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Hybrid origin of a parthenogenetic genus?

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Abstract

The origin of the obligate-parthenogenetic New Zealand stick insect genus *Acanthoxyla* was investigated using cytogenetics and sequencing of nuclear and mitochondrial DNA. Little mitochondrial DNA sequence variation (COI-II) was found among seven species of the genus *Acanthoxyla* and we found no evidence for monophyly of the morphologically distinguished lineages. In contrast, two distinct clades of nuclear sequence (ITS) were obtained, one is restricted to the genus *Acanthoxyla*, while the other includes sequences obtained from its sister genus *Clitarchus*. Although *Acanthoxyla* appears to be diploid (2n = 36–38), it has two ill-matched chromosome pairs. We hypothesize that two or more hybridization events involving the parental sexual species *Clitarchus hookeri* and an unknown taxon probably resulted in the formation of the parthenogenetic genus *Acanthoxyla*. However, the karyotype of *Acanthoxyla* bears little resemblance to the karyotype of the putative paternal species *C. hookeri* so the exact nature of *Acanthoxyla* remains in question.

Keywords: *Acanthoxyla*, hybrid origin, hybridization, parthenogenesis, phasmid, speciation

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Introduction

Speciation can be characterized as a cessation of gene flow between populations and is commonly summarized phylogenetically as a splitting of lineages. On the other hand, hybridization between species can coalesce very divergent genomes, create new species, and yield a reticulate evolutionary pattern. The frequency at which hybrid species are formed varies among groups and with the degree of similarity between the parental participants, but is frequently aided by a switch to asexual reproduction.

Hybridization is relatively common in plants, and molecular genetic data have shown that plant hybrids are often components of polyploid complexes and frequently have multiple origins (Trewick *et al*. 2002; Cronn & Wendel 2004; Soltis *et al*. 2004). Hybridization is apparently a less common mediatory step in animal speciation (Dowling & Secor 1997), but here too, examples exist in a wide range of groups (e.g. reptiles, Kizirian & Cole 1999; corals, McFadden & Hutchinson 2004). Parthenogenesis has also been documented across a wide array of eukaryote groups (Judson & Normark 1996; Lushai *et al*. 2003) often associated with the formation of hybrid species. In some instances multiple origins of parthenogens are inferred among closely related species (nematodes, Hugall *et al*. 1999; snails, Johnson & Bragg 1999; aphids, Delmotte *et al*. 2001).

Among animals, hybrid speciation and parthenogenesis both appear to be unusually common phenomena in stick insects (phasmids) (Bullini 1994). Although many hybrid species are also parthenogens this is not always the case, and parthenogenetic populations and species often arise without hybridization. The prevalence of parthenogenesis in stick insects may be due to a pre-adaptation of the egg pro-nucleus that has allowed repeated loss of the usual sperm contribution (Scali *et al*. 2003). The relative ease with which parthenogenesis arises in stick insects may explain the high frequency of hybrid speciation in the group as it provides a mechanism by which composite genomes can reproduce themselves.

In the North American stick insect genus *Timema*, independent evolution of parthenogenesis has given rise to five lineages (Law & Crespi 2002). In Europe, repeated interspecific hybidization of bisexual stick insects of the genus *Bacillus* has resulted in lineages that reproduce via either parthenogenesis, hybridogenesis or androgenesis (Scali *et al*. 2003). In New Zealand there are parthenogenetic populations of otherwise sexual species of *Clitarchus* and *Argosarchus* (Salmon 1991). However, unusual among animals (including stick insects), the New Zealand stick insect

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genus, *Acanthoxyla* is entirely parthenogenetic. It comprises eight species (Jewell & Brock 2002) with no males and no closely related bisexual species. Furthermore, the eight *Acanthoxyla* species exhibit a degree of morphological diversity not evident in any other New Zealand stick insect genus. It is not known whether *Acanthoxyla* is automictic or apomictic and the issue of species concepts in an apparently single parthenogenetic lineage has not been addressed.

We explored *Acanthoxyla* using karyology and nuclear and mitochondrial DNA sequence data in order to identify the origin of this morphological diversity. It is possible that the eight morphological species of this asexual genus arose via (i) a series of sexual species all of which have become extinct, (ii) asexual evolution since the lineage (*Acanthoxyla*) became parthenogenetic, or (iii) eight independent hybridization events producing hybrid parthenogens. Of course, a combination of these explanations is possible. Differentiating between these hypotheses might be problematical using just phylogenetics as all three scenarios could result in monophyly of each *Acanthoxyla* species. However, if the parental taxa are extant, a hybrid origin would result in nuclear alleles from the hybrid forming clades with both parental taxa. Even when neither parent species is sampled a case for hybrid origin can be made if alleles within one parthenogenetic lineage fail to form monophyletic clades (Hugall *et al*. 1999). In addition, the number of chromosomes and their degree of homology has been important in characterizing hybrid stick insect species (Bullini 1994). Some hybrid phasmids are triploid and some are diploid with poorly matching chromosome pairs.

Materials and methods

Taxonomy

Nine genera in two subfamilies (Phasmatinae and Pachymorphinae) make up New Zealand's stick insect fauna. We focus on the genus *Acanthoxyla* (Phasmatinae) and survey the two other genera of the Phasmatinae that have common species: two species of both *Clitarchus* (*Clitarchus hookeri* and *Clitarchus tuberculatus*) and *Argosarchus* (*Argosarchus horridus* and *Argosarchus spiniger*). Female *C. hookeri* and *Acanthoxyla inermis* are morphologically similar and members of these genera are often found in the same habitat. In contrast, *Argosarchus* spp. are much bigger, morphologically dissimilar and rarely found on the same tree species as *Acanthoxyla*. The only other member of the New Zealand Phasmatinae is the monotypic genus *Pseudoclitarchus*. *Pseudoclitarchus sentus* is a sexual species restricted to a single, small, offshore island at the northern extremity of New Zealand where it is protected and not available for study.

During the course of this study a possible hybrid origin for *Acanthoxyla* was inferred so we extended our search for potential maternal taxa by sequencing additional species. The New Zealand phasmid species are all endemic and geographically isolated from their nearest neighbours (Australia and New Caledonia) by 1200 km of sea. The Australian phasmid fauna is regarded as monophyletic (Bradler 2001) and preliminary phylogenetic analysis suggests the New Zealand fauna, too, is monophyletic (Trewick unpublished data). We sequenced mtDNA from five New Zealand pachymorphinid species, representing all the New Zealand genera in this subfamily (*Micrarchus hystriculeus*, *Tectarchus huttoni*, *Niveaphasma annulata*, *Asteliaphasma jucunda*, and *Spinotectarchus acornutus*), seeking near relatives of *Acanthoxyla*. Three of these genera are monotypic and this study includes 73% (16/22) of the described New Zealand phasmid species.

Sampling

We collected stick insects throughout New Zealand, focusing on the lower North Island (Table 1, Fig. 1). With one exception, animals that were not adult when collected were raised in captivity until they were, to aid identification. We required a broad representation of *Acanthoxyla* species for our analysis; these species are diverse, ranging in length from 72 to 110 mm, coloured grey, brown or green, and equipped to varying degrees with spines and/or foliaceous lobes. Species identification was based on number, size and distribution of spines on the head, thorax and abdomen and size and shape of a ventral spine near the subgenital plate (operculum; Hutton 1899; Salmon 1991). Sampling encompassed the geographical range of *Acanthoxyla*, and we sought *Acanthoxyla* and *Clitarchus* at the same locations, as well as collecting from locations where only one taxon occurs. In several instances three *Acanthoxyla* species were collected from a single plant. Although Salmon (1991) reported captive *C. hookeri* males attempting to mate with females of *Acanthoxyla* and other taxa, this has not been observed in the wild. And, despite extensive collecting and captive culturing, males of *Acanthoxyla* have never been seen (Salmon 1991; personal observation). In contrast, adult *C. hookeri* in sexual populations are frequently found in copula.

DNA extraction, amplification and sequencing

Muscle tissue from fresh, frozen or alcohol-preserved specimens was removed from a leg for genomic DNA extraction using a salting-out method (Sunnucks & Hale 1996). Tissue was macerated and incubated with 5 μL of 10 mg/mL proteinase K in 600 μL of TNES buffer (20 mM EDTA, 50 mM Tris, 400 mM NaCl, 0.5% SDS) at 50 °C for 1–4 h, 10% 5 M NaCl was added and the extractions shaken vigorously for 20 s followed by spinning at 16 000 *g* for 5 min. The supernatant was removed and precipitated with an equal volume of cold 100% ethanol. DNA was

Table 1 Summary of locations, identification and sequence variation in New Zealand stick insects studied, animals used for cytogenetics are indicated (#), ITS clades were diagnosed using phylogenetic analysis of the full sequence except those in italics where only fragment length was used

Genus	Species	Code	Location	Colour	ITS1-2 Clade	ITS1-2 Sequence	COI-COII Haplotype
Acanthoxyla	*geisovii*	Dun-1	Dunedin	green	I	e	H
Acanthoxyla	*geisovii*	Ax.Mak-142	Levin	green	I	h	B
Acanthoxyla	*geisovii*	Ap.Khd-10	Wellington	green	I	a	C
Acanthoxyla	*geisovii*	Ap.Pm-40	Wellington	green	I	a	
Acanthoxyla	*geisovii*	Ap.Pm-41	Wellington	green	I	a	
Acanthoxyla	*geisovii/huttoni*	Ap.Khd-12	Wellington	juvenile			C
Acanthoxyla	nr *geisovii*	Ap.PN-16	Palmerston North	green	II	i	N
Acanthoxyla	nr *geisovii*	Khd-3	Wellington	brown	I	b	C
Acanthoxyla	nr *geisovii*	Ap.Otr-31	Wellington	brown	I	e	B
Acanthoxyla	*huttoni*	Ap.Otr-30	Wellington	green	I	a	D
Acanthoxyla	*inermis*	Ax.Bid-105	Bideford	brown	II	i	A
Acanthoxyla	*inermis*	Ax.Bid-145	Bideford	green			M
Acanthoxyla	*inermis*	Ax.Omo-110	Omori	green	II	i	B
Acanthoxyla	*inermis*	Ax.Omo-109	Omori	green	II	i	
Acanthoxyla	*inermis*	Ap.PN-17	Palmerston North	brown	II	i	A
Acanthoxyla	*inermis*	PN Ac-1 #	Palmerston North	brown	I & II	c & i	K
Acanthoxyla	*inermis*	Khd-15	Wellington	green	II	i	
Acanthoxyla	*inermis*	Ap.Rn-1	Wellington	green	II	i	A
Acanthoxyla	*intermedia*	Ax.Bid-104	Bideford	green	I	d	M
Acanthoxyla	*intermedia*	Ax.Otk-146	Otaki	green	I	e	F
Acanthoxyla	*intermedia*	Ap-PN-2 #	Palmerston North	brown	I	g	J
Acanthoxyla	*intermedia*	Ap.Khd-11	Wellington	brown	I	d	E
Acanthoxyla	*intermedia*	Ap.Khd-75	Wellington	brown			F
Acanthoxyla	*prasina*	Ap.Opa-103	Auckland	green	I & II	c & i	A
Acanthoxyla	*prasina*	Ax.Opa-136	Auckland	green	I	f	L
Acanthoxyla	*prasina*	Ap.Bal-32	Balance	green	I & II	c & i	B
Acanthoxyla	*prasina*	Ap.Bal-1	Balance	green	I & II	c & i	B
Acanthoxyla	*prasina*	Stf-3	Bream Bay	green	I & II	e & t	G
Acanthoxyla	*prasina*	Ax.Dun-148	Dunedin	green	II	i	B
Acanthoxyla	*prasina*	Ap.Dun-47	Dunedin	brown	I	e	I
Acanthoxyla	*prasina*	Ax.Him-138	Himatangi	fawn/green	II	k	B
Acanthoxyla	*prasina*	Ax.Him-139	Himatangi	fawn/green			B
Acanthoxyla	*prasina*	Ap.Otr-29	Wellington	green	I	e	B
Acanthoxyla	*speciosa*	Ap.Bid-86	Bideford	grey	I	d	M
Acanthoxyla	*speciosa*	Ap.Khd-14	Wellington	grey	I	e	F
Acanthoxyla	*suteri*	Ap.Opa-102	Auckland	brown	*I*		B
Acanthoxyla	*suteri*	Ax.Opa-137	Auckland	green	I	f	L
Clitarchus	*hookeri*	Ch.How-66	Auckland	green	*II*		2
Clitarchus	*hookeri*	Ch.Opa-67	Auckland	green	II	q	2
Clitarchus	*hookeri*	Arw-3	Awaroa	brown	II	s	2
Clitarchus	*hookeri*	Ch.Bal-141	Balance	green	II	r	2
Clitarchus	*hookeri*	Ch.Bid-144	Bideford	green		r	2
Clitarchus	*hookeri*	Ch.Bid-20	Bideford	brown	II	r	2
Clitarchus	*hookeri*	HhH-2	Houhora Heads	brown	II	l	2
Clitarchus	*hookeri*	Ch.Cam-77	Karapiro	green			2
Clitarchus	*hookeri*	Ch.GB-34	Katherine Bay	green	II	o	
Clitarchus	*hookeri*	Ch.GB-35	Katherine Bay	green	II	m	2
Clitarchus	*hookeri*	Ch.GB-36	Katherine Bay	brown	II	p	
Clitarchus	*hookeri*	Ch.GB-37	Katherine Bay	brown	II	n	2
Clitarchus	*hookeri*	Ch.Ot-2 #	Otaki	brown			2
Clitarchus	*hookeri*	Ot.Ch-1	Otaki	brown	II	i	
Clitarchus	*hookeri*	Cth.Wn-43	Wellington	brown	*II*		2
Clitarchus	*hookeri*	Cth.Wn-44	Wellington	brown	*II*		2
Clitarchus	*hookeri*	Ch.Khd-61	Wellington	brown	II	i	2

Table 1 *Continued*

Genus	Species	Code	Location	Colour	ITS1-2 Clade	ITS1-2 Sequence	COI-COII Haplotype
Clitarchus	*hookeri*	Ch.Rn-62	Wellington	green	II	I	2
Clitarchus	*hookeri*	Ch.Wil.19	Wellington	brown	II	i	2
Clitarchus	*hookeri*	Ch.wby-45	Wellington	green	II	j	2
Clitarchus	*tuberculatus*	Arw-1	Awaroa	brwn	II	s	2
Clitarchus	*tuberculatus*	Ct.Bid-60	Bideford	brown	II	r	2
Argosarchus	*horridus*	Arg.Bal-111	Balance	grey			3
Argosarchus	*horridus*	Arg.Khd-64	Wellington	grey	III	*	3
Argosarchus	*spiniger*	Arg.Bal112	Balance	grey			3
Argosarchus	*spiniger*	Arg.Bal.33	Balance	grey	III	*	
Argosarchus	*spiniger*	Arg.PN.18	Palmerston North	grey	III	*	
Argosarchus	*spiniger*	Arg.Khd-65	Wellington	grey	III	*	3

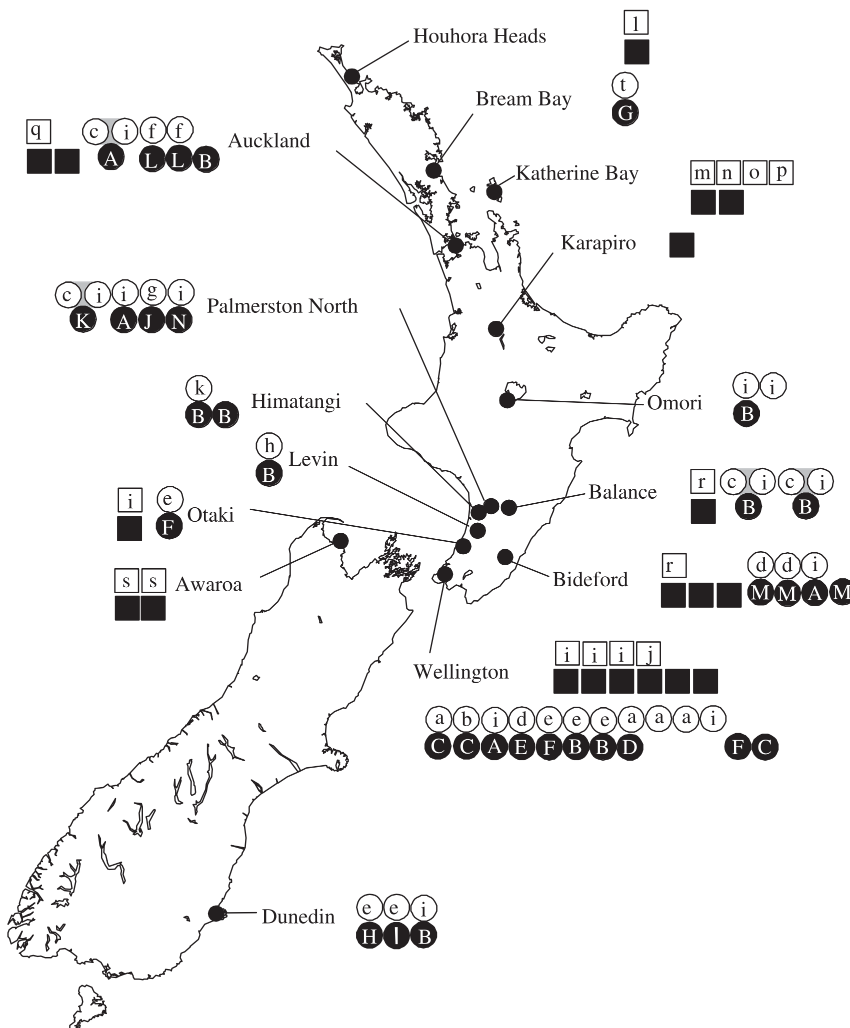

Fig. 1 Sampling locations of stick insects in New Zealand with distribution of sequence variants from COI-II (filled shapes, upper-case lettering), ITS1-2 (open shapes, lower-case lettering), *Acanthoxyla* (circles), *Clitarchus* (squares).

collected by spinning, washed with 70% ethanol, dried and dissolved in water.

We amplified and sequenced mitochondrial and nuclear DNA. The mitochondrial fragment, comprising the 3′ end of the cytochrome oxidase I (COI), tRNA-Leucine, and cytochrome oxidase II (COII), was amplified using the primers C1-J-2195 and TK-N3785 (Simon *et al*. 1994). Nuclear sequences were from the internal transcribed

spacers (ITS1 and ITS2) of the rRNA cluster and the intervening 5.8S, using the primers ITS4 and ITS5 (White *et al*. 1990). However, ITS PCR from five *Acanthoxyla* DNA templates products of two sizes (nominated slow and fast). We designed a primer that binds near the 18S/ITS1 junction within an insertion in the slow ITS sequence (STITS5F – GCCTCCCTGCTTGGGTTTCCG). Amplification with primers ITS4 and STITS5F with DNA templates that had given two products yielded just one of the expected size. A combination of three primers enabled us to sequence both classes of ITS from such templates.

Polymerase chain reaction (PCR) used standard conditions (Trewick *et al*. 2000). Amplification products were treated to Shrimp Alkaline Phophotase/Exonuclease I digestion. Cycle sequencing with the PCR primers used BigDye chemistry (PE) following the manufacturer's protocols, with automated reading on an ABI3730. Consensus sequences were obtained using SEQUENCHER version 4.1 (ABI, PE), and aligned using SEAL version 2.0a3 (Rambaut 1996).

Sequence analysis

Nucleotide diversity was estimated and evidence of isolation by distance was sought using a matrix correlation analysis of pairwise genetic (based on COI-II) vs. linear geographical distances for the *Acanthoxyla* ($n = 33$) and *Clitarchus* ($n = 19$) individuals, separately. The Mantel test implemented by ARLEQUIN version 2.0 (Schneider *et al*. 2000) used 1000 permutations to test for significant correlations. We searched for evidence of gene conversion in ITS using GENECONV version 1.81 (Sawyer 1999), a method that utilizes information from indels in addition to nucleotide sequence and performed well in a comparison of methods (Posada 2002). The global permutation P values < 0.05 (based on BLAST-like global scores with 10 000 replicates) were considered as evidence of gene conversion (or recombination). A multiple comparison correction is built into these P values. We used PAUP *4.0b10 (Swofford 2002) to calculate genetic distances, and to implement neighbour-joining (NJ) and maximum-parsimony (MP) methods for phylogenetic analyses. Insertions and deletions (indels) of DNA sequence from ITS1 and ITS2 were not used as characters for phylogenetic analyses of the full data set but were included in the minimum-spanning networks.

Cytogenetics

Cytogenetic methods were those applied successfully to orthoptera (Morgan-Richards 1997). Stick insects were injected with colchicine 16 h before killing them. Ovarian follicles were given a hypotonic treatment then fixed in fresh 3:1 methanol : acetic acid. Chromosome slides were air dried and stained with 8% Giemsa's stain (Gurr®) in Sorensen's buffer (pH 6.8; Gurr®). Chromosome spreads were photographed using an Olympus microscope under 1000× magnification with images manipulated using MAGNIFIRE and IMAGEPRO-PLUS.

Results

Mitochondrial cytochrome oxidase

We aligned COI-tRNALeucine-COII (COI-II) sequences of 1448 bp including the 67 bp tRNA Leucine. tRNA Leucine was identified by comparison with published sequences and confirmation of secondary structure using DOGMA (Wyman *et al*. 2004), and excluded from analyses. COI-II sequences were translated to amino acids to check for stop codons and shifts in reading frame that might indicate the presence of nuclear mitochondrial copies. No evidence of such copies was found. Reference sequences have been deposited at GenBank [Accession nos AY940428–AY940431 (COI-II) and AY943645–AY943648 (ITS)]. Observed genetic distances reached 0.022 among 14 distinct *Acanthoxyla* haplotypes ($n = 33$), and 0.026 among 15 distinct *Clitarchus* haplotypes ($n = 19$). Average genetic distances [Kimura 2-parameter correction (K2P)] between haplotypes from the three Phasmatinae genera were approximately 10% (0.098, 0.109, 0.113; Table 2). Comparisons among taxa including

Table 2 Average pairwise genetic distances (K2P) between eight lineages (from eight genera) of New Zealand stick insects based on 1448 bp of COI-II (mtDNA)

	Acan.	*Clit.*	*Argo.*	*Tech.*	*Micr.*	*Nive.*	*Spin.*	*Astel.*
Acanthoxyla spp.								
Clitarchus spp.	0.109							
Argosarchus spp.	0.098	0.113						
Tectarchus huttoni	0.111	0.116	0.140					
Micrarchus hystericuleus	0.133	0.135	0.147	0.140				
Niveaphasma annulata	0.105	0.108	0.112	0.102	0.140			
Spinotechtarchus acornatus	0.138	0.141	0.155	0.147	0.160	0.157		
Asteliaphasma jacunda	0.114	0.122	0.132	0.134	0.163	0.120	0.148	

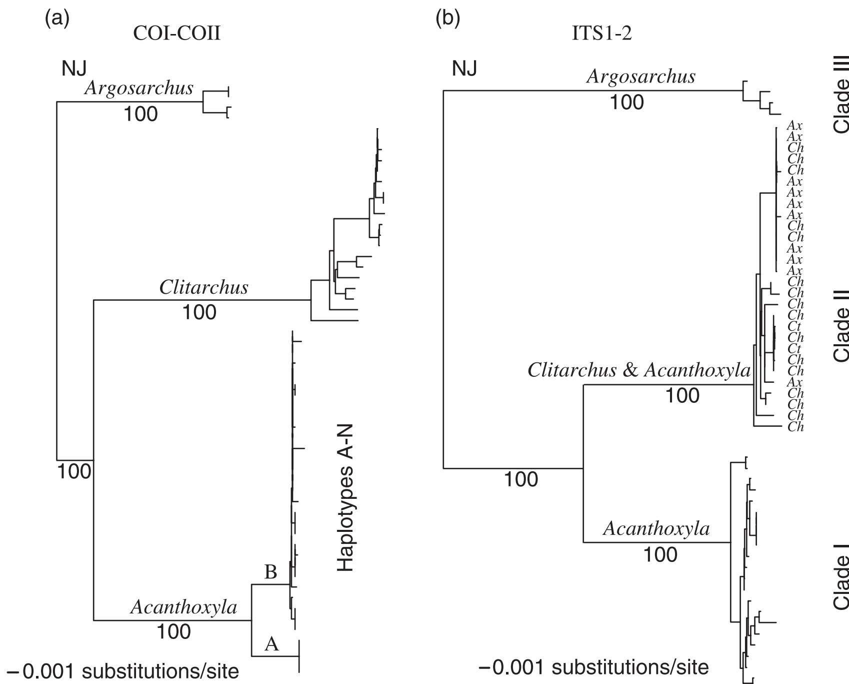

Fig. 2 Neighbour-joining trees for three genera of New Zealand stick insects from (a) COI-II and (b) ITS1-2 DNA sequences, with results of 1000 MP bootstrap replicates.

the five Pachymorphinae genera (*Micrarchus*, *Tectarchus*, *Niveaphasma*, *Asteliaphasma*, *Spinotectarchus*) were similar; all genetic distances were 10% or more (range 0.102–0.160; Table 2). A putative maternal species was not identified and the five New Zealand Pachymorphinae species were therefore excluded from further analyses. All analyses found high statistical support for the reciprocal monophyly of the three Phasmatinae genera (100% bootstrap support with NJ and MP; Fig. 2).

A minimum-spanning network for the 14 *Acanthoxyla* COI-II haplotypes (A to N) was constructed using the 46 variable sites, with a single backmutation inferred (Fig. 3a). This revealed two lineages consisting of haplotype A alone ($n = 4$), and the remainder, haplotypes B–N ($n = 29$), 23 steps apart. Average nucleotide diversity among all *Acanthoxyla* haplotypes (A to N) was low (0.0054 ± 0.0029). The most common haplotype (B) was found in 10 *Acanthoxyla* individuals (*A. inermis*, *A. geisovii*, *A.* nr *geisovii*, *A. prasina*, *A. suteri*). Twelve other haplotypes (C–N) differed from B by 1–3 single nucleotide polymorphisms (SNPs) and were found distributed among seven species (Table 1, Fig. 3). Haploype A was found in *A. inermis* ($n = 3$) and *A. prasina* ($n = 1$). We found little concordance between haplotype, species and location (Table 1). Four haplotypes were found at more than one location, for example, B was found at five locations up to 800 km apart. Where haplotypes were unique to a location they often occurred in a number of species, for example, haplotype M was found in three *Acanthoxyla* species at Bideford. No evidence of isolation by distance was detected in *Acanthoxyla*.

Observed nucleotide diversity within *Clitarchus* ($n = 19$) (*C. hookeri* and *C. tuberculatus* combined as they are not reciprocally monophyletic; Fig. 2) was higher (0.011 ± 0.006) than that found in the larger sample of *Acanthoxyla* (Student's *t*-test, $P < 0.001$). Mantel tests revealed a significant correlation between genetic and geographical distance in *Clitarchus* ($P < 0.001$).

Internal transcribed spacer

ITS PCR products from New Zealand Phasmatinae using primers ITS4 and ITS5 were of three sizes, as revealed by their rate of migration under agarose electrophoresis. Five *Acanthoxyla*, but no samples of *Clitarchus* nor *Argosarchus*, yielded two PCR products that differed in length by approximately 100 bp. The use of a specifically designed primer enabled us to get near-complete DNA sequences of both the slow and fast running ITS1-2 products from polymorphic individuals of *Acanthoxyla*.

Approximate lengths of unaligned ITS1 and ITS2 sequences (i.e. without gaps) from Phasmatinae were *Acanthoxyla* (slow) 858 bp and 516 bp, *Clitarchus* (fast) 757 bp and 518 bp, and *Argosarchus* 914 bp and 617 bp, respectively. The position and length (160 bp) of the *5.8S* gene was determined by comparison with published DNA sequences. ITS1-2 length polymorphism reflected the presence of a small number of large indels: a 142-bp indel in ITS2 distinguishing *Argosarchus* from *Acanthoxyla* and *Clitarchus*, and 92-bp, 70-bp, and 61-bp indels in ITS1 distinguishing *Acanthoxyla* and *Clitarchus*. These indels were not included in the phylogenetic analyses (Fig. 2).

Fig. 3 Minimum-spanning networks for (a) *Acanthoxyla* mitochondrial haplotypes using COI-II sequence (filled shapes, upper-case lettering), (b) *Acanthoxyla* nuclear sequences from ITS1-2 clade I, and (c) *Acanthoxyla* (circles), and *Clitarchus* (squares), nuclear sequences from ITS1-2 clade II. ITS1-2 (open shapes, lower-case lettering). Black bars on edges indicate character state changes, thick bar indicates probably backmutation.

Analysis with GENECONV found no evidence of gene conversion or recombination among 21 unique ITS1-2 DNA sequences (10 *Acanthoxyla* sequences, 9 *Clitarchus*, 1 found in both *Acanthoxyla* and *Clitarchus* and 1 *Argosarchus*).

Phylogenetic analysis of ITS1-2 sequences revealed a similar pattern of relationships between Pachymorphinae (outgroup, not shown) and Phasmatinae, and within Phasmatinae, to that identified by analysis of COI-II, but with one prominent difference. While clade I consisted only of ITS1-2 sequences from *Acanthoxyla*, clade II comprised all sequences from *Clitarchus* and some sequences from *Acanthoxyla* (Fig. 2). Indeed, some *Acanthoxyla* and *Clitarchus* individuals had identical clade II sequences.

We constructed separate minimum-spanning networks for clade I and II sequence variants; both lack homoplasy. Clade I comprised eight unique ITS1-2 sequences (a–h) that were variable at just 15 sites (Fig. 3b). Twelve unique clade II sequences (i–t) were variable at 37 sites including five single nucleotide indels and one 6-bp indel (Fig. 3c). Within clade II are three sequences (i, k, t) found in 14 *Acanthoxyla* individuals (Table 1). Twelve *Acanthoxyla* and four *Clitarchus* had sequence i, which forms a clade with sequences j and k that differs from all other clade II sequences by at least 10 SNPs and indels. *Acanthoxyla* sequence t is most similar to r from *Clitarchus* ($n = 4$; Fig. 3c). Five *Acanthoxyla* individuals had ITS sequences in both clade I and II. Of the four *Acanthoxyla* individuals with mitochondrial haplotype A, one had both ITS clade sequences and three had just clade II sequences. The *A. prasina* from Bream Bay, the only stick insect with ITS sequence t, also had ITS sequence e and mitochondrial haplotype G (Table 1).

Cytogenetics

Chromosome counts of mitotic cells show *Clitarchus* and *Acanthoxyla* to be similar; *C. hookeri* 2n = 36, *A. inermis* 2n = 36, *A. intermedia* 2n = 38. These chromosome counts are identical to those found in other New Zealand stick insects (Parfitt 1980). The size of the chromosomes cannot be directly compared due to variation in chromosome constriction among mitotic cells (within and among individuals), but a comparison of gross morphology is informative. Of the four largest chromosome pairs of *Clitarchus* only one pair has a centromere near the middle (metacentric pair 3; Fig. 4a). The largest *Clitarchus* chromosome pair is submetacentric and was identified as the sex chromosomes (males XO; Parfitt 1980). In contrast, all four of the largest pairs of chromosomes of *Acanthoxyla* are metacentric. The karyotype of *Acanthoxyla* can be paired, as expected of a diploid, with the exception of two of the eight largest pairs, which are consistently heterozygous in shape (pairs 3 and 6; Fig. 4b) and may not in fact be homologous chromosomes. The variation in number of chromosomes between *A. inermis* and *A. intermedia* was due to the inclusion/exclusion of an extra pair of small autosomes. Otherwise, in the shape of their other chromosomes and lack of homology of pairs 3 and 6, these *Acanthoxyla* species were identical using plain stained chromosome preparations.

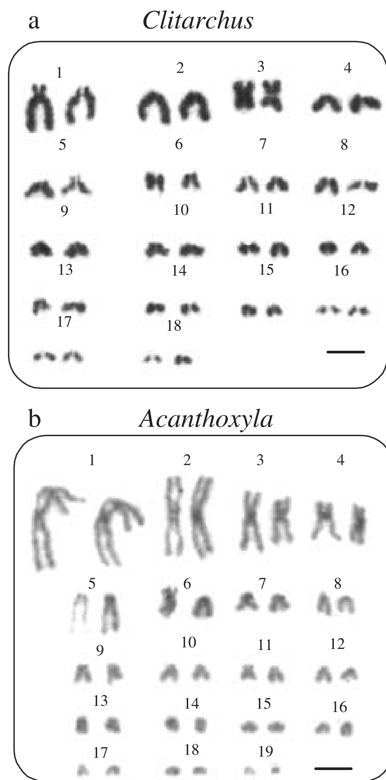

Fig. 4 Karyotypes of representative (a) *Clitarchus hookeri* and (b) *Acanthoxyla inermis*, scale bars = 5 μm.

Discussion

Cytochrome oxidase genes are relatively rapidly evolving regions of insect mitochondria, and have been widely used in intraspecific studies of insects (Caterino *et al*. 2000). The COI-II sequence diversity of the seven species of *Acanthoxyla* in our study is extremely low. Given the extent of morphological and taxonomic diversity surveyed, the minimal sequence evolution is unusual. Because the mitochondrial genome is nonrecombining and maternally inherited we can apply methods developed for mitochondrial data sets to this parthenogenetic genus. The sequence variation as represented by the minimum-spanning network (excluding haplotype A) shows a pattern characteristic of an expanding population (Slatkin & Hudson 1991) with the central, most common haplotype (B) found from Auckland to Dunedin (a distance of approximately 800 km). In contrast to *Clitarchus hookeri*, we found no evidence of isolation by distance in our *Acanthoxyla* sample. The limited mitochondrial genetic diversity within the *Acathoxyla* clade was not concordant with morphological diversity as expressed by their taxomony. Six of the seven *Acanthoxyla* species included in this study did not have unique haplotypes, the seventh being represented by a single individual. We found the same haplotypes in widely spaced locations and morphologically distinct individuals. Conversely we also found the most divergent haplotypes in morphologically similar individuals. Therefore, we see no evidence in our mitochondrial sequence data that the species of *Acanthoxyla* are monophyletic.

There are multiple copies of the nuclear transcribed spacers (ITS) in eukaryote genomes but in most studies little variation is detected within sexual species (Álvarez & Wendel 2003). Hybrid species have previously been identified using ITS sequences (e.g. Peonies, Sang *et al*. 1995; *Arabidopsis*, O'Kane *et al*. 1996; *Meloidogyne* nematodes, Hugall *et al*. 1999; coral, McFadden & Hutchinson 2004). Because homogenization of ITS repeats can occur rapidly via concerted evolution (Hillis *et al*. 1991; Elder & Turner 1995) the presence of a single ITS sequence does not confirm the absence of reticulate evolution. However, a mixture of divergent ITS sequences in one 'species' when variants are otherwise found in distinct taxa is compelling evidence for hybridization.

Two distinct classes of ITS sequences were found in *Acanthoxyla* individuals (Fig. 2, clades I and II), whereas all *Clitarchus* ITS sequences belong to clade II. From this we infer that *Acanthoxyla* is the product of one or more hybridization events involving *Clitarchus*. The placement of *Acanthoxyla* ITS clade II sequences in two regions within the minimum-spanning network cannot be explained by recombination or gene conversion, and suggests instead either two (or more) hybridization events or a single event involving a heterozygous *Clitarchus* male. The incongruity of mitochondrial and nuclear lineages within *Acanthoxyla* could be evidence of additional hybridization events. For example, three stick insects with mtDNA haplotype A, which is 23 steps divergent from all other *Acanthoxyla* haplotypes, did not have distinctive ITS sequences with a similar degree of divergence from other *Acanthoxyla* ITS sequences. Mitochondrial haplotypes from *Acanthoxyla* formed a monophyletic clade *c*. 11% divergent from the monophyletic *Clitarchus* mitochondrial clade. From this we conclude that *Clitarchus* was the paternal parent in the hybridization yielding *Acanthoxyla*. None of the nine species of New Zealand stick insects that we sequenced for COI-II provided evidence of a putative maternal taxon for *Acanthoxyla*. Of the nine genera of endemic phasmids in New Zealand only *Pseudoclitarchus* has not been included in this study so it is a possible maternal taxon. Some parthenogenetic species of hybrid origin are thought to have competitively excluded one or both bisexual parental taxa (Bullini 1994), so an alternative possibility is that the maternal *Acanthoxyla* taxon is extinct.

Chromososome counts indicate that *Acanthoxyla inermis* and *Acanthoxyla intermedia* are diploid as four sexual New Zealand phasmid species studied also have 2n = 36 or 38 (Parfitt 1980). Furthermore, 16/18 chromosome pairs match, so the *Acanthoxyla* karyotype is surprisingly well balanced

for a putative hybrid species (Scali & Marescalchi 1987; Bullini 1994) and unlike that of the putative paternal species, *Clitarchus*. If, as the DNA sequence data indicate, some or all *Acanthoxyla* species are the product of matings between the predominantly acrocentric karyotpe of *Clitarchus*, and some other species, then substantial karyotype remodelling has occurred since that event. Rapid karyotype differentiation of this type has been described in parthenogens and in hybrids. For example, asexual ants have rapidly increased the number of repetitive sequences in their genomes (Blackman *et al*. 2000), and wallaby hybrids show retro-element activation and chromosome remodelling in a single generation (Waugh O'Neill *et al*. 1998). Extensive chromosome evolution in hybrid parthenogenetic grasshoppers since two independent hybridizations has resulted in many karyotype lineages (Webb *et al*. 1972; Honeycutt & Wilkinson 1989). The relatively large DNA content of *Acanthoxyla* cell nuclei compared to *Clitarchus* and other New Zealand stick insect species provides additional evidence and a mechanism for chromosome shape change (Parfitt 1980; unpublished).

In conclusion, one can infer from our sequence data that *Clitarchus* is the paternal species of the hybrid genus *Acanthoxyla*, but this contrasts with the karyotypes of *Acanthoxyla* which do not look like a mixture of chromosomes from *Clitarchus* and an unknown mother, as expected under a simple hybrid model. Three scenarios might explain our results:

1. The identical ITS sequences in *Clitarchus hookeri* and *Acanthoxyla* could be the result of retention of an ancestral state. Concerted evolution might have acted to stop further mutations in some lineages. *Acanthoxyla*, under this explanation, would be a nonhybrid parthenogen like the North American parthenogenetic stick insect species within the genus *Timema*. Twenty-seven percent (27%) of our sample of *Acanthoxyla* only had ITS sequences that were identical or nearly identical to *Clitarchus* ITS sequences (clade II), 58% only had ITS sequences *c*. 10% divergent (clade I), but 15% had both ITS sequences. The 'retention' of *Clitarchus*-like ITS sequences was not restricted to a particular *Acanthoxyla* mitochondrial haplotype or lineage.
2. Rare mating between female *Acanthoxyla* and male *Clitarchus* might produce new parthenogenetic lineages. This could explain the morphological diversity within *Acanthoxyla*. The genus would consist of some hybrid lines and some nonhybrid parthenogenetic *Acanthoxyla* lines as seen in the European stick insect genus *Bacillus*. For example, in our sample of 33 *Acanthoxyla*, 19 individuals (representing seven species) had only the ITS sequence from clade I and may have nonhybrid origins. However, within a single individual the sequence data and chromosomes contrast: *Acanthoxyla* with 2n = 38 (*A. inermis* PN.Ac-1) has ITS sequences from both clades I and II and an apparently diploid karyotype with little in common with the *Clitarchus* karyotype.
3. The whole genus *Acanthoxyla* may have arisen via two or more hybridizations involving the paternal species *Clitarchus hookeri*. The distribution of the two clades of ITS in *Acanthoxyla* has resulted from concerted evolution post hybridization (or PCR bias). The maternal species, which we have not sampled, may be *Pseudoclitarchus sentus*, or an extinct sexual *Acanthoxyla*. The distinctive but apparently diploid karyotype of *Acanthoxyla* must then be explained by rapid chromosome remodelling.

We favour the third hypothesis but whichever explanation is correct a number of observations are clear: the seven species of *Acanthoxyla* are poorly differentiated genetically, may not be monophyletic, and have probably undergone recent range expansion. The question of how so much morphological diversity arose so rapidly within this parthenogenetic lineage remains to be answered.

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References

Álvarez I, Wendel JF (2003) Ribosomal ITS sequences and plant phylogenetic inference. *Molecular Phylogenetics and Evolution*, **29**, 417–434.

Blackman RL, Spence JM, Normark BB (2000) High diversity of structural heterozygous karyotypes and rDNA arrays in parthenogenetic aphids of the genus *Trama* (Aphididae: Lachninae). *Heredity*, **84**, 254–260.

Bradler S (2001) The Australian stick insects — a monophyletic group within the Phasmatodea? *Zoology*, **104**, 69.

Bullini L (1994) Origin and evolution of animal hybrid species. *Trends in Ecology & Evolution*, **9**, 422–426.

Caterino MS, Cho S, Sperling FAH (2000) The current state of insect molecular systematics: a thriving Tower of Babel. *Annual Review of Entomology*, **45**, 1–54.

Cronn R, Wendel JF (2004) Cryptic trysts, genomic mergers, and plant speciation. *New Phytologist*, **161**, 133–142.

Delmotte F, Leterme N, Bonhomme J, Rispe C, Simon JC (2001) Multiple routes to asexuality in an aphid species. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **268**, 2291–2299.

Dowling TE, Secor CL (1997) The role of hybridization and introgression in the diversification of animals. *Annual Review of Ecology and Systematics*, **28**, 593–619.

Elder JF, Turner BJ (1995) Concerted evolution of repetitive DNA sequences in eukaryotes. *Quarterly Review of Biology*, **70**, 297–320.

Hillis DM, Moritz C, Porter CA, Baker RJ (1991) Evidence of biased gene conversion in concerted evolution of ribosomal DNA. *Science*, **251**, 308–310.

Honeycutt RL, Wilkinson P (1989) Electrophoretic variation in the parthenogenetic grasshopper *Warramba virgo* and its sexual relatives. *Evolution*, **43**, 1027–1044.

Hugall A, Stanton J, Moritz C (1999) Reticulate evolution and the origins of ribosomal internal transcribed spacer diversity in apomictic *Meloidogyne*. *Molecular Biology and Evolution*, **16**, 157–164.

Hutton FW (1899) Revison of the New Zealand Phasmidae. *Transactions of the New Zealand Institute*, **31**, 50–59.

Jewell T, Brock PD (2002) A review of the New Zealand stick insects: new genera and synonymy, keys, and a catalogue. *Journal of Orthoptera Research*, **11**, 189–197.

Johnson SG, Bragg E (1999) Age and polyphyletic origins of hybrid and spontaneous parthenogenetic *Campeloma* (Gastropoda: Viviparidae) from the southeastern United States. *Evolution*, **53**, 1769–1781.

Judson OP, Normark BB (1996) Ancient asexual scandals. *Trends in Ecology & Evolution*, **11**, 41–46.

Kizirian DA, Cole CJ (1999) Origin of the unisexual lizard *Gymnothalmus underwoodi* (Gymnothalmidae) inferred from mitochondrial DNA nucleotide sequences. *Molecular Phylogenetics and Evolution*, **11**, 394–400.

Law JH, Crespi BJ (2002) Recent and ancient asexuality in *Timema* walkingsticks. *Evolution*, **56**, 1711–1717.

Lushai G, Loxdale HD, Allen JA (2003) The dynamic clonal genome and its adaptive potential. *Biological Journal of the Linnean Society*, **79**, 193–208.

McFadden CS, Hutchinson MB (2004) Molecular evidence for the hybrid origin of species in the soft coral genus *Alcyonium* (Cnidaria: Anthozoa: Octocorallia). *Molecular Ecology*, **13**, 1495–1505.

Morgan-Richards M (1997) Intraspecific karyotype variation is not concordant with allozyme variation in the Auckland tree weta of New Zealand, *Hemideina thoracica* (Orthoptera: Stenopelmatidae). *Biological Journal of the Linnean Society*, **60**, 423–442.

O'Kane SL, Jr, Schaal BA, Al-Shehbaz I (1996) The origins of *Arabidopsis suecica* (Brassicaceae), as indicated by nuclear rDNA. *Systematic Botany*, **21**, 559–566.

Parfitt RG (1980) *The cytology and feulgen-DNA microdensitometry of some New Zealand stick insect species (Phasmatodea: Phasmatidae)*. MSc Thesis, Victoria University of Wellington.

Posada D (2002) Evaluation of methods for detecting recombination from DNA sequences: Empirical data. *Molecular Biology and Evolution*, **19**, 708–717.

Rambaut A (1996) *SE-AL: Sequence Alignment*. Available at http://evolve.zoo.ox.ac.uk/

Salmon JT (1991) *The Stick Insects of New Zealand*. Reed, Singapore.

Sang T, Crawford DJ, Stuessy TF (1995) Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer sequences of nuclear ribosomal DNA: implications for biogeography and concerted evolution. *Proceedings of the National Academy of Sciences of the United States of America*, **92**, 6813D6817.

Sawyer SA (1999) *GENECONV: A computer package for the statistical detection of gene conversion*. Distributed by the Author. Department of Mathematics, Washington University in St. Louis. Available at http://www.math.wustl.edu/~sawyer.

Scali V, Marescalchi O (1987) Karyology and cytotaxonomy of Phasmatodea. In: *Stick Insects: Phylogeny and Reproduction* (eds. Mazzini M, Scali V), pp. 211–222. University of Siena & University of Bologna, Italy.

Scali V, Passamonti M, Marescalchi O, Mantovani B (2003) Linkage between sexual and asexual lineages: genome evolution in *Bacillus* stick insects. *Biological Journal of the Linnean Society*, **79**, 137–150.

Schneider S, Roessli D, Excoffier L (2000) *ARLEQUIN: a software for population genetics data analysis Version 2*. Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva, Switzerland.

Simon C, Frati F, Beckenbach A, Crespi BJ, Liu H, Flook P (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America*, **87**, 651–701.

Slatkin M, Hudson RR (1991) Pairwise comparisons of mitochondrial DNA sequences in stable and exponentially growing populations. *Genetics*, **129**, 555–562.

Soltis DE, Soltis PS, Tate JA (2004) Advances in the study of polyploidy since *Plant speciation*. *New Phytologist*, **161**, 173–191.

Sunnucks P, Hale DF (1996) Numerous transposed sequences of mitochondrial cytochrome oxidase I–II in aphids of the genus *Sitobion* (Hemiptera: Aphididae). *Molcular Biology and Evolution*, **13**, 510–524.

Swofford DL (2002) *PAUP*. Phylgenetic Analysis Using Parsimony (*and Other Methods), Version 4*. Sinauer Associates, Sunderland, Massachusetts.

Trewick SA, Wallis GP, Morgan-Richards M (2000) Phylogeographical pattern correlates with Pliocene mountain building in the alpine scree weta (Orthoptera, Anostostomatidae). *Molecular Ecology*, **9**, 657–666.

Trewick SA, Morgan-Richards M, Henderson S *et al.* (2002) Polyploidy, phylogeography and Pleistocene refugia of the rockfern *Asplenium ceterach*: evidence from cpDNA. *Molecular Ecology*, **11**, 2003–2012.

Waugh O'Neill RJ, O'Neill MJ, Marshall Graves JA (1998) Undermethylation associated with retro-element activation and chromosome remodelling in an interspecific mammalian hybrid. *Nature*, **393**, 68–72.

Webb GC, White MJD, Contreras N, Cheney J (1972) Cytogenetics of the parthenogenetic grasshopper *Warramaba* (formerly *Maraba*) *virgo* and its bisexual relatives. IX. Chromosome banding studies. *Chromosoma*, **67**, 309–339.

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols, A Guide to Methods and Applications* (eds. Innes MA, Gelfand DH, Sninsky JJ, White TJ), pp. 315–322. Academic Press, San Diego, California.

Wyman SK, Jansen RK, Boore JL (2004) Automatic annotation of organellar genomes with DOGMA. *Bioinformatics*, **20**, 3252–3255.

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