3	0.000	0.688	0.875	0.944	0.000	0.000	0.111	0.222	0.000	0.778	0.000	0.444	0.778	0.111	1.000
			4	0.944			0.000			0.000	0.000	1.000	0.111	1.000	0.000	0.000	0.500	0.000
			5	0.056						0.000	0.778	0.000	0.111	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.389	
			8														0.000	
	AM	3	1	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.167	0.333	0.000	0.000
			2	0.000	0.000	0.167	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.500	0.000	0.000
			3	0.000	1.000	0.833	1.000	0.000	0.000	0.000	0.167	0.000	0.167	0.000	0.833	0.167	0.000	1.000
			4	1.000			0.000			0.000	0.000	1.000	0.833	0.000	0.000	0.000	0.500	0.000
			5	0.000						0.000	0.833	0.000	0.000	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.500	
			8														0.000	

Population	Site Code	N	Allele	*Acyc*	*Ak1*	*Ak2*	*Argk*	*Fum*	*Got1*	*Hex*	*Idh2*	*Mdh2*	*Me*	*Mpi*	*PepA*	*PepC*	*PepD1*	*Pgm*
*viatica*17 - east	AN	2	1	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.250	0.000	0.000
			2	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.500	0.000	0.000
			3	0.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.750	0.000	1.000	0.250	0.000	1.000
			4	0.500			0.000			0.000	0.000	1.000	0.250	0.000	0.000	0.000	0.500	0.000
			5	0.500						0.000	1.000	0.000	0.000	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.500	
			8														0.000	
	D	6	1	0.000	0.000	0.000	0.000	1.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.333	0.000	0.083
			3	0.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.417	0.000	1.000	0.667	0.000	0.917
			4	0.833			0.000			0.000	0.000	1.000	0.500	1.000	0.000	0.000	0.583	0.000
			5	0.167						0.000	1.000	0.000	0.083	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.417	
			8														0.000	
	E	6	1	0.000	0.000	0.000	0.000	1.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.500	0.000	0.000
			2	0.000	0.100	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.167	0.000	0.000
			3	0.000	0.900	1.000	0.917	0.000	0.000	0.000	0.000	0.000	0.583	0.000	0.750	0.333	0.000	1.000
			4	0.667			0.083			0.000	0.000	0.833	0.333	1.000	0.250	0.000	0.667	0.000
			5	0.333						0.000	1.000	0.000	0.083	0.000	0.000		0.000	
			6								0.000	0.167	0.000	0.000			0.000	
			7									0.000					0.333	
			8														0.000	

Population	Site Code	N	Allele	*Acyc*	*Ak1*	*Ak2*	*Argk*	*Fum*	*Got1*	*Hex*	*Idh2*	*Mdh2*	*Me*	*Mpi*	*PepA*	*PepC*	*PepD1*	*Pgm*
*viatica*17 - east	F	2	1	0.000	0.000	0.000	0.000	0.000	0.250	1.000	0.000	0.000	0.000	0.000	0.000	0.750	0.000	0.000
			2	0.000	0.000	0.000	0.000	0.000	0.750	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			3	0.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.250	0.250	0.000	1.000	0.250	0.250	1.000
			4	1.000			0.000			0.000	0.000	0.750	0.250	0.000	0.000	0.000	0.750	0.000
			5	0.000						0.000	1.000	0.000	0.500	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.000	
			8														0.000	
	Q	3	1	0.000	0.000	0.000	0.000	0.000	0.167	1.000	0.000	0.000	0.000	0.000	0.000	0.333	0.000	0.000
			2	0.000	0.167	0.000	0.167	0.000	0.833	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			3	0.000	0.833	1.000	0.833	0.000	0.000	0.000	0.000	0.000	0.667	0.000	1.000	0.667	0.000	1.000
			4	1.000			0.000			0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.500	0.000
			5	0.000						0.000	1.000	0.000	0.333	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.500	
			8														0.000	
	R	6	1	0.000	0.000	0.000	0.000	1.000	0.000	1.000	0.000	0.000	0.000	0.000	0.083	0.333	0.000	0.000
			2	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.167	0.000	0.167
			3	0.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.333	0.000	0.917	0.500	0.250	0.833
			4	1.000			0.000			0.000	0.000	1.000	0.083	1.000	0.000	0.000	0.250	0.000
			5	0.000						0.000	1.000	0.000	0.583	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.500	
			8														0.000	

Population	Site Code	N	Allele	*Acyc*	*Ak1*	*Ak2*	*Argk*	*Fum*	*Got1*	*Hex*	*Idh2*	*Mdh2*	*Me*	*Mpi*	*PepA*	*PepC*	*PepD1*	*Pgm*
*viatica*17 - east	W	3	1	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.833	0.000	0.000
			2	0.000	0.000	0.000	0.167	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.167	0.000	0.000	0.000
			3	0.000	1.000	1.000	0.833	0.000	0.000	0.000	0.000	0.000	0.833	0.000	0.833	0.167	0.167	1.000
			4	0.167			0.000			0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.333	0.000
			5	0.833						0.000	1.000	0.000	0.167	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.500	
			8														0.000	
	X	3	1	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.333	0.000	0.000
			2	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.167
			3	0.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.167	0.000	1.000	0.667	0.667	0.833
			4	1.000			0.000			0.000	0.000	1.000	0.333	0.000	0.000	0.000	0.167	0.000
			5	0.000						0.000	1.000	0.000	0.500	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.167	
			8														0.000	
	Y	3	1	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.500	0.000	0.000
			2	0.000	0.167	0.000	0.167	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.333	0.000	0.000
			3	0.000	0.833	1.000	0.833	0.000	0.000	0.000	0.167	0.000	0.667	0.000	1.000	0.167	0.167	1.000
			4	1.000			0.000			0.000	0.000	1.000	0.167	0.000	0.000	0.000	0.833	0.000
			5	0.000						0.000	0.833	0.000	0.167	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.000	
			8														0.000	

Population	Site Code	N	Allele	*Acyc*	*Ak1*	*Ak2*	*Argk*	*Fum*	*Got1*	*Hex*	*Idh2*	*Mdh2*	*Me*	*Mpi*	*PepA*	*PepC*	*PepD1*	*Pgm*
*viatica*17-south	AO	9	1	0.000	0.000	0.000	0.000	1.000	0.000	0.944	0.000	0.000	0.000	0.056	0.000	0.000	0.000	0.000
			2	0.167	0.000	0.500	0.000	0.000	0.944	0.056	0.000	0.000	0.000	0.889	0.000	0.000	0.000	0.444
			3	0.000	1.000	0.500	1.000	0.000	0.056	0.000	0.111	0.000	0.056	0.000	1.000	1.000	0.000	0.556
			4	0.833			0.000			0.000	0.000	1.000	0.944	0.056	0.000	0.000	1.000	0.000
			5	0.000						0.000	0.889	0.000	0.000	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.000	
			8														0.000	
	AQ	1	1	0.000	0.000	0.000	0.000	1.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000
			3	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	0.000	1.000
			4	1.000			0.000			0.000	0.000	1.000	1.000	0.000	0.000	0.000	1.000	0.000
			5	0.000						0.000	1.000	0.000	0.000	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.000	
			8														0.000	
	AT	1	1	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.500	0.000	0.000	0.000	0.000	1.000	1.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.500
			3	0.000	1.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	0.000	0.500
			4	0.500			0.000			0.000	0.000	1.000	1.000	0.000	0.000	0.000	1.000	0.000
			5	0.000						0.000	1.000	0.000	0.000	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.000	
			8														0.000	

Population	Site Code	N	Allele	*Acyc*	*Ak1*	*Ak2*	*Argk*	*Fum*	*Got1*	*Hex*	*Idh2*	*Mdh2*	*Me*	*Mpi*	*PepA*	*PepC*	*PepD1*	*Pgm*
*viatica*17-south	AU	8	1	0.000	0.000	0.000	0.000	1.000	0.000	0.438	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.000	0.000	0.786	0.000	0.000	1.000	0.563	0.000	0.000	0.000	1.000	0.938	0.000	0.000	0.188
			3	0.000	1.000	0.214	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.063	1.000	0.000	0.813
			4	1.000			0.000			0.000	0.000	1.000	1.000	0.000	0.000	0.000	1.000	0.000
			5	0.000						0.000	1.000	0.000	0.000	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.000	
			8														0.000	
	AY	7	1	0.000	0.000	0.000	0.000	1.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.143	0.000	0.167	0.000	0.000	1.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.357
			3	0.000	1.000	0.833	1.000	0.000	0.000	0.000	0.071	0.000	0.071	0.000	1.000	1.000	0.000	0.643
			4	0.857			0.000			0.000	0.000	1.000	0.786	0.000	0.000	0.000	1.000	0.000
			5	0.000						0.000	0.857	0.000	0.143	0.000	0.000		0.000	
			6								0.071	0.000	0.000	0.000			0.000	
			7									0.000					0.000	
			8														0.000	
	AZ	1	1	0.000	0.000	0.000	0.000	1.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.500	0.000	1.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.500	0.000	0.000	0.000	0.500
			3	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	0.000	0.500
			4	0.500			0.000			0.000	0.000	1.000	1.000	0.500	0.000	0.000	0.500	0.000
			5	0.000						0.000	1.000	0.000	0.000	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.500	
			8														0.000	

Population	Site Code	N	Allele	*Acyc*	*Ak1*	*Ak2*	*Argk*	*Fum*	*Got1*	*Hex*	*Idh2*	*Mdh2*	*Me*	*Mpi*	*PepA*	*PepC*	*PepD1*	*Pgm*
*viatica*17-south	BA	2	1	0.000	0.000	0.000	0.000	1.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.750
			3	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	0.000	0.250
			4	1.000			0.000			0.000	0.000	1.000	1.000	0.000	0.000	0.000	1.000	0.000
			5	0.000						0.000	1.000	0.000	0.000	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.000	
			8														0.000	
	BC	7	1	0.000	0.000	0.000	0.000	1.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.500	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.714
			3	0.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.071	0.000	1.000	1.000	0.000	0.286
			4	0.500			0.000			0.000	0.000	1.000	0.786	0.000	0.000	0.000	1.000	0.000
			5	0.000						0.000	1.000	0.000	0.143	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.000	
			8														0.000	
	BD	7	1	0.000	0.000	0.000	0.000	1.000	0.000	0.929	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.214	0.250	0.000	0.000	0.000	1.000	0.071	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.929
			3	0.000	0.750	0.000	1.000	0.000	0.000	0.000	0.071	0.000	0.000	0.000	1.000	1.000	0.000	0.071
			4	0.786			0.000			0.000	0.000	0.643	0.857	0.000	0.000	0.000	1.000	0.000
			5	0.000						0.000	0.714	0.000	0.143	0.000	0.000		0.000	
			6								0.214	0.214	0.000	0.000			0.000	
			7									0.143					0.000	
			8														0.000	

Population	Site Code	N	Allele	*Acyc*	*Ak1*	*Ak2*	*Argk*	*Fum*	*Got1*	*Hex*	*Idh2*	*Mdh2*	*Me*	*Mpi*	*PepA*	*PepC*	*PepD1*	*Pgm*
*viatica*17-south	BF	1	1	0.000	0.000	0.000	0.000	1.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.500
			3	0.000	1.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	1.000	1.000	0.000	0.500
			4	1.000			0.000			0.000	0.000	1.000	0.000	0.000	0.000	0.000	1.000	0.000
			5	0.000						0.000	1.000	0.000	0.000	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.000	
			8														0.000	
	BN	17	1	0.000	0.000	0.000	0.000	1.000	0.000	0.941	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.324	0.000	0.571	0.000	0.000	0.971	0.059	0.000	0.000	0.000	0.971	0.000	0.000	0.000	0.176
			3	0.000	1.000	0.429	1.000	0.000	0.029	0.000	0.088	0.000	0.147	0.000	1.000	1.000	0.000	0.824
			4	0.676			0.000			0.000	0.000	1.000	0.853	0.029	0.000	0.000	0.971	0.000
			5	0.000						0.000	0.735	0.000	0.000	0.000	0.000		0.000	
			6								0.176	0.000	0.000	0.000			0.000	
			7									0.000					0.029	
			8														0.000	
	BO	2	1	0.000	0.000	0.000	0.000	1.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.500	0.000	1.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000
			3	0.000	1.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.250	0.000	1.000	1.000	0.000	1.000
			4	0.500			0.000			0.000	0.000	1.000	0.750	0.000	0.000	0.000	1.000	0.000
			5	0.000						0.000	1.000	0.000	0.000	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.000	
			8														0.000	

Population	Site Code	N	Allele	*Acyc*	*Ak1*	*Ak2*	*Argk*	*Fum*	*Got1*	*Hex*	*Idh2*	*Mdh2*	*Me*	*Mpi*	*PepA*	*PepC*	*PepD1*	*Pgm*
*viatica*17-south	BR	1	1	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	1.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000
			3	0.000	1.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	0.000	1.000
			4	0.000			0.000			0.000	0.000	1.000	1.000	0.000	0.000	0.000	1.000	0.000
			5	0.000						0.000	0.000	0.000	0.000	0.000	0.000		0.000	
			6								1.000	0.000	0.000	0.000			0.000	
			7									0.000					0.000	
			8														0.000	
	BV	1	1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	0.000	0.000	0.500	0.000	0.000	1.000	1.000	0.000	0.000	0.000	1.000	0.500	0.000	0.000	0.000
			3	0.000	1.000	0.500	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.500	1.000	0.000	1.000
			4	1.000			0.000			0.000	0.000	1.000	1.000	0.000	0.000	0.000	1.000	0.000
			5	0.000						0.000	1.000	0.000	0.000	0.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.000	
			8														0.000	
*viatica*19	AD	1	1	0.000	0.000	0.500	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000
			2	1.000	1.000	0.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000
			3	0.000	0.000	0.500	1.000	1.000	0.000	0.500	1.000	0.000	0.000	0.000	0.500	0.500	0.500	0.500
			4	0.000			0.000			0.500	0.000	0.000	0.000	0.500	0.500	0.500	0.500	0.500
			5	0.000						0.000	0.000	0.000	0.000	0.500	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.000	
			8														0.000	

Population	Site Code	N	Allele	*Acyc*	*Ak1*	*Ak2*	*Argk*	*Fum*	*Got1*	*Hex*	*Idh2*	*Mdh2*	*Me*	*Mpi*	*PepA*	*PepC*	*PepD1*	*Pgm*
*viatica*19	AV	4	1	0.125	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.250	0.000	0.000	0.000	0.000	0.125
			2	0.875	1.000	0.000	0.000	0.125	1.000	0.000	0.000	1.000	0.750	0.000	0.000	0.000	0.000	0.125
			3	0.000	0.000	1.000	1.000	0.875	0.000	0.125	1.000	0.000	0.000	0.000	0.250	0.625	0.000	0.750
			4	0.000			0.000			0.750	0.000	0.000	0.000	0.125	0.750	0.375	0.875	0.000
			5	0.000						0.125	0.000	0.000	0.000	0.875	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.125	
			8														0.000	
	AW	4	1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.250	0.000	0.000	0.000	0.000	0.000
			2	1.000	1.000	0.000	0.000	0.000	1.000	0.000	0.000	1.000	0.750	0.000	0.000	0.000	0.000	0.000
			3	0.000	0.000	0.000	1.000	1.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.750	0.000	1.000
			4	0.000			0.000			1.000	0.000	0.000	0.000	0.250	1.000	0.250	0.750	0.000
			5	0.000						0.000	0.000	0.000	0.000	0.750	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.250	
			8														0.000	
	AX	2	1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.250
			2	1.000	1.000	0.000	0.000	0.000	1.000	0.000	0.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000
			3	0.000	0.000	1.000	1.000	1.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.500	0.000	0.750
			4	0.000			0.000			1.000	0.000	0.000	0.000	0.000	0.750	0.500	1.000	0.000
			5	0.000						0.000	0.000	0.000	0.000	1.000	0.250		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.000	
			8														0.000	

Population	Site Code	N	Allele	*Acyc*	*Ak1*	*Ak2*	*Argk*	*Fum*	*Got1*	*Hex*	*Idh2*	*Mdh2*	*Me*	*Mpi*	*PepA*	*PepC*	*PepD1*	*Pgm*
*viatica*19	BE	6	1	0.333	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.167	0.000	0.000	0.000	0.000	0.000
			2	0.667	1.000	0.000	0.000	0.000	1.000	0.000	0.000	0.917	0.833	0.000	0.000	0.000	0.000	0.000
			3	0.000	0.000	0.000	1.000	1.000	0.000	0.083	1.000	0.000	0.000	0.000	0.083	0.667	0.000	1.000
			4	0.000			0.000			0.833	0.000	0.000	0.000	0.000	0.917	0.333	1.000	0.000
			5	0.000						0.083	0.000	0.083	0.000	1.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.000	
			8														0.000	
	BH	7	1	0.071	0.071	0.000	0.000	0.000	0.000	0.071	0.071	0.000	0.143	0.000	0.000	0.000	0.000	0.000
			2	0.929	0.929	0.000	0.000	0.000	1.000	0.000	0.000	1.000	0.857	0.000	0.000	0.000	0.000	0.000
			3	0.000	0.000	1.000	1.000	1.000	0.000	0.143	0.929	0.000	0.000	0.000	0.000	0.857	0.000	1.000
			4	0.000			0.000			0.786	0.000	0.000	0.000	0.500	1.000	0.143	0.857	0.000
			5	0.000						0.000	0.000	0.000	0.000	0.500	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.143	
			8														0.000	
	BI	2	1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
			2	1.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000
			3	0.000	0.000	0.000	1.000	1.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	1.000	0.000	1.000
			4	0.000			0.000			1.000	0.000	0.000	0.000	0.250	1.000	0.000	1.000	0.000
			5	0.000						0.000	0.000	0.000	0.000	0.750	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.000	
			8														0.000	

Population	Site Code	N	Allele	*Acyc*	*Ak1*	*Ak2*	*Argk*	*Fum*	*Got1*	*Hex*	*Idh2*	*Mdh2*	*Me*	*Mpi*	*PepA*	*PepC*	*PepD1*	*Pgm*
*viatica*19	BJ	5	1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.100
			2	1.000	1.000	0.000	0.000	0.000	1.000	0.000	0.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000
			3	0.000	0.000	1.000	1.000	1.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.400	0.000	0.900
			4	0.000			0.000			1.000	0.000	0.000	0.000	0.100	1.000	0.600	0.800	0.000
			5	0.000						0.000	0.000	0.000	0.000	0.900	0.000		0.100	
			6								0.000	0.000	0.000	0.000			0.100	
			7									0.000					0.000	
			8														0.000	
	BL	3	1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.167	0.167	0.333	0.000	0.000	0.000	0.000	0.333
			2	1.000	1.000	0.000	0.000	0.000	1.000	0.000	0.167	0.833	0.667	0.000	0.000	0.000	0.000	0.000
			3	0.000	0.000	1.000	1.000	1.000	0.000	0.000	0.667	0.000	0.000	0.000	0.000	0.500	0.000	0.500
			4	0.000			0.000			0.833	0.000	0.000	0.000	0.000	1.000	0.500	1.000	0.167
			5	0.000						0.167	0.000	0.000	0.000	1.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.000	
			8														0.000	
	BM	2	1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.250	0.000
			2	1.000	1.000	0.000	0.000	0.000	1.000	0.000	0.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000
			3	0.000	0.000	0.000	1.000	1.000	0.000	0.000	1.000	0.000	0.000	0.000	0.500	0.500	0.000	1.000
			4	0.000			0.000			1.000	0.000	0.000	0.000	0.250	0.500	0.500	0.750	0.000
			5	0.000						0.000	0.000	0.000	0.000	0.500	0.000		0.000	
			6								0.000	0.000	0.000	0.250			0.000	
			7									0.000					0.000	
			8														0.000	

Population	Site Code	N	Allele	*Acyc*	*Ak1*	*Ak2*	*Argk*	*Fum*	*Got1*	*Hex*	*Idh2*	*Mdh2*	*Me*	*Mpi*	*PepA*	*PepC*	*PepD1*	*Pgm*
*viatica*19	BS	6	1	0.000	0.000	0.000	0.000	0.167	0.000	0.000	0.000	0.000	0.167	0.000	0.000	0.000	0.000	0.000
			2	1.000	1.000	0.000	0.000	0.167	1.000	0.000	0.000	1.000	0.833	0.000	0.000	0.000	0.167	0.000
			3	0.000	0.000	1.000	1.000	0.667	0.000	0.000	1.000	0.000	0.000	0.000	0.167	0.583	0.000	1.000
			4	0.000			0.000			1.000	0.000	0.000	0.000	0.000	0.833	0.417	0.750	0.000
			5	0.000						0.000	0.000	0.000	0.000	1.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.083	
			8														0.000	
	Z	6	1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.083	0.000	0.000	0.000	0.000	0.000
			2	1.000	0.667	0.000	0.000	0.000	1.000	0.000	0.000	1.000	0.917	0.000	0.000	0.000	0.000	0.000
			3	0.000	0.333	1.000	1.000	1.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	1.000	0.000	0.917
			4	0.000			0.000			1.000	0.000	0.000	0.000	0.000	1.000	0.000	0.833	0.083
			5	0.000						0.000	0.000	0.000	0.000	1.000	0.000		0.000	
			6								0.000	0.000	0.000	0.000			0.000	
			7									0.000					0.167	
			8														0.000	

Appendix Fig. 5.1 Sample haplotype frequencies and variable sites in the sequence alignment of 61 haplotypes of *COI* fragment (637 bp) found in the three chromosomal races of *Vandiemenella* on Kangaroo Island (populations: *viatica*19 [V19]; *viatica*17 [V17] East and South; P24(XY) East and North). Identical nucleotides with the haplotype 1 are marked as dots.

		Frequency					Nucleotide positions	
		V19	V17		P24(XY)		1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 2 2 2 3 3 3 3 3 3 3 3 3 3 3 3 3 4 4 4 4 4 4 4 4 4 4 4 4 5 5 5 5 5 5 5 5 5 5 5 6 6 6 6 6 6	
			East	South	East	North	1 2 2 2 3 3 7 7 0 0 1 3 7 7 8 9 9 0 0 1 2 3 3 4 5 6 7 7 9 0 1 1 3 3 3 4 5 6 6 7 7 8 9 9 0 0 0 0 1 1 2 2 6 7 7 7 8 9 0 0 0 4 4 4 5 5 6 6 9 1 1 2 2 3 3	
Haplotype	Phylogroup						8 4 5 7 0 9 5 8 5 8 4 9 1 7 0 7 8 1 2 3 5 1 4 6 9 7 6 8 8 0 2 8 6 7 9 8 2 0 9 2 6 2 3 9 2 5 8 4 7 4 9 4 1 2 4 6 1 1 4 7 0 3 6 0 8 1 2 2 2 5 1 4 3 6	Accession No.
1	Mid	4		6	12		G A G A T A A G G C T G A C A T A C T G G T C C T C A C A G G T T C A T G A G G G C T A T G T A A C A G C A T C C C A A A C A G T A G C C T T A G T	EU121420
2	Mid			1			. G	EU121421
3	Mid			1			. . . . . G	EU121422
4	Mid	1					. . . . . A	EU121423
5	Mid	4			7		. . . . . C	EU121424
6	Mid	3					. . . . G	EU121425
7	Mid	1					. . . . . A	EU121426
8	Mid	1					. . . . . T	EU121427
9	Mid	1					. . . . . G	EU121428
10	Mid				1		. . . . . G	EU121429
11	Mid	2					. . . . . A .	EU121430
12	Mid			5			. . . . . G	EU121431
13	Mid				1		. . . . . A	EU121432
14	Mid				1		. . . . . T	EU121433
15	Mid	1					. . . . A . . . . C	EU121434
16	Mid				2		. . . . . C . . . . C . . . . . A	EU121435
17	Mid	2					. . . . . C . . . . . T	EU121436
18	Mid				1		. . . . . A . . . . . C G	EU121437
19	Mid				2		. . . . . A . . . . . C . . A	EU121438
20	Mid	1					. . . . . T . . T . . . . . C	EU121439
21	Mid	2					. . . . . T . . T . . . . . C . . . . . A	EU121440
22	Mid	1					. . . . . A . . T . . T . . . . C . . . . . C . . . . . A	EU121441
23	Mid				1		. . . . . T G . . . . . C . . . . . T . . . . . C . . .	EU121442
24	Mid			1			. . . . . A . . . . . T . . . . . T . . . . . C	EU121443
25	Mid	1					. . . . . G . . . . . T C . . . . . A . . . . . G	EU121444
26	Mid	2					. . . . . C . . . . . G	EU121445
27	Mid	1					. . . . . A . . . . . T . . . . . A .	EU121446
28	Mid	1					. . . . . G . . . . . A .	EU121447
29	Mid	1					. . . . . G . . . . A	EU121448
30	Mid	3					. . . . . A . . . . . G	EU121449
31	Mid	1					. . . . . A . . . . . A . . . . . G	EU121450
32	Mid			2			. . . . . C . . . . . G . . . G	EU121451
33	Mid	2					. . . . . G . . . . . G	EU121452
34	Mid	2					. . . . . G . . . . . C	EU121453
35	Mid			1			. . . . . A . . . . . G . . . . . C	EU121454
36	Mid			2			. . . . . T . . . . . G . . . . . C . . . . . A	EU121455
37	Mid	2					. . . . . C . . . . . G	EU121456

Haplotype	Phylogroup	Frequency V19	Frequency V17 East	Frequency V17 South	Frequency P24(XY) East	Frequency P24(XY) North	Nucleotide positions	Accession No.
38	East		23		1		A.TC...A....T.....CT...G...A...G.....C.....C.....T..GG.....G..	EU121457
39	East				2		A.TC...AA....T.....CT...G...A...G.....C.....C.....T..GG.....G..	EU121458
40	East		1				..TC...A....T.....CT...G...A...G.....C.....CA.....T..GG.....G..	EU121459
41	East		1				A.TC...A....T....C..CT...G...A...G.....C.....C.....T..GG.....G..	EU121460
42	East		1				A.TC...A....T....C..CT...G...A...GC.....C.....C.....T..GG.....G..	EU121461
43	East				1		A.TC...A....T.....CT...G...A...G.....C..A...A....C.....T..GG.....G..	EU121462
44	East		1				A.TT...A....T.....CT...G...A...G.....C.....C.....T..GG.....G..	EU121463
45	East		3				A..C...A....T.....CT...G...AC..G....A..C.....C..T...T..GG.....G..	EU121464
46	East		1				A..C...A....T.....CT...G...A...G.....AC.....C..T...T..GG.....G..	EU121465
47	East		2				A..C...A....T.....CT...G...A...G.....AC.....C..T...T..GG....CG..	EU121466
48	East		1				A..C...A....T.....CT...G.G.A...G.....CT.....C.....T..GG.....G..	EU121467
49	East		1				A..C...A....T.....CT...G...A...G.....C.....C.....T..G.....G..	EU121468
50	East		1				A..C...A....T...T..CT...G...A...G.....C.....C.....T..G.....G..	EU121469
51	East		1				A..C...A....T.....CT..TG...A...G.....C.....C.....T..GG.....G..	EU121470
52	East		1				A..C...A....T.....CT..TG...A.....C.....C.....T..GG.....G..	EU121471
53	East		2				A..C...A....T.....CTT..G...A.C.....C.....C.....T..GG...C.G..	EU121472
54	North					3	.....T...T..A.....G..AA.....C....C.....GT.....C	EU121473
55	North					1	.....T...T..A.....G...A.....C....C.....GT.....C	EU121474
56	North					1	.....T.C.T..A.....G..AA.....C....C.....GT.....C	EU121475
57	North					1	.....T...T..A.....G..AA.....CA...C.....GT.....C	EU121476
58	North					1	.....T...T..AC....G..AA.C....C....C.....GT.....C	EU121477
59	North					3	....A.G.....T...T..A.....G..AA.....C.A..C.....GT.....C	EU121478
60	North					2	.....G...T..A.....G..AA.....C....C.....T.....GT.....C	EU121479
61	North					2	.....T...T..A.....G..AA.....C....C.....A.....GT.....C	EU121480

Appendix Fig. 5.2 Allele frequencies and variable sites in the sequence alignment of 22 haplotypes of *EF*-1α fragment (825 bp) found in the three chromosomal races of *Vandiemenella* on Kangaroo Island (populations: *viatica*19 [V19]; *viatica*17 [V17] East and South; P24(XY) East and North). Identical nucleotides with the allele1 are marked as dots. Nucleotide sites in exon regions are marked in bold.

		Frequency					Nucleotide positions	
		V19	V17		P24(XY)			
			East	South	East	North	**1**111111112**2 3 3**44444**6 6 7 7**7777777777777778888 **6 7 2**5567788009**0 3**0113**4 6 0 0**3335555778889900012 **0 8 9**1200467031**7 6**4891**0 4 3 9**6790124295674525 9 1	
Allele	Phylogroup							Accession No.
1	Viatica19	49					**CCT**TAATGAAAA**GTA**GTGG**CGGC**AAG - - - AATTCTCGGCGT	EU121486
2	Viatica19	2					. . . . . C . . . . . - - -	EU121487
3	Viatica19	1					. . . . . **T** . . . . . - - -	EU121488
4	Viatica19	1					. . . . . **C** . . . . . - - -	EU121489
5	Viatica19	4					. . . . . **A** . . . . . - - -	EU121490
6	Viatica19	2					. . . . . A . . . . . A - - -	EU121491
7	Viatica19	1					. . . . T . . . . . - - -	EU121492
8	Viatica19	1					. . . C . . . . . - - -	EU121493
9	Viatica19	2					. . . . . A	EU121494
10	Viatica19	5					. . . . . T	EU121495
11	Viatica17		26	29			**T** . . . . . C . . . **G** . . . . . - - - C . G . . . T . . A . .	EU121496
12	Viatica17		1				**T** . . . . . C . . . **G** . - - - . . . . . - - - C . G . . . T . . A . .	EU121497
13	Viatica17		2				**T** . . . . . CG . . **G** . . . . . - - - C . G . . . T . . A . .	EU121498
14	Viatica17		13		2		**T** . . . . . CG . . **G** . . . . . - - - C . G . . . T . AA . .	EU121499
15	Viatica17		3				**TT** . . . . . CG . . **G** . . . . . - - - C . G . . . T . AA . .	EU121500
16	Viatica17			1			**T** . . . . . C . . . **G** . . . . . **A** . . . . . - - - C . G . . . T . . A . .	EU121501
17	Viatica17		1				**T** . . . . . - - C . **A** . **G** . . . . . - - - C . G . . . T . . A . .	EU121502
18	P24(XY)		6		15	19	. . . . . TC . . . C . . . **G** . . . . . **A** . G . . TGT . TG - - - T . . ATA	EU121503
19	P24(XY)				1	1	. . . . . TCA . . C . . . **G** . . . . . **A** . G . . TGT . TG - - - T . . ATA	EU121504
20	P24(XY)				2		. . . . . TCA . . C . . . **G** . . . . **A** . **A** . G . . TGT . TG - - - T . . ATA	EU121505
21	P24(XY)					6	. . . . . TC . . . C . . . **G** . . . . . **A** . - - - . TGT . TG - - - T . . ATA	EU121506
22	P24(XY)				2		. . **C** . . TC . . . C . . . **G** T . . . . . G . . TGT . TG - - - T . . . TA	EU121507

Appendix Table 6.1 List of localities studied. Locality numbers indicated in Fig. 6.1 (No.), Taxa, locality names, Australian States, number of samples (*N*, total; N_A, allozyme; N_C, *COI*; N_E, *EF*-1α; N_M, *Mvia11*).

No.	Taxa	Locality	State*	*N*†	N_A	N_C	N_E	N_M	Latitude (S), Longitude (W)
1	viatica17	Penneshaw	SA (KI)	5	5	4	4	2	35.7192, 137.9413
2		Dudley Peninsula	SA (KI)	8	6	6	7	2	35.8325, 137.9293
3		Pelican Lagoon	SA (KI)	29 (6)	0	26	13	6	35.8470, 137.7911
4		Cape Willoughby	SA (KI)	5	2	4	4	0	35.8576, 138.0972
5		Seal Bay	SA (KI)	21	3	21	15	1	35.9782, 137.2573
6		Gawler	SA	6	3	2	4	4	34.7075, 138.9610
7		Cape Jervis	SA	4	3	2	2	1	35.6084, 138.0954
8		Geranium	SA	6	5	2	4	4	35.5131, 140.2598
9		Tintinara	SA	4	2	2	2	0	35.9605, 139.8716
10		Keith-north	SA	9 (2)	2	8	8	6	35.9751, 140.4325
11		Policemans Point	SA	4	2	4	2	2	36.1012, 139.6612
12	viatica19	Lathami Cons. Park	SA (KI)	6	1	5	4	0	35.6440, 137.2587
13		Beyeria Cons. Park	SA (KI)	4	1	4	3	0	35.7802, 137.5924
14		Edward Lagoon	SA (KI)	6	0	6	5	0	35.7957, 137.1662
15		Harriet River	SA (KI)	4	1	4	4	0	35.9317, 137.0725
16		Point Ellen	SA (KI)	12	0	12	11	0	35.9744, 137.2760
17		Point Tinline	SA (KI)	8	5	3	3	2	36.0023, 137.6065
18		Hanson Bay	SA (KI)	6	2	4	3	0	36.0123, 136.8534
19		Keith south	SA	7 (4)	2	5	6	2	36.0879, 140.4399
20		Weampa	SA	2	0	2	2	0	36.2330, 140.1177
21		Coorong Nat. Park	SA	6 (2)	2	5	5	5	36.3942, 139.7657
22		Padthaway	SA	4	1	2	4	2	36.5894, 140.5056
23		Penola	SA	3	2	2	2	2	37.5020, 140.8178
24		Bendigo	VIC	3	3	3	3	2	36.7612, 144.2789
25		Halls Gap	VIC	6	2	2	5	2	37.1331, 142.4797
26		Grampians	VIC	10	0	2	10	2	37.3970, 142.2806
27		Portland	VIC	1	1	1	1	0	38.3580, 141.4498
28		Geelong	VIC	1	1	0	1	1	38.4100, 144.1850
29		Coles Bay	TAS	3	3	2	2	1	42.1340, 148.2920
30	P24(XO)	Mt Remarkable	SA	5	4	2	2	2	32.8228, 138.1836
31		Thompson Beach	SA	2	0	2	2	2	34.4762, 138.2663
32		Port Gawler	SA	4	3	2	2	1	34.6516, 138.4458
33		Aldinga Beach	SA	4	2	2	1	2	35.2886, 138.4454
34	P24(XY)	Streaky Bay	SA	6	3	4	4	3	33.0156, 134.1914
35		Arno Bay	SA	5	3	4	4	2	33.8316, 136.5548
36		Balgowan	SA	5	4	2	2	2	34.3231, 137.4961
37		Ardrossan	SA	5	5	2	2	2	34.4169, 137.9271
38		Port Julia	SA	3	2	2	2	2	34.6612, 137.8815
39		Edithburgh	SA	5	5	2	2	2	35.0938, 137.7450
40		Innes Nat. Park	SA	12	12	5	2	1	35.2689, 136.8902
41		Emu Bay	SA (KI)	6	2	6	5	0	35.5945, 137.5253
42		Stokes Bay	SA (KI)	9	1	8	8	2	35.6179, 137.2808
43		American River	SA (KI)	41 (9)	13	32	11	8	35.8517, 137.7504
44	P24(XY)-Transl	Mt Dutton Bay	SA	5	4	0	2	2	34.5119, 135.3945
45		Coffin Bay	SA	5	4	0	2	2	34.6566, 135.3655
46	P25(XY)	Alligator Gorge	SA	7	7	2	2	1	32.7416, 138.0779
47	P45b(XO)	Port Bonython	SA	6	5	2	2	1	32.9359, 137.7539
48		Port Germein	SA	11	5	8	8	8	32.9617, 137.9911
49		Talowe Gorge	SA	5	3	2	2	2	33.0257, 138.1016
50		Port Broughton	SA	4	3	2	2	2	33.5914, 137.9301
51	P45b(XY)	Kimba	SA	5	4	2	2	1	33.2718, 136.4533
52		Lucky Bay	SA	4	4	2	2	1	33.7060, 137.0528
53	P45c	Sheringa Beach	SA	3	3	2	2	2	33.8722, 135.1699
54		Lake Hamilton	SA	3	3	2	1	2	34.0118, 135.2680
55		Mt Hope	SA	4	3	2	2	2	34.2212, 135.3353
56	P50	Peebinga	SA	3	3	2	3	2	34.9164, 140.7636
57		Peebinga CP	SA	2	2	1	2	2	34.9164, 140.7636
58	V. pichirichi	Flinders Ranges	SA	10	5	5	4	1	32.3397, 137.9674
59	Keyacris P110a	Winninowie	SA	4	3	2	1	2	32.6283, 137.9163

*: Kangaroo Island (KI) in South Australia
†: numbers in brackets indicate specimens that were collected from sites within 1 km of presumed centre of contact zones

Appendix Table 6.2 Locus names, enzymes, enzyme commission (EC) numbers, and buffer conditions fpr allozyme electrophoresis used in this study. The nomenclature for referring to allozymes and multiple loci follows Maryan *et al.* (2007).

	Locus	Enzymes	EC number	Buffer*
1	*Acon1*	aconitase hydratase	4.2.1.3	I
2	*Acon2*	aconitase hydratase	4.2.1.3	A
3	*Acyc*	aminoacylase	3.5.1.14	I & C
4	*Ada*	adenosine deaminase	3.5.4.4	B
5	*Ak1*	adenylate kinase	2.7.4.3	C
6	*Ak2*	adenylate kinase	2.7.4.3	C
7	*Ald*	fructose-bisphosphate aldolase	4.1.2.13	B
8	*Argk*	arginine kinase	2.7.3.3	C & I
9	*Dia*	diaphorase	1.6.99.	C
10	*Enol*	enolase	4.2.1.11	B
11	*Est*	esterase	3.1.1	C
12	*Fdp*	fructose-bisphosphatase	3.1.3.11	B
13	*Fum*	fumarate hydratase	4.2.1.2	A
14	*Glo*	lactoylglutathione lyase	4.4.1.5	C
15	*Got1*	aspartate aminotransferase	2.6.1.1	A
16	*Got2*	aspartate aminotransferase	2.6.1.1	A
17	*Gpd*	glycerol-3-phosphate dehydrogenase	1.1.1.8	B
18	*Gpi*	glucose-6-phosphate isomerase	5.3.1.9	C
19	*Idh1*	isocitrate dehydrogenase	1.1.1.42	A
20	*Idh2*	isocitrate dehydrogenase	1.1.1.42	A
21	*Ldh*	L-lactate dehydrogenase	1.1.1.27	B
22	*Mdh1*	malate dehydrogenase	1.1.1.37	A & B
23	*Mdh2*	malate dehydrogenase	1.1.1.37	B
24	*Me*	"malic" enzyme	1.1.1.40	B
25	*Mpi*	mannose-6-phosphate isomerase	5.3.1.8	I
26	*Ndpk*	nucleoside-diphosphate kinase	2.7.4.6	C
27	*PepA*	dipeptidase (val-leu)	3.4.13.	C
28	*PepC*	dipeptidase (lys-leu)	3.4.13.	C
29	*PepD*	proline dipeptidase	3.4.13.	I
30	*Pgam*	phosphoglycerate mutase	5.4.2.1	C
31	*6Pgd*	phosphogluconate dehydrogenase	1.1.1.44	B
32	*Pgk*	phosphoglycerate kinase	2.7.2.3	C
33	*Pgm*	phosphoglucomutase	5.4.2.2	C
34	*Pk*	pyruvate kinase	2.7.1.40	A & I
35	*Tpi*	triose-phosphate isomerase	5.3.1.1	A

* Buffer code follows Table 9.2 in Richardson *et al.* (1986)

Appendix Table 6.3 Allele frequencies of 35 allozyme loci at 53 sampling sites, and global statistics of polymorphisms. Loci marked with astarisk (*) are monomorphic within the *Vandiemenella* taxa according to Ayala's (1976) 99% polymorphism criterion.

Taxa	Site	*N*	Allele	*Aco1*	*Aco2*	*Acyc*	*Ada*	*Akl*	*Ak2*	*Ald*	*Argk*	*Dia*	*Enol**	*Est*	*Fdp*	*Fum*	*Glo**	*Gotl*	*Got2*	*Gpd**	*Gpi*	*Idh1*	*Idh2*	*Ldh*	*Mdh1*	*Mdh2*	*Me*	*Mpi*	*Ndpk**	*PepA*	*PepC*	*PpD1*	*6Pgd*	*Pgam*	*Pgk*	*Pgm*	*Pk*	*Tpi**
*viaitca*17	1	5	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.10	0.00	0.00	1.00
			2	0.00	0.10	0.00	0.90	0.00	1.00	0.00	1.00	0.00		1.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.00	0.80	0.00	0.10	1.00	0.00	0.90	0.00	0.00		0.00	1.00	0.00		0.00	0.00		0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.80	0.10	0.00	
			4	1.00	0.00	0.00	0.00	0.00	0.00	0.10					0.00	0.00		0.00	1.00		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.30	0.00	0.90	0.00	0.00	0.00	1.00	
			5	0.00	0.10	0.80				0.00					0.00	1.00		0.00	0.00				1.00		0.00	0.00	0.00	0.00		1.00	0.70	0.60	0.00	0.00	0.10	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	1.00		0.90	0.00	
			7	0.00		0.00									0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.20										0.00			0.00				0.00		0.00		0.00	1.00		0.00		0.40	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					0.50	0.00				0.00							
			11																								0.50												
			12																								0.00												
			13																								0.00												
			14																								0.00												
	2	6	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00
			2	0.00	0.00	0.00	0.75	0.10	1.00	0.00	0.92	0.00		1.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.75	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.00	1.00	0.00	0.25	0.90	0.00	0.83	0.08	1.00		0.00	1.00	0.00		0.00	0.00		0.00	1.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.67	0.00	0.00	0.00	1.00	0.00	0.00	
			4	1.00	0.00	0.08	0.00	0.00	0.00	0.17					0.00	0.00		0.00	1.00		0.00	0.00	0.00		0.00	0.25	0.00	0.00		0.00	0.08	0.00	1.00	0.00	0.00	0.00	1.00	
			5	0.00	0.00	0.58				0.00					0.00	1.00		0.00	0.00				1.00		0.00	0.00	0.00	0.00		0.75	0.25	0.67	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	1.00		1.00	0.00	
			7	0.00		0.00									0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.25	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.33										0.00			0.00				0.00		0.00		0.00	1.00		0.00		0.33	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					0.67	0.00				0.00							
			11																								0.25												
			12																								0.08												
			13																								0.00												
			14																								0.00												
	4	2	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00
			2	0.00	0.25	0.00	1.00	0.00	1.00	0.00	1.00	0.00		1.00	0.00	0.00	0.75	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.00	0.25	0.00	0.00	1.00	0.00	0.75	0.00	1.00		0.00	1.00	0.00		0.00	0.00		0.00	1.00	0.25	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.00	1.00	0.00	0.00	
			4	1.00	0.50	0.00	0.00	0.00	0.00	0.25					0.00	0.00		0.00	1.00		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.25	0.00	0.75	0.00	0.00	0.00	1.00	
			5	0.00	0.00	0.25				0.00					0.00	1.00		0.00	0.00				0.75		0.00	0.00	0.00	0.00		1.00	0.50	1.00	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	1.00		1.00	0.00	
			7	0.00		0.00									0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.25	0.00		0.00		
			8	0.00		0.75										0.00			0.00				0.00		0.00		0.00	1.00		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					1.00	0.00				0.00							
			11																								0.00												
			12																								0.00												
			13																								0.00												
			14																								0.00												
	5	3	1	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	1.00	0.33	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00
			2	0.00	0.00	0.00	1.00	0.00	0.83	0.00	1.00	0.00		0.67	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.83	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.00	1.00	0.00	0.00	1.00	0.00	0.33	0.00	1.00		0.00	1.00	0.00		0.00	0.00		0.00	1.00	0.33	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.33	0.00	0.00	0.00	1.00	0.33	0.00	
			4	1.00	0.00	0.00	0.00	0.00	0.00	0.67					0.00	0.00		0.00	1.00		0.00	0.00	0.00		0.00	0.17	0.00	0.00		0.00	0.00	0.00	0.83	0.00	0.00	0.00	1.00	
			5	0.00	0.00	1.00				0.00					0.00	1.00		0.00	0.00				0.67		0.00	0.00	0.00	0.00		0.67	1.00	1.00	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	1.00		0.00	0.00	0.00	0.17	1.00		0.67	0.00	
			7	0.00		0.00									0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.00		0.00	0.00		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					0.00	0.00				0.00							
			11																								1.00												
			12																								0.00												
			13																								0.00												
			14																								0.00												

Taxa	Site ID	*N*	Allele	*Aco1*	*Aco2*	*Acyc*	*Ada*	*Ak1*	*Ak2*	*Ald*	*Argk*	*Dia*	*Eno1**	*Est*	*Fdp*	*Fum*	*Glo**	*Got1l*	*Got2*	*Gpd**	*Gpi*	*Idh1*	*Idh2*	*Idh*	*Mdh1*	*Mdh2*	*Me*	*Mpi*	*Ndpk**	*PepA*	*PepC*	*PpD1*	*6Pgd*	*Pgam*	*Pgk*	*Pgm*	*Pk*	*Tpi**
*viatica*17	6	3	1	0.00	0.17	0.00	0.00	0.00	0.17	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.83	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.67	0.00	0.00	1.00
			2	0.00	0.50	0.00	1.00	0.00	0.83	0.00	1.00	0.00		1.00	0.00	0.00	1.00	1.00	0.17	0.17	0.00	0.33	0.00	0.00	0.00	0.50	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.00
			3	0.00	0.17	0.00	0.00	1.00	0.00	0.33	0.00	1.00		0.00	1.00	0.00		0.00	0.00		0.00	0.67	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.33	0.17	0.00	
			4	1.00	0.17	0.17	0.00	0.00	0.00	0.50					0.00	0.00		0.00	0.83		0.00	0.00	0.00		0.00	0.50	0.00	0.00		0.00	0.33	0.00	1.00	0.17	0.00	0.00	1.00	
			5	0.00	0.00	0.83				0.00					0.00	1.00		0.00	0.00				0.83		0.00	0.00	0.00	0.00		1.00	0.50	0.67	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.17					0.00	0.00			0.00				0.17		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.67		0.67	0.00	
			7	0.00		0.00									0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.00		0.00	0.83		0.00		0.33	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.17		0.00		0.00		0.17				
			10			0.00																			0.33		0.00	0.00				0.00						
			11																						0.50													
			12																						0.17													
			13																						0.00													
			14																						0.00													
	7	5	1	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.20	0.20	0.00	0.90	0.50	1.00	0.00	1.00	0.20		1.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.70	0.80	0.10	0.10	0.50	0.00	1.00	0.00	0.80		0.00	0.90	0.00		0.00	0.00		0.00	1.00	0.30	1.00	1.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	
			4	0.00	0.00	0.60	0.00	0.00	0.00	0.00					0.00	0.00		0.00	1.00		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.10	0.00	0.25	0.00	0.00	0.10	1.00	
			5	0.00	0.00	0.20				0.00					0.10	0.70		0.00	0.00				0.60		0.00	0.00	0.00	0.00		0.90	0.90	0.90	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.10		0.00		0.00	0.80		0.00	0.00	0.00	0.75	0.80		0.80	0.00	
			7	0.00		0.00									0.00	0.30			0.00				0.00		0.00		0.60	0.00		0.00	0.00	0.00	0.00	0.20		0.00		
			8	0.00		0.10										0.00			0.00				0.00		0.00		0.40	0.00		0.10		0.10	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.10		0.00		0.00		0.00				
			10			0.00																			0.00		0.00	0.00				0.00						
			11																						0.00													
			12																						0.00													
			13																						0.00													
			14																						0.00													
	8	3	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.83	0.00	0.00	1.00
			2	0.00	0.33	0.00	1.00	0.00	1.00	0.00	0.83	0.00		1.00	0.00	0.00	1.00	1.00	0.67	0.00	0.00	0.00	0.00	0.00	0.00	0.67	0.00	0.00	1.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.17	0.67	0.00	0.00	1.00	0.00	0.83	0.00	1.00		0.00	1.00	0.00		0.00	0.00		0.00	1.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.67	0.00	0.00	0.00	0.17	0.33	0.00	
			4	0.67	0.00	0.00	0.00	0.00	0.00	0.17					0.00	0.00		0.00	0.33		0.00	0.00	0.00		0.00	0.33	0.00	0.00		0.00	0.17	0.00	0.83	0.00	0.00	0.00	1.00	
			5	0.00	0.00	1.00				0.00					0.00	1.00		0.00	0.00				1.00		0.00	0.00	0.00	0.00		1.00	0.00	0.33	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	1.00		0.50	0.00	
			7	0.17		0.00									0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.17		0.17		
			8	0.00		0.00										0.00			0.00				0.00		0.00		0.00	0.67		0.00		0.67	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.33		0.00		0.00		0.00				
			10			0.00																			0.83		0.00	0.00				0.00						
			11																						0.00													
			12																						0.17													
			13																						0.00													
			14																						0.00													
	9	2	1	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00
			2	0.00	0.00	0.00	0.75	0.25	1.00	0.00	1.00	0.25		1.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.75	1.00	0.00	0.25	0.75	0.00	1.00	0.00	0.75		0.00	0.75	0.00		0.00	0.00		0.00	1.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
			4	0.00	0.00	1.00	0.00	0.00	0.00	0.00					0.00	0.25		0.00	1.00		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.50	0.00	0.00	0.00	1.00	
			5	0.00	0.00	0.00				0.00					0.25	0.50		0.00	0.00				1.00		0.00	0.00	0.00	0.00		1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	1.00		0.00	0.00	0.00	0.50	1.00		1.00	0.00	
			7	0.00		0.00									0.00	0.25			0.00				0.00		0.00		0.50	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.00		0.50	0.00		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																			0.00		0.00	0.00				0.00						
			11																						0.00													
			12																						0.00													
			13																						0.00													
			14																						0.00													

Taxa	Site ID	*N*	Allele	*Aco1*	*Aco2*	*Acyc*	*Ada*	*Ak1*	*Ak2*	*Ald*	*Argk*	*Dia*	*Eno1**	*Est*	*Fdp*	*Fum*	*Glo**	*Got1l*	*Got2*	*Gpd**	*Gpi*	*Idh1*	*Idh2*	*Idh*	*Mdh1*	*Mdh2*	*Me*	*Mpi*	*Ndpk**	*PepA*	*PepC*	*PpD1*	*6Pgd*	*Pgam*	*Pgk*	*Pgm*	*Pk*	*Tpi**
viatica 17	10	2	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.75	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.25	0.00	0.00	0.50	0.00	1.00	0.00	1.00	0.00		1.00	0.00	0.00	1.00	0.75	0.00	0.00	0.25	0.00	0.25	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.75	1.00	0.00	0.00	1.00	0.00	1.00	0.00	1.00		0.00	1.00	0.00		0.25	0.00		0.00	1.00	0.00	1.00	0.75	0.00	0.00	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.00	
			4	0.00	0.00	0.50	0.50	0.00	0.00	0.00					0.00	0.00		0.00	1.00		0.00	0.00	0.00		0.00	0.00	0.25	0.00		0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	
			5	0.00	0.00	0.50				0.00					0.00	1.00		0.00	0.00				0.50		0.00	0.00	0.00	0.00		1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00		0.00	0.00				0.25		0.00		0.00	0.75		0.00	0.00	0.00	0.00	1.00		0.75	0.00	
			7	0.00		0.00									0.00	0.00		0.00	0.00				0.00		0.00		0.25	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00		0.00	0.00				0.00		0.25		0.50	0.00		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					0.00	0.00				0.00						
			11																								0.00											
			12																								0.00											
			13																								0.00											
			14																								0.00											
	11	2	1	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.00	0.00	0.00	0.25	1.00	0.00	1.00	0.00		1.00	0.00	0.00	1.00	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.50	1.00	0.75	0.00	0.75	0.00	0.75	0.00	0.00		0.00	1.00	0.00		0.50	0.00		0.00	1.00	0.00	1.00	0.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
			4	0.00	0.00	0.25	0.00	0.00	0.00	0.25					0.00	0.00		0.25	1.00		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	
			5	0.00	0.00	0.00				0.00					0.00	0.50		0.00	0.00				1.00		0.00	0.00	0.00	0.00		1.00	0.25	1.00	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.50		0.00	0.75	0.00	0.00	1.00		0.75	0.00	
			7	0.00		0.00									0.00	0.50			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.25		
			8	0.00		0.00										0.00			0.00				0.00		0.25		1.00	0.50		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					0.00	0.00				0.00						
			11																								0.00											
			12																								0.00											
			13																								0.00											
			14																								0.00											
viatica 19	12	1	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00
			2	0.00	0.00	0.00	0.00	1.00	1.00	0.00	1.00	0.00		1.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.00	1.00	1.00	0.00	0.00	0.00	1.00	0.00	1.00		0.00	1.00	0.00		0.00	0.00		0.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	
			4	1.00	0.00	0.00	1.00	0.00	0.00	0.00					0.00	0.00		0.00	1.00		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.50	1.00	0.00	1.00	0.00	0.00	
			5	0.00	0.00	0.00				0.00					0.00	0.00		0.00	0.00				0.00		1.00	0.00	0.00	0.00		0.00	1.00	0.50	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			7	0.00		0.00									0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.00		0.00	0.00		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	1.00		0.00		0.00		0.00				
			10			0.00																					0.00	0.00				0.00						
			11																								0.00											
			12																								0.00											
			13																								0.00											
			14																								0.00											

Taxa	Site ID	*N*	Allele	*Aco1*	*Aco2*	*Acyc*	*Ada*	*Ak1*	*Ak2*	*Ald*	*Argk*	*Dia*	*Eno1**	*Est*	*Fdp*	*Fum*	*Glo**	*Got1*	*Got2*	*Gpd**	*Gpi*	*Idh1*	*Idh2*	*Idh*	*Mdh1*	*Mdh2*	*Me*	*Mpi*	*Ndpk**	*PepA*	*PepC*	*PpD1*	*6Pgd*	*Pgam*	*Pgk*	*Pgm*	*Pk*	*Tpi**
*viatica*19	15	1	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00
			2	0.00	0.50	0.00	0.00	1.00	1.00	0.00	1.00	0.50		1.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.00	0.50	1.00	0.00	0.00	0.00	1.00	0.00	0.50		0.00	1.00	0.00		0.00	0.00		0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	
			4	1.00	0.00	0.00	1.00	0.00	0.00	0.00					0.00	0.00		0.00	1.00		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	
			5	0.00	0.00	0.00				0.00					0.00	0.00		0.00	0.00				0.00		0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	1.00		1.00	0.00	
			7	0.00		0.00									0.00	0.00			0.00				0.00		0.00		1.00	0.00		1.00	1.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										1.00			0.00				0.00		0.00		0.00	0.00		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	1.00		0.00		0.00		0.00				
			10			0.00																					0.00	0.00				0.00						
			11																								0.00											
			12																								0.00											
			13																								0.00											
			14																								0.00											
	17	5	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00
			2	0.00	0.00	0.00	0.00	0.70	1.00	0.00	1.00	0.00		1.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.90	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.00	0.80	1.00	0.00	0.30	0.00	0.50	0.00	0.00		0.00	1.00	0.00		0.00	0.00		0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	
			4	1.00	0.20	0.00	1.00	0.00	0.00	0.00					0.00	0.00		0.00	1.00		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	
			5	0.00	0.00	0.00				0.50					0.00	0.00		0.00	0.00				0.00		1.00	0.00	0.10	0.00		0.00	1.00	0.90	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	1.00		0.90	0.00	
			7	0.00		0.00									0.00	0.00			0.00				0.00		0.00		0.90	0.00		1.00	0.00	0.00	0.00	0.00		0.10		
			8	0.00		0.00										1.00			0.00				0.00		0.00		0.00	0.00		0.00		0.10	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	1.00		0.00		0.00		0.00				
			10			0.00																					0.00	0.00				0.00						
			11																								0.00											
			12																								0.00											
			13																								0.00											
			14																								0.00											
	18	2	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00
			2	0.00	0.00	0.00	0.00	1.00	1.00	0.00	1.00	0.00		1.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.00	0.00
			3	0.00	1.00	1.00	0.00	0.00	0.00	0.75	0.00	0.00		0.00	1.00	0.00		0.00	0.00		0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	
			4	1.00	0.00	0.00	1.00	0.00	0.00	0.00					0.00	0.00		0.00	1.00		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	
			5	0.00	0.00	0.00				0.25					0.00	0.00		0.00	0.00				0.00		1.00	0.00	0.00	0.00		0.00	0.50	1.00	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	1.00		0.75	0.00	
			7	0.00		0.00									0.00	0.00			0.00				0.00		0.00		1.00	0.00		0.75	0.50	0.00	0.00	0.00		0.00		
			8	0.00		0.00										1.00			0.00				0.00		0.00		0.00	0.00		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	1.00		0.25		0.00		0.00				
			10			0.00																					0.00	0.00				0.00						
			11																								0.00											
			12																								0.00											
			13																								0.00											
			14																								0.00											
	19	2	1	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25	1.00	0.00	0.25	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.00	0.00	0.25	0.00	1.00	0.00	1.00	0.00		1.00	0.00	0.00	1.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.75	1.00	0.00	0.75	0.50	0.00	0.00	0.00	0.75		0.00	0.75	0.25		0.50	0.00		0.00	1.00	0.00	1.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
			4	0.00	0.00	0.75	0.00	0.50	0.00	0.50					0.00	0.00		0.00	0.75		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.50	0.00	0.00	0.00	1.00	
			5	0.00	0.00	0.25				0.50					0.00	0.50		0.00	0.25				1.00		0.00	0.00	0.00	0.00		1.00	0.75	0.00	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.75		0.00	0.25	1.00	0.50	1.00		1.00	0.00	
			7	0.00		0.00									0.00	0.00			0.00				0.00		0.00		0.50	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.25			0.00				0.00		0.25	0.25	0.25	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			9			0.00																			0.25	0.25	0.00	0.00		0.00		0.00		0.00				
			10			0.00																					0.00	0.00				0.00						
			11																								0.00											
			12																								0.00											
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			14																								0.00											

Taxa	Site ID	*N*	Allele	*Aco1*	*Aco2*	*Acyc*	*Ada*	*Ak1*	*Ak2*	*Ald*	*Argk*	*Dia*	*Eno1**	*Est*	*Fdp*	*Fum*	*Glo**	*Got1*	*Got2*	*Gpd**	*Gpi*	*Idh1*	*Idh2*	*Idh*	*Mdh1*	*Mdh2*	*Me*	*Mpi*	*Ndpk**	*PepA*	*PepC*	*PpD1*	*6Pgd*	*Pgam*	*Pgk*	*Pgm*	*Pk*	*Tpi**
*viatica*19	21	2	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.25	0.00	1.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.00	0.00	0.00	0.75	1.00	0.00	1.00	1.00		1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	1.00	1.00	0.00	1.00	0.25	0.00	1.00	0.00	0.00		0.00	1.00	0.00		0.75	0.00		0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
			4	0.00	0.00	1.00	0.00	0.00	0.00	0.00					0.00	0.00		0.00	1.00		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.50	0.00	0.00	0.00	1.00	
			5	0.00	0.00	0.00				0.00					0.00	0.25		0.00	0.00			1.00		0.00	0.00	0.00	0.00		1.00	0.50	1.00	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.25			0.00			0.00			0.00		0.00		0.00	0.50	0.00	0.25	1.00		1.00	0.00	
			7	0.00		0.00									0.00	0.25			0.00			0.00			0.00		0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.25			0.00			0.00			1.00		0.50	1.00		0.00		0.25	0.00		0.00		
			9			0.00																			0.00		0.50	0.00		0.00		0.00		0.00			
			10			0.00																					0.00	0.00				0.00					
			11																								0.00										
			12																								0.00										
			13																								0.00										
			14																								0.00										
	22	1	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.00	0.00	1.00	1.00	1.00	0.00	1.00	0.50		1.00	0.00	0.00	1.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	1.00	1.00	0.50	0.00	0.00	0.00	1.00	0.00	0.50		0.00	0.50	0.00		1.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.50	0.00	
			4	0.00	0.00	0.50	0.00	0.00	0.00	0.00					0.00	0.00		0.00	0.50		0.00	1.00	0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	
			5	0.00	0.00	0.00				0.00					0.00	0.00		0.00	0.00			1.00		0.00	0.00	0.00	0.00		1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.50			0.00			0.00			0.00		0.00		0.00	0.00	0.00	0.00	0.00		0.50	0.00	
			7	0.00		0.00									0.50	0.00			0.00			0.00			0.00		0.50		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.50			0.00			0.00			1.00		0.50	0.50		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00			
			10			0.00																					0.00	0.00				0.00					
			11																								0.00										
			12																								0.00										
			13																								0.00										
			14																								0.00										
	23	2	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00		1.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.00	0.00
			3	1.00	1.00	0.00	0.00	1.00	0.00	0.50	0.00	1.00		0.00	1.00	1.00		0.00	0.00		0.00	1.00	0.00	1.00	0.25	0.00	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.00	0.00	0.00	
			4	0.00	0.00	0.75	0.00	0.00	0.00	0.50					0.00	0.00		0.00	1.00		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.25	0.00	0.75	0.00	0.00	0.00	0.75	
			5	0.00	0.00	0.25				0.00					0.00	0.00		0.00	0.00			1.00		0.00	0.00	0.00	0.00		1.00	0.50	1.00	0.00	0.00	0.00	0.00	0.25	
			6	0.00		0.00				0.00					0.00	0.00			0.00			0.00			0.00		0.00		0.00	0.25	0.00	0.00	0.00	0.00	1.00		0.25	0.00
			7	0.00		0.00									0.00	0.00			0.00			0.00			0.00		0.00		0.00	0.00	0.00	0.00	0.00		0.50		
			8	0.00		0.00										0.00			0.00			0.00			0.75		0.00	0.75		0.00		0.00	0.00	0.00		0.25		
			9			0.00																			0.00		0.00	0.00		0.00		0.00					
			10			0.00																					1.00	0.00				0.00					
			11																								0.00										
			12																								0.00										
			13																								0.00										
			14																								0.00										
	24	3	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.17	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.00	0.00	1.00
			2	0.00	0.00	0.00	1.00	0.00	0.67	0.00	1.00	0.00		1.00	0.00	0.00	1.00	0.67	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	1.00	1.00	0.00	0.00	1.00	0.33	0.83	0.00	1.00		0.00	1.00	0.00		0.33	0.00		0.00	0.33	0.00	1.00	0.50	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.67	0.00	0.00	
			4	0.00	0.00	1.00	0.00	0.00	0.00	0.17					0.00	0.00		0.00	1.00		0.00	0.33	0.00		0.17	0.00	0.00	0.00		0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	
			5	0.00	0.00	0.00				0.00					0.00	0.00		0.00	0.00			1.00		0.00	0.00	0.00	0.00		0.83	0.00	0.33	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00			0.00			0.00		0.00		0.00	0.17	0.00	0.00	1.00		1.00	0.00	
			7	0.00		0.00									0.00	0.50			0.00			0.00			0.00		0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.50			0.00			0.00			0.17		1.00	0.83		0.00		0.33	0.00	0.00		0.00		
			9			0.00																			0.17		0.00	0.00		0.00		0.00		0.00			
			10			0.00																					0.00	0.00				0.33					
			11																								0.00										
			12																								0.00										
			13																								0.00										
			14																								0.00										

Taxa	Site ID	*N*	Allele	*Aco1*	*Aco2*	*Acyc*	*Ada*	*Ak1*	*Ak2*	*Ald*	*Argk*	*Dia*	*Eno1**	*Est*	*Fdp*	*Fum*	*Glo**	*Got1*	*Got2*	*Gpd**	*Gpi*	*Idh1*	*Idh2*	*Idh*	*Mdh1*	*Mdh2*	*Me*	*Mpi*	*Ndpk**	*PepA*	*PepC*	*PpD1*	*6Pgd*	*Pgam*	*Pgk*	*Pgm*	*Pk*	*Tpi**
*viatica*19	25	2	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.75	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.00	0.00	1.00	0.00	1.00	0.00	1.00	0.25		1.00	0.00	0.00	1.00	0.75	0.00	0.00	0.25	0.25	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.75	1.00	0.25	0.00	1.00	0.00	1.00	0.00	0.75		0.00	1.00	0.00		0.25	0.00		0.00	0.75	0.00	1.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
			4	0.00	0.00	0.75	0.00	0.00	0.00	0.00					0.00	0.00		0.00	1.00		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.75	0.00	0.00	0.50	1.00	
			5	0.00	0.00	0.00				0.00					0.00	0.00		0.00	0.00				1.00		0.00	0.00	0.00	0.00		1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.25	0.75		0.50	0.00	
			7	0.25		0.00									0.00	0.75			0.00				0.00		0.00		0.25	0.00		0.00	0.00	0.00	0.00	0.25		0.00		
			8	0.00		0.00										0.25			0.00				0.00		0.50		0.00	1.00		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.75	0.00		0.00		0.00		0.00				
			10			0.00																					0.00	0.00				0.00						
			11																								0.00											
			12																								0.00											
			13																								0.00											
			14																								0.00											
	27	1	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00
			2	0.50	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00		1.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.50	1.00	0.00	0.00	1.00	0.00	1.00	0.00	1.00		0.00	1.00	1.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
			4	0.00	0.00	0.00	0.00	0.00	0.00	0.00					0.00	0.00		0.00	1.00		0.00	0.00	0.00		1.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	
			5	0.00	0.00	0.00				0.00					0.00	0.00		0.00	0.00				1.00		0.00	0.00	0.00	0.00		1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	1.00		1.00	0.00	
			7	0.00		0.00									0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.00		0.00	1.00		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.50	0.00		0.00		0.00		0.00				
			10			0.00																					0.50	0.00				0.00						
			11																								0.00											
			12																								0.00											
			13																								0.00											
			14																								0.00											
	28	1	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00
			2	0.50	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00		1.00	0.00	0.00	1.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.50	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00		0.00	1.00	0.00		0.50	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	
			4	0.00	0.00	0.00	0.00	0.00	0.00	0.00					0.00	0.00		0.00	1.00		0.00	0.00	0.00		1.00	0.00	0.00	0.00		0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	
			5	0.00	0.00	0.00				0.00					0.00	0.00		0.00	0.00				0.00		0.00	0.00	0.00	0.00		1.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	1.00		0.00	0.00	
			7	0.00		0.00									0.00	1.00			0.00				0.00		0.00		1.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.00		0.00	1.00		0.00		0.50	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					0.00	0.00				0.00						
			11																								0.00											
			12																								0.00											
			13																								0.00											
			14																								0.00											
	29	3	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.33	0.00	0.00	0.17	0.00	0.00	0.00	0.00	1.00
			2	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.17		0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.67	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	1.00	1.00	0.00	1.00	1.00	0.00	1.00	0.00	0.83		1.00	1.00	0.00		0.00	0.00		0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.00	1.00	0.67	0.00	
			4	0.00	0.00	1.00	0.00	0.00	0.00	0.00					0.00	0.00		0.00	0.67		0.00	0.00	0.00		0.00	1.00	0.00	0.00		0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	
			5	0.00	0.00	0.00				0.00					0.00	0.00		0.00	0.00				1.00		1.00	0.00	0.00	0.00		1.00	1.00	0.33	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	1.00			0.00				0.00		0.00		1.00	0.00		0.00	0.00	0.00	0.00	1.00		0.33	0.00	
			7	0.00		0.00									0.00	0.00			0.33				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.00		0.00	1.00		0.00		0.33	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					0.00	0.00				0.00						
			11																								0.00											
			12																								0.00											
			13																								0.00											
			14																								0.00											

Taxa	Site ID	*N*	Allele	*Aco1*	*Aco2*	*Acyc*	*Ada*	*Ak1*	*Ak2*	*Ald*	*Argk*	*Dia*	*Enol**	*Est*	*Fdp*	*Fum*	*Glo**	*Got1*	*Got2*	*Gpd1**	*Gpi*	*Idh1*	*Idh2*	*Ldh*	*Mdh1*	*Mdh2*	*Me*	*Mpi*	*Ndpk**	*PepA*	*PepC*	*PpD1*	*6Pgd*	*Pgam*	*Pgk*	*Pgm*	*Pk*	*Tpi**
P24(XO)	30	4	1	0.00	0.25	0.00	0.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.88	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.50	0.00	1.00	0.38	0.00	0.00	0.00	0.00		1.00	0.00	0.00	1.00	1.00	0.13	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.63	0.25	0.00	0.00	0.63	0.00	1.00	0.00	1.00		0.00	1.00	0.13		0.00	0.00		0.00	1.00	1.00	1.00	0.13	0.00	0.00	0.00	0.00	0.75	0.63	0.00	0.00	0.00	0.00	0.75	0.00	
			4	0.00	0.00	0.00	0.00	0.00	0.00	0.00					0.00	0.00		0.00	0.13		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	
			5	0.38	0.00	0.00				0.00					0.00	0.88		0.00	0.75				0.00		0.00	0.00	0.00	0.00		0.25	0.38	0.88	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.50		0.00	0.00	0.00	0.00	1.00		0.25	0.00	
			7	0.00		1.00									0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.00		0.00	0.50		0.00		0.13	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					0.00	0.00				0.00						
			11																								0.00												
			12																								0.88												
			13																								0.13												
			14																								0.00												
	32	3	1	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.67	0.00	0.83	1.00	0.00	0.00	0.00	0.00		1.00	0.17	0.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.00
			3	0.50	0.33	0.00	0.17	0.00	0.00	1.00	0.00	1.00		0.00	0.83	0.00		0.00	0.00		0.00	1.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
			4	0.50	0.00	0.00	0.00	0.00	0.00	0.00					0.00	0.00		0.00	0.00		0.00	0.00	0.00		0.00	0.83	0.00	0.00		0.00	1.00	0.00	0.83	0.00	0.00	0.00	1.00	
			5	0.00	0.00	0.00				0.00					0.00	1.00		0.00	0.00				1.00		0.00	0.17	0.00	0.00		0.67	0.00	0.67	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.50		1.00	0.00	
			7	0.00		0.00									0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.00		0.00	0.83		0.00		0.33	0.00	0.50		0.00		
			9			0.00																			0.00		0.17	0.00		0.00		0.00		0.00				
			10			0.00																					0.67	0.17				0.00						
			11																								0.00												
			12																								0.17												
			13																								0.00												
			14																								0.00												
	33	2	1	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	1.00	0.00	0.75	1.00	0.00	0.00	0.00	0.25		1.00	0.00	0.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.00	0.00	0.00	0.25	0.00	0.00	1.00	0.00	0.75		0.00	1.00	0.25		0.00	0.00		0.00	1.00	0.75	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.75	0.00	0.00	0.00	0.00	0.50	0.00	
			4	1.00	0.00	0.00	0.00	0.00	0.00	0.00					0.00	0.00		0.00	0.00		0.00	0.00	0.00		0.00	1.00	0.00	0.00		0.00	0.25	0.00	1.00	0.00	0.00	0.00	0.75	
			5	0.00	0.00	0.00				0.00					0.00	0.75		0.00	0.00				0.25		0.00	0.00	0.00	0.00		1.00	0.00	0.25	0.00	0.00	0.00	0.00	0.25	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	1.00		0.50	0.00	
			7	0.00		0.00									0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.00		0.00	1.00		0.00		0.75	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					0.50	0.00				0.00						
			11																								0.00												
			12																								0.50												
			13																								0.00												
			14																								0.00												
P24(XY)	34	3	1	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.33	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.17	0.00	0.83	1.00	0.00	0.00	1.00	0.00		1.00	0.00	0.00	0.67	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00
			3	0.33	0.83	0.00	0.17	0.00	0.00	1.00	0.00	1.00		0.00	1.00	0.67		0.00	0.00		0.00	1.00	0.67	1.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
			4	0.67	0.00	1.00	0.00	0.00	0.00	0.00					0.00	0.00		0.00	0.00		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.83	0.00	0.00	0.00	0.00	0.00	1.00	
			5	0.00	0.00	0.00				0.00					0.00	0.33		0.00	0.00				0.17		0.00	0.00	0.00	0.00		0.33	0.17	1.00	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	1.00		1.00	0.00	
			7	0.00		0.00									0.00	0.00			0.00				0.17		0.00		0.00	0.00		0.00	0.00	0.00	0.50	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.00		0.00	1.00		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					0.83	0.00				0.00						
			11																								0.00												
			12																								0.17												
			13																								0.00												
			14																								0.00												

Taxa	Site ID	*N*	Allele	*Aco1*	*Aco2*	*Acyc*	*Ada*	*Ak1*	*Ak2*	*Ald*	*Argk*	*Dia*	*Enol**	*Est*	*Fdp*	*Fum*	*Glo**	*Got1*	*Got2*	*Gpd1**	*Gpi*	*Idh1*	*Idh2*	*Ldh*	*Mdh1*	*Mdh2*	*Me*	*Mpi*	*Ndpk**	*PepA*	*PepC*	*PpD1*	*6Pgd*	*Pgam*	*Pgk*	*Pgm*	*Pk*	*Tpi**
P24(XY)	35	3	1	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.17	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.67	0.00	1.00	1.00	0.00	0.00	0.83	0.00		1.00	0.00	0.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.33	0.17	0.00	0.00	0.00	0.00	0.00
			3	0.00	0.33	0.00	0.00	0.00	0.00	0.67	0.00	1.00		0.00	1.00	0.50		0.00	0.00		0.00	1.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
			4	1.00	0.00	1.00	0.00	0.00	0.00	0.33					0.00	0.00		0.00	0.00		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.67	0.00	1.00	0.00	0.00	0.00	1.00	
			5	0.00	0.00	0.00				0.00					0.00	0.50		0.00	0.00				0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.83	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	1.00		1.00	0.00	
			7	0.00		0.00									0.00	0.00			0.00				0.83		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.17		0.00		0.00	1.00		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.17	0.00		0.00		0.00		0.00				
			10			0.00																					0.83	0.00				0.00						
			11																								0.00											
			12																								0.00											
			13																								0.00											
			14																								0.00											
	36	4	1	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.63	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.00	0.00	0.00	0.13	0.88	0.00	0.00	1.00
			2	0.00	0.13	0.00	1.00	1.00	0.00	0.00	0.38	0.00		1.00	0.00	0.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.17	0.00	0.75	0.00	0.00	1.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.00
			3	0.00	0.88	0.00	0.00	0.00	0.00	0.25	0.00	1.00		0.00	1.00	0.00		0.00	0.00		0.00	1.00	0.13	0.83	1.00	0.00	0.00	0.00	0.00	0.88	0.00	0.00	0.00	0.00	0.13	0.00	0.00	
			4	1.00	0.00	1.00	0.00	0.00	0.00	0.75					0.00	0.00		0.00	0.00		0.00	0.00	0.00		0.00	0.25	0.00	0.00		0.00	0.88	0.00	0.83	0.13	0.00	0.00	1.00	
			5	0.00	0.00	0.00				0.00					0.00	1.00		0.00	0.00				0.00		0.00	0.00	0.00	0.00		0.00	0.13	1.00	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.13		0.00	0.00	0.00	0.00	0.63		1.00	0.00	
			7	0.00		0.00									0.00	0.00			0.00				0.88		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.00		0.00	0.88		0.00		0.00	0.00	0.13		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					0.38	0.00				0.00						
			11																								0.00											
			12																								0.63											
			13																								0.00											
			14																								0.00											
	37	5	1	0.00	0.00	0.00	0.00	0.00	0.90	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.30	0.00	0.60	1.00	0.10	0.00	0.00	0.00		1.00	0.00	0.00	1.00	1.00	0.80	0.00	0.00	0.00	0.00	0.13	0.00	1.00	0.00	0.00	1.00	0.00	0.30	0.00	0.20	0.00	0.00	0.00	0.00	0.00
			3	0.10	0.70	0.00	0.40	0.00	0.00	0.80	0.00	1.00		0.00	1.00	0.00		0.00	0.00		0.00	1.00	0.60	0.88	1.00	0.00	0.00	0.00	0.00	0.90	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
			4	0.90	0.00	1.00	0.00	0.00	0.00	0.20					0.00	0.00		0.00	0.20		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.70	0.00	0.80	0.20	0.00	0.00	1.00	
			5	0.00	0.00	0.00				0.00					0.00	0.90		0.00	0.00				0.00		0.00	0.00	0.00	0.00		0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.80		1.00	0.00	
			7	0.00		0.00									0.00	0.10			0.00				0.40		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.00		0.00	0.60		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					0.80	0.40				0.00						
			11																								0.10											
			12																								0.10											
			13																								0.00											
			14																								0.00											
	38	2	1	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.25	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.25	0.00	1.00	1.00	0.00	0.00	0.75	0.00		1.00	0.00	0.00	1.00	1.00	0.75	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.25	0.00	0.25	0.00	0.00	0.00	0.00	0.00
			3	0.00	0.75	0.00	0.00	0.00	0.00	0.50	0.00	1.00		0.00	1.00	0.00		0.00	0.00		0.00	0.75	0.25	1.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.50	0.00	
			4	1.00	0.00	1.00	0.00	0.00	0.00	0.50					0.00	0.00		0.00	0.25		0.00	0.25	0.00		0.00	0.00	0.00	0.00		0.00	0.75	0.00	0.75	0.50	0.00	0.00	1.00	
			5	0.00	0.00	0.00				0.00					0.00	1.00		0.00	0.00				0.00		0.00	0.00	0.00	0.00		0.25	0.00	1.00	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.50		0.50	0.00	
			7	0.00		0.00									0.00	0.00			0.00				0.75		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.00		0.00	1.00		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					0.50	0.00				0.00						
			11																								0.00											
			12																								0.50											
			13																								0.00											
			14																								0.00											

Taxa	Site ID	*N*	Allele	*Aco1*	*Aco2*	*Acyc*	*Ada*	*Ak1*	*Ak2*	*Ald*	*Argk*	*Dia*	*Enol**	*Est*	*Fdp*	*Fum*	*Glo**	*Got1*	*Got2*	*Gpd**	*Gpi*	*Idh1*	*Idh2*	*Ldh*	*Mdh1*	*Mdh2*	*Me*	*Mpi*	*Ndpk**	*PepA*	*PepC*	*PpD1*	*6Pgd*	*Pgam*	*Pgk*	*Pgm*	*Pk*	*Tpi**
P24(XY)	39	5	1	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.40	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.30	0.00	1.00	1.00	0.00	0.00	0.60	0.00		1.00	0.00	0.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.00	0.00
			3	0.10	0.60	0.00	0.00	0.00	0.00	0.30	0.00	1.00		0.00	1.00	0.00		0.00	0.00		0.00	0.90	0.70	1.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
			4	0.90	0.10	1.00	0.00	0.00	0.00	0.70					0.00	0.00		0.00	0.00		0.00	0.10	0.00		0.00	0.00	0.00	0.00		0.00	1.00	0.00	0.75	0.10	0.00	0.00	1.00	
			5	0.00	0.00	0.00				0.00					0.00	1.00		0.00	0.00				0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.90	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.90		1.00	0.00	
			7	0.00		0.00								0.00	0.00	0.00			0.00				0.30		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.00		0.00	1.00		0.00		0.10	0.00	0.00		0.00		
			9			0.00																			0.00		0.20	0.00		0.00		0.00		0.00				
			10			0.00																					0.50	0.00				0.00						
			11																								0.00											
			12																								0.30											
			13																								0.00											
			14																								0.00											
	40	12	1	0.00	0.00	0.00	0.00	0.04	1.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.04	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.04	0.00	1.00
			2	0.00	0.00	0.00	0.96	0.96	0.00	0.00	1.00	0.00		1.00	0.00	0.00	0.96	1.00	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.00	1.00	0.00	0.04	0.00	0.00	0.17	0.00	1.00		0.00	1.00	0.00		0.00	0.00		0.00	0.96	1.00	0.68	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.96	0.00	
			4	1.00	0.00	0.00	0.00	0.00	0.00	0.83					0.00	0.00		0.00	0.79		0.00	0.04	0.00		0.00	1.00	0.00	0.00		0.04	0.00	0.00	0.83	0.46	0.00	0.00	1.00	
			5	0.00	0.00	0.00				0.00					0.00	0.96		0.00	0.00				0.00		0.00	0.00	0.00	0.00		0.88	0.00	0.92	0.17	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.54		0.00	0.00	
			7	0.00		1.00								0.00	0.04				0.04				0.00		0.00		0.00	0.00		0.08	0.00	0.08	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.00		0.00	0.92		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.08		0.00		0.00		0.00				
			10			0.00																					0.88	0.00				0.00						
			11																								0.08											
			12																								0.00											
			13																								0.00											
			14																								0.04											
	41	2	1	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	1.00
			2	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00		1.00	0.00	0.00	1.00	0.75	0.75	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00		0.00	1.00	0.00		0.25	0.00		0.00	1.00	0.75	1.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.25	0.00	
			4	1.00	0.00	1.00	0.00	0.00	0.00	1.00					0.00	0.00		0.00	0.25		0.00	0.00	0.00		0.00	0.50	0.00	0.00		0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	
			5	0.00	0.00	0.00				0.00					0.00	1.00		0.00	0.00				0.00		0.00	0.00	0.00	0.00		0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	1.00		0.75	0.00	
			7	0.00		0.00								0.00	0.00	0.00			0.00				0.25		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.00		0.00	1.00		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					1.00	0.00				0.00						
			11																								0.00											
			12																								0.00											
			13																								0.00											
			14																								0.00											
	42	1	1	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.50	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.50	0.00		1.00	0.00	0.00	1.00	1.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	1.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	1.00		0.00	1.00	0.00		0.00	0.00		0.00	1.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00	1.00	0.00	
			4	1.00	0.00	1.00	0.00	0.00	0.00	0.50					0.00	0.00		0.00	0.50		0.00	0.00	0.00		0.00	0.50	0.00	0.00		0.00	1.00	0.00	1.00	0.00	0.00	0.00	1.00	
			5	0.00	0.00	0.00				0.00					0.00	1.00		0.00	0.00				0.00		0.00	0.00	0.00	0.00		0.00	0.50	0.00	0.50	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	1.00		0.00	0.00	
			7	0.00		0.00								0.00	0.00				0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.00		0.00	1.00		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					0.50	0.00				0.00						
			11																								0.00											
			12																								0.50											
			13																								0.00											
			14																								0.00											

Taxa	Site ID	*N*	Allele	*Aco1*	*Aco2*	*Acyc*	*Ada*	*Ak1*	*Ak2*	*Ald*	*Argk*	*Dia*	*Enol**	*Est*	*Fdp*	*Fum*	*Glo**	*Got1*	*Got2*	*Gpd**	*Gpi*	*Idh1*	*Idh2*	*Ldh*	*Mdh1*	*Mdh2*	*Me*	*Mpi*	*Ndpk**	*PepA*	*PepC*	*PpD1*	*6Pgd*	*Pgam*	*Pgk*	*Pgm*	*Pk*	*Tpi**
P24(XY)	43	13	1	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.35	0.00	1.00	0.04	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.12	0.00	0.00	1.00
			2	0.00	0.27	0.00	0.96	1.00	0.00	0.00	0.65	0.00		0.96	0.00	0.00	1.00	1.00	0.42	0.00	0.00	0.00	0.00	0.00	0.00	0.88	0.00	0.00	1.00	0.00	0.08	0.00	0.00	0.00	0.00	0.19	0.00
			3	0.15	0.73	0.00	0.04	0.00	0.00	0.42	0.00	1.00		0.00	1.00	0.00		0.00	0.00		0.00	1.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.96	0.00	0.00	0.00	0.00	0.88	0.00	0.00	
			4	0.85	0.00	1.00	0.00	0.00	0.00	0.58					0.00	0.00		0.00	0.58		0.00	0.00	0.00		0.00	0.12	0.00	0.00		0.00	0.92	0.00	0.91	0.15	0.00	0.00	0.81	
			5	0.00	0.00	0.00				0.00					0.00	1.00		0.00	0.00				0.00		0.00	0.00	0.00	0.00		0.04	0.00	0.73	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.85		1.00	0.00	
			7	0.00		0.00									0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.15	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.00		0.00	1.00		0.00		0.12	0.09	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																			0.00		0.46	0.00				0.00						
			11																								0.04											
			12																								0.50											
			13																								0.00											
			14																								0.00											
P24(XY)-Trans	44	4	1	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.13	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.13	0.00	1.00	1.00	0.00	0.38	0.88	0.00		1.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.75	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.00	0.88	0.00	0.00	0.00	0.00	0.63	0.00	1.00		0.00	1.00	1.00		0.00	0.00		0.00	1.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
			4	1.00	0.00	1.00	0.00	0.00	0.00	0.00					0.00	0.00		0.00	0.38		0.00	0.00	0.00		0.00	0.25	0.00	0.00		0.00	0.00	0.00	1.00	0.00	0.00	0.00	1.00	
			5	0.00	0.00	0.00				0.00					0.00	0.00		0.00	0.00				0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.88	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.63	0.13		0.00	0.00	0.00	0.00	1.00		1.00	0.00	
			7	0.00		0.00									0.00	0.00			0.00				1.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.63				0.00		0.00		0.00	0.88		0.00		0.13	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																			0.00		0.25	0.00				0.00						
			11																								0.00											
			12																								0.13											
			13																								0.00											
			14																								0.00											
	45	4	1	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.13	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.13	0.00	0.75	1.00	0.00	0.00	0.88	0.00		1.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00	0.00	0.13	0.00	0.00	0.00	0.00
			3	0.00	0.88	0.00	0.25	0.00	0.00	1.00	0.00	1.00		0.00	1.00	1.00		0.00	0.13		0.00	1.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
			4	1.00	0.00	0.75	0.00	0.00	0.00	0.00					0.00	0.00		0.00	0.75		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.88	0.00	0.00	0.00	1.00	
			5	0.00	0.00	0.25				0.00					0.00	0.00		0.00	0.00				0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.63	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.25	0.00		0.00	0.00	0.00	0.00	1.00		1.00	0.00	
			7	0.00		0.00									0.00	0.00			0.00				1.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.13				0.00		0.00		0.00	1.00		0.00		0.38	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																			0.00		0.25	0.00				0.00						
			11																								0.00											
			12																								0.50											
			13																								0.00											
			14																								0.00											
P25(XY)	46	7	1	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.71	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.57	0.00	1.00	0.00	0.00	0.00	0.00	0.00		1.00	0.00	0.00	1.00	1.00	0.14	0.00	0.29	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.07	0.43	0.00	0.00	1.00	0.00	1.00	0.00	1.00		0.00	0.93	0.14		0.00	0.00		0.00	1.00	0.93	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.93	0.17
			4	0.43	0.00	0.00	0.00	0.00	0.00	0.00					0.00	0.00		0.00	0.21		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.92	0.00	0.00	0.00	0.83	
			5	0.00	0.00	0.00				0.00					0.00	0.86		0.00	0.57				0.07		0.00	0.00	0.00	0.00		0.00	1.00	1.00	0.00	1.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.07	0.00			0.00				0.00		0.00		0.00	0.64		0.00	0.00	0.00	0.08	0.00		0.07	0.00	
			7	0.50		1.00									0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.07				0.00		0.00		0.00	0.36		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																			0.00		0.00	0.00				0.00						
			11																								0.00											
			12																								0.93											
			13																								0.00											
			14																								0.07											

Taxa	Site ID	*N*	Allele	*Aco1*	*Aco2*	*Acyc*	*Ada*	*Ak1*	*Ak2*	*Ald*	*Argk*	*Dia*	*Enol**	*Est*	*Fdp*	*Fum*	*Glo**	*Got1*	*Got2*	*Gpd**	*Gpi*	*Idh1*	*Idh2*	*Ldh*	*Mdh1*	*Mdh2*	*Me*	*Mpi*	*Ndpk**	*PepA*	*PepC*	*PpD1*	*6Pgd*	*Pgam*	*Pgk*	*Pgm*	*Pk*	*Tpi**
P45b(XO)	47	5	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.90	0.00	0.10	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.30	0.00	0.90	1.00	1.00	0.00	1.00	0.00		1.00	0.00	0.00	1.00	0.90	0.10	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.00	0.60	0.00	0.10	0.00	0.00	1.00	0.00	1.00		0.00	1.00	1.00		0.10	0.10		0.00	1.00	0.80	0.75	0.60	0.10	0.00	0.90	0.00	0.00	0.10	0.00	0.25	0.00	0.00	0.00	1.00	
			4	0.00	0.10	0.00	0.00	0.00	0.00	0.00					0.00	0.00		0.00	0.80		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.20	0.00	0.50	0.00	0.00	0.00	0.00	
			5	0.00	0.00	1.00				0.00					0.00	0.00		0.00	0.00				0.10		0.00	0.00	0.00	0.00		1.00	0.70	0.50	0.25	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.10		0.00		0.00	0.00		0.00	0.00	0.00	0.00	1.00		1.00	0.00	
			7	1.00		0.00									0.00	0.00			0.00				0.00		0.00		0.10	0.00		0.00	0.00	0.20	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.40		0.80	0.00		0.00		0.30	0.00	0.00		0.00		
			9			0.00																			0.00		0.10	0.00		0.00		0.00		0.00				
			10			0.00																					0.00	0.00				0.00						
			11																								0.00											
			12																								0.00											
			13																								0.00											
			14																								0.00											
	48	5	1	0.00	0.00	0.00	0.00	0.10	0.33	0.00	0.10	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.30	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.00	0.00	0.00	0.80	0.67	0.00	0.90	0.00		1.00	0.00	0.10	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.80	0.00	0.00	1.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.00	1.00	0.00	1.00	0.10	0.00	1.00	0.00	1.00		0.00	1.00	0.90		0.00	0.00		0.00	1.00	0.13	1.00	1.00	0.00	0.00	0.90	0.00	0.20	1.00	0.20	0.00	0.00	0.00	0.00	1.00	
			4	0.10	0.00	1.00	0.00	0.00	0.00	0.00					0.00	0.00		0.00	0.90		0.00	0.00	0.00		0.00	0.20	0.00	0.00		0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	
			5	0.00	0.00	0.00				0.00					0.00	0.00		0.00	0.10				0.88		0.00	0.00	0.00	0.00		0.70	0.00	0.50	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	1.00		1.00	0.00	
			7	0.80		0.00									0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.10		0.00										0.00			0.00				0.00		0.00		0.90	0.00		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.10	0.00		0.00		0.00		0.00				
			10			0.00																					0.00	0.00				0.00						
			11																								0.00											
			12																								0.00											
			13																								0.00											
			14																								0.00											
	49	3	1	0.00	0.00	0.00	0.00	0.00	0.83	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.67	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.00	0.00	0.00	1.00	0.17	0.00	1.00	0.00		1.00	0.00	0.17	1.00	1.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.00	1.00	0.00	1.00	0.00	0.00	1.00	0.00	1.00		0.00	1.00	0.83		0.00	0.00		0.00	1.00	0.00	1.00	1.00	0.00	0.00	1.00	0.00	0.33	1.00	0.00	0.00	0.00	0.00	0.00	1.00	
			4	0.00	0.00	1.00	0.00	0.00	0.00	0.00					0.00	0.00		0.00	0.83		0.17	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	
			5	0.00	0.00	0.00				0.00					0.00	0.00		0.00	0.17				1.00		0.00	0.00	0.00	0.00		0.67	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	1.00		1.00	0.00	
			7	1.00		0.00									0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.00		1.00	0.00		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					0.00	0.00				0.00						
			11																								0.00											
			12																								0.00											
			13																								0.00											
			14																								0.00											
	50	3	1	0.00	0.00	0.00	0.00	0.00	0.33	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.00	0.00	0.17	1.00	0.67	0.00	1.00	0.00		1.00	0.17	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.25	0.00	0.83	0.00	0.00	1.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00
			3	0.00	1.00	0.00	0.83	0.00	0.00	1.00	0.00	1.00		0.00	0.83	1.00		0.00	0.50		0.00	1.00	0.33	0.75	1.00	0.00	0.00	1.00	0.00	0.33	1.00	0.00	0.00	0.00	0.00	0.00	1.00	
			4	0.17	0.00	1.00	0.00	0.00	0.00	0.00					0.00	0.00		0.00	0.50		0.00	0.00	0.00		0.00	0.17	0.00	0.00		0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	
			5	0.00	0.00	0.00				0.00					0.00	0.00		0.00	0.00				0.67		0.00	0.00	0.00	0.00		0.67	0.00	0.50	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.17	0.00		0.00	0.00	0.00	0.00	1.00		1.00	0.00	
			7	0.83		0.00									0.00	0.00			0.00				0.00		0.00		0.33	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.00		0.33	0.00		0.00		0.50	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					0.00	0.00				0.00						
			11																								0.17											
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			14																								0.00											

Taxa	Site ID	*N*	Allele	*Aco1*	*Aco2*	*Acyc*	*Ada*	*Ak1*	*Ak2*	*Ald*	*Argk*	*Dia*	*Enol**	*Est*	*Fdp*	*Fum*	*Glo**	*Got1*	*Got2*	*Gpd**	*Gpi*	*Idh1*	*Idh2*	*Ldh*	*Mdh1*	*Mdh2*	*Me*	*Mpi*	*Ndpk**	*PepA*	*PepC*	*PpD1*	*6Pgd*	*Pgam*	*Pgk*	*Pgm*	*Pk*	*Tpi**
P45b(XY)	51	4	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.13	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.13	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.00	0.00	0.75	0.75	1.00	0.00	1.00	0.00		1.00	0.00	0.00	1.00	0.75	0.00	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.00	1.00	0.13	0.00	0.00	0.75	0.00	0.00	0.00	0.00	0.00
			3	0.00	0.88	0.00	0.13	0.25	0.00	1.00	0.00	1.00		0.00	1.00	0.88		0.25	0.00		0.00	1.00	0.00	0.75	0.00	0.00	0.00	1.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.50	
			4	0.00	0.13	1.00	0.13	0.00	0.00	0.00					0.00	0.00		0.00	1.00		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.38	0.00	0.25	0.00	0.00	0.00	0.00	
			5	0.00	0.00	0.00				0.00					0.00	0.00		0.00	0.00				1.00		0.00	0.00	0.00	0.00		0.88	0.00	0.88	0.00	0.00	0.00	0.00		
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.88		1.00	0.50	
			7	1.00		0.00									0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		1.00		1.00	0.00		0.00		0.13	0.00	0.13		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					0.00	0.00				0.00						
			11																								0.00											
			12																								0.00											
			13																								0.00											
			14																								0.00											
	52	4	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.88	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.00	0.00	1.00	1.00	1.00	0.00	1.00	0.00		1.00	0.00	0.00	1.00	0.38	0.00	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00		0.00	1.00	1.00		0.63	0.00		0.00	1.00	0.00	0.75	0.50	0.00	0.00	1.00	0.00	0.00	0.13	0.38	0.00	0.00	0.00	0.25	0.75	
			4	0.00	0.00	1.00	0.00	0.00	0.00	0.00					0.00	0.00		0.00	1.00		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	
			5	0.00	0.00	0.00				0.00					0.00	0.00		0.00	0.00				1.00		0.00	0.00	0.00	0.00		0.88	0.00	0.63	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.13	0.00	0.00	0.00	1.00		0.75	0.25	
			7	1.00		0.00									0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.50		1.00	0.00		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					0.00	0.00				0.00						
			11																								0.00											
			12																								0.00											
			13																								0.00											
			14																								0.00											
P45c	53	3	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00		1.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	1.00	1.00	1.00	1.00	0.00	0.00	1.00	0.00	1.00		0.00	0.00	0.00		0.00	0.00		0.00	1.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.83	0.00	0.00	0.00	0.00	0.00	1.00	
			4	0.00	0.00	0.00	0.00	0.00	1.00	0.00					1.00	0.00		0.00	1.00		0.00	0.00	0.50		0.00	0.00	0.00	0.00		0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	
			5	0.00	0.00	0.00				0.00					0.00	0.00		0.00	0.00				0.50		0.00	0.00	1.00	0.00		1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	1.00			0.00				0.00		0.00		1.00	1.00		0.00	0.00	0.00	0.00	1.00		1.00	0.00	
			7	0.00		0.00									0.00	0.00			0.00				0.00		1.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.00		0.00	0.00		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					0.00	0.00				0.00						
			11																								0.00											
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			13																								0.00											
			14																								0.00											
	54	3	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00		1.00	0.00	0.00	1.00	1.00	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00
			3	1.00	1.00	1.00	1.00	0.00	0.00	1.00	0.00	1.00		0.00	0.00	0.50		0.00	0.00		0.00	1.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	
			4	0.00	0.00	0.00	0.00	0.00	1.00	0.00					1.00	0.00		0.00	0.83		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	
			5	0.00	0.00	0.00				0.00					0.00	0.00		0.00	0.00				1.00		0.00	0.00	0.00	0.00		1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.50			0.00				0.00		0.00		1.00	1.00		0.00	0.00	0.00	0.00	1.00		1.00	0.00	
			7	0.00		0.00									0.00	0.00			0.00				0.00		1.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00			0.00				0.00		0.00		0.00	0.00		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					0.00	0.00				0.00						
			11																								0.00											
			12																								0.00											
			13																								0.00											
			14																								0.00											

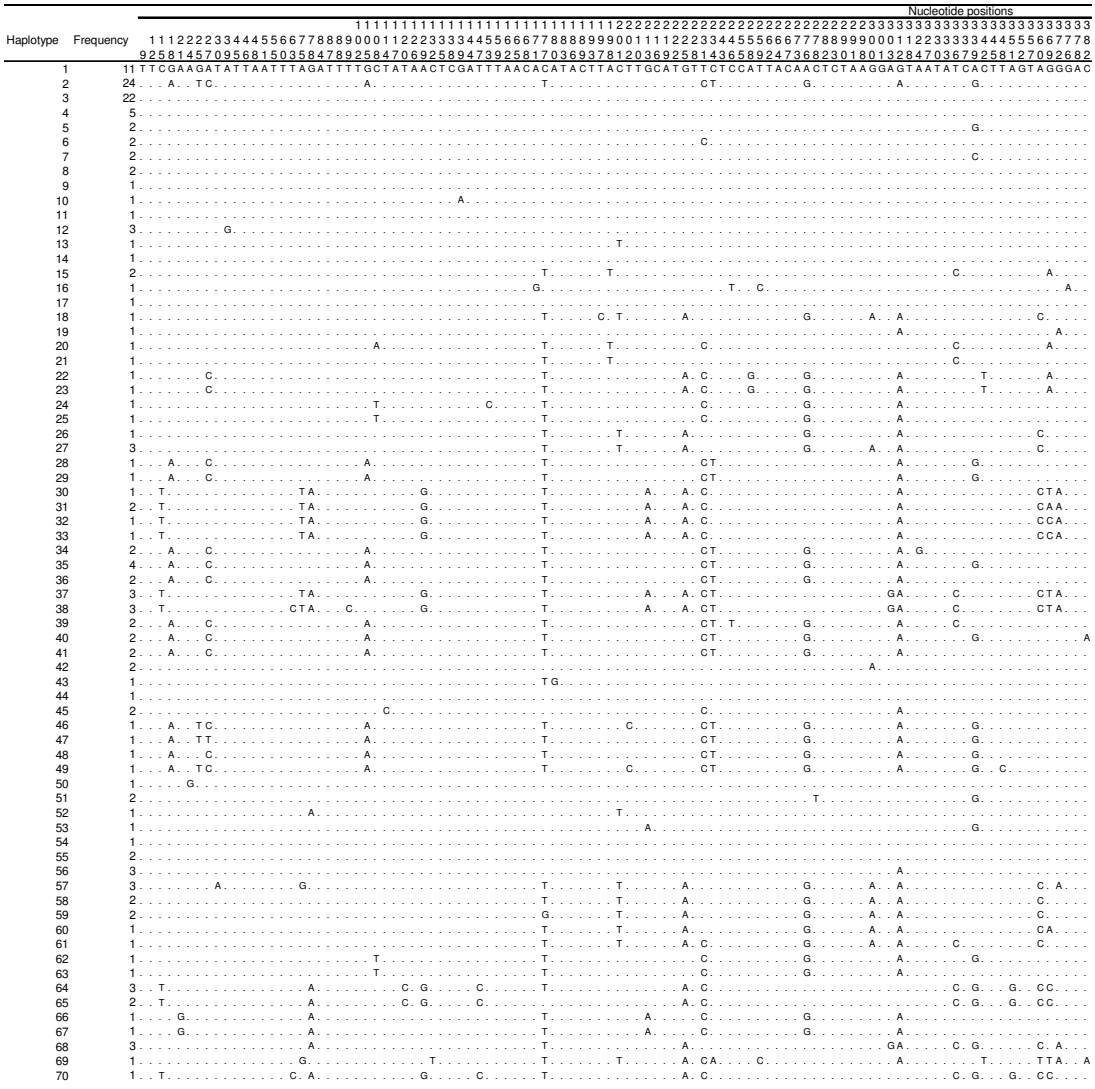
Taxa	Site ID	*N*	Allele	*Aco1*	*Aco2*	*Acyc*	*Ada*	*Ak1*	*Ak2*	*Ald*	*Argk*	*Dia*	*Enol**	*Est*	*Fdp*	*Fum*	*Glo**	*Got1*	*Got2*	*Gpd**	*Gpi*	*Idh1*	*Idh2*	*Ldh*	*Mdh1*	*Mdh2*	*Me*	*Mpi*	*Ndpk**	*PepA*	*PepC*	*PpD1*	*6Pgd*	*Pgam*	*Pgk*	*Pgm*	*Pk*	*Tpi**
P45c	55	3	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.17	1.00	0.83	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.17	0.00	0.33	1.00	0.00	0.00	1.00	0.00		1.00	0.00	0.00	1.00	1.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.33	0.00	0.00	1.00	0.00	0.33	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.67	0.83	0.67	0.67	0.00	0.00	1.00	0.00	1.00		0.00	0.33	0.17		0.00	0.00		0.00	1.00	0.00	1.00	0.67	0.67	0.00	0.00	0.00	0.33	0.67	0.00	0.00	0.00	0.00	0.00	0.67	
			4	0.33	0.00	0.33	0.00	0.00	1.00	0.00					0.67	0.00		0.00	0.67		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.33	
			5	0.00	0.00	0.00				0.00					0.00	0.33		0.00	0.00				0.67		0.00	0.00	0.00	0.00		0.67	0.00	0.83	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.50		0.00					0.00		0.00		0.67	0.67		0.00	0.00	0.00	0.00	1.00		1.00	0.00	
			7	0.00		0.00									0.00	0.00		0.00					0.33		0.33		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.00										0.00		0.00					0.00		0.00		0.00	0.33		0.00		0.17	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					0.17	0.00				0.00							
			11																								0.00												
			12																								0.17												
			13																								0.00												
			14																								0.00												
P50	56	3	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
			2	0.00	0.00	0.00	0.50	0.17	1.00	0.00	1.00	0.00		1.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	1.00	1.00	0.00	0.50	0.83	0.00	1.00	0.00	1.00		0.00	1.00	0.17		0.00	0.00		0.00	1.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
			4	0.00	0.00	0.33	0.00	0.00	0.00	0.00					0.00	0.17		0.00	0.67		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.83	0.00	0.00	0.00	1.00	
			5	0.00	0.00	0.67				0.00					0.00	0.50		0.00	0.00				1.00		0.00	0.00	0.00	0.00		1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.83		0.00	0.00	0.00	0.00	1.00		1.00	0.00	
			7	0.00		0.00									0.00	0.17		0.00					0.00		0.00		0.67	0.00		0.00	0.00	0.00	0.17	0.00		0.00		
			8	0.00		0.00										0.00		0.33					0.00		0.00		0.00	0.00		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.33	0.17		0.00		0.00		0.00				
			10			0.00																					0.00	0.00				0.00							
			11																								0.00												
			12																								0.00												
			13																								0.00												
			14																								0.00												
	57	2	1	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00
			2	0.00	0.00	0.00	1.00	0.25	1.00	0.00	1.00	0.00		1.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			3	0.75	1.00	0.00	0.00	0.75	0.00	1.00	0.00	1.00		0.00	1.00	0.00		0.00	0.00		0.00	1.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.00	
			4	0.00	0.00	0.75	0.00	0.00	0.00	0.00					0.00	0.25		0.00	0.75		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	
			5	0.00	0.00	0.25				0.00					0.00	0.50		0.00	0.25				1.00		0.00	0.00	0.00	0.00		1.00	1.00	0.75	0.00	0.00	0.00	0.00	0.00	
			6	0.00		0.00				0.00					0.00	0.00			0.00				0.00		0.00		0.00	0.75		0.00	0.00	0.00	0.00	1.00		0.75	0.00	
			7	0.00		0.00									0.00	0.00		0.00					0.00		0.00		0.50	0.00		0.00	0.00	0.00	0.00	1.00		0.00		
			8	0.00		0.00										0.25		0.00					0.00		0.00		0.00	0.25		0.00		0.00	0.00	0.00		0.00		
			9			0.00																			0.00		0.50	0.00		0.00		0.25		0.00				
			10			0.00																					0.00	0.00				0.00							
			11																								0.00												
			12																								0.00												
			13																								0.00												
			14																								0.00												
V.pichirichi	58	5	1	0.00	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.70	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.00	1.00
			2	0.00	0.00	0.00	0.00	1.00	0.75	0.00	1.00	1.00		1.00	0.10	0.00	1.00	1.00	0.00	0.00	0.00	0.00	0.90	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.10	0.10	1.00	0.00	0.00	0.00
			3	0.30	1.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00		0.00	0.90	1.00		0.00	0.00		0.00	1.00	0.00	1.00	0.00	0.30	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	1.00	
			4	0.50	0.00	0.25	0.00	0.00	0.00	0.00					0.00	0.00		0.00	1.00		0.00	0.00	0.10		0.00	0.00	0.00	1.00		0.00	0.00	0.00	0.80	0.20	0.00	0.00	0.00	
			5	0.00	0.00	0.00				0.00					0.00	0.00		0.00	0.00				0.00		0.00	0.00	0.00	0.00		0.00	1.00	0.80	0.00	0.10	0.00	0.00	0.00	
			6	0.00		0.38				0.00					0.00	0.00			0.00				0.00		1.00		0.00	0.00		0.00	0.00	0.00	0.10	0.60		0.30	0.00	
			7	0.20		0.00									0.00	0.00			0.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.13										0.00			0.00				0.00		0.00		0.00	0.00		0.00		0.20	0.00	0.00		0.00		
			9			0.00																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.25																					0.00	0.00				0.00							
			11																								0.80												
			12																								0.10												
			13																								0.10												
			14																								0.00												

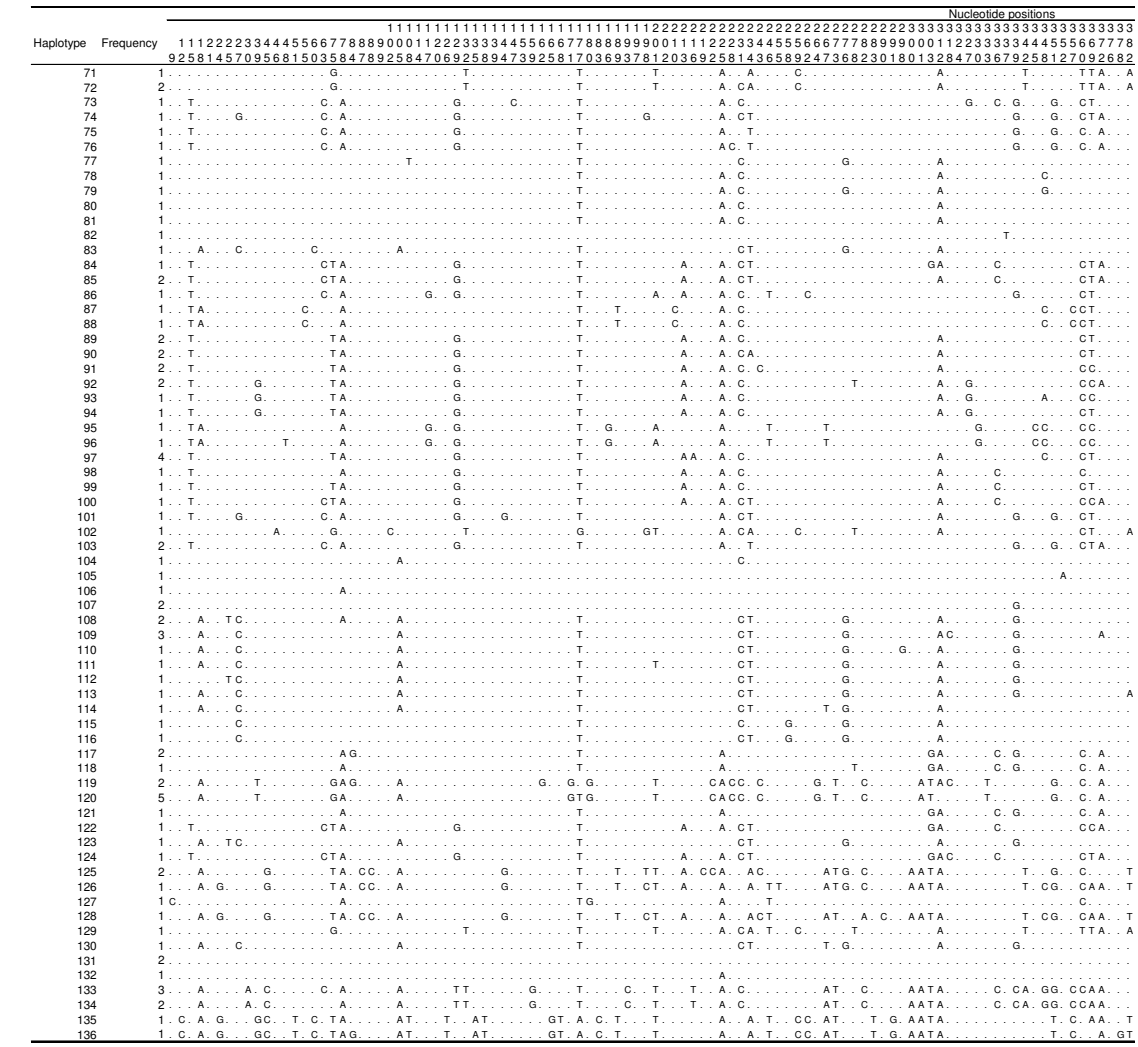
Taxa	Site ID	*N*	Allele	*Aco1*	*Aco2*	*Acyc*	*Ada*	*Ak1*	*Ak2*	*Ald*	*Argk*	*Dia*	*Enol**	*Est*	*Fdp*	*Fum*	*Glo**	*Got1*	*Got2*	*Gpd**	*Gpi*	*Idh1*	*Idh2*	*Ldh*	*Mdh1*	*Mdh2*	*Me*	*Mpi*	*Ndpk**	*PepA*	*PepC*	*PpD1*	*6Pgd*	*Pgam*	*Pgk*	*Pgm*	*Pk*	*Tpi**
Keyacris	59	3	1	0.00	0.00	0.00	1.00	0.00	0.83	0.17	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.83	0.00	0.67	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00
P110a			2	0.00	1.00	0.00	0.00	1.00	0.17	0.00	1.00	0.00		1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.33	0.00	0.50	0.00	0.33	0.17	1.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	1.00
			3	0.00	0.00	0.00	0.00	0.00	0.00	0.83	0.00	1.00		0.00	1.00	0.00		0.83	0.00		0.17	0.00	0.00	0.00	0.50	1.00	0.33	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00	
			4	0.00	0.00	0.00	0.00	0.00	0.00	0.00					0.00	0.00		0.00	0.00		0.00	0.00	0.00		0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.67	0.00	0.00	0.00	0.00	
			5	0.00	0.00	0.00				0.00					0.00	0.00		0.17	0.00				0.00		0.00	0.00	0.17	0.50		1.00	1.00	0.50	0.17	0.00	0.00	1.00	0.00	
			6	1.00		0.00				0.00					0.00	0.00			1.00				0.00		0.00		0.00	0.00		0.00	0.00	0.00	0.00	1.00		0.00	0.00	
			7	0.00		0.00									0.00	1.00			0.00				0.00		0.00		0.00	0.33		0.00	0.00	0.00	0.00	0.00		0.00		
			8	0.00		0.50										0.00			0.00				0.00		0.00		0.00	0.00		0.00		0.00	0.00	0.00		0.00		
			9			0.50																			0.00		0.00	0.00		0.00		0.00		0.00				
			10			0.00																					0.00	0.00				0.00						
			11																								0.00											
			12																								0.00											
			13																								0.00											
			14																								0.00											
Total		180	1	0.02	0.01	0.03	0.02	0.01	0.47	0.00	0.20	0.00	1.00	0.01	0.00	0.00	0.01	0.00	0.00	1.00	0.97	0.00	0.01	0.04	0.06	0.30	0.00	0.01	0.01	0.01	0.02	0.03	0.00	0.00	0.73	0.01	0.02	0.98
			2	0.01	0.17	0.00	0.69	0.67	0.48	0.01	0.80	0.07		0.98	0.01	0.01	0.99	0.91	0.23	0.00	0.02	0.02	0.03	0.03	0.01	0.46	0.01	0.00	0.99	0.01	0.14	0.01	0.08	0.00	0.02	0.01	0.01	0.02
			3	0.25	0.79	0.11	0.22	0.32	0.01	0.75	0.00	0.92		0.02	0.93	0.26		0.08	0.02		0.00	0.96	0.38	0.93	0.69	0.07	0.01	0.14	0.00	0.35	0.18	0.03	0.00	0.01	0.19	0.20	0.25	
			4	0.53	0.02	0.50	0.07	0.00	0.05	0.21					0.04	0.01		0.00	0.63		0.00	0.02	0.01		0.01	0.17	0.00	0.03		0.00	0.25	0.00	0.82	0.07	0.05	0.01	0.69	
			5	0.01	0.00	0.16				0.02					0.01	0.52		0.00	0.06				0.43		0.07	0.00	0.01	0.01		0.56	0.36	0.77	0.02	0.04	0.00	0.02	0.01	
			6	0.02		0.01				0.00					0.00	0.06			0.02				0.01		0.03		0.08	0.19		0.00	0.03	0.01	0.04	0.86		0.74	0.02	
			7	0.16		0.13									0.00	0.06			0.01				0.12		0.04		0.11	0.01		0.07	0.01	0.02	0.02	0.01		0.01		
			8	0.00		0.04										0.08			0.03				0.00		0.09		0.18	0.54		0.00		0.13	0.01	0.01		0.00		
			9			0.01																			0.01		0.05	0.07		0.00		0.00		0.00				
			10			0.01																					0.29	0.01				0.01						
			11																								0.08											
			12																								0.16											
			13																								0.01											
			14																								0.01											

Taxa	Site ID	*N*	Allele	*Aco1*	*Aco2*	*Acyc*	*Ada*	*Ak1*	*Ak2*	*Ald*	*Argk*	*Dia*	*Enol**	*Est*	*Fdp*	*Fum*	*Glo**	*Got1*	*Got2*	*Gpd**	*Gpi*	*Idh1*	*Idh2*	*Ldh*	*Mdh1*	*Mdh2*	*Me*	*Mpi*	*Ndpk**	*PepA*	*PepC*	*PpD1*	*6Pgd*	*Pgam*	*Pgk*	*Pgm*	*Pk*	*Tpi**
Total		177	1	0.02	0.01	0.03		0.01	0.46		0.20	0.00	1.00	0.01	0.00	0.00	0.01	0.00	0.00	1.00	0.97	0.00		0.04	0.06	0.30		0.01	0.01	0.01	0.02	0.03	0.00	0.00	0.72	0.01		1.00
(Excluding Keyacris)			2	0.01	0.16	0.00	0.70	0.67	0.48	0.01	0.80	0.07		0.97	0.01	0.01	0.99	0.93	0.24	0.00	0.02	0.02	0.03	0.03		0.47			0.99	0.01	0.15	0.01	0.08	0.00	0.02	0.01	0.01	
			3	0.26	0.81	0.11	0.23	0.32	0.01	0.75	0.00	0.92		0.02	0.93	0.27		0.07	0.02			0.96	0.39	0.93	0.69	0.06		0.14	0.00	0.36	0.19	0.02	0.00	0.01	0.19	0.21	0.26	
			4	0.54	0.02	0.51	0.07	0.00	0.05	0.22					0.05	0.01		0.00	0.64		0.00	0.02	0.01		0.01	0.17	0.00	0.03		0.00	0.25	0.00	0.83	0.07	0.05	0.01	0.71	
			5	0.01	0.00	0.16				0.02					0.01	0.53			0.06				0.44		0.07	0.00	0.01			0.55	0.34	0.78	0.01	0.04	0.00		0.01	
			6			0.01				0.00					0.00	0.06							0.01		0.03		0.08	0.19		0.00	0.03	0.01	0.05	0.85		0.75	0.02	
			7	0.16		0.13									0.00	0.05			0.01				0.12		0.04		0.12			0.07	0.01	0.02	0.02	0.01		0.01		
			8	0.00		0.03										0.08			0.03				0.00		0.09		0.18	0.55		0.00		0.13	0.01	0.01		0.00		
			9																						0.01		0.05	0.07		0.00		0.00		0.00				
			10			0.01																					0.30	0.01				0.01						
			11																								0.08											
			12																								0.17											
			13																								0.01											
			14																								0.01											
Global statistics			*N**	177	175	172	161	171	164	176	177	147	176	177	175	176	176	176	159	146	177	152	174	88	177	177	177	177	177	177	177	176	153	177	167	177	173	177
(Excluding Keyacris)			*Na*	7	5	9	3	4	4	5	3	3	1	3	7	8	2	4	7	2	3	4	7	3	8	5	11	7	3	9	7	10	8	9	5	7	5	1
			H_{obs}	0.18	0.21	0.14	0.13	0.09	0.04	0.19	0.11	0.06	0.00	0.02	0.05	0.19	0.01	0.09	0.25	0.01	0.03	0.07	0.15	0.10	0.08	0.14	0.36	0.15	0.02	0.14	0.23	0.25	0.19	0.14	0.07	0.15	0.06	0.00
			H_{exp}	0.62	0.33	0.68	0.45	0.45	0.55	0.39	0.33	0.15	0.00	0.05	0.13	0.64	0.02	0.13	0.53	0.01	0.05	0.08	0.65	0.14	0.50	0.66	0.82	0.64	0.02	0.57	0.76	0.38	0.31	0.27	0.44	0.39	0.44	0.00

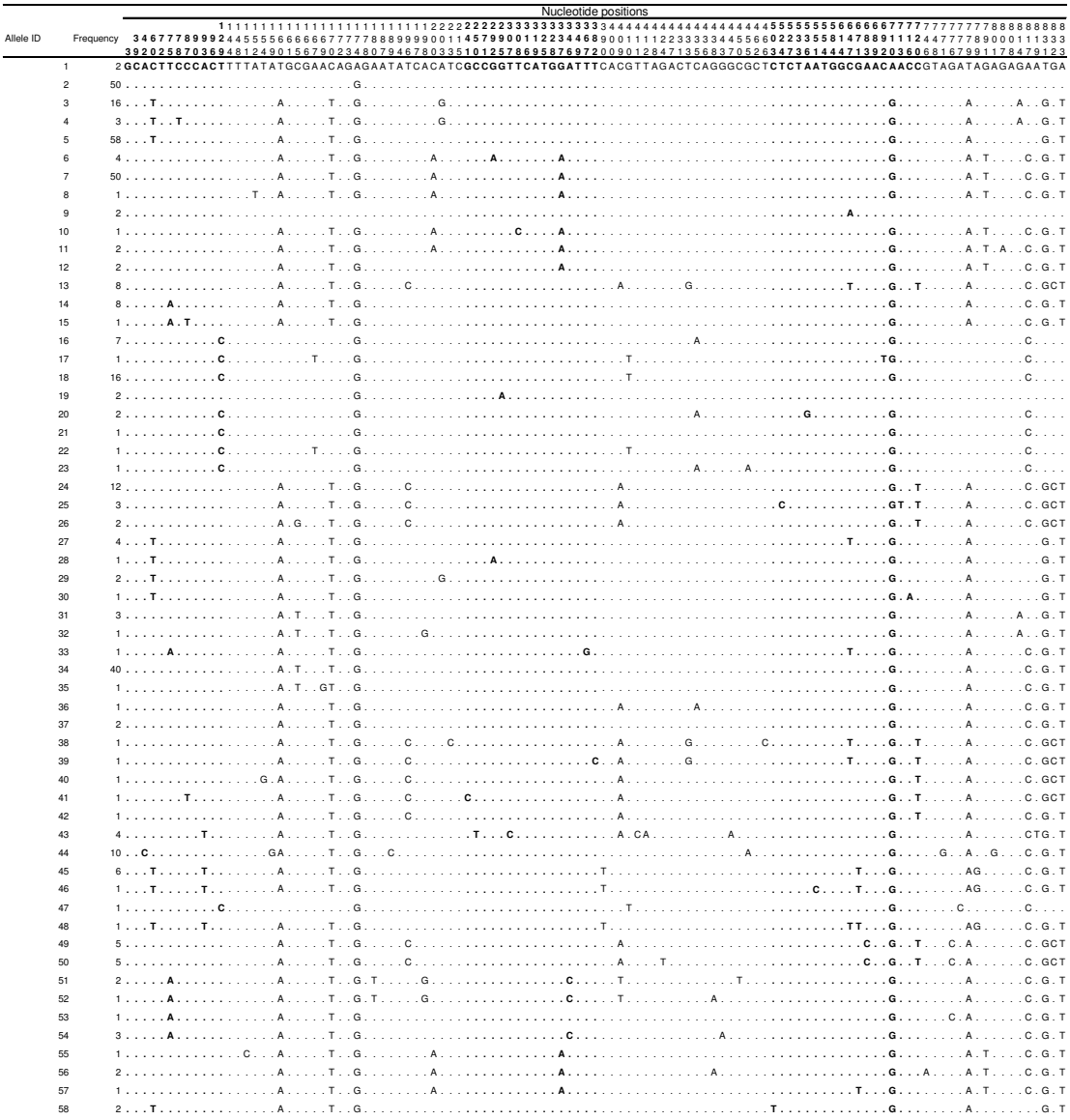
N: number of individuals
*N**: number of individuals successfully scored for each locus
N_a: number of alleles
H_{obs}: observed heterozygosity
H_{exp}: expected heterozygosity

Appendix Fig. 6.1 Sample haplotype frequencies and variable sites in the sequence alignment of 136 haplotypes of *COI* fragment (637 bp) found in the 10 *Vandiemenella* taxa and outgroup *Keyacris* P110a. Identical nucleotides with the haplotype-1 are marked as dots.




Nucleotide positions
Haplotype
Frequency

Appendix Fig. 6.2 Allele frequencies and variable sites in the sequence alignment of 85 alleles of *EF*-1α fragment (806 - 837) found in the 11 *Vandiemenella* taxa and outgroup *Keyacris* P110a. Identical nucleotides with the allele1 are marked as dots. Nucleotide sites in exon regions are marked in bold.



Appendix Fig. 6.3 Allele frequencies and variable sites in the sequence alignment of 77 alleles of *Mvia11* fragment (511 - 643) found in the 11 *Vandiemenella* taxa and outgroup *Keyacris* P110a. Identical nucleotides with the allele1 are marked as dots. Nucleotide sites in exon regions are marked in bold.