Species status and population genetic structure of grapevine eriophyoid mites

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Abstract

Bud mite, blister mite (*Colomerus vitis* Pagenstecher), and rust mite (*Calepitrimerus vitis* Nalepa) (Acari: Eriophyoidae) are recognized grapevine pests. Much of the biology and ecology of these pests is poorly understood. We used two types of molecular markers to gain further insight into the breeding biology and population structure of these mites, using individuals collected from sites around south-eastern Australia. Patterns of genetic variation observed using PCR-RFLP of ITS 1 (Internal Transcribed Spacer 1) confirmed the separate species status of the rust mite, and resolved the species status of bud and blister mites, revealing two closely related but distinct species. Microsatellite markers revealed extensive genetic differentiation between bud mite populations and blister mite populations even at micro-geographical levels, suggesting low movement in these species. The findings indicate that separate control strategies are needed against bud and blister mites, and that localized control strategies are likely to be effective given their limited dispersal.

Introduction

The eriophyoid mite group includes many species that are economically important plant pests. Previous studies have focused on the biology and control of a limited number of species based entirely on observational data (Lindquist et al., 1996; Davies et al., 2001). We are unaware of molecular research on this group, despite the fact that molecular markers can rapidly provide insight into cryptic species, genetic structure, mode of reproduction, and migration rate (Sunnucks, 2001), which can be particularly useful in helping to devise control strategies against pest organisms including mites (Navajas & Fenton, 2000; Weeks et al., 2000).

The bud mite and blister mite are members of the family Eriophyoidae (Acari) and have long been recognized as grapevine pests (Smith & Stafford, 1948). Although morphologically identical, they are thought to represent two strains of one species, *Colomerus vitis* (Pagenstecher), based on the type of damage that they cause to the grapevine, *Vitis vinifera* (L.) (Vitaceae). Bud mite damage includes distortion on basal leaves, shortening of the basal internode, and extensive 'bubbling' of the tissue around basal shoots. High infestations may result in witches broom shoot growth, zigzag shoots, and, in severely infected plants, the death of buds (Smith & Stafford, 1948; Buchanan & Amos, 1992; Bernard et al., 2000). Blister mite damage differs from that of the bud mite, and involves the formation of hemispherical galls on grapevine leaves. Plant hairs grow densely within the concave cavity of galls on the underside of the leaves. These hairs are initially white but change to a reddish brown colour through the growing season. High infestations can result in most leaves being galled and premature leaf drop (Smith & Stafford, 1948; Buchanan & Amos, 1992). Although extensive damage may occur as a result of bud mite and blister mite infestation, economic loss is thought to be limited to certain grapevine varieties and to young plants when infestation levels are high (Buchanan & Amos, 1992; Lindquist et al., 1996).

The life cycles of the bud mite and blister mite have been described by a number of workers, based on biological observations (Smith & Stafford, 1948; Whitehead et al., 1978; Buchanan & Amos, 1992; Bernard et al., 2000). In brief, both bud and blister mites reside in buds during winter as adults, where they remain active and feed on the dormant buds. Egg production begins prior to bud burst. The development of egg to adult can take 2 weeks under favourable conditions, allowing numerous generations per year (Buchanan & Amos, 1992). After bud burst, the

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behaviour of each type of mite differs. Bud mites move towards newly developing buds. As the population increases, the mites invade deeper to feed on the developing bud. The resulting damage to the bud becomes apparent in mid-summer to early autumn (Bernard et al., 2000). In contrast, blister mites do not continue to feed on the buds past bud burst. Mites that have penetrated inner leaf scales are carried up to infect the developing shoot, while those on the outer scales affect the leaves of basal shoots. Blister mites feed on the underside of leaves, resulting in characteristic gall formation which provides shelter for the feeding mites. During autumn, the mites migrate from galls back to buds for the winter (Buchanan & Amos, 1992). Males in these species have not been described, although they may be indistinguishable from females, as is suggested for other eriophyoid mites (Lindquist et al., 1996).

Although the type of damage and control measures of the bud and blister mites are well described in the literature, there is a lack of information on the basic biology and ecology of these species. For instance, (i) do they represent different strains of the same species or different species? (ii) what level of genetic differentiation exists among distinct populations? and (iii) is there extensive gene flow within and between vineyards? The use of molecular markers has proven useful to address such questions (Corrie et al., 2002). For instance, the Internal Transcribed Spacer (ITS) region has been used in a range of taxa to examine genetic variation and population structure both within and between populations, and also in species identification including mites (Lohse et al., 2002). With highly conserved regions among many taxa, primers can be developed for DNA amplification that works on species where there is little knowledge of the DNA sequence (Hillis & Dixon, 1991). However, in the past decade microsatellite markers have surpassed many other molecular markers systems by providing a detailed insight into the population structure at many levels. The main advantages with microsatellite markers are that they are mostly neutral (with regard to selection), have a relatively high level of polymorphism, and co-dominant (Mendelian) inheritance patterns (Sunnucks, 2001).

Here we describe the development of molecular markers for eriophyoid mites and with these markers gain insight into the biology of mites from several vineyards of grapes. Specifically, we use the ITS 1 region to suggest that bud and blister mites are separate species. We also describe the isolation of microsatellites from the blister mite and use these markers to investigate the population structure of both bud and blister mite, focusing specifically on differences among mites collected from local infestations. The implications of these findings for control of these mites are discussed.

Methods

Collections

Blister mite, bud mite (*Colomerus vitis* Pagenstecher), and the closely related rust mite (*Calepitrimerus vitis* Nalepa), were collected during outbreaks from July 1998 to December 2002 in south-eastern Australia and assessed for ITS 1 RFLP variation (Figure 1 and Table 1). The sampling

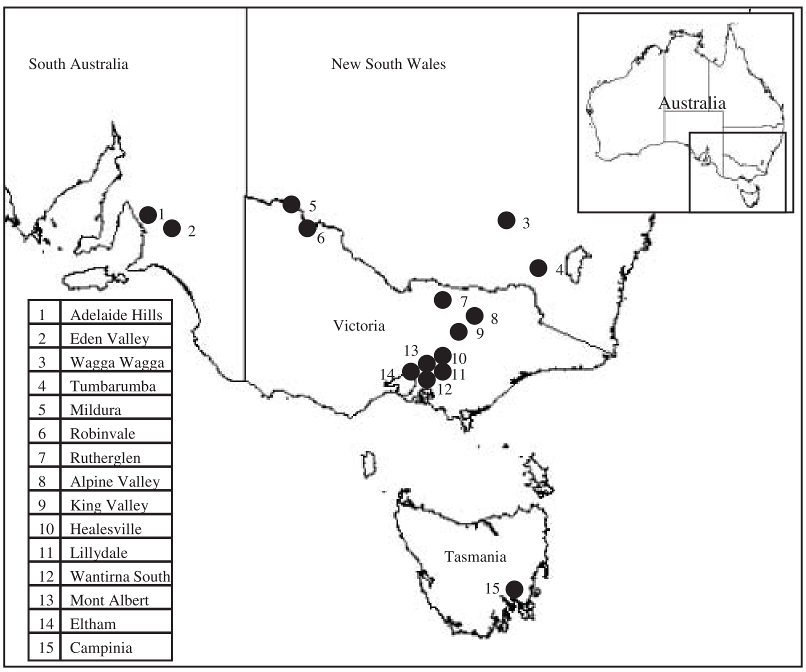

Figure 1 Sites in south-eastern Australia where rust, bud, and blister mites were collected. For mite types collected from each location, see Table 1.

Table 1 Bud mite, blister mite, and rust mite collections, along with the distribution of ITS 1 genotypes in these mites based on RFLPs with the restriction enzymes *Alu*I, *Hae*III, and *Hin*fI. Locations are mapped in Figure 1

Location	State	Mite type	Mites sampled	ITS 1 genotype (number of individuals)
Wantirna South	Victoria	Bud	7	G4
Healesville	Victoria	Bud	7	G4
King Valley	Victoria	Bud	15	G4
Campinia	Tasmania	Bud	19	G3 (9), G4 (10)
Tumbarumba	New South Wales	Bud	5	G3 (4), G4 (1)
Adelaide Hills	South Australia	Bud	10	G3 (6), G4 (4)
Eden Valley	South Australia	Bud	14	G4
Wagga Wagga	New South Wales	Blister	6	G2
Mont Albert	Victoria	Blister	6	G2
Eltham	Victoria	Blister	12	G2
Mildura	Victoria	Blister	5	G2 (4), G5 (1)
Robinvale	Victoria	Blister	21	G2 (17), G6 (3), G7 (1)
Rutherglen	Victoria	Blister	10	G2 (9), G6 (1)
Alpine Valley	Victoria	Blister	6	G2
Tumbarumba	New South Wales	Rust	11	G1
Lilydale	Victoria	Rust	10	G1
Healesville	Victoria	Rust	2	G1
Eden Valley	South Australia	Rust	2	G1

regime encompassed mites taken from different vineyards and geographical regions (Figure 1). Unfortunately, we were unable to collect sympatric populations of *Colomerus* mites, as no outbreaks of both mites occurred in the same vineyard.

Mites were also sampled intensively from a few vineyards for microsatellite analysis. In these vineyards, the varieties were planted in different rows of trellised vines separated by 3.5 m. Supports for the trellis were 7.5 m apart, separating rows into panels each with 3–4 vines. For the bud mite, Healesville and Wantirna South were sampled intensively (Table 2). At Healesville, mites were sampled from two to three buds from two canes of a single plant. The Wantirna South sample consisted of mites from 32 buds sampled from two grape varieties (22 from Cabernet Sauvignon/Merlot, 10 from Pinot Noir). These varieties were planted in adjacent rows; buds were taken from the entire 90 m along these rows.

Blister mites were intensively sampled from Rutherglen and Eltham (Table 2). For the Eltham sample, seven blisters

Table 2 Levels of sampling for bud and blister mite populations for microsatellite analysis

Location	Mite type	Mites sampled	Number of vineyards	Tissues sampled
Campinia	Bud	37	1	6 buds
Eden Valley	Bud	16	1	2 buds
Tumbarumba	Bud	11	1	2 buds
Healesville	Bud	97	1	2 canes*, 8 buds
Adelaide Hills	Bud	33	1	not known
Lilydale	Bud	30	1	5 buds
King Valley	Bud	34	1	6 buds
Wantirna South	Bud	176	1	32 buds from 2 varieties
Wagga Wagga	Blister	6	1	1 blister
Mont Albert	Blister	7	1	1 blister
Mildura	Blister	11	1	not recorded
Eltham	Blister	126	1	2 leaves, 19 buds
Robinvale	Blister	134	6	not recorded
Alpine Valley	Blister	15	2	not recorded
Rutherglen	Blister	124	1	3 varieties, 10 plants, 2 sampled intensively (4 leaves/6 blisters, and 5 leaves/12 blisters)

*Canes are defined as horizontal stems containing numerous dormant buds.

from one leaf and 12 blisters from an adjacent leaf of the same plant were sampled. The Rutherglen sample consisted of 10 plants of three grape varieties, Riesling, Shiraz, and Pedro Ximenes, from different rows 330 m apart. For two of the varieties (Riesling, Shiraz), one plant was sampled intensively by taking several leaves (5–17 cm apart) from an 80 cm section of a shoot. Blisters (1.5–10 cm apart) were sampled from the same leaf. Mites were also taken from other blisters collected from plants in different panels (three in the case of Riesling, four for Shiraz) within 20 m of the intensively sampled plant.

DNA extraction of mites

A variation of the Chelex® protocol (Walsh et al., 1991) was used to extract DNA from mites for Polymerase Chain Reactions (PCRs). Individual mites were placed into 0.5 ml microcentrifuge tubes and spun at 20 800 ***g*** for 5 min. Tubes were placed on ice and 5 μl of proteinase K was added. Mites were crushed using a clean 0.5 ml pestle and 100 μl of 5% Chelex® solution was added. The pestle was removed and the tubes were incubated at 55 °C for 30–60 min followed by 100 °C for 8 min. Extractions were stored at −20 °C until required. Prior to PCR, the extractions were spun at 20 800 ***g*** for 2 min. The supernatant taken from just above the Chelex® resin was then used in the PCR amplifications.

Internal Transcribed Spacer 1 PCR

Amplifications of the Internal Transcribed Spacer (ITS) 1 were made using the primers 18s and 5.8s rev (Navajas et al., 1998). PCRs were carried out in 25 μl volumes, with 5 μl of template DNA, 200 mM of each dNTP, 1.5 mM $MgCl_2$, 0.5 μM 18s primer, 0.5 μM 5.8s rev primer, 1 × PCR buffer, and 0.5 units of *Taq* polymerase (Gibco BRL). PCRs were completed in an Applied Biosystem 9700 Thermocycler using a touch down profile. The profile consisted of an initial denaturation of 93 °C for 2 min followed by 12 cycles of touch down, 93 °C for 20 s, 55 °C decreasing to 43 °C (decreasing by 1 degree each cycle), 72 °C for 1 min followed by 25 cycles of 93 °C for 20 s, 43 °C for 30 s, and 72 °C for 1 min (increasing elongation by 10 s each cycle). PCR amplifications were run on 1.5% agarose gels, stained with ethidium bromide, and visualized with UV light. PCR product sizes were estimated with a ϕX174 (*Hae*III) ladder.

Restriction analysis of ITS 1

ITS 1 PCR amplification products were initially digested with six restriction enzymes, *Alu*I, *Bam*HI, *Hae*III, *Hinc*II, *Hin*fI, and *Rsa*I. Restriction digests were run on 3% agarose gels, stained with ethidium bromide, and visualized with UV light. Fragment sizes were estimated with a ϕX174 (*Hae*III) ladder.

Isolation of microsatellites

Approximately 400 blister mites were collected from the Eltham vineyard and pooled into a single 0.5 ml microcentrifuge. Purified genomic DNA was isolated using a CTAB-based extraction method incorporating an RNase step (Sambrook et al., 1989). An Amplified Length Fragment Polymorphism (AFLP) library was constructed from the DNA extract, to the pre-amplification stage according to the Gibco BRL AFLP Analysis System II manual. The pre-amplification was run on 1% low melt agarose gel and products between 300 and 800 bp were excised from the gel and cleaned using wizard PCR preps (Promega), followed by drying down to a 12 μl volume. Size-selected digest products were ligated into a pGEM-T vector according to the Promega pGEM-T vector manual. Ligations were transformed into *Epicurian coli*® XL2-Blue ultracompetent cells according to the Stratagene instruction manual. Colonies were lifted onto N+ hybond membranes. Microsatellite probes $(AC)_{10}$ and $(AG)_{10}$ were end-labelled with [γ^{33}P] ATP and hybridized to membranes overnight at 50 °C. Membranes were exposed to autoradiograph film and the film was aligned back to the colonies after exposure. Positive colonies were re-screened to confirm their status. Inserts in positive clones were sequenced, and primers were designed to amplify PCR products of 90–200 bp. Levels of variation were determined by screening 48 mites from six populations for both bud and blister mite. Primer pairs that showed sufficient variation (H_o>0.3) and no high frequency null alleles were used in further analysis.

Microsatellite PCR

Polymerase chain reactions were carried out in 10 μl volumes, with 3 μl of template DNA (from Chelex extractions), 167 μM dNTPs, 1.5 mM $MgCl_2$, 0.1 μM of unlabelled forward primer, 0.03–0.06 μM of forward primer end-labelled with [γ^{33}P] ATP, 0.4 μM of unlabelled reverse primer, 5 μg of bovine serum albium (BSA), 1 × *Taq* Buffer, and 0.4 units of *Taq* polymerase (Promega). Thermocycling was the same for all primer pairs, with an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 47–52 °C (see Table 3 for annealing temperatures) for 30 s, and 72 °C for 30 s. PCR products were electrophorised on 5% polyacrylamide denaturing gels and initially sized with a pUC19 sequence. PCR products run on subsequent gels were sized using pUC19 sized PCR products.

Analysis of microsatellite data

It remains unknown if *Colomerus* mites produce haploid males, and, consequently, it is unclear if haploid individuals were sampled in this study. To prevent possible biases in

Table 3 Restriction fragment length polymorphism (RFLP) fragment sizes revealed by the enzymes *Alu*I, *Hae*III, and *Hinf* I for rust, bud, and blister mite

ITS 1 genotype	Mite type	PCR product size (bp)	Estimated fragment sizes (bp)		
			*Alu*I	*Hae*III	*Hinf* I
G1	Rust	610	270, 250, 90	390, 220	340, 150, 120
G2	Blister	660	660, 460, 200	300, 240, 130	330, 230, 120
G3	Bud	650	360, 200, 130	290, 240, 120	340, 190, 120
G4	Bud	690	370, 200, 120	320, 240, 130	360, 190, 120
G5	Blister	660	660, 460, 200	420, 240	330, 230, 120
G6	Blister	660	660	270, 240, 140	330, 230, 120
G7	Blister	660	660, 600	270, 240, 140	330, 230, 120

our analyses that might arise if some fraction of sampled mites were haploid, a two-step analysis of the microsatellite data was pre-formed in several cases. First, we conducted analyses on the distribution of genetic variation, assuming all mites were diploid. Second, we used a sampling program to randomly select a single allele from each locus of each individual, thereby creating a new data set consisting of haploid genotypes. This reduced data set was also used to examine patterns of genetic structure within populations. The haploid data set could not be used to estimate non-random associations of alleles within individuals (i.e., true inbreeding), but yielded estimates of genetic structure at higher levels unbiased by individual ploidy level.

Allele frequencies were calculated using the program GENEPOP (version 3.1) (Raymond & Rousset, 1995a; Raymond & Rousset, 1995b). GENEPOP was also used to measure non-random associations of alleles within individuals, quantified by the f statistic (analogous to F_{IS}), and the significance of these non-random associations, which were obtained using a probability test (Guo & Thompson, 1992). In addition, we also tested for deviations from Hardy–Weinberg equilibrium using a reduced data set consisting only of individuals that were heterozygous for at least one locus (i.e., individuals that were clearly diploid).

The significance of non-random associations between alleles at different loci (genotypic disequilibrium for the diploid data sets and 'gametic' disequilibrium for the haploid data sets) and allelic differentiation between populations were estimated using probability tests. In addition, the magnitude of differentiation between populations of mites from distinct sampling locations was estimated from the haploid data sets as quantified by F statistics (analogous to F_{ST}) (Weir & Cockerham, 1984) through the program GDA (Lewis & Zaykin, 1999). The significance of the differentiation was obtained through permutation tests (Raymond & Rousset, 1995a; Raymond & Rousset, 1995b). In cases where mites were sampled in a hierarchical fashion (from distinct plants within vineyards or from distinct blisters within leaves), the program Arlequin (Schneider et al., 2000) was used in conjunction with the haploid data sets to estimate the magnitude and significance of the differentiation, using the methods of Weir & Cockerham (1984) and Excoffier et al. (1992).

Results

PCR-RFLP analysis of Internal Transcribed Spacer 1

Amplifications of ITS 1 in the mites produced different sized PCR products. PCR products in blister mites were 660 bp in length, bud mites were 690 or 650 bp in length, and rust mites were 610 bp in length (Figure 2A). Restriction analysis of the PCR-amplified ITS products with the enzymes *Hae*III (Figure 2B), *Hin*fI, or *Alu*I revealed additional sequence variation (Table 3). *Alu*I digests in blister mite revealed at least two amplified copies of the ITS 1 region, with the sum of fragments exceeding the total product length. This was not apparent in the other species or using the other restriction enzymes.

The variation in PCR product length and RFLP profiles demonstrated a complete genetic differentiation between rust, bud, and blister mite, with no groups sharing any PCR product sizes or RFLP profiles.

Microsatellites

Ten microsatellite primers were developed from the blister mite library. Primer pairs were tested on the bud mite and blister mite to determine which primers were polymorphic. Four primer pairs (Cvit 3, Cvit 4, Cvit 13, Cvit 15) were polymorphic in the blister mite and three primer pairs (Cvit 4, Cvit 15, Cvit 22) were polymorphic in the bud mite (Table 4).

Analysis of microsatellite data

Tables 5 and 6 show the allele frequencies of the bud and blister mites at each of the microsatellite loci. Typically, within each of the two strains of bud and blister mites, one or a few alleles predominated at each locus. However, there

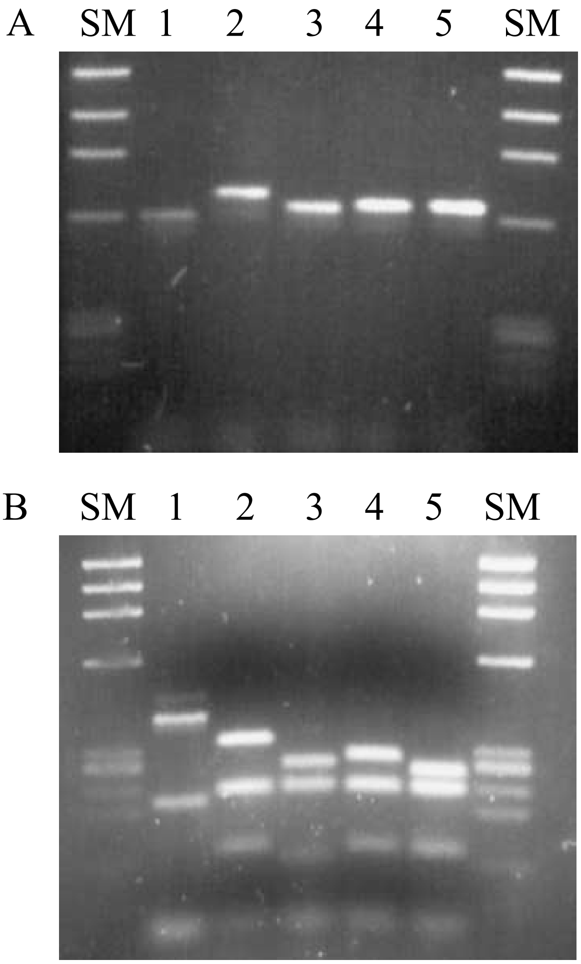

Figure 2 (A) Length differences in mite ITS amplifications. (B) Mite ITS genotypes after being cut with a restriction enzyme (*Hae*III). SM: Size Marker (ϕX174 *Hae*III), 1: rust mite (G1), 2: bud mite (G4), 3: bud mite (G3), 4: blister mite (G2), 5: blister mite (G6).

was substantial variation between the two mite types. Bud and blister mites were not necessarily polymorphic for the same loci, and they showed substantial differences in allele frequencies, particularly at locus Cvit 3, where their allelic ranges did not overlap (Table 4).

Both bud and blister mite populations exhibited strong Hardy–Weinberg disequilibrium ($P<0.001$ for all loci and all populations combined within each mite strain and $P<0.05$ for each population tested independently with the exception of Mont Albert blister mites). Overall estimates of f for the bud and blister mites were 0.379 and 0.502, respectively. An analysis of individuals that were heterozygous at one locus or more revealed that there was still an excess of homozygotes ($P<0.001$) (95% heterozygous at one locus for blister mite and 84% heterozygous at one locus for bud mite) when all populations of either bud or blister mite were considered independently ($f = 0.071$ and 0.311). Consequently, the genetic data strongly suggest inbreeding or null alleles within populations of *Colomerus* mites.

Tests of linkage disequilibrium were performed on the bud and blister mite data as both a diploid and haploid data set. Both data sets showed no genotypic disequilibrium in the bud mite populations ($P>0.05$ for all pair-wise comparisons in all populations combined). However, a strong genotypic disequilibrium was observed in blister mite populations, with strong associations between all loci ($P<0.001$ for all pairs of loci in both haploid and diploid data sets). The disequilibrium appeared to arise primarily from the populations of Robinvale and Rutherglen. Other blister mite populations did not possess a substantial linkage disequilibrium.

Strong genetic differentiation was evident among *Colomerus* mite populations. Using the haploid data set, all but three of the 28 pair-wise comparisons between bud mite populations showed significant pair-wise genetic differentiation ($P<0.05$), and the overall estimate was highly significant ($F = 0.133$, $P<0.0001$). Similar patterns of genetic differentiation were observed in the blister mites. Eighteen of the 21 pair-wise estimates of significance of differentiation fell below 0.05 using the blister mite haploid data set, and the overall estimate, $F = 0.126$, was large, and led to highly significant genetic differentiation ($P<0.001$).

Nested analyses revealed that population structuring existed at various geographical levels in both species. For example, we compared the levels of differentiation of bud mites sampled from populations in the state of South Australia (Eden Valley and Adelaide Hills) and the populations in the state of Victoria (Healesville, Lilydale, King Valley, and Wantirna South). Hierarchical analysis of genetic differentiation revealed no significant differentiation between states ($F = -0.003$, $P = 0.374$), although populations within states did show significant differentiation ($F = 0.139$, $P<0.001$).

On a smaller scale, we examined bud mites sampled from different grape varieties and buds. At Wantirna South, bud mites were sampled from two varieties, Pinot Noir and Cabernet Sauvignon / Merlot. Analysis of the haploid data set revealed a slight but significant differentiation between mites sampled from these two varieties ($F = 0.02$, $P = 0.05$). Finally, we examined mites sampled from two to three buds from each of two canes at Healesville. There was no genetic differentiation between the canes ($F = 0.001$, $P = 0.597$) or among buds within canes ($F = -0.025$, $P = 0.804$). Bud mites in a single bud sometimes contained more than four alleles at a locus, indicating that they must have originated from more than two parental lineages.

Table 4 Characteristics of polymorphic microsatellite loci

Locus	Primer sequence 5′–3′	Repeat motif in clone	Annealing temperature (°C)	Number of alleles		Allelic size range (bps)		Genbank accession no. for clone
				Bud mite	Blister mite	Bud mite	Blister mite	
Cvit 3	F-ACTGCTGATGCGACTCTTGAC R-GCAGATGTGACTTTGAATCCG	$(CT)_{12}$	50	3	4	86–92	104–114	AY485981
Cvit 4	F-AGCCAGAGCTTATGTAATATGAAG R-GCTAACCACAAAATTGCCAC	$(CT)_{24}$	50	NA*	14	NA*	123–157	AY485982
Cvit 13	F-ATGTCATGTTCTAGCCTCAAGC R-ATCACGTTTCCGCAGTTCG	$(GT)_{10}$	50	1	8	114	102–124	AY485983
Cvit 15	F-GATGTCGGTGATAGATTGTATGTG R-AAACTCAGCTAAACCATCATAGTG	$(CA)_{10}$	50	8	8	103–121	109–125	AY485984
Cvit 22	F-AATATTGGGTATCGAAGTTAATGG R-GTGCTAAGGTGCTAAACGAATTAC	$(GA)_{9}$	47	14	1	69–96	87	AY485985

*NA = Null alleles present.

To determine the hierarchical structuring of blister mites, we examined the distribution of genetic variation from six vineyards in the Robinvale area several km apart. We found strong and significant structure among these six subpopulations ($F = 0.278$, $P<0.0001$). We then examined the small-scale structure of blister mites. At Rutherglen, blister mites were sampled from three distinct grape varieties, Riesling, Shiraz, and Pedro Ximenes. We found a

Table 5 Allele frequencies of bud mite from eight populations at three polymorphic microsatellite loci. Estimates obtained from haploid data sets are given in parentheses. Rare alleles (frequency <3%) are excluded. Sample sizes are given in Table 2

Locus and allele	Population							
	Adelaide Hills	Campinia	Eden Valley	Healesville	King Valley	Lilydale	Tumbarumba	Wantirna South
Cvit 13								
106	0.015 (0.000)	0.014 (0.000)	0.031 (0.000)	0.013 (0.013)	0.048 (0.048)			0.009 (0.018)
108		0.014 (0.000)	0.063 (0.000)	0.087 (0.107)	0.071 (0.095)	0.054 (0.071)	0.111 (0.222)	0.021 (0.024)
110	0.197 (0.212)	0.069 (0.028)	0.281 (0.313)	0.240 (0.253)	0.143 (0.143)	0.089 (0.071)	0.167 (0.111)	0.151 (0.169)
112	0.348 (0.364)	0.097 (0.056)	0.250 (0.375)	0.527 (0.493)	0.690 (0.667)	0.179 (0.179)		0.193 (0.193)
114	0.394 (0.394)	0.806 (0.917)	0.375 (0.313)	0.100 (0.107)	0.048 (0.048)	0.679 (0.679)	0.667 (0.667)	0.617 (0.584)
116	0.015 (0.000)			0.013 (0.013)			0.056 (0.000)	0.003 (0.000)
Cvit 15								
103		0.161 (0.194)						0.039 (0.039)
109				0.028 (0.022)		0.111 (0.111)	0.090 (0.091)	0.019 (0.013)
111		0.081 (0.097)				0.037 (0.037)		0.016 (0.019)
113				0.006 (0.011)		0.037 (0.037)		0.016 (0.006)
115	0.500 (0.433)	0.258 (0.194)	0.115 (0.154)	0.022 (0.022)		0.074 (0.037)		0.149 (0.169)
117	0.067 (0.067)				0.091 (0.091)			0.029 (0.019)
119	0.033 (0.033)							0.003 (0.000)
121	0.400 (0.467)	0.500 (0.516)	0.885 (0.846)	0.944 (0.944)	0.909 (0.909)	0.741 (0.778)	0.909 (0.909)	0.727 (0.734)
Cvit 22								
75					0.125 (0.156)			0.003 (0.000)
77				0.015 (0.015)			0.222 (0.222)	
83				0.341 (0.318)	0.063 (0.031)	0.060 (0.080)		0.023 (0.018)
84					0.016 (0.031)			
85	0.120 (0.207)	0.219 (0.125)	0.250 (0.286)	0.083 (0.076)	0.063 (0.063)	0.160 (0.120)		0.104 (0.107)
87	0.879 (0.793)	0.734 (0.844)	0.679 (0.643)	0.515 (0.515)	0.688 (0.688)	0.780 (0.800)	0.778 (0.778)	0.869 (0.876)
89		0.047 (0.031)	0.071 (0.071)					

Table 6 Allele frequencies of blister mite from seven populations at four polymorphic microsatellite loci. Estimates obtained from haploid data sets are given in parentheses. Rare alleles (frequency <3%) are excluded. Sample sizes given in Table 2

Locus and allele	Population						
	Alpine Valley	Eltham	Mildura	Mont Albert	Robinvale	Rutherglen	Wagga Wagga
Cvit 3							
112	0.933 (0.933)	0.640 (0.627)	1.000 (1.000)	1.000 (1.000)	0.706 (0.702)	0.788 (0.814)	0.500 (0.500)
113	0.067 (0.067)	0.360 (0.373)			0.290 (0.298)	0.136 (0.127)	0.333 (0.333)
114						0.076 (0.059)	0.167 (0.167)
Cvit 4							
123	0.143 (0.071)				0.010 (0.010)		
125	0.036 (0.071)					0.376 (0.385)	0.083 (0.167)
129					0.026 (0.010)	0.009 (0.018)	
131		0.021 (0.026)			0.097 (0.112)		
133	0.036 (0.071)	0.509 (0.513)	0.350 (0.300)	0.214 (0.143)	0.398 (0.388)	0.046 (0.055)	
135		0.017 (0.026)	0.150 (0.200)		0.041 (0.051)	0.014 (0.018)	
137	0.036 (0.000)	0.406 (0.376)	0.150 (0.100)	0.714 (0.714)		0.023 (0.018)	0.583 (0.500)
139	0.750 (0.786)			0.071 (0.143)		0.046 (0.046)	
141		0.021 (0.026)	0.250 (0.300)		0.260 (0.255)	0.069 (0.073)	
143		0.017 (0.026)	0.100 (0.100)		0.117 (0.112)	0.179 (0.156)	0.250 (0.333)
145					0.051 (0.061)	0.083 (0.073)	0.083 (0.000)
147						0.142 (0.147)	
Cvit 13							
110			0.091 (0.182)		0.015 (0.000)	0.025 (0.042)	
112	0.867 (0.800)	0.774 (0.762)	0.818 (0.818)	0.214 (0.143)	0.690 (0.679)	0.729 (0.708)	0.833 (0.833)
114	0.133 (0.200)	0.111 (0.095)	0.091 (0.000)	0.071 (0.143)	0.284 (0.313)	0.225 (0.225)	0.167 (0.167)
118		0.111 (0.135)			0.007 (0.007)		
124				0.714 (0.714)			
Cvit 15							
109	0.036 (0.071)					0.005 (0.009)	0.167 (0.167)
111	0.250 (0.214)	0.045 (0.036)			0.148 (0.160)	0.084 (0.075)	0.083 (0.167)
115	0.321 (0.286)	0.241 (0.255)	0.318 (0.364)	0.167 (0.167)	0.444 (0.456)	0.257 (0.243)	0.333 (0.333)
117		0.386 (0.391)			0.016 (0.008)	0.047 (0.047)	
119	0.393 (0.429)	0.327 (0.318)	0.682 (0.636)	0.833 (0.833)	0.380 (0.360)	0.519 (0.533)	0.417 (0.333)
125						0.065 (0.075)	

significant differentiation among blister mites sampled from these varieties ($F = 0.138$, $P<0.001$). In addition, blister mites were sampled from nine panels within three rows at Rutherglen. There was no structure among rows ($F = 0.017$, $P = 0.257$), but there was some differentiation among panels along the rows ($F = 0.089$, $P = 0.012$).

At Rutherglen and Eltham, mites were sampled from individual blisters on leaves. At Rutherglen, mites from 19 blisters were sampled from nine different leaves. Hierarchical analyses suggested that there was a substantial differentiation between leaves ($F = 0.188$, $P<0.0001$), but no differentiation among mites in blisters found within the leaves ($F = 0.027$, $P = 0.320$). A direct examination of the genotypes of mites from individual blisters also revealed the presence of more than four alleles in some cases, indicating that mites from within blisters originated from more than two parents. In the population of Eltham, mites from 19 blisters were sampled from two leaves. In contrast to the Rutherglen samples, we found no differentiation between leaves ($F = 0.007$, $P = 0.246$), but substantial structure among blisters in leaves ($F = 0.098$, $P<0.0001$). In Eltham, no more than four alleles were present within groups of mites in blisters, although the polymorphism of the loci in Eltham was lower than in Rutherglen, reducing our power to detect multiple lineages.

Discussion

RFLP analysis of the ITS 1 region indicates that complete reproductive isolation exists between the rust mite and the morphologically similar bud and blister mites (*Colomerus* mites), which is apparent despite the large geographical distance between collection sites. This confirms the classification of the rust mite as a separate species, which was previously based on morphological differences (Buchanan & Amos, 1992). Bud and blister mites are currently classified as the same species with behavioural differences used to discriminate between them, as they are morphologically indistinguishable (Smith & Stafford, 1948). Our data suggest that this classification is incorrect, as it appears likely that the bud mite and blister mite 'strains' may represent two distinct but closely related species. This is strongly evident, despite not collecting *Colomerus* mites in sympatry. It is unclear why these mites were not found in the same vineyards, but sympatric populations may have gone unnoticed due to either small localized infestations, particularly in the bud mite, or a result of damage occurring at different times of year. Finally, the use of PCR-RFLP of the ITS 1 region could be routinely used as a rapid diagnostic tool for confirming the species of mite present in a vineyard.

Although the PCR-RFLP of the ITS 1 region proved useful for species discrimination, it lacked the resolution to determine potential modes of reproduction, the level of genetic diversity that exists in bud and blister mites, or the relationship of infestations of these mites within and between vineyards. We used microsatellite markers to investigate these questions.

The main reproductive mode described for the eriophyoid mites is haplodiploidy (arrhenotoky). However, these descriptions are based on a limited number of species, and with the diversity of reproductive modes exhibited by mites, other modes of reproduction may exist in this family (Wrensch & Ebbert, 1993; Lindquist et al., 1996). It is therefore difficult to interpret the large excess of homozygous individuals in both bud and blister mite populations without family based studies to confirm likely reproductive modes. Homozygote excesses in multiple loci can be attributed to null alleles, inbreeding effects, or haploid males (Hartl & Clark, 1997). High frequency null alleles are unlikely in this study, as homozygous excesses are present at all loci, a pattern that usually reflects genome-wide, rather than locus-specific, evolutionary processes. An analysis of individuals that were heterozygous at more than one locus revealed that there was still a homozygous excess, suggesting that the observed homozygous excess in the entire data set could not be attributed to haploid individuals alone. However, inbreeding and haplodiploidy are often closely associated in mites (Wrensch & Ebbert, 1993), so whether inbreeding alone or inbreeding with haplodiploid reproduction is occurring remains unclear. The lack of linkage disequilibrium in bud mite populations does indicate that this species is probably reproducing sexually. However unlike bud mite, strong linkage was observed in some blister mite populations. This disequilibrium was largely attributed to the Rutherglen and Robinvale populations, indicating either the existence of a cryptic population structure or parthenogenetic reproduction.

Various patterns of genetic structure were observed in *Colomerus* mites. Strong differentiation was observed among populations in both bud and blister mites. This suggests a restricted movement of mites over large areas. Although bud mite populations were differentiated, there was no significant differentiation in bud mites from different states, which may reflect an initial infestation from a common source. Bud mites from different varieties, canes, or buds were not differentiated, suggesting some movement of the mites within a vineyard. Bud mites from individual buds possessed as many as six alleles, suggesting the presence of offspring from several parents within a bud.

The Rutherglen data suggest that blister mites are not randomly distributed across vineyards. These mites were collected early in the growing season when the infestation first appeared, and it would be worthwhile sampling the same vines throughout the season and across different years. Genetic differentiation was also observed at finer levels, either at the level of blisters (Eltham) or leaves (Rutherglen). These patterns may reflect the colonization and subsequent reproduction of mite populations in different leaves or different parts of the leaf surface.

Because blister mites and bud mites from different vineyards and even different parts of the vineyard are genetically distinct, the mites are probably not widely dispersed, and outbreaks may possibly result from localized overwintering populations or perhaps the transfer of grapevine material. This result is in contrast to rust mites, where wind is known to play a major role in dispersal (Duffner et al., 2001).

We have shown that the different types of eriophyoid mites are separate species. Thus, they should be treated as separate control problems, and infestations of vineyards with blister mite will not result in outbreaks of bud mite. Given that genetic differentiation occurs on a local scale, there may be a limited movement of mites within a vineyard, and localized control strategies could be sufficient. Finally, to fully decipher the life history of these mites, family studies are still required.

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