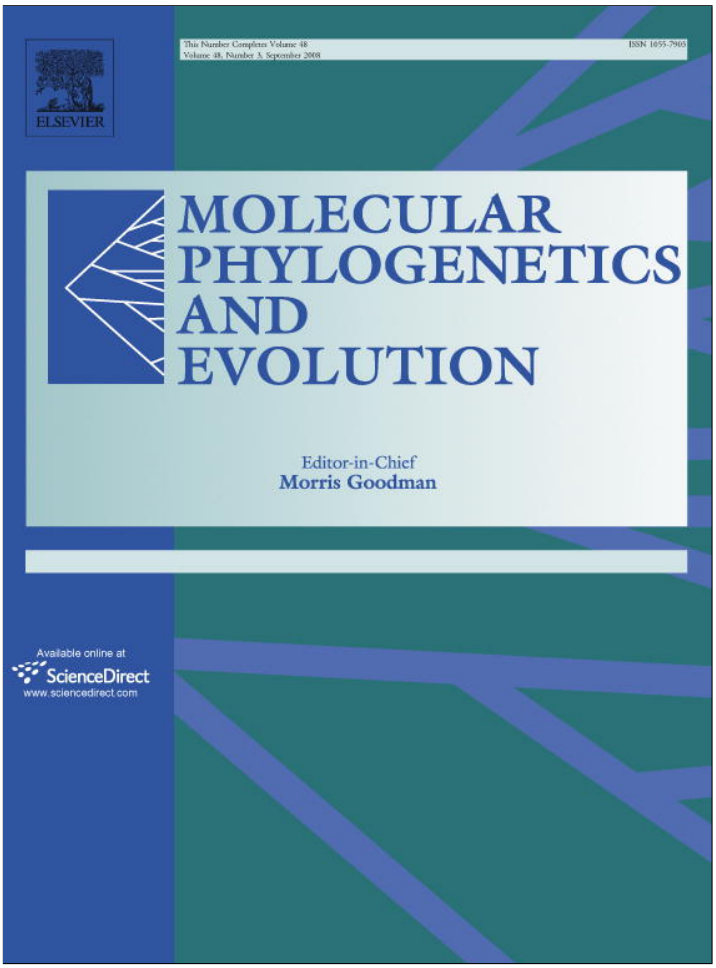

Molecular Phylogenetics and Evolution 48 (2008) 799–808

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Are you my mother? Phylogenetic analysis reveals orphan hybrid stick insect genus is part of a monophyletic New Zealand clade

Steve A. Trewick [a,*], Mary Morgan-Richards [a], Lesley J. Collins [b]

[a] Allan Wilson Centre for Molecular Ecology & Evolution, Ecology Group PN 624, Institute of Natural Resources, Massey University, Private Bag 11-222, Palmerston North, New Zealand
[b] Allan Wilson Centre for Molecular Ecology & Evolution, Institute of Molecular Biosciences, Massey University, New Zealand

ARTICLE INFO

Article history:
Received 23 February 2007
Revised 6 March 2008
Accepted 20 May 2008
Available online 27 May 2008

Keywords:
Hybrid genesis
Phasmid
Parthenogenesis
mtDNA
rDNA
BLAST search
Outgroup

ABSTRACT

The hybrid stick insect genus *Acanthoxyla* Uvarov 1944 is unusual for an obligate parthenogen, in the extreme morphological diversity it exhibits that has led to eight species being recognised. The New Zealand sexual species *Clitarchus hookeri* [White, A. 1846. The zoology of the Voyage of H.M.S. Erebus and Terror. In: 1 Insects of New Zealand. E.W. Janson, London.] is the putative parental species in the hybridization that gave rise to the hybrid lineage *Acanthoxyla*. In an effort to identify the maternal ancestor of *Acanthoxyla* we sequenced nuclear 28S rDNA and/or mtDNA COI & COII of all nine endemic New Zealand stick insect genera, representing 17 of the 22 described species. We also sequenced 28S from eight non-New Zealand stick insects to supplement published 28S sequence data that provided a taxonomically and geographically broad sampling of the phasmids. We applied a novel search algorithm (SeqSSi = Sequence Similarity Sieve) to assist in selection of outgroup taxa for phylogenetic analysis prior to alignment. Phylogenetic reconstructions resolved an exclusively New Zealand clade to which the maternal lineage of *Acanthoxyla* belonged, but did not support existing higher level taxonomy of stick insects. We did not find a sexual maternal species for *Acanthoxyla* but phylogenetic relationships indicate that this species lived in New Zealand and could be classified among the New Zealand Phasmatinae. Among the available taxa, the nearest evolutionary neighbours to the New Zealand phasmid fauna as a whole were predominantly from the New Zealand region (Fiji, Australia, New Guinea, New Caledonia and South America). As it appears to be an orphan, it is interesting to speculate that a combination of parthenogenetic reproduction and/or hybrid vigour in *Acanthoxyla* may have contributed to the extinction of its mother.

1. Introduction

Hybrid speciation is rare in animals (Coyne and Orr, 2004) because reproductive isolation from parental taxa requires a combination of traits that are uncommon. However, stick insects have such a flexible reproductive pathway that hybrid lineages have arisen many times using diverse strategies to by-pass the routine recombination and gamete fusion of sexual diploids (Bullini, 1994). The adoption of parthenogenetic reproduction is common in stick insects and especially well documented in the genera *Timema* (Law and Crespi, 2002) and *Bacillus* (Scali et al., 2003). The best-studied group from the perspective of hybrid speciation are the Sicilian *Bacillus* complex of stick insects where a diverse range of reproductive mechanisms is implicated in the emergence and maintenance of hybrid species (Scali et al., 2003). Asexual species of the North American genus *Timema* have arisen without hybridisation, and some show (and some do not) colour and host differentiation from their sexual ancestors (Crespi and Sandoval, 2000). However, in *Bacillus* where asexual species have arisen with and without hybridisation there is little ecological or morphological variations among parthenogentic species and their sexual ancestors. The situation among a genus of New Zealand stick insects is strikingly different (Morgan-Richards and Trewick, 2005).

The genus *Acanthoxyla* Uvarov 1944 is the most speciose of the nine endemic New Zealand stick insect genera (Jewell and Brock, 2002). All eight species of *Acanthoxyla* are obligate parthenogens, entirely lacking males but with females routinely producing "fertile" eggs. The extent of morphological diversity within *Acanthoxyla* is unusual and includes variation in colour, the number and size of spines and development of abdominal flanges. These morphologically distinct parthenogenetic lineages appear to share a common origin, whereas other hybrid animal species appear to result in single morphological daughter lineages (Bullini, 1994). In comparison to other taxa, the high diversity of cuticle colour and texture expressed in *Acanthoxyla*, contrasts with low mitochondrial DNA sequence variation (<2% over 1448 bp of COI and COII) within the genus (Morgan-Richards and Trewick, 2005). Analysis of mitochondrial and nuclear DNA sequences, in addition to evidence from allozyme data, karyology and flowcytometry (Morgan-Richards and

* Corresponding author. Fax: +64 6 350 5623.
E-mail address: s.trewick@massey.ac.nz (S.A. Trewick).

1055-7903/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2008.05.025

Trewick, 2005) indicate that the *Acanthoxyla* lineage most probably originated from one or few hybridization(s) between male *Clitarchus hookeri* (White, 1846) and another, unknown, species (see Table 1).

Evidence from DNA sequence data could be interpreted as indicating more than one hybridization event in *Acanthoxyla* but there is no direct evidence for eight separate events to match the species number. The presence of two distinct and size polymorphic classes of ITS nuclear DNA sequences within *Acanthoxyla* demonstrates its hybrid origin (Clades I and II; Fig. 1). *Acanthoxyla* individuals have ITS DNA sequences belonging to one or both clades, and individuals of the same morpho-species express these alternative conditions. One clade of ITS sequences evidently originated in *Clitarchus* as the identical sequence occurs in both *Clitarchus hookeri* individuals and some *Acanthoxyla* individuals. PCR assays indicate the operation of concerted evolution in homogenising the nuclear rDNA gene cluster in some lineages (Morgan-Richards and Trewick, 2005). A combination of morphological (taxonomic), ITS (nuclear) and mtDNA (maternal) data reveals a complex though apparently shallow history (Fig. 1). Significantly, these data cast doubt on the validity of the current taxonomic treatment, and instead indicate that numerous parthenogenetic lineages have independently converged on a range of similar morphotypes. Identifying both parental species would simplify estimation of the timing and number of hybridization events and facilitate exploration of genomic processes that have resulted in the morphological diversity, distinct karyotype and increased DNA content that characterise this genus (Morgan-Richards and Trewick, 2005; unpublished data). We would expect the maternal ancestor to have ITS sequences identical (or almost identical) to the ITS sequences that form the clade unique to *Acanthoxyla* (Clade I), because the putative paternal ancestor (*Clitarchus hookeri*) and *Acanthoxyla* share identical ITS sequences in Clade II (Fig. 1).

If the maternal ancestor of *Acanthoxyla* is extant, where should we look for it? All New Zealand stick insects lack wings and New Zealand is separated by at least 1200 km of ocean from other major landmasses. Isolation of the New Zealand continent (Zealandia) by continental drift started about 80 MA and as a consequence it is has been widely assumed that the biota of New Zealand is similarly ancient and isolated (Goldberg et al., in press; Trewick and Morgan-Richards, in press). However, recent molecular work has revealed that many New Zealand endemic plants and animals share common ancestors with Australian and Pacific taxa only a few million years ago and are thus not monophyletic (Waters et al., 2000; Chambers et al., 2001; Winkworth et al., 2002; Vink and Paterson, 2003; Arensburger et al., 2004; and see Trewick et al., 2007). In order to focus our search for a maternal ancestor for *Acanthoxyla* we need to know if the New Zealand phasmid fauna is monophyletic.

Table 1
Genomic anatomy of *Acanthoxyla*, the New Zealand obligate parthenogenetic stick insect genus

Taxonomy	Genus of eight species recognised on morphological grounds[a]
Reproduction	Obligate parthenogens, males never recorded, no females observed *in copula*[b]
mtDNA	Distinct lineage with little sequence divergence within genus[c]
Nuclear DNA sequence	At least two nuclear sequence-lineages, one shared with *Clitarchus hookeri*, the others are unique
Karyotype	Diploid number same as *Clitarchus hookeri* but karyotype dominated by metacentrics which are absent from *C. hookeri* karyotype[c,d]
Allozymes	Fixed heterozygosity at 1 locus (*Gpi*) in some *Acanthoxyla* lineages. All 8 alleles at 8 loci found in *Clitarchus* occur in *Acanthoxyla*, but *Acanthoxyla* also has 3 alleles not found in *Clitarchus*[e]

[a] Jewell and Brock (2002).
[b] Salmon (1991).
[c] Morgan-Richards and Trewick (2005), Buckley et al. (2008).
[d] Parfitt (1980).
[e] Buckley (1995).

The wingless New Zealand fauna are thought to be related to Australian phasmids. The Australian phasmid fauna comprises some 200 species placed within ten subfamilies, including the two (Pachymorphinae and Phasmatinae; Appendix 1) in which the New Zealand species are currently placed, and the New Zealand monotypic genus *Micrachus hystriculeus* (Westwood, 1859) was formerly placed within the Australian genus *Pachymorpha* (Salmon, 1991). Hence, taxonomy implies that the New Zealand phasmid do not form a monophyletic group. In general, higher level taxonomy of stick insects is widely regarded as in need of work (Otte and Brock, 2003). Constraints of morphology imposed by crypsis (stick mimicry) have restricted cladistic resolution, and the solution has been the identification of one or few key synapomorphies. Thus, we made few assumptions as to the likely relatives of the New Zealand fauna.

The identification of appropriate outgroup taxa for phylogenetic analysis is both important and problematic (Lyons-Weiler et al., 1998; Milinkovitch and Lyons-Weiler, 1998; Holland et al., 2003). Even where it is possible to obtain sequence data from a wide diversity of potential outgroup taxa, the severest limitations on computing efficiency time for phylogenetic analysis arise from taxon number. A compromise between increasing taxon sampling to reduce phylogenetic error (Zwickl and Hillis, 2002) and minimising taxon sampling to reduce computing time is sought. A further problem with an unconstrained sampling scheme is that sequence alignment is frequently more demanding and perhaps less reliable where length-polymorphic genes are concerned (e.g. components of the rDNA cluster). Sequence alignment can be critical for reliable phylogenetic inference, therefore it is preferable to work with a relatively small number of taxa that minimises the number of likely alternative alignments (Ogden and Rosenberg, 2006). In addition, sequence alignment should not be subject to circular reasoning by the assumption of evolutionary relationships. The New Zealand stick insects provide an exemplary case where neither taxonomy nor biogeography can be relied upon for outgroup selection. Indeed it is possible that some putative outgroup taxa (i.e. non-New Zealand species) could in fact be unidentified ingroups. Therefore we devised an efficient and convenient method (SeqSSi) to minimise the assumptions in outgroup identification. For our study we were in the convenient position of being able to take advantage of existing data representing stick insect diversity worldwide (Whiting et al., 2003).

2. Materials and methods

2.1. Rationale

To investigate the evolutionary relationships within the New Zealand fauna Morgan-Richards and Trewick (2005) sequenced protein coding mitochondrial genes (COI and COII) and nuclear internal transcribed spacers (ITS) from an extensive sample of individuals and species representing all New Zealand genera except *Pseudoclitarchus* (Salmon, 1991). The distribution of genetic diversity within the *Acanthoxyla* clade was not concordant with morphological diversity implying that the currently recognised obligate parthenogenetic species are not monophyletic lineages. From analysis of nuclear (ITS) and mitochondrial (COI, COII) DNA sequence data it was inferred that the mitochondrial genome of *Acanthoxyla* came from a maternal bisexual ancestral species (mother) and that two nuclear lineages (mother and father) remain in the nuclear rDNA gene cluster of this genus (Morgan-Richards and Trewick, 2005) (Fig. 2). These nuclear lineages are the signatures of the two ancestral (maternal and paternal) species from which *Acanthoxyla* originated.

However, because of the limited capacity of these mitochondrial protein coding genes to accumulate phylogenetic signal at

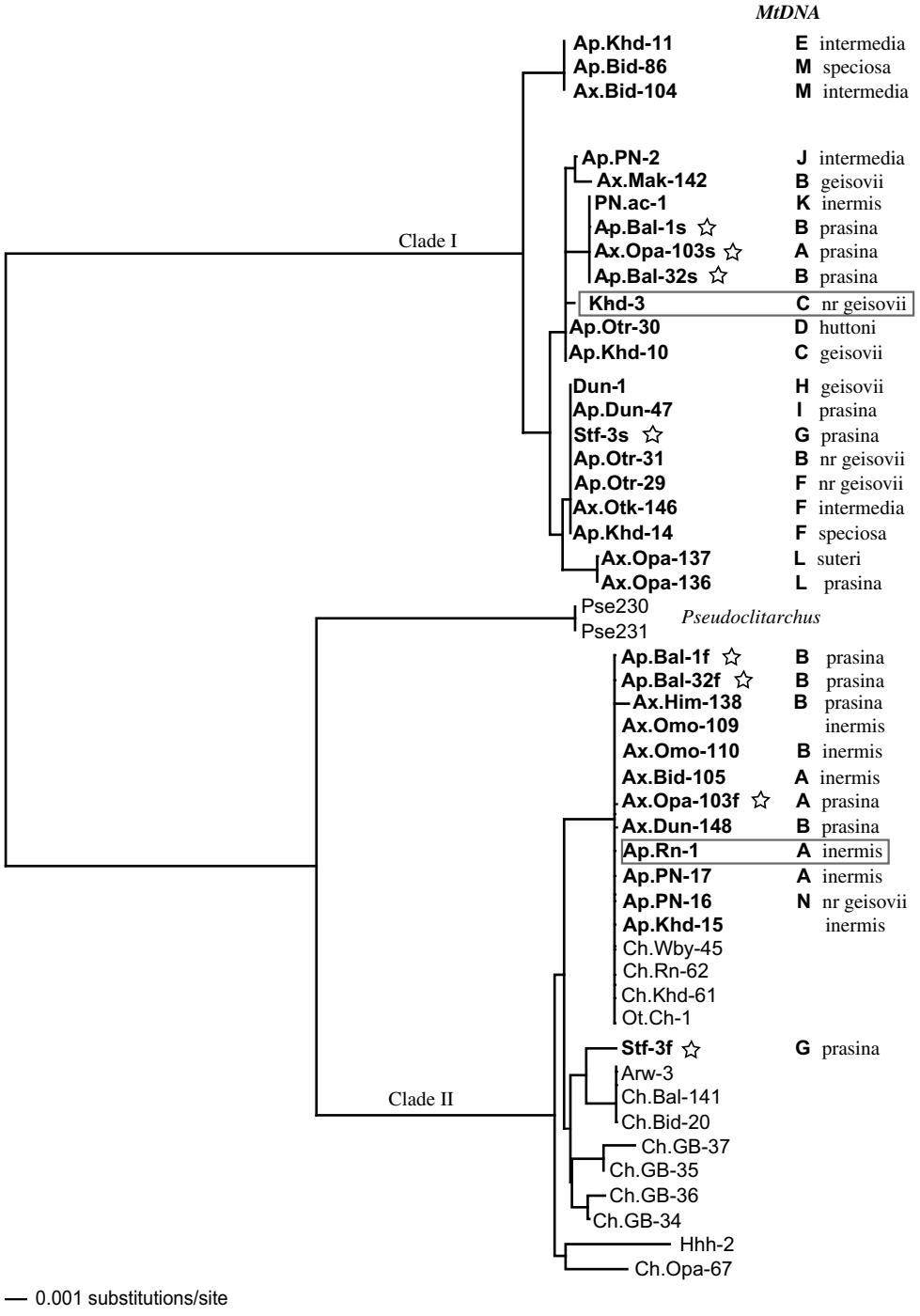

Fig. 1. Evidence from nuclear (rDNA) sequence that *Clitarchus hookeri* maybe one parent in hybrid origin of *Acanthoxyla* (Morgan-Richards and Trewick, 2005). Midpoint rooted Neighbor Joining (NJ) tree of ITS1&2 (internal transcribed spacers) nuclear DNA sequences comprising three clades: *Aconthoyxla* (Clade I), *Pseudoclitarchus* (Pse), and *Clitarchus* plus *Acanthoxyla* (Clade II). *Acanthoxyla* individuals are labelled with bold lettering, and annotated with species name (indicative of different morphologies) and COI–COII mtDNA haplotype (bold uppercase letter). *Acanthoxyla* individuals found to have both classes of ITS sequence are indicated by star, and the two individuals (Khd-3, Ap.Rn-1) used in other analyses presented here are indicated by grey boxes.

deeper taxonomic levels (Szymura et al., 1996; Paton and Baker, 2006) we have here further utilised the nuclear rDNA gene cluster. In their analysis of DNA sequences from stick insects and other insect orders where 28S represented a little less than 50% of the sequence data, Whiting et al. (2003) found that it provided some 62% of the phylogenetic signal and provided resolution throughout the tree topology. Hence our present analysis focuses on this gene, which provides a good chance of phylogenetic resolution, the opportunity to incorporate an existing extensive resource of data, and take advantage of the presence of both the maternal and paternal lineages in *Acanothoxyla* already identified by ITS sequences (Fig. 2).

2.2. Taxon sampling

Twenty-two species and nine genera of New Zealand stick insect are recognised by Jewell and Brock (2002). The genera (and number of species in brackets) for each subfamily are: Pachymorphinae—*Micrarchus* (1), *Niveaphasma* (1), *Asteliaphasma* (2), *Tectarchus* (4), *Spinotectarchus* (1); Phasmatinae—*Acanthoxyla* (8),

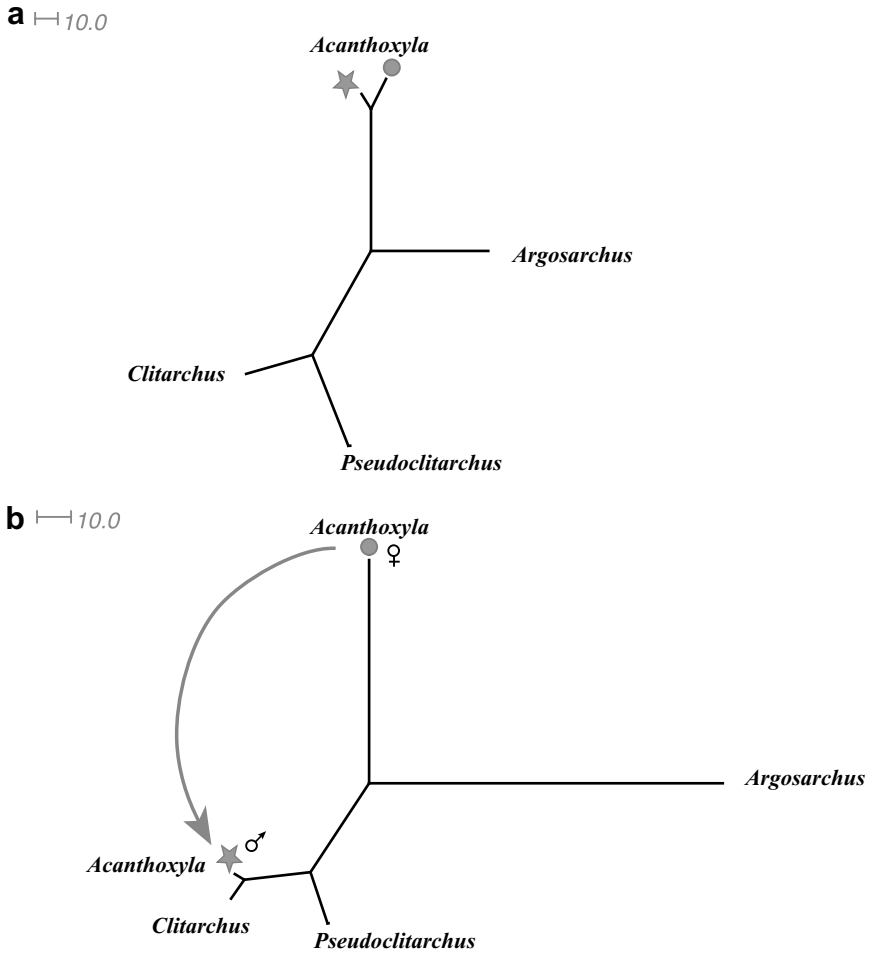

Fig. 2. Unrooted MP trees for (a) COI–COII mitochondrial, and (b) 28S and ITS nuclear DNA sequence data for representatives of the New Zealand "Phasmatinae". A single shortest tree was returned in each case. The grey arrow highlights the placement of *Acanthoxyla*'s two distinct 28S and ITS DNA sequence variants. Male and female symbols indicate inferred paternal and maternal lineages that gave rise to the *Acanthoxyla* lineage.

Argosarchus (2), *Clitarchus* (2), *Pseudoclitarchus* (1). Of these, we have sampled 17 species for the present analysis, representing all genera (Appendix 1).

The species not included in the present study are unlikely to contribute to the current analysis, and comprise: (1) one species each from two genera (*Clitarchus* and *Argosarchus*) that were recently inferred from DNA sequence data to be monotypic (Trewick et al., 2005). (2) Despite several attempts, nobody has found *Asteliaphasma naomi* (Salmon, 1991) since its description from a single specimen. However, we have collected *Asteliaphasma jucunda* (Salmon, 1991) from the type location of *A. naomi*. *A. naomi* may have been an unusual morph of *A. jucunda*. (3) Three of the four *Tectarchus* (Salmon, 1948) species (*T. huttoni* (Brunner, 1907), *T. ovobessus* Salmon, 1948, *T. salebrosus* (Hutton, 1899)) are included but not the rare *T. semilobatus* (Salmon, 1948). (4) The present analyses include seven of the eight described species of *Acanthoxyla* (Fig. 1). Individuals representing two *Acanthoxyla* species (*A. geisovii, A inermis*) were selected for analysis of 28S as representing the extremes of COI–COII (1.8%) and ITS sequence diverge (Morgan-Richards and Trewick, 2005; see summary in Fig. 1).

We include additional geographic isolates of *Micrarchus hystriculeus* and *Niveaphasma annulata* (the latter including a putative new species identified by Tony Jewell) and the monotypic sexual species *Pseudoclitarchus sentus* Salmon, 1991. *Pseudoclitarchus sentus* is restricted to a single offshore island that is a New Zealand Scientific Reserve (Great King Island, Three Kings Islands) and as a consequence was previously not available for analysis (Morgan-Richards and Trewick, 2005). The inclusion of *Pseudoclitarchus* in the present analysis means that all New Zealand Phasmatinae (the subfamily that includes *Acanthoxyla*) are now represented. Although Salmon (1948) suggested *Acanthoxyla* was taxonomically near *Clitarchus* and *Pseudclitarchus*, *Acanthoxyla* species combine a suite of features including highly sculptured egg capsules, presence of abdominal flanges, well-developed thoracic spines and a distinctive opercular spine, and colours not seen in other stick insects in New Zealand.

Non-New Zealand stick insects were sampled using published sequences for a set of species reported by Whiting et al. (2003) and available from GenBank, with additional taxa from the West Pacific: Australia (3 species), New Caledonia (3 species), Fiji (2 species) and the Solomon Islands (2 species) (see Appendix). Identification of Pacific island and Australian stick insects was provided by Paul D. Brock (Natural History Museum, London) and Geoff Monteith (Brisbane Museum, Australia), respectively. Together these taxa comprise representatives of a range of subfamilies including those (Pachymorphinae, Phasmatinae) found in New Zealand (Appendix 1).

2.3. DNA extraction, amplification and sequencing

In most instances muscle tissue from fresh, frozen, or alcohol preserved specimens was removed from a leg for genomic DNA extraction using a salting-out method (Sunnucks and 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 14,000 rpm for 5 min. The supernatant was removed and precipitated with an equal volume of cold 100% ethanol. DNA was collected by spinning and washed with 70% ethanol, then dried and dissolved in water. In the case of pinned museum specimens (from Australia and New Caledonia), DNA extraction used incubation at 55 °C with Proteinase-K and a CTAB buffer (2% Hexadecyltrimethylammonium bromide, 100 mM Tris–HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA), followed by a combined phenol/chloroform/isoamyl alcohol (25:24:1) cleanup.

2.4. PCR

To confirm the relationship and expected consistency of ITS and 28S sequences within *Acanothoxyla* we sequenced these genes plus the mitochondrial fragment comprising partial COI and COII genes from representatives of *Acanthoxyla* species (morphotypes) and the other New Zealand Phasmatinae (*Argosarchus*, *Clitarchus*, *Pseudoclitarchus*). We compiled COI–COII data from representatives of all New Zealand genera to assess the level of genetic diversity among them. All taxa were subjected to PCR and sequencing targeting the nuclear rDNA gene 28S.

The mitochondrial fragment, comprising the 3′ end of 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). The internal transcribed spacers (ITS regions 1 and 2) and the intervening 5.8S gene were amplified using the primers ITS4, ITS5 (White et al., 1990) and STITS5F (Morgan-Richards and Trewick, 2005). For the majority of samples, 28S sequences were amplified using the primers 1.2a, 28Sb and 28Sa, 7.1b (Whiting et al., 1997), although older material required amplification and sequencing with additional internal primers (4.2, 4.8, 5b) targeting shorter fragments.

PCR used standard conditions (Trewick et al., 2000). Amplification products were treated to Shrimp Alkaline Phosphotase/Exonuclease I digestion prior to sequencing. 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 v4.1 (ABI, PE), and aligned using SeAl v2.0a3 (Rambaut, 1996).

2.5. SeqSSi

We devised a targeted BLAST-based (Altschul et al., 1997) search algorithm to identify suitable outgroup and previously unidentified ingroup taxa for our analyses of the New Zealand stick insects, instead of using phylogeny or taxonomy based approaches. This is particularly useful for the analysis of DNA with INDELS as outgroup selection is done before multiple DNA sequences are aligned. Ribosomal genes frequently contain many INDELS and are thus sensitive to alignment bias.

The SeqSSi (Sequence Similarity Sieve) program is a Perl script developed to search a group of sequences for those that are the most similar to a nominated ingroup and thus locate possible outgroups for further phylogenetic analysis. SeqSSi uses the standalone gapped BLAST package (Altschul et al., 1997) available from NCBI (http://www.ncbi.nih.nlm.gov). Sequences are input in a single FASTA formatted file with the nominated ingroup of sequences in a block at the end of this file. SeqSSi firstly formats the input file (calling the program formatdb.exe) as a database for subsequent searching by the blastall.exe program, then searches all of the sequences against every other sequence in the input file. SeqSSi can use nucleotide or protein sequences as sequence type (aa or nt) as nominated by the user. Our study used the BLAST default parameters with the input sequences in nucleotide format (blastn program option). Importantly, instead of searching GenBank in its entirety, the SeqSSi procedure uses prior information, so that ingroup and potential outgroup DNA sequences are matched for locus coverage and broad taxonomic relevance (e.g. in this case 28S gene, and phasmids).

The BLAST program is a widely used tool for searching protein and nucleotide sequence databases for similar sequence (Altschul et al., 1997). Scores are produced for each similarity hit to indicate the level of similarity between the query (input from user) and subject (from database) sequences. In this case the subject and query sequences are homologous as they represented the full sequence of a single gene. After the BLAST search, SeqSSi records each score from the generated output file. For each sequence in the input file, SeqSSi records the difference between the top score (which is always the 100% match between this sequence and itself found in the database) and the scores for all the other sequences. Thus, similar sequences will have very low difference values and dissimilar sequences higher difference scores.

For each DNA sequence in the ingroup, the difference scores are compared at different ranges to produce a rating of the outgroup sequences based on their similarity to ingroup sequences. For our data we used the following category bounds: category A (1–500 difference score), category B (501–1000 difference score), category C (1001–2000 difference score) and category D (above 2001 difference score). Outgroup sequences are then ranked according to this rating and how many times each sequence appears in each category. This creates a list of outgroup sequence codes, starting with those that appear most frequently in category A, then B then C (D should contain all the sequences and is used as a control). We summarise this output as a table comprising differences scores (expressed as A–C rank codes for simplicity) for comparisons of ingroup species sequences versus outgroup sequences (Table 2). The incidence of A and B category difference scores was used to select sequences of suitable outgroup species that were aligned with ingroup sequences for phylogenetic analysis.

Statistical analysis of SeqSSi results was performed with the *R* statistics software V2.4.0 (Ihaka and Gentleman, 1996). Difference scores from outgroups against ingroup taxa were plotted as density distributions so that the histogram had a total area of 1. Difference scores for individual outgroup sequences were plotted against the original histograms to obtain 'SeqSSi-plots' that express the distribution of difference scores for specific outgroup taxa in the context of the overall score distribution. Outgroup taxa that showed a higher proportion of SeqSSi score differences in the lower ranges (SeqSSi score difference <1000, i.e. category A and B in Table 2) were included in subsequent analysis (Fig. 3).

2.6. Phylogenetic analysis

We used PAUP* 4.0b9 (Swofford, 2002) to implement phylogenetic analyses with Neighbor Joining (NJ), Maximum Parsimony (MP), and Maximum Likelihood (ML) criteria, and MrBayes v3.1.2. (Ronquist and Huelsenbeck, 2003) for Bayesian analysis. To select among alternative models of DNA evolution for the ML and NJ analyses we used Modeltest version 3.06 (Posada and Crandall, 1998).

For MP we used unweighted data, while for ML and NJ analysis we used Modeltest v3.06 to separately estimate parameters under the GTR+I+Γ model selected for the 28S and COI–COII data sets, respectively. Bayesian analyses of 28S data used a GTR model with gamma-distributed rate variation across sites (with four categories), a proportion of invariant sites and default priors. We used two independent, simultaneous runs with three heated chains and a burnin of first 25% of trees.

Table 2
Summary of SeqSSi analysis (Sequence Similarity Sieve algorithm) of 28S DNA sequence data, to find suitable outgroup taxa for the New Zealand stick insects

SeqSSi no.& code	*Species*	Subfamily	Locatation	*Extatosoma tiaratum* 4	*Ctenomorphodes briareus* 5	*Eurycnema goliath* 3	*Tropidoderus childrenii* 2	*Graeffea crouanii* 43	*Paraphasma rufipes* 8	*Agathemera crassa* 10	*Lamponius guerini* 13	*Eurycantha insularis* 14	*Cnipsus rachis* 39	*Pachymorpha sp.* 41	*Ophicrania leveri* 42	*Haaniella dehaanii* 11	*Neohirasea sp.WS29* 12	*Neohirasea maerens* 15	*Sungaya inexpectata* 7	*Nisyrus amphibius* 38	*Sipyloidea sipylus* 16	*Phyllium bioculatum* 17	*Carausius morosus* 6	*Chitoniscus brachysoma* 40
57 Otr-5	*Argosarchus horridus*	Phas.	Wellington	A	A	B	A	B	B	B	B	C	C	C	C	C	C	C	C	C	C	C	C	C
55 Khd-3	*Acanthoxyla nr geisovii*	Phas.	Welllington	A	A	B	A	C	B	B	B	B	B	B	C	C	C	C	C	C	C	C	C	C
56 Ap.Rn-1	*Acanthoxyla inermis*	Phas.	Wellington	A	A	B	A	B	B	B	B	B	B	B	C	C	C	C	C	C	C	C	C	C
68 Ax.Opa-137	*Acanthoxyla suteri*	Phas.	Auckland	A	A	A	A	C	B	B	B	B	B	B	B	C	C	C	C	C	C	C	C	C
69 Ax.Dun-148	*Acanthoxyla prasina*	Phas.	Dunedin	A	A	B	A	B	B	B	B	B	B	B	C	C	C	C	C	C	C	C	C	C
59 HhH-2	*Clitarchus hookeri*	Phas.	Far North	A	A	B	A	B	B	B	B	B	B	B	C	C	C	C	C	C	C	C	C	C
67 Ct.Bid-60	*Clitarchus h. tuberculatus*	Phas.	Bideford	A	A	A	A	B	B	B	B	B	B	B	C	C	C	C	C	C	C	C	C	C
62 Pse-230	*Pseudoclitarchus sentus*	Phas.	Three Kings Is.	A	A	A	A	B	B	B	B	B	B	B	C	C	C	C	C	C	C	C	C	C
49 Niv.Pis-126	*Niveaphasma sp.*	Pach.	Pisa Range	B	B	B										C				C				
48 Mi.Bid-87	*Micrarchus hystriculeus*	Pach.	Bideford	C		B														C				
64 Khd2	*Tectarchus huttoni*	Pach.	Wellington	A	A		A	B			B	C	B	B	C	C	C	C	C	C	C	C	C	
66 Ts.PH-1	*Tectarchus salebrosus*	Pach.	Christchurch	A	A	B	B	B	C	C	B	C			C		C			C		C	C	
65 Tec.Rg-241	*Tectarchus ovobessus*	Pach.	Rangiwahia	A	A	B	A	B	C	C	B	B	B	B	C	C	C	C	C	C	C	C	C	
54 Sp.Co-238	*Spinotectarchus acornutus*	Pach.	Coromandel	C	B	C	C		C	C	C	C			C	C	C	C	C					
52 Ast.LWk-152	*Asteliaphasma jucunda*	Pach.	Waikaremoana	C	C	C	C	C					C	C	C									

Letters A, B and C indicate ranked similarity score (A > B > C) for each outgroup/ingroup taxon combination. Missing entries indicate instances where similarity ranking was lower than category C. For clarity only the summarised results of the top 21 of 44 potential outgroup taxa and one representative of each ingroup species are shown. Shading indicates highest ranked taxa used in subsequent phylogenetic analysis.

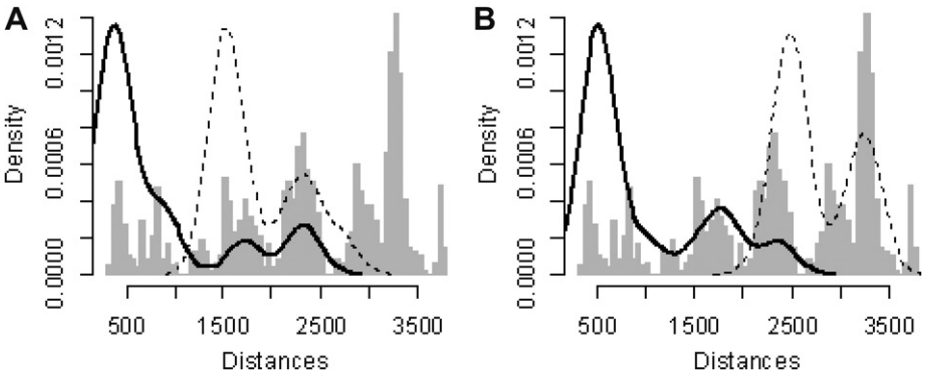

Fig. 3. Example 'SeqSSi-plots' of difference scores obtained with SeqSSi (Sequence Similarity Sieve). The background histogram plots the probability densities of the total SeqSSi score differences and is identical for the two plots shown here. Solid lines represent the relative densities of taxa that were included as outgroups in our phylogenetic analysis. The taxa represented by dotted lines had profiles typical of those not used in our subsequent analysis. Outgroup taxa selected had a higher proportion of SeqSSi score differences in the lower ranges (A and B in Table 3) whereas excluded taxa had a higher proportion of their scores in the higher ranges (C and above). (A) The proportional density of the difference scores obtained with Outgroup 5-*Ctenomorphodes* (solid line) compared with those obtained with Outgroup 12-*Neohirasea* (dotted line). (B) The proportional density of the difference scores obtained with Outgroup 3-*Eurycnema* (solid line) compared with those obtained with Outgroup 22-*Gratida* (dotted line).

Analysis of COI–COII sequence data was undertaken with tRNA Leucine excluded. Analysis of 28S DNA sequences used outgroup taxa selected by SeqSSi analysis that received a minimum of two hits in the category B ranking (this is an arbitrary cut off chosen to provide an optimal number of outgroup taxa for this study). Then 28S sequences for these outgroup (non-New Zealand) taxa were aligned with those representing all New Zealand taxa, either manually using SeAl v2.0a3 (Rambaut, 1996) or employing the computer programs Muscle v3.6 (Edgar, 2004a, 2004b) and ClustalW v1.82 (Higgins et al., 1994). For comparison we also generated alignments of the 28S sequences for the complete taxon set using Muscle v3.6 (Edgar, 2004a,b) and ClustalW v1.82 (Higgins et al., 1994) with default settings as implemented by Geneious v3.5.4 (Drummond et al., 2007). Output alignments were then subjected to Bayesian analysis with no data excluded.

Tree outputs were manipulated for display using TreeViewX v0.5 (Page, 1996).

3. Results

The aligned COI–COII, ITS including the 5.8S, and 28S sequences were 1380, 1806, and 2260 bp in length, respectively. New data reported here are deposited on GenBank (Appendix). Aligned sequences from New Zealand Phasmatinae (*Acanthoxyla*, *Clitarchus*, *Pseudoclitarchus*, *Argosarchus*) yielded a data set with 3966 bp of nuclear (28S and ITS) and 1380 bp of mitochondrial (COI, COII) DNA sequence. Pairwise genetic distances estimated using a GTR+I+Γ model applied to the COI–COII mtDNA data for representatives of New Zealand taxa are high (max 0.45), and comparisons with non-New Zealand stick insects yielded similar high distances indicating saturation.

Genetic distances (COI–COII) among the New Zealand Phasmatinae (*Argosarchus*, *Clitarchus*, *Pseudoclitarchus*, *Acanthoxyla*) were similar to between family distances (Phasmatinae–Pachymorphinae) within New Zealand. Either (a) the systematics of these stick

insects is inappropriate and/or (b) the information content of the mtDNA data at this level is negligible. The lowest intergeneric distance observed was between *Pseudoclitarchus* and *Clitarchus* (0.08); a relationship confirmed by phylogenetic analysis (Fig. 2a). Interspecific genetic distances from spatially separated samples were typical of those observed in New Zealand insects (0.02–0.05, Table 3).

The high functional constraint operating on the protein coding genes (COI–COII) imposes a major limitation on their phylogenetic utility and precludes their use for analysis of the relationships of stick insects at deeper taxonomic levels. Analysis of the 28S and ITS sequences together and apart revealed the same signal for two distinct nuclear lineages within *Acanthoxyla* (Fig. 2b). Given the close physical and thus heritable association of these two genes in the rDNA cluster this congruence is as expected. The addition of 28S sequence data confirms the previous observation of two (parental) lineages in *Acanthoxyla* (one close to *Clitarchus hookeri*), and the inclusion of new nuclear sequence data (28S and ITS) from *Pseudoclitarchus sentus* reveals that this species was not the source of either of these (Figs. 1 and 2).

SeqSSi results facilitated the selection of 11 putative outgroup/unidentified ingroup species (from a set of 44) with a ranked similarity score of A or B with at least one New Zealand stick insect species (Table 2). Examples of SeqSSi-plots are shown in Fig. 3. In these plots, two outgroups are plotted onto each graph to contrast the difference between sequences that were included in our analysis and those which were excluded. The 11 outgroup species selected with SeqSSi originated from Australia (5), Fiji (1), New Caledonia (1), New Guinea (1), South America (2) and the Caribbean (1). Analyses of 28S with New Zealand taxa and the 11 species selected by SeqSSi for the outgroup reveal two important features, (a) the New Zealand stick insects form a monophyletic clade in most cases (see below), and (b) *Acanthoxyla* forms a well-supported clade with *Clitarchus* and *Pseudoclitarchus*. The monophyly of the New Zealand taxa is supported by Bayesian analysis implemented by MrBayes and ML implemented by PAUP. In these analyses *Spinotectarchus acornutus* is placed as basal to the other New Zealand taxa. Bayesian posterior probabilities indicate strong support for this monophyletic grouping (Fig. 4), as does bootstrap resampling (10,000 replicates) using NJ distance criteria with a GTR+I+Γ model. To save computing time, a reduced data set of 12 taxa comprising representatives of the New Zealand fauna plus just 3 outgroup taxa, selected on the basis of previous analysis were subjected to ML (PAUP) bootstrap analysis (1000 replicates) employing a GTR+I+Γ model. This also revealed strong support for the monophyly of the New Zealand taxa (Fig. 5).

Bayesian analyses of 28S sequences in both, the full taxon set and the SeqSSi selection, each aligned using Muscle v3.6 and ClustalW v1.82, were entirely consistent with the observation above. There was high statistical support (posterior probabilities of 0.99 or 1.00) for monophyly of New Zealand stick insects, with, in all cases *Spinotectarchus* being sister to all other New Zealand species. These analyses also supported the grouping of *Acanthoxyla*, *Clitarchus* and *Pseudoclitarchus* (as above), although internal edge lengths for this clade were disproportionately short in trees that included all taxa (i.e. including very distant relatives in the outgroup). It was clear that resolution of ingroup structure was enhanced by exclusion of least-similar sequences.

However, NJ using HKY and simpler models of DNA evolution, and MP using unweighted data, applied to the full 28S data set, each returned trees with a topology that differed in the placement of *Spinotectarchus* among the outgroup taxa. MP bootstrap resampling (10,000) replications gave 84% for the New Zealand clade without *Spinotectarchus*. These latter results most likely reflect the high degree of variation among taxa (a tendency for long branch attraction) and model misspecification. In contrast, the clade comprising *Acanthoxyla*, *Clitarchus* and *Pseudoclitarchus* was returned by all analyses.

4. Discussion

The SeqSSi method of outgroup selection was extremely useful in reducing the number of taxa that might be a suitable outgroup before alignment of the INDEL-rich rDNA sequences. Outgroup selection prior to phylogenetic analysis can be difficult when there are a large number of species to choose from, taxonomy is unreliable or incomplete and alignment error or bias may result in misleading topologies or poor tree resolution. While BLAST results should generally be treated with caution in any evolutionary analysis, the use of BLAST to select outgroup taxa in the targeted manner performed by SeqSSi, has potential. Although SeqSSi is still under development and is undergoing further testing and evaluation, we note that in this case the ingroup identified using SeqSSi was also revealed by Bayesian analysis of all available taxa. This supports our inference that SeqSSi correctly identified the most-suitable outgroup taxa (by excluding sequences with lowest

Table 3
Pairwise genetic distances using a GTR+I+Γ model for COI–COII mtDNA nucleotide sequence data (excluding tRNALeu), for representatives of the New Zealand stick insects

		Spinotectarchus acornutus	*Spinotectarchus acornutus*	*Micrarchus hystriculeus*	*Micrarchus hystriculeus*	*Niveaphasma sp.*	*Niveaphasma annulata*	*Asteliaphasma jucunda*	*Asteliaphasma jucunda*	*Tectarchus huttoni*	*Tectarchus ovobessus*	*Tectarchus salebrosus*	*Argosarchus horridus*	*Acanthoxyla nr geisovii*	*Clitarchus hookeri*	*Pseudoclitarchus sentus*
Spinotectarchus acornutus	Sp.Opa70															
Spinotectarchus acornutus	Sp.Co238	0.03														
Micrarchus hystriculeus	BluS246	0.39	0.38													
Micrarchus hystriculeus	Mi.Bid87	0.45	0.43	0.05												
Niveaphasma sp.	Niv.Pis126	0.42	0.37	0.30	0.32											
Niveaphasma annulata	nadun42	0.43	0.40	0.31	0.32	0.05										
Asteliaphasma jucunda	Ast.Opa72	0.39	0.36	0.32	0.37	0.25	0.23									
Asteliaphasma jucunda	Ast.LWk152	0.42	0.39	0.33	0.35	0.25	0.22	0.02								
Tectarchus huttoni	Khd2	0.35	0.33	0.26	0.27	0.18	0.18	0.22	0.21							
Tectarchus ovobessus	Tec.Rg241	0.38	0.36	0.29	0.30	0.19	0.21	0.25	0.24	0.06						
Tectarchus salebrosus	Ts.PH1	0.42	0.39	0.36	0.40	0.22	0.24	0.29	0.29	0.24	0.27					
Argosarchus horridus	Arg.Omo159	0.40	0.37	0.34	0.34	0.21	0.20	0.25	0.25	0.21	0.21	0.24				
Acanthoxyla nr geisovii	Khd3	0.32	0.31	0.29	0.28	0.21	0.20	0.24	0.22	0.19	0.19	0.24	0.18			
Clitarchus hookeri	HhH2	0.36	0.34	0.29	0.29	0.22	0.21	0.25	0.25	0.22	0.24	0.26	0.20	0.21		
Pseudoclitarchus sentus	Pse232	0.37	0.35	0.25	0.28	0.20	0.22	0.24	0.25	0.21	0.22	0.27	0.20	0.20	0.08	

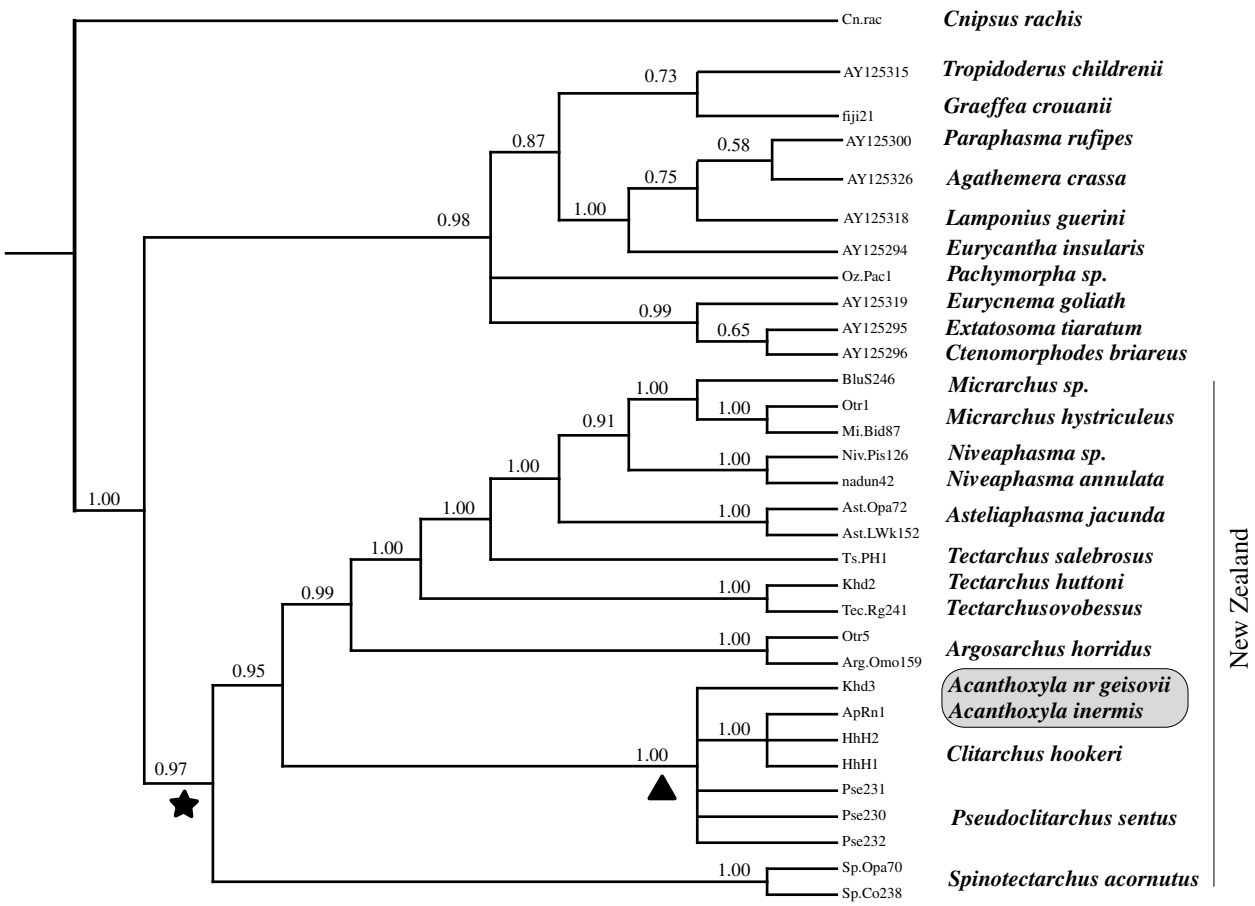

Fig. 4. Consensus tree from Bayesian analysis of 28S rDNA stick insect data using GTR+I+Γ with outgroup taxa selected by SeqSSi. Posterior probabilities estimated after removal of 25% burnin are given at nodes. The star indicates the base of clade comprising New Zealand stick insects, triangle indicates clade that includes *Acanthoxyla*.

similarity). The value of SeqSSi is evident in enhanced phylogenetic resolution of the ingroup in analysis of the SeqSSi sample, and this probably reflects better alignments and enhanced model specification obtained from a more appropriate sample set.

The present data do not support monophyly of the subfamilies Phasmatinae and Pachymorphinae, neither within New Zealand nor across the geographic/taxonomic sample as a whole. The representatives of the two New Zealand subfamilies appear to be polyphyletic, including poor support for the grouping of *Argosarchus* with the other New Zealand Phasmatinae (*Acanthoxyla*, *Clitarchus*, *Pseudoclitarchus*). However, there is good evidence that the New Zealand fauna as a whole form a monophyletic group. The monotypic genus *Spinotectarchus* was consistently returned as sister to the other New Zealand taxa and as such these data support the classification of the genus *Asteliaphasma* Jewell and Brock (2002), splitting *A. jucundus* from *S. acornutus*; species that are clearly not sister taxa as implied by the previous classification (Salmon, 1991). Interestingly, the only New Zealand genus apart from *Acanthoxyla* with more than two described species in this study (*Tectarchus*) consistently failed to form a monophyletic clade, emphasising the inherent difficulty of resolving relationships within a group where morphology is extremely constrained for crypsis.

We cannot reject the hypothesis that the parthenogenetic genus *Acanthoxyla* is part of an exclusively New Zealand clade. We have included all described New Zealand genera and most species of New Zealand stick insects. It is possible that species remain undescribed, but it is less likely that genera remain unnoticed. Although two new New Zealand stick insect genera were recently erected, the species they comprise were already well characterised (Jewell and Brock, 2002). We have failed to find a mother for *Acanthoxyla*, but know where to look; New Zealand.

If a mother existed, we expected the unique *Axanthoxyla* ITS sequences of Clade I to be nested within its sexual maternal species in much the same way as the *Acanthoxyla* Clade II sequences are nested within *Clitarchus hookeri* diversity. In addition, mitochondrial DNA sequences from a maternal species would form part of the existing *Acanthoxyla* mtDNA clade. Salmon (1991) proposed, on the basis of morphological evidence that the genus "*Pseudoclitarchus* forms an evolutionary link between *Clitarchus* and *Acanthoxyla*". Indeed, *Pseudoclitarchus sentus* was originally described by Salmon (1948) as an *Acanthoxyla* species (*Acanthoxyla senta*). Although a clade consisting of these three genera is supported by all DNA data, all analyses show that the sexual taxon *Pseudoclitarchus sentus* is not the maternal ancestor of *Acanthoxyla*. (Buckley et al., 2008). On the basis of mtDNA nucleotide data, *Pseudoclitarchus* is genetically closer to *Clitarchus* than *Acanthoxyla*.

The sexual mother of *Acanthoxyla* appears to have been sister to *Clitarchus* and *Pseudoclitarchus* (Fig. 2). This relationship, indicated by 28S sequence data (Figs. 4 and 5) is corroborated by analysis of mtDNA sequence indicating that the *Acanthoxyla* maternal lineage is genetically closer to these than any other taxa known. This suggests that the parthenogenetic *Acanthoxyla* and, more specifically, the half of the genome represented by the unique 28S/ITS sequences are all that is left of the sexual maternal species. An alternative explanation is that the presence of *Clitarchus* 28S/ITS sequences in the genome of *Acanthoxlya* may be the result of current, opportunistic mating between male *Clitarchus* and *Acanthoxyla* (Buckley et al., 2008). This would require that *Acanothyxla* is a facultative parthenogen rather than an obligate parthenogen.

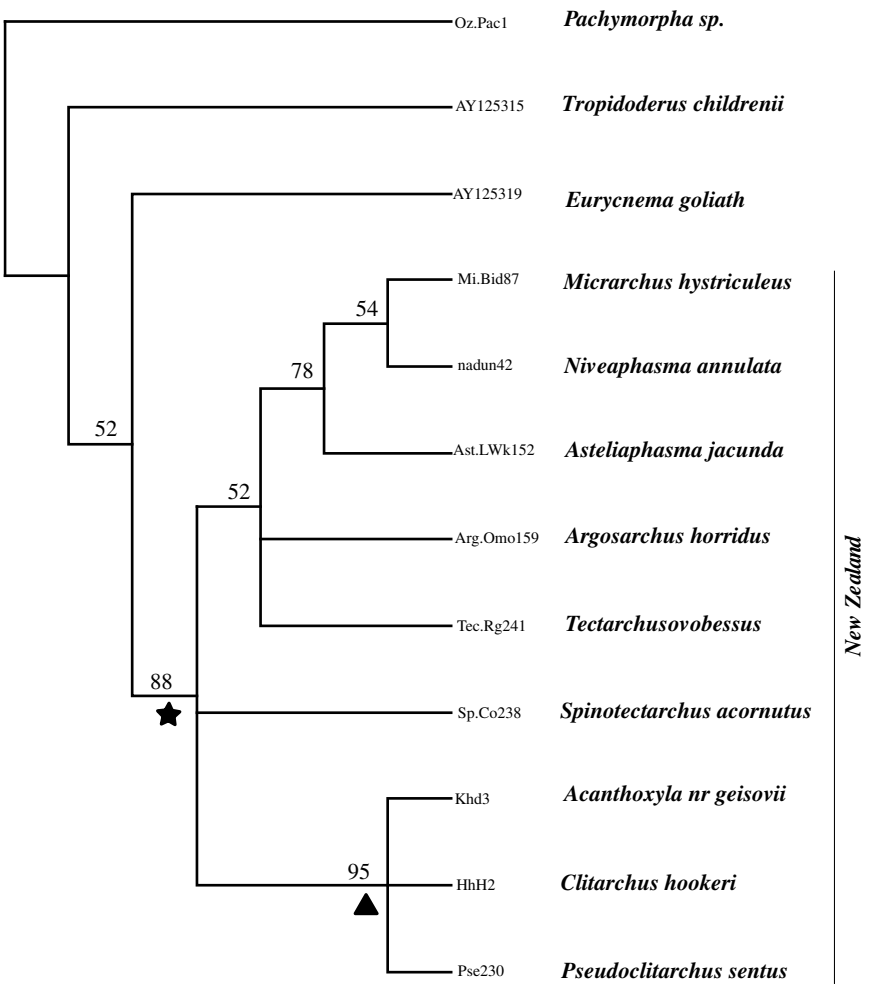

Fig. 5. Bootstrap consensus tree from Maximum Likelihood (ML) analysis of 28S rDNA stick insect data for a reduced taxon set, using GTR+I+Γ with 1000 replications. Bootstrap values are given at nodes. The star indicates the base of clade comprising New Zealand stick insects, triangle indicates clade that includes *Acanthoxyla*.

An inter-specific reproductive strategy of this type (hybridogenesis—Bullini, 1994) is known among Sicillian stick insects (*Bacillus*). In that case, successful egg production requires mating with males of the paternal species each generation but the paternal genome is discarded prior to gametogenesis, and the taxa involved (*B. rossius-grandii*) are congenerics (Mantovani and Scali, 1992; Mantovani et al., 2001). Karyological evidence for New Zealand stick insects indicates such a situation is unlikely (Morgan-Richards and Trewick, 2005; Unpublished data). The karotypes of some *Acanthoxyla* species (lineages) are diploid and distinct from that of *Clitarchus*. Furthermore, the karyotype of *Acanthoxyla* with a single 28S/ITS sequence variant is the same as that of *Acanthoxyla* individuals bearing both 28S/ITS sequences (i.e. including the *Clitarchus* type). This would be an unlikely condition if hybridisation is ongoing. Further evidence against this interpretation comes from extensive field and captive observations; although sexual *Clitarchus* pairs are a common sight, and *Clitarchus* and *Acanthoxyla* are often sympatric, inter-generic coupling has never been observed.

Therefore, *Acanthoxyla* is probably an orphan, and it is interesting to speculate that it may have competed with its bisexual mother species and contributed to 'her' extinction. The success of parthenogens is commonly associated with occupation of range margins (geographic parthenogenesis) and has been explained by avoidance of the homogenising effect of gene flow allowing adaptation to marginal habitats (Peck et al., 1998). In addition, the numerical advantage of parthenogenetic reproduction during range expansion associated with Pleistocene climate cycling may have given *Acanthoxyla* an advantage over bisexual species. For *Acanthoxyla* it will be difficult to distinguish pathenogenetic advantage from hybrid advantage (Kearney, 2005), but either and both conditions could have given the genus an adaptive advantage over a sexual maternal species. It is therefore intriguing that *Acanthoxyla* is widely sympatric with its paternal species, *Clitarchus hookeri*, which is sexual in northern New Zealand and frequently asexual at its southern range limits. One expression of hybrid vigour may be the food range exhibited by *Acanthoxyla*. All *Acanthoxyla* species eat a very wide range of plant species; from New Zealand endemics including rata (Myrtaceae) and totara (Podocarpaceae) to introduced rose (Roseacea), pine (Pinaceae), and cypress (Cupressaceae). This dietary plasticity has allowed the successful invasion of *Acanthoxyla* into the United Kingdom (Brock, 1987). In contrast, paternal *Clitarchus* has a much more limited range of host plants and a narrower species range (Trewick and Morgan-Richards, 2005; Trewick, 2007).

Acknowledgments

Paul Brock assisted us with identification. Tony Jewell, Geoff Monteith, Dinah & Scott Dunavan, Craig Morley, Rhys Richards, Ted and Bee Trewick, Judy and Llyn Richards, Mary and Ralph Powlesland, Richard Murray and family of Bluff Station, and Julia Goldberg, provided stick insects. Michael Whiting's lab (Brigham Young University) provided details of internal 28S primers. Matt Philips, Martyn Kennedy, Barbara Holland and Klaus Schliep helped with

analysis. The New Zealand Department of Conservation provided permits and we are particularly grateful for the assistance of Tony Beauchamp, Andrea Booth, Donna Stuthridge and Dave King. Specimens of *Pseudoclitarchus sentus* were collected on the Three King's Islands Nature Reserve by DoC staff under permit NO-13868-FAU, with permission of the tangata whenua, Ngati Kuri and Te Aupouri. This work was partly funded by Massey University Research Fund grant (to MMR; MURF 05/2044) and Marsden grant (to SAT:GNS302).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2008.05.025.

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Schnaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Arensburger, P., Buckley, T.R., Simon, C., Moulds, M., Holsinger, K.E., 2004. Biogeography and phylogeny of the New Zealand cicada genera (Hemiptera: Cicadidae) based on nuclear and mitochondrial DNA data. J. Biogeogr. 31, 557–569.

Brock, P.D., 1987. A third New Zealand stick-insect (Phasmatodea) established in the British Isles, with notes on the other species, including a correction. In: Mazzini, M., Scali, V. (Eds.), 1st International Symposium on Stick Insects. University of Siena, pp. 125–132.

Buckley, T., 1995. An allozyme survey of stick insects (Insecta: Phasmida) from the Wellington region. Unpublished Honours thesis, Victoria University of Wellington.

Buckley, T.R., Attanayake, D., Park, D.-C., Ravindran, S., Jewell, T.R., Normak, B.B., 2008. Investigating hybridization in the parthenogenetic New Zealand stick insect *Acanthoxyla* (Phasmatodea) using single-copy nuclear loci. Mol. Phylogen. Evol 48, 335–349.

Bullini, L., 1994. Origin and evolution of animal hybrid species. Trends Ecol. Evol. 9, 422–426.

Chambers, G.K., Boon, M.W., Buckley, T.R., Hitchmough, R.A., 2001. Using molecular methods to understand the Gondwanan affinities of the New Zealand biota: three case studies. Aust. J. Bot. 49, 377–387.

Coyne, J.A., Orr, H.A., 2004. Speciation. Sinauer Associates, Sunderland, MA.

Crespi, B.J., Sandoval, C.P., 2000. Phylogenetic evidence for the evolution of ecological specialization in *Timema* walking-sticks. J. Evol. Biol. 13, 249–262.

Drummond, A.J., Ashton, B, Cheung, M., Heled, J., Kearse, M., Moir, R., Stones-Havas, S., Thierer, T., Wilson, A., 2007. Geneious v3.5, Available from: <http://www.geneious.com/>.

Edgar, R.C., 2004a. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797.

Edgar, R.C., 2004b. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinform. 5, 113.

Goldberg, J., Trewick, S.A., Paterson, A.M., in press. Evolution of New Zealand's terrestrial fauna: A review of molecular evidence. Philosophical Transcations of the Royal Society, London.

Higgins, D., Thompson, J., Gibson, T., Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties, and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Holland, B.R., Penny, D., Hendy, M.D., 2003. Outgroup misplacement and phylogenetic inaccuracy under a molecular clock—a simulation study. Syst. Biol. 52, 229–238.

Ihaka, R., Gentleman, R., 1996. R: a language for data analysis and graphics, J. Comput. Graph. Stat. 5, 299–314. See, R Development Core Team. 2006. R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria. ISBN: 3-900051-07-0 <http://www.R-project.org>.

Jewell, T., Brock, P.D., 2002. A review of the New Zealand stick insects: new genera and synonymy, keys, and a catalogue. J. Orthopt. Res. 11, 189–197.

Kearney, M., 2005. Hybridization, glaciation and geographical parthenogenesis. Trends Ecol. Evol. 20, 495–502.

Law, J.H., Crespi, B.J., 2002. Recent and ancient asexuality in *Timema* walkingsticks. Evolution 56, 1711–1717.

Lyons-Weiler, J., Hoelzer, G.A., Tausch, R.J., 1998. Optimal outgroup analysis. Biol. J. Linn. Soc. 64, 493–511.

Mantovani, B., Scali, V., 1992. Hybridogenesis and androgenesis in the stick insect *Bacillus rossius-grandii benazzii* (Insects, Phasmatodea). Evolution 46, 783–796.

Mantovani, B., Passamonti, M., Scali, V., 2001. The mitochondrial cytochrome oxidase II gene in *Bacillus* stick insects: ancestry of hybrids, androgenesis, and phylogenetic relationships. Mol. Phylogenet. Evol. 19, 157–163.

Milinkovitch, M.C., Lyons-Weiler, J., 1998. Finding optimal ingroup topologies and convexities when the choice of outgroups is not obvious. Mol. Phylogenet. Evol. 9, 348–357.

Morgan-Richards, M., Trewick, S.A., 2005. Hybrid origin of a parthenogenetic genus? Mol. Ecol. 14, 2133–2142.

Ogden, T.H., Rosenberg, M.S., 2006. Multiple sequence alignment and phylogenetic inference. Syst. Biol. 55, 314–328.

Otte, D., Brock, P., 2003. Phasmida species file: a catalog of the stick and leaf insects of the world. ISBN:1-929014-03-1.

Page, R.D.M., 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12, 357–358. <http://darwin.zoology.gla.ac.uk/~rpage/treeviewx/index.html>.

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

Paton, T.A., Baker, A.J., 2006. Sequences from 14 mitochondrial genes provide a well-supported phylogeny of the Charadriiform birds congruent with the nuclear RAG-1 tree. Mol. Phylogen. Evol. 39, 657–667.

Peck, J.R., Yearsley, J.M., Waxman, D., 1998. Explaining the geographic distributions of sexual and asexual populations. Nature 391, 889–892.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Rambaut, A., 1996. Se-Al: Sequence Alignment Editor. Available from: <http://evolve.zoo.ox.ac.uk/>.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Salmon, J.T., 1948. New genera, species and records of Orthoptera from the Three Kings Islands New Zealand. Rec. Auckland Inst. Mus. 3, 291–311.

Salmon, J.T., 1991. The Stick Insects of New Zealand. Reed, Singapore.

Scali, V., Passamonti, M., Marescalchi, O., Mantovani, B., 2003. Linkage between sexual and asexual lineages: genome evolution in *Bacillus* stick insects. Biol. J. Linn. Soc. 79, 137–150.

Simon, C., Frati, F., Beckenbach, A., Crespi, B.J., Liu, H., Flook, P., 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. 87, 651–701.

Sunnucks, P., Hale, D.F., 1996. Numerous transposed sequences of mitochondrial cytochrome oxidase I–II in aphids of the genus Sitobion (Hemiptera: Aphididae). Mol. Biol. Evol. 13, 510–524.

Swofford, D.L., 2002. PAUP*. Phylogenetic analysis using parsimony (* and other methods). Version 4. Sinauer Associates, Sunderland, MA.

Szymura, J.M., Lunt, D.H., Hewitt, G.M., 1996. The sequence and structure of the meadow grasshopper (Chorthippus parallelus) mitochondrial srRNA, ND2, COI, COII, ATPase8 and 9 tRNA genes. Insect Mol. Biol. 5, 127–139.

Trewick, S., 2007. Stick insects, Te Ara – the Encyclopedia of New Zealand, updated 21-sep-2007. Available from: <http://www.TeAra.govt.nz/TheBush/InsectsAndOtherInvertebrates/StickInsects/en>

Trewick, S.A., Morgan-Richards, M., 2005. New Zealand Wild: Stick Insects. Reed Publishing, Hong Kong.

Trewick, S.A., Morgan-Richards, M., in press. New Zealand biology. In: Gillespie, R., Clague, E. (Eds.), Encyclopaedia of Islands. University of California Press, Berkeley.

Trewick, S.A., Morgan-Richards, M., Wallis, G.P., 2000. Phylogeographic pattern correlates with Pliocene mountain building in the alpine scree weta (Orthoptera: Anostostomatidae). Mol. Ecol. 9, 657–666.

Trewick, S.A., Goldberg, J., Morgan-Richards, M., 2005. Fewer species of *Argosarchus* and *Clitarchus* stick insects (Phasmida, Phasmatinae): evidence from nuclear and mitochondrial DNA sequence data. Zool. Scr. 34, 483–491.

Trewick, S.A., Paterson, A.M., Campbell, H.J., 2007. Hello New Zealand. J. Biogeogr. 34, 1–6.

Vink, C.J., Paterson, A.M., 2003. Combined molecular and morphological phylogenetic analyses of the New Zealand wolf spider genus *Anoteropsis* (Araneae: Lycosidae). Mol. Phylogen. Evol. 28, 576–587.

Waters, J.M., Andres-Lopez, J., Wallis, G.P., 2000. Molecular phylogenetics and biogeography of galaxiid fishes (Osteichthyes: Galaxiidae): dispersal, vicariance, and the position of *Lepidogalaxias salamandroides*. Syst. Biol. 49, 777–795.

White, A., 1846. The zoology of the voyage of H.M.S. Erebus and Terror. In 1 Insects of New Zealand. E.W. Janson, London.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, Inc., New York, NY, pp. 315–322.

Whiting, M.F., Carpenter, J.C., Wheeler, Q.D., Wheeler, W.C., 1997. The *Strepsiptera* problem: phylogeny of the holometabolous insect orders inferred from 18S and 28S ribosomal DNA sequences and morphology. Syst. Biol. 46, 1–68.

Whiting, M.F., Bradler, S., Maxwell, T., 2003. Loss and recovery of wings in stick insects. Nature 421, 264–267.

Winkworth, R.C., Wagstaff, S.J., Glenny, D., Lockhart, P.J., 2002. Plant dispersal NEWS from New Zealand. Trends Ecol. Evol. 17, 514–520.

Zwickl, D.J., Hillis, D.M., 2002. Increased taxon sampling greatly reduces phylogenetic error. Syst. Biol. 51, 588–598.