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

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The hybrid stick insect genus \textit{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 \textit{Citarchus 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 \textit{Acanthoxyla}. In an effort to identify the maternal ancestor of \textit{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 phasmsids. 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 \textit{Acanthoxyla} belonged, but did not support existing higher level taxonomy of stick insects. We did not find a sexual maternal species for \textit{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 \textit{Acanthoxyla} may have contributed to the extinction of its mother.

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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 \textit{Timema} (Law and Crespi, 2002) and \textit{Bacillus} (Scali et al., 2003). The best-studied group from the perspective of hybrid speciation are the Sicilian \textit{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 \textit{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 \textit{Bacillus} where asexual species have arisen with and without hybridisation there is little ecological or morphological variation among parthenogenetic 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 \textit{Acanthoxyla} Uvarov 1944 is the most speciose of the nine endemic New Zealand stick insect genera (Jewell and Brock, 2002). All eight species of \textit{Acanthoxyla} are obligate parthenogens, entirely lacking males but with females routinely producing “fertile” eggs. The extent of morphological diversity within \textit{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 \textit{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...
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 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.

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 (Pachy- morphinae and Phasmatinae; Appendix 1) in which the New Zealand species are currently placed, and the New Zealand monotypic genus Micrachus hystericulus (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).

Table 1

<table>
<thead>
<tr>
<th>Taxonomy</th>
<th>Reproduction</th>
<th>mtDNA Nuclear DNA sequence</th>
<th>Karyotype</th>
<th>Allozymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genus of eight species recognised on morphological grounds</td>
<td>Obligate parthenogens, males never recorded, no females observed in copula</td>
<td>Distinct lineage with little sequence divergence within genus</td>
<td>Diplod number same as Clitarchus hookeri, the others are unique</td>
<td>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</td>
</tr>
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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
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 Acanthoxyla 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), Pseudoclitarchus (Pse), Clitarchus (Clad I), Acanthoxyla (Clad II).
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 Pseudoclitarchus, 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 Montefith (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 decane and dissolved in water. In the case of pinned museum specimens (from Australia and New Caledonia), DNA extraction used decane and dissolved in water.

2.4. PCR

To confirm the relationship and expected consistency of ITS and 28S sequences within Acanthoxya we sequenced these genes plus the mitochondrial fragment comprising partial COI and COII genes from representatives of Acanthoxya species (morphotypes) and the other New Zealand Phasmatinae (Argosarchus, Citarchus, Pseudociliarchus). 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, 28b 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 stand-alone gapped BLAST package (Altschul et al., 1997) available from NCBI (http://www.ncbi.nlm.nih.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 phasmoids).

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 distant or different 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.
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 DNA sequence data 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+C 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. 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.
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 DNA 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 was also revealed by Bayesian analysis of all available taxa. This 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 DNA 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

<table>
<thead>
<tr>
<th>Species</th>
<th>mtDNA Distance</th>
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<tbody>
<tr>
<td>Spinotectarchus acornutus</td>
<td>0.03</td>
</tr>
<tr>
<td>Micrarchus hystrixculus</td>
<td>0.38</td>
</tr>
<tr>
<td>Micrarchus hystrixculus</td>
<td>0.37</td>
</tr>
<tr>
<td>Niveaphasma sp.</td>
<td>0.37</td>
</tr>
<tr>
<td>Niveaphasma annulata</td>
<td>0.32</td>
</tr>
<tr>
<td>Astelaphasma jacunda</td>
<td>0.37</td>
</tr>
<tr>
<td>Acanthoxyla</td>
<td>0.36</td>
</tr>
<tr>
<td>Tectarchus huttoni</td>
<td>0.23</td>
</tr>
<tr>
<td>Tectarchus ovobesus</td>
<td>0.23</td>
</tr>
<tr>
<td>Tectarchus salubrosus</td>
<td>0.22</td>
</tr>
<tr>
<td>Argocharus hornum</td>
<td>0.21</td>
</tr>
<tr>
<td>Acanthoxyla nr goswellii</td>
<td>0.21</td>
</tr>
<tr>
<td>Clitarchus hookeri</td>
<td>0.20</td>
</tr>
<tr>
<td>Pseudoclitarchus sentus</td>
<td>0.20</td>
</tr>
</tbody>
</table>
are clearly not sister taxa as implied by the previous classifications, 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 Acanthoxyla 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) propounded, 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 sentus). 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 Acanthoxyla may be the result of current, opportunistic mating between male Clitarchus and Acanthoxyla (Buckley et al., 2008). This would require that Acanthoxyla is a facultative parthenogen rather than an obligate parthenogen.
An inter-specific reproductive strategy of this type (hybridogenesis—Bullini, 1994) is known among Sicilian 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 karyotypes 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).

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**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.
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;GNS202).

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


Jackson, S.T., 1988. Finding optimal ingroup topologies and outgroup 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;GNS202).

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References


