M r a a a *Acanthoxyla*

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A a . 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.

K . Acanthoxyla, asexual, biodiversity, chromosome mosaic, hybrid species, phasmids, triploids.

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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 suspecsexual29enw4.77978.966-429.5(general)-213.

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

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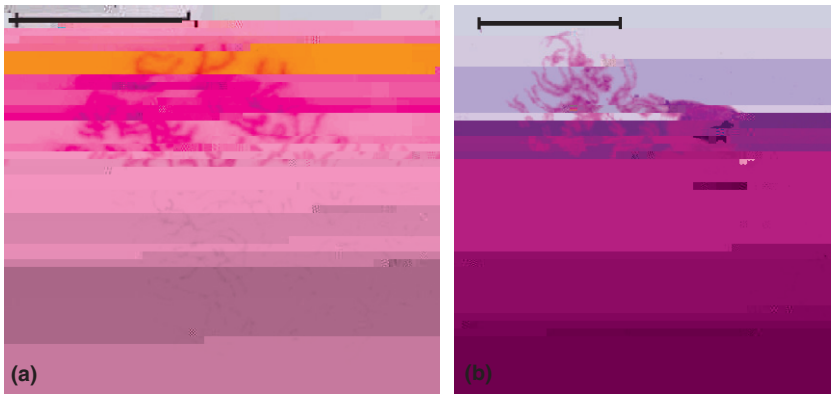
Sampling

Nineteen adult Acanthoxyla stick insects were collected

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

Ta. **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



F . 2. Chromosome spreads obtained from ovariole tips of Acanthoxyla prasina specimen Ax.Wes-408 exhibiting both diploid and triploid cells, scale bar = 20 μm. (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-

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.

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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.

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

(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

3. 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 Gen-

Zealand.