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Investigating hybridization in the parthenogenetic New Zealand stick insect *Acanthoxyla* (Phasmatodea) using single-copy nuclear loci

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Abstract

The New Zealand stick insect genus *Acanthoxyla* Uvarov is extremely unusual among higher taxa of animals in that all known species are obligate parthenogens. We have used a combination of the mitochondrial DNA genes *cytochrome oxidase subunits I* and *II*, *28S* nuclear ribosomal RNA, and the two single-copy nuclear genes *elongation factor 1α* and *phosphoglucose isomerase* to test hypotheses on the role of hybridization in the evolution of this genus. Alleles at the single-copy nuclear loci in three sampled species of *Acanthoxyla* were resolved by cloning the PCR products. Analysis of multilocus genotypes shows that most sampled individuals of *Acanthoxyla* possess three alleles at the single-copy nuclear loci, which we have interpreted to indicate triploidy. Because most of the alleles from *Acanthoxyla* form a monophyletic group, including sets of alleles possessed by the putative triploids, we have inferred that the extant parthenogenetic lineages formed via hybridization between species of *Acanthoxyla*, at least one of which must have been sexual. More recently, there have been multiple introgression events from the related species *Clitarchus hookeri* White, although *C. hookeri* does not appear to be involved with the origin of parthenogenesis in *Acanthoxyla*. Our study demonstrates the utility of cloning alleles from multiple single-copy nuclear genes for resolving the origins of parthenogenetic lineages.

Keywords: Hybridization; Introgression; Polyploidy; Asexual reproduction; Mitochondrial DNA; Nuclear genes

1. Introduction

The New Zealand stick insect genus *Acanthoxyl*a Uvarov contains eight recognized species (Salmon, 1955, 1991; Jewell and Brock, 2002) all of which are obligately parthenogenetic. No male *Acanthoxyla* has ever been reported. A completely parthenogenetic genus is something of an anomaly, and invites speculation about the possibility of ancient asexuality (Mayr, 1963, p. 411; Judson and Normark, 1996). A recent molecular study (Morgan-Richards and Trewick, 2005) suggested a relatively recent hybrid origin for *Acanthoxyla*, but left many questions unanswered. Here we follow up on that study, using single-copy nuclear markers and an additional outgroup, to provide a better-resolved view of the origin and history of this unusual group of insects.

Species of *Acanthoxyla* are widespread throughout large areas of New Zealand and are sympatric with one another. They most commonly feed on endemic podocarp trees such as *Podocarpus* and *Dacrydium* but also angiosperms such as *Leptospermum scoparium* and *Metrosideros*. They have survived human environmental modification by feeding on adventive plants such as *Rosa*, *Rubus*, and ornamental

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conifers. Their success on adventive plants has allowed them to survive translocation to the British Isles (Brock, 1987).

Acanthoxyla are large insects (7–9 cm) and are most commonly green or brown and are often covered in black tipped spines. There is significant morphological diversity among the species with variation in body size, number, density and arrangement of spines, shape and size of the cerci, and operculum (Salmon, 1955, 1991). The taxonomy of the genus has had a confusing history with different authors recognizing different numbers of species and subspecies (e.g., Salmon, 1955, 1991; Jewell and Brock, 2002). Many of the species are very similar and are differentiated by subtle differences in spination and egg sculpturing. The genitalia are virtually identical among all of the species (Salmon, 1955, 1991), including the structure and arrangement of the gonaphyses (Buckley, unpubl.), which indicates potential taxonomic oversplitting.

The closest relatives of *Acanthoxyla* are *Clitarchus hookeri* White and *Pseudoclitarchus senta* Salmon (Buckley et al., unpubl.), which are also endemic to New Zealand (Salmon, 1948, 1955, 1991; Jewell and Brock, 2002). *C. hookeri* is widespread throughout large areas of New Zealand. It includes populations with both sexes as well as populations with females only (Buckley, unpubl.), and is apparently a geographic parthenogen. *P. senta* has a single sexual population restricted to the Three Kings Islands, off the northern tip of New Zealand (Salmon, 1948).

Morgan-Richards and Trewick (2005) found that *Acanthoxyla* had a unique group of mitochondrial DNA (mtDNA) haplotypes (at *cytochrome oxidase I* and *II*), that were 10% divergent from haplotypes of *C. hookeri*. However, there was relatively little divergence ($\leqslant$2.2%) between any two species of *Acanthoxyla*. Most strikingly, Morgan-Richards and Trewick (2005) found that *Acanthoxyla* shared haplotypes with *C. hookeri* at the *Internal Transcribed Spacer* (*ITS*) of ribosomal DNA (rDNA). Based on this evidence, these authors proposed the hypothesis that two or more hybridization events between *C. hookeri* and an unknown taxon led to the origin of *Acanthoxyla*. They suggested *P. senta* or an extinct sexual lineage of *Acanthoxyla* as a possible maternal ancestor, although *P. senta* was not sampled. One line of evidence against this hypothesis was the karyotypes they presented for *Acanthoxyla*, which did not show the expected evidence of hybridity or structural match to *C. hookeri*. To explain this, Morgan-Richards and Trewick (2005) invoked rapid chromosome remodeling in *Acanthoxyla*.

When used in isolation, neither of the loci used by Morgan-Richards and Trewick (2005) is an ideal marker for tracing a history of hybridization. mtDNA provides evidence of only one of the parental species (the maternal). Ribosomal DNA (rDNA), including *ITS*, occurs in many copies and is subject to unusual evolutionary dynamics. In some parthenogenetic hybrids, the rDNA contribution of one of the parental species is preferentially erased due to biased gene conversion (Hillis et al., 1991). To test the hypotheses advanced by Morgan-Richards and Trewick (2005), and further explore the evolutionary history of hybridization and parthenogenesis in the group, we have amplified and sequenced the alleles of two single-copy nuclear loci (scnDNA) from 11 individuals across three species of *Acanthoxyla*. The potential power of this class of molecular data for elucidating the evolutionary history of parthenogenetic lineages has long been discussed (Birky, 1996; Judson and Normark, 1996; Normark et al., 2003), but there are still very few taxa for which such data have yet been obtained (Delmotte et al., 2003; Mark Welch and Meselson, 2000). In addition to expanding the molecular sampling to include this crucial new class of markers, we have also expanded taxonomic sampling to include the crucial taxon *P. senta*. Because this sexual species was originally placed in *Acanthoxyla* by Salmon (1948), it clearly has the potential to be an ancestor.

2. Methods

2.1. Species sampling

Morgan-Richards and Trewick (2005) noted the lack of correspondence between morphological and molecular diversity in *Acanthoxyla*. We independently made a similar observation (Buckley and Jewell, in prep.) and designed our sampling for this study to maximize molecular, rather than morphological or taxonomic diversity. We sampled 11 individuals from the genus *Acanthoxyla* including 3 individuals of *A. inermis* Salmon, 1 individual of *A. intermedia* Salmon and 7 individuals of *A. geisovii* Kaup (Table 1 and Fig. 1). Although there exist five additional nominate species of *Acanthoxyla* that are not included in this study, our sampling includes all major mtDNA lineages in the genus that we have identified to date. These three species also cover the broad range of morphological diversity in the genus, ranging from the spineless *A. inermis* to the often heavily spined *A. geisovii*. Furthermore, from the outset of this study we decided to focus resources on gathering sequence data from multiple loci, and sequence multiple clones per PCR product rather than a large number of individuals. This approach is justified because for a study such as this, sampling multiple loci and accurately resolving the multilocus genotypes through cloning provides more power than sampling large numbers of individuals for only a single locus such as mtDNA.

Species were identified principally using the color of spines on the dorsal surface of the head and relative sizes of spines on the mesonotum and abdominal tergites. These characters are more reliable than the key from Salmon (1991), which relies on characters such as foliaceous lobes, size and shape of the basal opercular spine, all of which are highly variable. The most recent revision (Salmon, 1991) of the genus *Clitarchus* recognized two species within New Zealand, *C. hookeri* and *C. tuberculatus* Salmon. However, we can find no diagnostic morphological characters to dif-