



# Multiple lines of evidence suggest mosaic polyploidy in the hybrid parthenogenetic stick insect lineage *Acanthoxyla*

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**Abstract.** 1. Although hybridisation is common in animals, it rarely results in speciation. Yet, many examples of hybrid species have been documented in one animal group, the stick insects (Phasmida).

2. The New Zealand stick insect *Acanthoxyla* is of particular interest as the entire genus is of hybrid origin and consists of eight morphological forms recognised as species, all of which are obligate parthenogens.

3. Using five complementary techniques on the same individuals, our study confirms that both triploids and diploids are present in *Acanthoxyla* populations, and further, that some individuals contain both diploid and triploid cells.

4. Chromosome spreads and estimates of relative DNA content from flow cytometry provided contrasting information about the ploidy of this unusual parthenogenetic genus.

5. Analysis of morphometric variation showed no correlation with ploidy level in *Acanthoxyla*, and also mtDNA sequence networks failed to distinguish morphospecies or ploidy level.

6. Unexpectedly, cloned sequences of a putatively single-copy nuclear marker were also unhelpful in distinguishing ploidy, instead indicating that phosphoglucose isomerase is likely to be a multiple copy gene.

7. We propose a mechanism for the evolution of the *Acanthoxyla* lineage and suggest that interpretation may be complicated by the presence of individuals that are diploid and triploid mosaics.

**Key words.** *Acanthoxyla*, asexual, biodiversity, chromosome mosaic, hybrid species, phasmids, triploids.

## Introduction

Hybridisation is the process of individuals from genetically distinct populations mating and producing viable offspring (Harrison, 1993) and it can be an important generator of biodiversity (Bullini, 1994; Arnold, 1997). Although hybridisation commonly results in homogenisation of populations, a new species can arise in a single

generation when hybrid offspring form a lineage that is reproductively isolated from their two parental taxa (Coyne & Orr, 2004). Parthenogenetic reproduction is one such mechanism by which animal interspecific hybrids can become reproductively isolated from their parental taxa. For example, in the aphid genus *Rhopalosiphum*, obligate parthenogenetic strains have arisen after hybridisation of two sexual species (Delmotte *et al.*, 2003). Interspecific hybridisation has been suspected as a general mechanism for the origins of unisexual species and is well documented in *Bacillus* stick insects (Mantovani *et al.*, 2001), *Otiorrhynchus* weevils (Tomiuk & Loeschcke, 1992), *Warramaba* grasshoppers (Honeycutt & Wilkinson, 1989), and *Calligrapha* beetles (Gómez-Zurita *et al.*, 2006).

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Many parthenogenetic lineages of stick insect (Phasmatodea) have originated via hybridisation (Bullini, 1994; Ghiselli *et al.*, 2007; Schwander & Crespi, 2009). In phasmids, changes in ploidy level sometimes accompany the origination of hybrid lineages. For example, the Spanish stick insect *Leptynia hispanica* is a complex of diploid bisexual populations and polyploid parthenogenetic lineages. The triploid lineage of *L. hispanica* has arisen via hybridisation independent of the tetraploid lineage within this complex (Ghiselli *et al.*, 2007). Individuals of hybrid taxa sometimes further hybridise with related bisexual individuals giving rise to further hybrid species, often with elevated ploidy (Bullini, 1994; Milani *et al.*, 2010). Yet, some parthenogenetic triploid stick insects have been inferred to have arisen without hybridisation (e.g. *Bacillus atticus* has both diploid and triploid parthenogenetic races, Scali *et al.*, 2003).

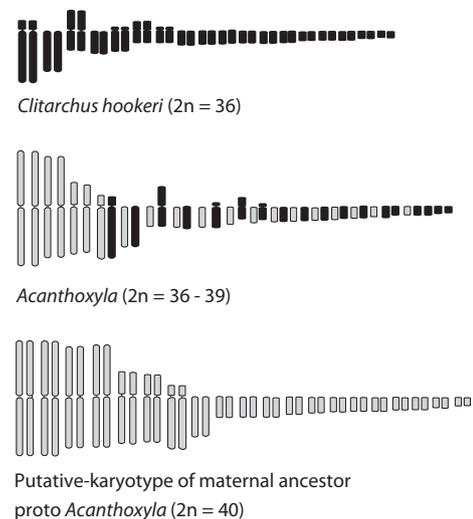
In New Zealand, the stick insect genus *Acanthoxyla* is entirely female and thus obligatorily parthenogenetic. Eight morphological variants have been defined as subspecies or species (Salmon, 1955, 1991; Jewell & Brock, 2002), although evidence for correlation between morphology and parthenogenetic lineages is equivocal (Morgan-Richards & Trewick, 2005). These morphological variants differ in their degree of spination, ranging from *Acanthoxyla huttoni* (Salmon) with numerous long, sharp, black tipped spines on head, thorax, and abdomen to *A. inermis* (Salmon) with only a few blunt bumps (tubercles) on head and thorax.

How the *Acanthoxyla* lineage arose is not well understood. Evidence for a hybrid origin for *Acanthoxyla* was provided by mtDNA and nuclear DNA sequence analysis (Morgan-Richards & Trewick, 2005; Buckley *et al.*, 2008). *Acanthoxyla* has low mitochondrial sequence diversity (cytochrome oxidase I and cytochrome oxidase II), different morphotypes do not form distinct mtDNA clades, and no maternal sexual species has been found (Trewick *et al.*, 2008). Nuclear ITS sequences suggested that *Clitarchus hookeri* (White) was the paternal ancestor (or descendant of the ancestor) and evolution of *Acanthoxyla* might have involved more than one hybridisation event (Morgan-Richards & Trewick, 2005). Also, individuals of *Acanthoxyla* have been found with two or more alleles of a putatively single-copy nuclear locus that differed from those found in *Clitarchus*. This is consistent with the idea that *Acanthoxyla* hybrid origins involved now extinct lineages of *Clitarchus*. Buckley *et al.* (2008) found that five of the nine *Acanthoxyla* individuals they studied possessed three alleles at a putatively single-copy nuclear locus leading to the conclusion that they were triploids, and a more complex pattern of hybridisation was inferred.

Chromosome counts of *Acanthoxyla* have recorded similar or identical chromosome numbers in *Acanthoxyla* and *Clitarchus* ( $2n = 36-39$ ; Parfitt, 1980; Morgan-Richards & Trewick, 2005), implying that *Acanthoxyla* are diploid. Triploid *Acanthoxyla* would be expected to have approximately 54 chromosomes (about 1.5 times the number in

*C. hookeri*). Karyotypes of *Acanthoxyla* and *Clitarchus* are very different. *Acanthoxyla* has 11 large metacentric (centromere in the middle) or submetacentric (centromere not in the middle, but distinctly biarmed) chromosomes, whereas *C. hookeri* has only four metacentric or submetacentric chromosomes (Morgan-Richards & Trewick, 2005). Using the *C. hookeri* karyotype as a guide, it is possible to hypothesise which chromosomes within *Acanthoxyla* are most likely to have come from each presumed parental species (Fig. 1).

Here, we further address this ploidy enigma and ask the question; does *Acanthoxyla* comprise diploid, triploid or mixed ploidy individuals? We approach the problem using three different forms of information for a set of *Acanthoxyla* individuals: estimates of relative DNA content; cytogenetic chromosome counts; and nuclear sequence variants [phosphoglucose isomerase (PGI)]. These methods should provide an empirical basis for deciphering an organism's ploidy level, and used in combination should have sufficient power to resolve the ploidy of *Acanthoxyla*. In addition, morphometrics and mitochondrial DNA sequencing (COI and COII) of the same individuals are used to determine (i) whether there is a correlation between body size and inferred ploidy level, and (ii) whether inferred ploidy level groups are monophyletic. Obligate parthenogenetic reproduction renders the mtDNA locus highly appropriate as the gene tree will be the 'species' (or lineage) tree.



**Fig. 1.** Karyotype ideograms of New Zealand stick insects based on karyotypes presented in Morgan-Richards & Trewick, 2005. The predicted composition of *Acanthoxyla*'s maternal ancestor (proto *Acanthoxyla*) is inferred by subtracting a haploid *Clitarchus* karyotype from the *Acanthoxyla* karyotype, and doubling to create a diploid. The black chromosomes in the *Acanthoxyla* sp. karyotype correspond to a haploid set from the hypothesised parental species, *Clitarchus hookeri*. Note the presence of large metacentric chromosomes in *Acanthoxyla* (see also Fig. S1).

## Materials and methods

### Sampling

Nineteen adult *Acanthoxyla* stick insects were collected throughout New Zealand and make up the majority of biological material used in this study. Four morphotypes were represented (Salmon, 1991; Jewell & Brock, 2002); six *A. inermis* Salmon, 1955; three *A. intermedia* Salmon, 1955; six *A. prasina* (Westwood 1859), and four *A. nr-geisovii* (Kaup 1866) (Table S1). The species were distinguished on the basis of characteristics described by Salmon (1991) as follows: *A. inermis* without spines and tubercles sparse, operculum with broad basal tubercle tapering to a long thin point; *A. intermedia* with few spines and tubercles sparse, operculum with broad blunt spine, apex of operculum attenuated to a sharp point that reaches beyond tip of abdomen; *A. prasina* with moderate number of spines restricted to head, mesothorax and metathorax, operculum with broad short spine; *A. nr-geisovii* with many black spines on head, thorax, and abdomen, operculum boat shaped and lateral flanges on abdominal tergites IV to VI (largest on VI) unlike *A. prasina*, but differing from *A. geisovii* sensu Salmon (1991) by the lack a prominent pair of larger spines on the mesothorax. The probable paternal species, *C. hookeri* ( $n = 10$ ) was collected from a bisexual population near Lake Karapiro, Waikato (E1830670; S5796210) for comparison of genome size and morphology. Our strategy was to maximise the sampling of morphometric, cytogenetic, relative DNA content, and DNA sequence data from the same stick insects (Table S2).

### Cytogenetics

Chromosome preparation followed the procedure of Morgan-Richards (2005) without colchicine pre-treatment. Although colchicine can improve yields of chromosome spreads, it prevents spindle fibre formation so that the chromosomes do not separate. This can result in false polyploid cells, which would be misleading in a study of polyploidy. Previous experience has shown that colchicine pre-treatment does not produce significantly more chromosome spreads with *Acanthoxyla* (M. Morgan-Richards, unpubl. data). *Acanthoxyla* were held at  $-20^{\circ}\text{C}$  for 20–30 min to kill them, prior to dissection. Ovarian follicles were removed and submerged for 15 min in a hypotonic solution (0.144% NaCl). Microscope slides were prepared from ovariole tips after fixation in fresh methanol/acetic acid (3:1), teasing and dispersing in 45% acetic acid, drying on a hot plate ( $50^{\circ}\text{C}$ ) and, Giemsa staining. Resulting slides were surveyed systematically for mitotic cells with visible chromosomes that were spread sufficiently. Chromosomes were counted and photographed using a Zeiss Axiophot Compound light microscope in bright field, with attached DFC320 digital camera, and LAS version: 3.3.0 software (Leica Microsystems Ltd., Heerbrugg, Switzerland) was used to obtain

images. For each individual, a minimum of two spreads consisting of well resolved, non-overlapping chromosomes were recorded.

### Genome size estimates with flow cytometry

DNA flow cytometry is a rapid way of estimating DNA quantity in cell nuclei and has been a popular method for screening ploidy, mixoploidy, and aneuploidy as there is a close relationship between DNA content and chromosome number (Husband & Schemske, 2000; Dolezel & Bartos, 2005).

Nuclei were isolated from stick insect leg tissue of 16 *Acanthoxyla* and two *C. hookeri*. Samples were stored in 95% ethanol for periods ranging from 1 month to 2 years. Our protocol followed that of Galbraith *et al.* (1983) with modifications (Morgan-Richards, 2005). Samples consisting of 10 mm of tibia were cut from each stick insect and chopped in a Petri dish with nuclear isolation buffer. Commercial UV 2.5  $\mu\text{m}$  calibration beads (Partec code 05-4020) were used as an internal standard. Calibration beads provide a constant value, enable confirmation of flow stability and normalise fluorescence and cell scatter. The DNA stain DAPI is a non-intercalating dye that allows genome size to be estimated, and provides results similar to those obtained using other fluorochromes (Johnston *et al.*, 1999). Nuclei sizes were measured using a Partec PA-11 particle analysing system flow cytometer. This yielded a target peak value with coefficient of variation (CV), calibration bead peak value, and CV. The ratio of the target peak over standard peak provided information on relative DNA content that could be compared among samples. The CV is the standard deviation/peak mean  $\times 100$ , and unlike the standard deviation is not dependent on the value of the peak mean, so the precision of measurements with peaks at different positions may be directly compared (Dolezel & Bartos, 2005). A post hoc ANOVA implemented in Minitab version 5.0 (Minitab Inc., State College, PA, USA) was employed to compare relative nuclear DNA content.

### DNA sequencing: mtDNA and PGI

For each specimen, a leg was removed for DNA extraction using a salting-out method (Sunnucks & Hales, 1996; Trewick *et al.*, 2005). For 16 *Acanthoxyla*, partial mitochondrial cytochrome oxidase subunit I and II were amplified and sequenced (Morgan-Richards & Trewick, 2005) using primers C1-J-2195, L2-N-3014, TL2-J-3034, and TK-N-3785 (Simon *et al.*, 1994). For five *Acanthoxyla*, the putatively single-copy nuclear gene PGI was amplified using primers PGI-271 and PGI-2047 (Buckley *et al.*, 2008). Polymerase chain reactions were performed in 20  $\mu\text{l}$  containing: 200  $\mu\text{M}$  dNTPs, 2 mM  $\text{MgCl}_2$ , 1  $\mu\text{M}$  primers, and 0.20 U of RedHot DNA polymerase (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 1–10 ng template DNA. PCR cycling conditions were

94 °C for 1 min, followed by 35 cycles of 94 °C for 30 s, annealing temperatures of 55 °C (COI) or 53 °C (PGI) for 30 s, 72 °C for 80 s and one cycle of 72 °C for 10 min. Purified PCR products were sequenced using Big Dye chemistry (Perkin Elmer, Waltham, MA, USA) following manufacturer's protocols, with automated reading on an ABI3730 (Massey University Genome Service). Sequences were viewed and edited in Sequencher v.4.1 (ABI, PE), before being transferred into SeAl v.2.0a3 (Rambaut, 1996) for alignment.

Amplified PGI products were first cloned to ensure resolution of allelic diversity within *Acanthoxyla* individuals. This study focused on surveying sequence diversity within individuals, and therefore targeted a large number of clones from the same individual rather than PCR products from many individuals. A sample of stick insects was selected based on data from DAPI flow cytometry that indicated they were triploid (Ax.PN-381, Ax.Daw-471, Ax.Pun-412) or diploid (Ax.Owk-517, Ax.Azc-524). For each, two independent PCR products were ligated and cloned to minimise unrecognised Taq polymerase error. DNA cloning used pGEM-T vector and the pGEM-T cloning kit (Promega, Fitchburg, WI, USA). After ligation into the vector, transformation into competent cells (MAX efficiency DH5alpha; Invitrogen, Carlsbad, CA, USA) followed manufacturer's instructions. PCR was performed for 11–20 isolated clones for each stick insect DNA using standard conditions, M13 forward and reverse primers, and Red Hot DNA polymerase (ABgene). PCR cycling conditions were: denaturing cycle of 94 °C for 1 min, followed by 30 cycles of 94 °C for 20 s, 53 °C for 20 s, 72 °C for 1 min, and finally an extension step of 72 °C for 5 min. Cloned PCR products were sequenced as above.

Published sequences were downloaded from GenBank to complement the new mtDNA and PGI sequences (Tables S1 and S3). We constructed a minimum spanning network for the mtDNA haplotypes using the parsimony criterion. We used Splittree v4.1 (Huson & Bryant, 2006) to construct a network of PGI sequence variants. We tested for recombination in PGI using the DualBrothers programme (Minin *et al.*, 2005) as implemented by Geneious v5.5 (Drummond *et al.*, 2010), examining five sequences where recombination was suspected, and five which showed no obvious signs of recombination.

#### Morphometrics

Eight morphological characters were measured for 16 adult *Acanthoxyla* and 10 adult female *C. hookeri*, using vernier callipers accurate to 0.05 mm. Measurements were taken of head length, thorax length, thorax width where hind legs join, length of tibia and femur in left forelimb (or right if left was missing). Discriminate analyses implemented in SPSS v.18.0 (SPSS Inc., Chicago, IL, USA) were used to examine morphological variation among stick insects coded by genus and ploidy level as resolved

by flow cytometry, to test for any effect of genome size on body size.

## Results

### Cytogenetics

Mitotic cells from 16 *Acanthoxyla* had 37 or 38 chromosomes, consistent with previous counts for *Acanthoxyla* (38, 39: Parfitt, 1980; 36, 38: Morgan-Richards & Trewick, 2005). If *Acanthoxyla* is of hybrid origin and the two parental species had different numbers of chromosomes, then a resulting female diploid could have an uneven chromosome number. But three individuals in this study each had far more chromosomes than expected (Table 1). Cells in Ax.Wes-408 (*A. prasina*), Ax.PN-409 (*A. inermis*), and Ax.PN-381 (*A. intermedia*) had 53 or 54 chromosomes, up to 1.5 times greater than any previously published *Acanthoxyla* karyotypes and near the expected number for a hybrid triploid. Two of the specimens with these triploid chromosome counts (Ax.Wes-408, Ax.PN-409) also had mitotic cells with chromosome counts of 38 (Fig. 2), while all spreads observed for Ax.PN-381 had 53 chromosomes. The triploid karyotypes of these three stick insects included 18 large metacentric and submetacentric chromosomes, as expected if they consist of a diploid *Acanthoxyla* set plus a haploid *C. hookeri* set (Fig. 1; Fig. S1).

### mtDNA haplotypes

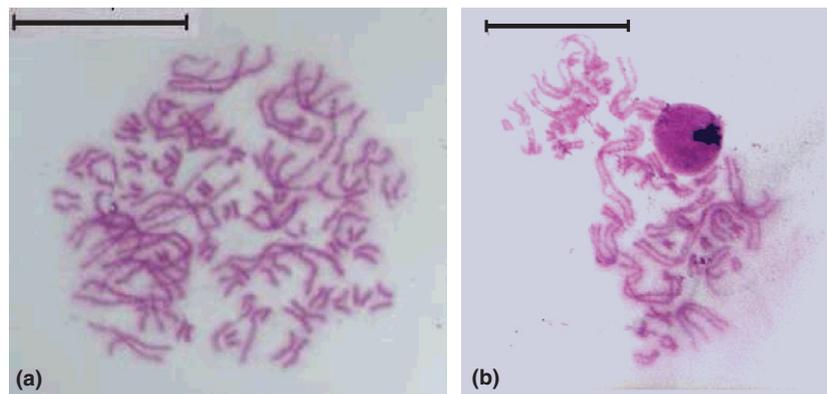
DNA sequences were obtained for a 1246 bp fragment comprising parts of the mitochondrial COI and COII genes. This fragment revealed 13 haplotypes among 16 *Acanthoxyla* specimens (Table S2). We combined these new mtDNA data with those from previous studies (Morgan-Richards & Trewick, 2005; Buckley *et al.*, 2008) to estimate the relationships and distribution of mtDNA lineages in 60 *Acanthoxyla* stick insects. A minimum spanning network revealed three major lineages (Fig. 3a and b). Two of these major lineages (haplogroups A and B), are as previously reported (Morgan-Richards & Trewick, 2005). Diploid *Acanthoxyla* identified by relative DNA content are within either haplogroup A or B. Triploids identified in this study had either A or B or C haplogroup mitochondrial sequences (Fig. 3a; Table S1). Stick insects within haplogroup A were predominantly smooth animals (*A. inermis*), and stick insects in haplogroup B were predominantly spiny forms (Fig. 3a). Haplogroup A and putatively diploid *Acanthoxyla* have not yet been collected from South Island New Zealand.

### Relative DNA content: flow cytometry with DAPI

All stick insects produced single peaks of cells in the same stage of cell cycle, from which relative DNA content

**Table 1.** Lack of concordance among methods makes inference about *Acanthoxyla* ploidy non-trivial. Ploidy inferred from estimates of relative DNA content from flow cytometry of leg tissue is inconsistent with direct chromosome counts of mitotic cells in ovarian follicles. Numbers of PGI sequence variants encountered by cloning PCR products are conservative and exclude sequence variation involving fewer than three substitutions in each 480 bp sequence, if detected in only one PCR product. mtDNA haplogroups are as in Fig. 3. Species identification: i.e. *A. inermis*; p *A. prasina*; it *A. intermedia*, g *A. nr-geisovii*.

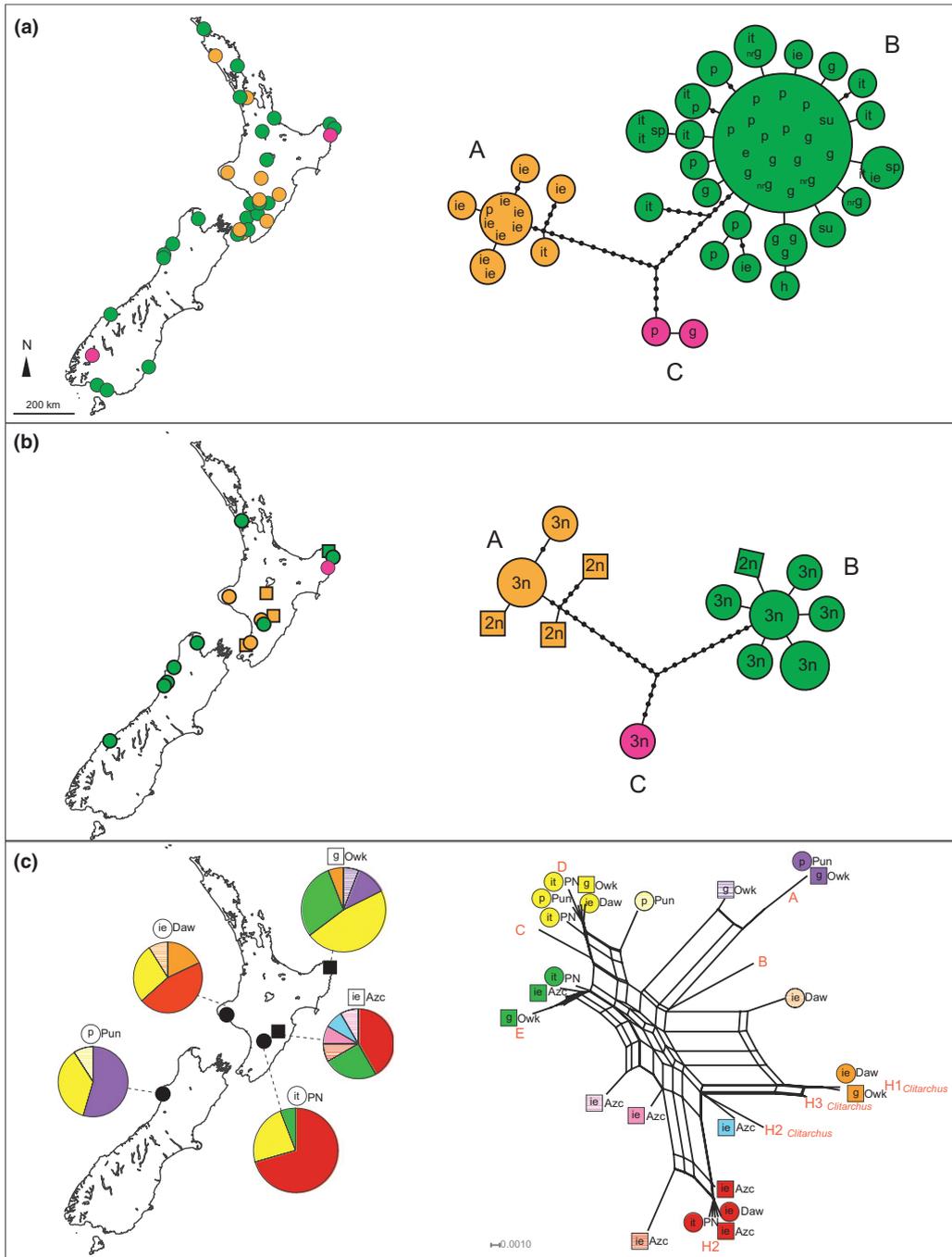
Stick insect sample code	Ploidy flowcytometry		Ploidy chromosomes		PGI sequence variants				mtDNA haplogroup			Species			
	2n	3n	2n	3n	3	4	5	6	A	B	C	ie	it	p	g
AxPN381		■		■	■						■				
Ax.Pun-412		■		■	■						■				
Ax.Daw-471		■		■			■				■				
Ax.Azc-524	■			■				■			■				
Ax.Owk-517	■			■				■							■
Ax.Wes-408		■		■							■			■	
Ax.PN-409		■		■							■				
Ax.Haa-399		■		■							■				■
Ax.Pun-398		■		■							■		■		
Ax.Ar-470		■		■							■				■
Ax.Ec-521		■		■							■				■
Ax.lot-510		■		■							■	■			
Ax.Ack-476		■		■							■				■
Ax.Par-504		■		■							■				■
Ax.Par-503	■			■							■				
Ax.Rtk-465	■			■							■			■	
Ax.PN-new	■			■							■				
Ax.PN-418		■		■										■	
Ax.Ack-477		■		■											■



**Fig. 2.** Chromosome spreads obtained from ovariole tips of *Acanthoxyla prasina* specimen Ax.Wes-408 exhibiting both diploid and triploid cells, scale bar = 20  $\mu$ m. (a) Cell with 54 chromosomes. (b) Cell from the same ovarian tissue with 38 chromosomes. Note the large metacentric chromosomes. Because of the large number of chromosomes per cell, all spreads obtained had chromosome overlap (as shown) which prevented construction of triploid karyotypes.

was estimated. Results were not sensitive to the age of samples or preservation in ethanol. The *Clitarchus* samples gave a peak ratio of 1.5 compared to the calibration bead standard. The DNA content values of the 16 *Acanthoxyla* individuals separated into two non-overlapping groups that differed significantly in relative DNA content (ANOVA  $F = 408$ , d.f. = 1,14,  $P < 0.001$ ). Five *Acanthoxyla* stick insects gave an average peak ratio of

1.67 compared to calibration beads and 11 gave an average peak ratio of 2.68. The smaller *Acanthoxyla* genome was indistinguishable in size from the genome of *Clitarchus* when we experimentally combined them in the same flow run, and was thus inferred to be diploid. The estimated relative DNA contents of 11 *Acanthoxyla* with the larger average peak ratio were approximately 1.5 times larger than the smaller *Acanthoxyla* value, and the *Clitar-*



**Fig. 3.** Distribution of genetic and morphology variation in the New Zealand stick insect *Acanthoxyla*; (a) Distribution of mtDNA (COI and COII) sequence variation in 60 *Acanthoxyla*. Colours represent three main haplogroups as illustrated with a minimum spanning network. Species identification based on morphological variation is indicated with letter codes: i.e. *A. inermis*; p *A. prasina*; it *A. intermedia*, g *A. geisovii*, sp *A. speciosa*, su *A. suteri*, nrg *A. nr-geisovii*; h *A. huttoni*. Haplotypes that were not sampled are represented by small black circles; large coloured circles are proportional to observed haplotype frequency in the sample. (b) Distribution of diploid (squares) and triploid (circles) *Acanthoxyla* stick insects inferred from genome-size estimates using DAPI stain flow cytometry. Evolutionary relationships of these parthenogenetic individuals are represented using a minimum spanning network of mtDNA sequences. (c) Sequence diversity of PGI from five *Acanthoxyla* individuals. Pie charts show relative frequency of main sequence variants obtained from cloning PCR products. Pie area is proportional to the number of clones sequenced per individual. Sequence variation was used to construct a Neighbour-net network revealing phylogenetic uncertainty (boxiness of internal edges) resulting from recombination. Colours indicate the main PGI sequence variants, labelled in scarlet with codes (*Acanthoxyla*, A–E; *Clitarchus* H1–H3) reported by Buckley *et al.*, 2008. Apparently recombinant sequences are indicated by hatched fill.

*chus* genomes, as expected for triploid individuals. Putative triploids identified in this way came from locations in both North Island and South Island (Fig. 3b). When considered by morphotype, we found among our flow cytometry sample that three *Acanthoxyla nr-geisovii* were triploid and one was diploid, three *A. prasina* were triploid, two *A. intermedia* were triploid and one was diploid, three *A. inermis* were diploid and three triploid (Table 1).

#### PGI sequences

We sequenced a minimum of 11 clones (480 bp) for each of five individual stick insects after amplification of PGI and transformation into plasmids. The total number of unique sequences per stick insect ranged from 4 to 11 including sequences obtained only once: Ax.PN-381 (3n from relative DNA content) had eight different PGI sequences; Ax.Owk-517 (2n) had eleven; Ax.Daw-471 (3n) five; Ax.Pun-412 (3n) four; and Ax.Azc-524 (2n) had six PGI sequences (Fig. 3c). A test for recombination using new and published sequences suggested recombination was likely to have produced a subset of sequences (Ax23Cl3, Ax.Owk-517\_Af28; Ax.Owk-517\_Af29; Ax.PN-381\_Ah4, and Ax.Daw-471.3). Analysis with the programme DualBrothers predicted the site of recombination to be 124 bp from the forward primer. Examination of the Splitstree network was consistent with this inference, with a number of sequences appearing to have mixed phylogenetic signal (hatched symbols; Fig. 3c).

Sequence alignments revealed that some variation involved single nucleotide substitutions that resulted in minor variation from one of the more common sequence types. As polymerase and cloning errors are plausible sources of some of the observed sequence variation we took a conservative approach and regarded sequences as unique only if they differed by more than three nucleotide substitutions, or were found in clones from different PCR products, or different individuals or different species (*C. hookeri*). This provided an estimate of PGI diversity of between three and six distinct sequences per stick insect: three in Ax.PN-381 (3n); five in Ax.Owk-517 (2n); four in Ax.Daw-471 (3n); three in Ax.Pun-412 (3n); and six in Ax.Azc-524 (2n) (Table 1; Fig 3c). Clusters of PGI sequences (regarded as distinct) are colour coded on the Splitstree network based on sequence similarity (Fig. 3c). The *Acanthoxyla* we studied had PGI sequences similar or identical to published sequences for this genus and *C. hookeri* (Buckley *et al.*, 2008), and thus we annotate these main variants on the network (Fig. 3c). PGI sequences are deposited in GenBank (Table S1).

#### Morphometrics

*Acanthoxyla* body length measured from the base of antennae to end of abdomen, ranged between 62 and 92.05 mm, with an average length of 77.84 mm

(SD = 8.9). Leg length ranged from 26.8 to 41.47 mm with an average of 35.67 mm (SD = 6.05). A stepwise discriminant analysis with cross validation conducted on body size data (combining 8 dimensions) for female *C. hookeri* and *Acanthoxyla* found significant differences between these two genera (Wilks'  $\lambda = 0.367$ ,  $\chi^2 = 1.242$ ,  $P < 0.01$ ). The analyses classified 23 of the 26 samples into the correct group, so additive size is a valid predictor of which genus a stick insect belongs to in 88% of cases. The feature that predicted species the best was thorax width. This is consistent with field observations that *Acanthoxyla* are 'wider' than *Clitarchus*.

Discriminant analysis with cross validation was conducted on 16 *Acanthoxyla* specimens, using the same character set and incorporating ploidy level as inferred from relative DNA content (DAPI flow cytometry). Four specimens were excluded from this analysis due to missing data. Of the eight measurements, there were no significant morphometric differences between the two ploidy levels (Wilks'  $\lambda = 0.883$ ,  $\chi^2 = 26.6$ ,  $P = 0.871$ ). Additive size was a poor predictor of ploidy with only 4 of 16 individuals being grouped correctly in this discriminate analysis.

#### Results summary

For 16 *Acanthoxyla* individuals, we were able to gather the four characters we set out to record (Table 1; Table S2); estimates of relative DNA content, chromosome counts, morphology, and mtDNA sequence data. For five of these individuals we also obtained PGI sequence data from at least 11 cloned PCR products each, but we found little consistency in ploidy level inference (Table 1). Estimates of relative DNA content provided the strongest evidence that our sample of 16 *Acanthoxyla* stick insects comprised five diploids and eleven triploid individuals. Although chromosome counts are usually considered robust indicators of ploidy, we observed that, unusually, some individuals had cells with diploid chromosome numbers and cells with triploid chromosome numbers. From these observations, we conclude that the direct counts of chromosomes give only partial information on ploidy for individuals of this genus. Of the 11 triploids inferred from relative DNA content, only one also had a triploid chromosome count and one had both triploid and diploid chromosome numbers, the remaining nine had diploid chromosome counts (Table 1). Neither morphology nor mtDNA haplotype data suggest that the apparently triploid and diploid individuals (identified by either relative DNA content or mitotic chromosome counts) are distinct lineages (Fig. 3). Additional data from sequencing clones of a putatively single-copy nuclear locus (PGI) for five of these stick insects also failed to provide the corroborating evidence expected; conservative estimates of the number of sequence variants per insect reached six in a diploid rather than the expected maximum of two.

## Discussion

The formation of plant and animal species through hybridisation is an important source of biodiversity (Bullini, 1994; Soltis & Soltis, 1999; Morgan-Richards *et al.*, 2009). The number of animal taxa that are now recognised as products of interspecific hybridisation is growing, fuelled by molecular evidence. Genetic data have the capacity to reveal fine details of species formation (Harrison, 1993; Noor & Feder, 2006) and the present study of *Acanthoxyla* exemplifies this. The genus *Acanthoxyla* is recognised as having eight species, and this degree of morphological variation is unusual for a lineage of obligate parthenogens. Mismatches between geographic distribution, gene trees, and morphology had indicated that unravelling the story of these insects will not be simple, but could improve our understanding of hybrid origins, parthenogenesis, and the origin and maintenance of sexual reproduction.

### *The ploidy level of Acanthoxyla*

All information so far indicates that the related New Zealand stick insect *C. hookeri* is diploid (Parfitt, 1980; Morgan-Richards & Trewick, 2005; Buckley *et al.*, 2008). Considering *C. hookeri* and *Pseudoclitarchus* are phylogenetically sister to *Acanthoxyla* (Morgan-Richards & Trewick, 2005; Buckley *et al.*, 2008; Trewick *et al.*, 2008; Buckley *et al.*, 2010), we would expect their genome size to be similar if *Acanthoxyla* is diploid, or proportionately equivalent if polyploid (Fig. 1). We thus expect a triploid *Acanthoxyla* genome would be approximately 1.5 times the size of the *Clitarchus* genome (i.e. equivalent to three sets of chromosomes). The precision of this estimate is dependent on the unknown exact karyotype of ancestral *Clitarchus* and proto *Acanthoxyla* diploids. It is not uncommon for triploids to have chromosome numbers that are not integer multiples of related diploids (e.g. Lachowska *et al.*, 2008; Milani *et al.*, 2010). Relative measures of genome size of *Acanthoxyla* using flow cytometry with DAPI stain did indeed reveal two distinct classes of DNA content; smaller genomes indistinguishable from *Clitarchus*, and genomes approximately 1.5 times as large. Using this information we inferred the existence of both diploid and triploid *Acanthoxyla* individuals. These measurements of relative DNA content indicated that 11 *Acanthoxyla* in our sample were triploid, but direct chromosome counts of mitotic cells identified only three triploid individuals. Although all triploids inferred from cytogenetics did have triploid sized genomes, others gave contrasting data (Table 1). In each of the tibia samples used for flow cytometry, the cell populations were in the same, single stage of the cell cycle and there was no evidence of mixed ploidy.

Chromosome spread analysis is the traditional and most straightforward method for determining ploidy number and has been applied extensively since the first

chromosomal study demonstrating different ploidy numbers in *Oenothera lamarckiana* (Lutz, 1907). Preparing chromosome spreads is relatively simple, but is a time consuming method, and requires fresh tissue, which can be challenging when collecting in remote areas. Using cytogenetics of ovarian follicles, we found that the majority of *Acanthoxyla* individuals in our sample were diploid ( $2n = 37$  or  $38$ ), while three individuals had chromosome numbers ( $53$  or  $54$ ) indicative of triploids. This pattern is consistent with other stick insects where hybridisation has resulted in triploid species, such as the Mediterranean genus *Bacillus*; triploid *B. lynceorum* ( $2n = 51$ ) have approximately 1.5 times the chromosome number of diploid *B. rossius* ( $2n = 36$ ), *B. grandii* ( $2n = 34$ ), and the parthenogenetic *B. whitei* ( $2n = 35$ ; Mantovani, 1998). In *Acanthoxyla*, triploid karyotypes were observed in specimens exhibiting three distinct external morphologies (*A. prasina*, *A. intermedia*, *A. inermis*). Significantly, and most surprisingly, triploid and diploid karyotypes were present within the same individual with both triploid and diploid cells found in the same tissue of two *Acanthoxyla*.

### *Embryos, endopolyploidy, and mosaics*

Chromosome preparations in this study came from the females' ovarioles, and thus the dividing cells might belong to the developing embryos of her offspring, not of the female herself. However, embryo development in these and other stick insects does not occur until after eggs are ejected. The majority of tissue in ovarian follicles contributes to the yolk rather than to the embryo. Studies of phasmid species using freshly deposited eggs show that the first embryo cell divisions occur long after leaving the tips of the ovarioles (Marescalchi & Scali, 2001). In *Acanthoxyla*, eggs dissected weeks after being deposited were entirely yolk filled, with embryos not visible until at least 8 weeks (M. Morgan-Richards, pers. observation). Embryo development is therefore unlikely to have contributed to the observations reported here.

Chromosome replication without cell division (endoreplication) results in polyploid cells. Many animals and plants have cell differentiation that involves endoreplication, resulting in elevated ploidy in particular tissues (Lee *et al.*, 2009). Endopolyploidy has been reported in a number of insect species (grasshoppers, beetles, flies pond skaters), and in all cases involves clearly delineated genome doublings ( $4\times$ ,  $8\times$ ,  $16\times$  etc.) and thus never results in triploid cells (White, 1977; Lee *et al.*, 2009). Endopolyploidy is therefore also unlikely to explain our observations.

Mosaics are organisms containing populations of genetically distinct cells, which have arisen from a common original zygote (unlike chimeras which arise from more than one zygote). Although ploidy mosaics can and do occur in bisexual organisms (e.g. Oysters, Nell, 2002), more commonly when a mosaic is produced a hermaphroditic

individual results that is unable to produce viable gametes (e.g. chickens, Lepidoptera, and fish, Abdel-Hameed & Shoffner, 1971; Razak *et al.*, 1999). Unisexual diplo-triploid mosaics are thought to be rare but have been detected using a combination of flow cytometry and chromosome counts (Dawley & Goddard, 1988; Goddard & Schultz, 1993; Guo *et al.*, 1996), as in our present study. Individual mosaics contain varying proportions of diploid and triploid cells among different tissues. For example, hybrid unisexual minnows, produced from crossing *Phoxinus eos* with *P. neogaeus*, produce both triploid and diploid chromosome counts from caudal fin tissue, but flow cytometry with erythrocytes indicates only triploids (Dawley & Goddard, 1988). It is likely that in these minnows, delayed syngamy accounts for diploid/triploid mosaics (Dawley & Goddard, 1988). Syngamy before the first egg cleavage yields the triploid cells, while diploids are derived from actual gynogenetic eggs (Mantovani & Scali, 1992). Although some *Acanthoxyla* had both triploid and diploid cells in their ovarian follicles, flow cytometry revealed only one ploidy level in tibia tissue. The most likely explanation for this is that these *Acanthoxyla* individuals are mosaics, analogous to hybrid *Phoxinus* minnows. Gynogenetic loaches (*Misgurnus anguillicaudatus*) include mosaics that produce small and large eggs. Small eggs contain both haploid and diploid progeny while large eggs produce triploid progeny, implying that mosaic diplo-triploid females form haploid, diploid, and triploid eggs simultaneously (Yoshikawa *et al.*, 2007). Finding both triploid and diploid karyotypes in *Acanthoxyla* ovarian material suggests that females might be capable of producing both diploid and triploid offspring without the contribution of male gametes.

#### mtDNA

Although within a parthenogenetic lineage one might expect mtDNA to be concordant with other characters, we found little correlation between haplogroup and other traits. Three mitochondrial haplogroups have been identified within parthenogenetic *Acanthoxyla*. Haplogroup A contained all but one of the putative diploids and is so far not represented in South Island New Zealand. Haplogroup B contained most of the putative triploid *Acanthoxyla* specimens collected throughout New Zealand. The third lineage, haplogroup C was represented by just two individuals, collected from locations approximately 1500 kms apart (Fig 3). The occurrence of apparently triploid individuals in all three haplogroups is consistent with triploidy being the ancestral state of the *Acanthoxyla* lineage.

#### Nuclear PGI

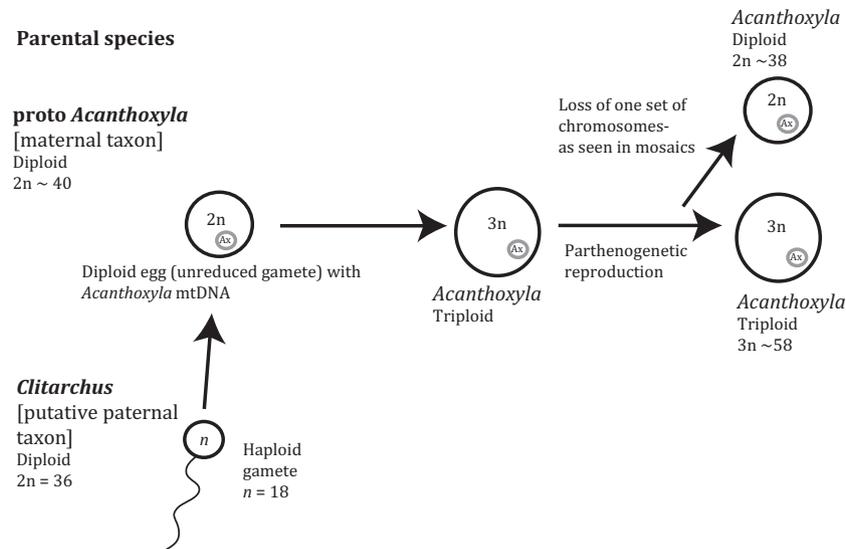
We explored allele diversity at the putatively single-copy PGI locus examined previously (Buckley *et al.*, 2008). We found that the putative diploid and triploid

*Acanthoxyla* individuals had a minimum of three, four, five or six PGI sequences. Since Ax.Azc-524 was diploid according to the chromosome counts and relative DNA content, yet had at least six distinct PGI sequences, it appears likely that PGI is a multicopy gene in these insects. Expansion of these data is required to fully resolve loci number. A number of other taxa have been shown to have two or more PGI loci (fish, Avise & Kitto, 1973; plants, Ghatnekar, 1999). In other studies of stick insects such as *Leptynia hispanica*, cloned and amplified PCR products of the nuclear gene EF1- $\alpha$  also revealed varying numbers of alleles among ploidy levels indicative of multiple copies of EF1- $\alpha$  (Ghiselli *et al.*, 2007). In the present study, we detected evidence of recombination in our dataset of PGI sequences. Although recombination can occur during PCR (e.g. Meyerhans *et al.*, 1990), this requires that the distinctive sequences occur in the same genome, which is not the situation observed here. In our dataset, the apparently recombined sequences involved sequences that had been found in other *Acanthoxyla* individuals, including one identified in a previous study in a different laboratory (Buckley *et al.*, 2008). Thus, the recombination identified is more likely to be the product of meiotic crossing over in *Acanthoxyla* or proto *Acanthoxyla* as might occur during either sexual reproduction or automictic parthenogenesis. We have evidence of recombination within a subset of distinct PGI sequences, indicating that these are from the same locus (i.e. true alleles), but as other distinct PGI sequences show no evidence of recombination, they might represent a separate locus or loci.

#### Hybrid and polyploid origins

In summary, we suggest that many triploid *Acanthoxyla* are mosaics with both diploid and triploid cells, and this explains how past cytogenetic studies could have inferred only diploidy for the genus (Parfitt, 1980; Morgan-Richards & Trewick, 2005). The morphological diversity within *Acanthoxyla* does not appear to be associated with ploidy level variation and we detected no body size effect associated with having a larger genome. Although the spiny morphotypes (*A. prasina*, *A. nr-geisovii*) are often triploid and the smooth stick insects (*A. inermis*) are often diploid, there are a number of exceptions even in the current sample.

A triploid stick insect lineage might result from a single hybridisation event involving a haploid egg and two haploid sperm (physiological polyspermy), or it might involve a two step origin through an intermediate diploid parthenogenetic hybrid with subsequent incorporation of an additional parental genome (Fig. 4). Both of these scenarios require a male *Acanthoxyla* (or proto *Acanthoxyla*) because the triploid *Acanthoxyla* karyotype appears to have only one set of *Clitarchus*-like chromosomes and two distinctive (metacentric rich) *Acanthoxyla* sets. Alternatively, the triploid *Acanthoxyla* lineage might have resulted from the single step of a non-reduced egg



**Fig. 4.** An evolutionary scenario for the origin of the parthenogenetic stick insect lineage *Acanthoxyla* based on current evidence. Hybridisation between two species has been inferred from DNA sequence data and both triploid and diploid individuals have been identified, along with evidence of ploidy mosaics.

( $2n$ ) fertilised by a haploid *Clitarchus* sperm (Fig. 4). More complex scenarios involving multiple hybridisations may also apply (Morgan-Richards & Trewick, 2005).

The evolution of a diplo-triploid mosaic genus is of general interest for its novelty as a reproductive and genetic system. This study highlights the value of employing multiple techniques in concert for such research, as these have revealed unexpected ploidy variation that needs to be explained. The occurrence of what appear to be mosaics among *Acanthoxyla* adds an extra element of complexity to this asexual system. It is possible that all triploid *Acanthoxyla* are mosaics, which would be a novel discovery in phasmids.

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### Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: doi: 10.1111/icad.12008:

**Figure S1.** Chromosome spreads obtained from seven *Acanthoxyla* specimens. The top three spreads have been interpreted as belonging to triploid individuals. The lower six spreads are from individuals which have been interpreted as diploid from chromosome spreads. Four of these spreads were from three individuals (Ax.Haa399, Ax.Pu398, and Ax.Daw471) whose leg tissue gave triploid results for flow cytometry. Ax.Rtk465 and Ax.Par503 were diploid according to both chromosome spreads and flow cytometry. *Specimen* codes are written in the top left of each picture, please refer to Table S1 for more information of each individual.

**Table S1.** Location, morphospecies identification and PGI GenBank accession numbers for *Acanthoxyla* stick insects from New Zealand used in this study.

**Table S2.** Sampling of *Acanthoxyla* stick insects for which we have multiple character data in this study (black) and previous work (grey).

**Table S3.** Stick insects (*Acanthoxyla* sp.) specimens used to infer minimum spanning network of mtDNA sequences (COI and COII), showing haplogroup designations (A, B or C) as in Fig. 3a. Unique sequences are lodged in GenBank, either as a concatenated accession (one GenBank number) or two separate accessions (two GenBank numbers) in the case of Buckley *et al.*, 2008. Where a specimen haplotype was identical to a published sequence the reference accession is indicated (sequence = xxxxxxx).

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