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*trn*L intron variation in New Zealand taxa of the *Asplenium obtusatum* Chloroplast Group

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ABSTRACT: Previous attempts to distinguish the tetraploid ferns *Asplenium oblongifolium* and *A. obtusatum* subsp. *obtusatum* with chloroplast DNA sequence data have been unsuccessful, although they have implicated one or other of these taxa as the chloroplast parents of the putative allopolyploids *A. lyallii* and *A. scleroprium*. Here we report new chloroplast DNA sequence data from the *trn*L intron, which are able to distinguish genetically the samples of *A. oblongifolium* and *A. obtusatum* subsp. *obtusatum* investigated. However, these data are insufficient to resolve which of the tetraploids were involved in the allopolyploid events producing *A. lyallii* and *A. scleroprium*.

KEYWORDS: *Asplenium*, *A. obtusatum*, *A. oblongifolium*, *A. lyallii*, *A. scleroprium*, New Zealand, chloroplast, DNA sequence, *trn*L intron, allopolyploidy.

Introduction

The *Asplenium* ferns of New Zealand appear to have had diverse biogeographic origins, but most of the species belong to a comparatively closely related 'Austral' group (Brownsey 1977a, Perrie & Brownsey 2004). There are no diploid *Asplenium* species in New Zealand (Dawson *et al.* 2000), but amongst the tetraploid representatives of the Austral group, three groups can be recognised by both morphological (Brownsey 1977a) and molecular (Perrie & Brownsey 2004) evidence: (i) the Bulbiferum Group of *A. bulbiferum* G.Forst. *sensu stricto* and *A. hookerianum* Colenso; (ii) the Flaccidum Group of *A. flaccidum* G.Forst. subsp. *flaccidum*, *A. flaccidum* subsp. *haurakiense* Brownsey, *A. chathamense* Brownsey, and *A. lamprophyllum* Carse; and (iii) the Obtusatum Group of *A. obtusatum* G.Forst. subsp. *obtusatum* and *A. oblongifolium* Colenso.

In addition to these tetraploid taxa, there are a similar number of octoploid taxa, and these appear to include examples of both auto- and allopolyploid evolution (Brownsey 1977b). The tetraploid chloroplast parents of the putative allo-octoploids *Asplenium lyallii* (Hook.f.) T.Moore and *A. scleroprium* Hombr. were recently determined to be either *A. oblongifolium* or *A. obtusatum* subsp. *obtusatum* (Perrie & Brownsey 2004). That study investigated variation in the DNA sequence of the *trn*L-*trn*F intergenic spacer of the chloroplast, which is maternally inherited in at least some European *Asplenium* (Vogel *et al.* 1998). However, which of the two tetraploids from the Obtusatum Group were involved in the respective polyploid events could not be distinguished as all four taxa had identical *trn*L-*trn*F sequences. Further, *A. oblongifolium* and *A. obtusatum* subsp. *obtusatum* were found to have identical sequences for the *rbc*L gene and the *psb*C-*trn*S intergenic spacer (Perrie & Brownsey 2004).

Asplenium oblongifolium is endemic to New Zealand, and occurs in coastal to lower montane areas of the North Island and northern South Island. In contrast, *A. obtusatum* subsp. *obtusatum* is considered to be restricted to coastal habitats, and occurs from Wellington to the subantarctic islands south of New Zealand. It is also found outside New Zealand, and probably has a circumantarctic distribution. However, while Tristan de Cunha plants have been demonstrated to be tetraploid, the majority of

Table 1 *Asplenium* material analysed, with collection locality, WELT (Museum of New Zealand Te Papa Tongarewa) herbarium voucher accession number, and GenBank accession numbers for the *trn*L intron and *trn*L-*trn*F intergenic spacer DNA sequences. Duplicate specimens are uniquely numbered.

Taxon	Collection locality	WELT voucher	*trn*L intron	*trn*L-*trn*F spacer
A. oblongifolium [1]	Paihia	P020508	AY538174	AY283216
A. oblongifolium [2]	Pohangina	P020509	AY538175	AY283216
A. obtusatum subsp. *obtusatum* [1]	Bluff	P020510	AY538176	AY283217
A. obtusatum subsp. *obtusatum* [2]	Haast	P020511	AY538177	AY283217
A. obtusatum subsp. *northlandicum*	Auckland	P020512	AY538178	AY283218
A. lyallii [1]	Castlepoint	P020507	AY538179	AY283215
A. lyallii [2]	Mangleton	P020552	AY538180	AY538180
A. scleroprium	Invercargill	P020516	AY538181	AY283222
A. bulbiferum	Pohangina	P020494	AY538182	AY283204
A. flaccidum subsp. *flaccidum*	Dunedin	P020501	AY538183	AY283210
A. hookerianum	Dannevirke	P020551	AY538184	AY538184
A. lamprophyllum	Auckland	P020506	AY538185	AY283214

putative populations outside New Zealand have not been investigated cytologically (Brownsey 1977a, Brownsey & Smith-Dodsworth 2000).

Asplenium oblongifolium and *A. obtusatum* subsp. *obtusatum* are morphologically very similar, but can usually be distinguished by the shape of their frond pinnae, the form of their stipe scales, and the ornamentation of their spores (Brownsey 1977a, Brownsey & Smith-Dodsworth 2000). The distinction is, however, less clear in the northwest of the South Island, where plants with *A. oblongifolium*-like frond morphology but *A. obtusatum*-like spore ornamentation and stipe scales occur inland, in some instances several kilometres from the coast (e.g. Pororari River, *c.* 1 km from the coast, Perrie *et al.*, 26 February 2002, WELT P020548; Karamea Bluff, *c.* 6 km from the coast, Perrie *et al.*, 28 February 2002, WELT P020549).

Asplenium lyallii occurs principally on calcareous substrates from coastal to subalpine areas, and is distributed from the Waikato to Stewart Island (Brownsey 1977a, Brownsey & Smith-Dodsworth 2000). Based on its morphology, Brownsey (1977b) suggested *A. lyallii* was an allopolyploid with the parentage of either *A. bulbiferum* x *obtusatum* subsp. *obtusatum* or *A. bulbiferum* x *oblongifolium*, although the involvement of *A. hookerianum* instead of *A. bulbiferum* should also be considered. A putative collection of *A. hookerianum* x *oblongifolium* (Masters Shelter, northeastern Ruahine Ranges, Shepherd *et al.*, 20 October 2002, WELT P020550) bears a striking resemblance to some of the morphological forms of *A. lyallii*.

Asplenium scleroprium occurs from Invercargill southwards, where it is mostly confined to coastal habitats, and is at its most prevalent on the Auckland Islands (Brownsey 1977a, Brownsey & Smith-Dodsworth 2000). Brownsey (1977b) indicated that *A. flaccidum* subsp. *flaccidum* x *A. obtusatum* subsp. *obtusatum* was the most likely allopolyploid parentage for *A. scleroprium. Asplenium obtusatum* subsp. *obtusatum* occurs sympatrically with *A. scleroprium*, but *A. oblongifolium* does not (Brownsey 1977a).

Here we report data from the *trn*L intron, a DNA sequence marker that has only recently been investigated in ferns, and which neighbours the *trn*L-*trn*F intergenic spacer (Trewick *et al.* 2002). We hoped these data would

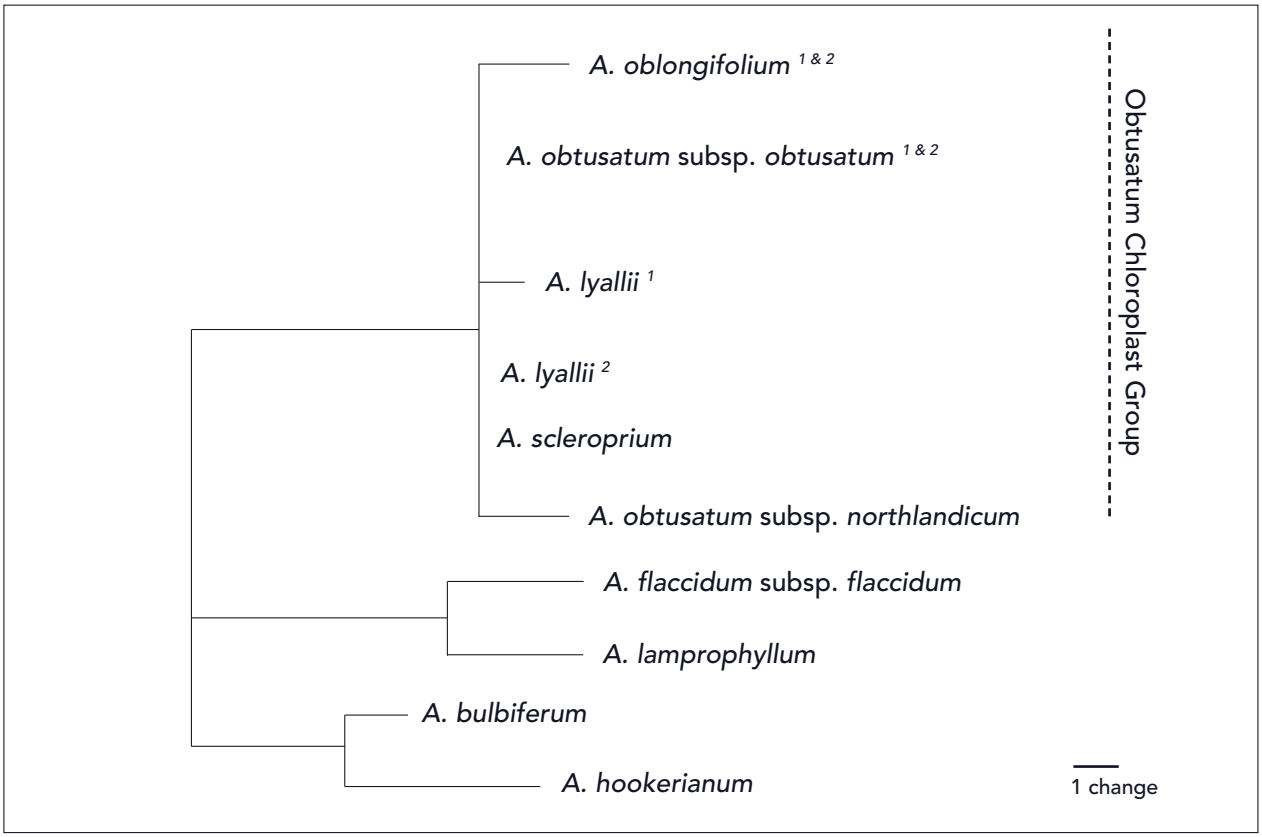

Fig. 1 Chloroplast phylogeny of the New Zealand members of the *Asplenium obtusatum* Chloroplast Group: phylogram of the single tree of 60 steps recovered from maximum parsimony analysis of *trn*L intron and *trn*L-*trn*F intergenic spacer DNA sequences. Superscript numbers identify duplicate samples (*see* Table 1).

allow us to distinguish genetically *Asplenium oblongifolium* and *A. obtusatum* subsp. *obtusatum*, and to identify which was the chloroplast parent in the allopolyploid events producing *A. lyallii* and *A. scleroprium*.

Methods

DNA was extracted from the samples listed in Table 1 as described by Perrie & Brownsey (2004). DNA sequence for the *trn*L intron was obtained using the PCR reagents of Perrie & Brownsey (2004), and the primers and thermocycling conditions of Trewick *et al.* (2002), except a primer annealing temperature of 65°C rather than 48°C was used.

PCR products were purified by incubation at 37°C for 30 minutes with 2 units Shrimp Alkaline Phosphatase and 10 units Exonuclease I (both USB Corporation), followed by heat inactivation of the enzymes at 80°C for 15 minutes. The purified PCR products were sequenced in both directions using an Applied Biosystems ABI3730 Genetic Analyser.

The edited DNA sequences for the *trn*L intron were combined with those from the *trn*L-*trn*F intergenic spacer (Perrie & Brownsey 2004) and aligned using ClustalX 1.8 (Thompson *et al.* 1997). PAUP 4.b10 (Swofford 2002) was used to conduct a phylogenetic analysis using a maximum parsimony criterion with a branch and bound search and sites encompassing insertion-deletion events treated as missing data. *Asplenium bulbiferum*, *A. flaccidum* subsp. *flaccidum*, *A. hookerianum*, and *A. lamprophyllum* were designated as the outgroup (Perrie & Brownsey 2004).

Results

A single tree of 60 steps was recovered from the maximum parsimony analysis (*see* Fig. 1), with a Consistency Index of 0.9833, a Retention Index of 0.9848, and a Rescaled

Table 2 Sequence polymorphisms within the New Zealand members of the *Asplenium obtusatum* Chloroplast Group for the *trn*L intron and *trn*L-*trn*F intergenic spacer. The character state(s) for these sites in the outgroup is also shown. Duplicate samples are identified by superscript numbers (*see* Table 1). The alignment positions are numbered from the first base sequence of the *trn*L intron.

Locus	***trn*L intron**			***trn*L-*trn*F spacer**	
Alignment position	**56**	**196**	**518**	**719**	**869**
A. oblongifolium[1]	C	A	C	T	A
A. oblongifolium[2]	C	A	C	T	A
A. obtusatum subsp. *obtusatum*[1]	A	G	C	T	A
A. obtusatum subsp. *obtusatum*[2]	A	G	C	T	A
A. lyallii[1]	A	G	T	T	A
A. lyallii[2]	A	G	C	T	A
A. scleroprium	A	G	C	T	A
A. obtusatum subsp. *northlandicum*	A	G	C	G	G
Outgroup	A/C	G	C	T/–	A

Consistency Index of 0.9684. The *trn*L intron sequence recovered from the two samples of *Asplenium oblongifolium* differs from those of the two samples of *A. obtusatum* subsp. *obtusatum* at two base-pair positions. The *A. scleroprium* sample and one of the *A. lyallii* samples have identical *trn*L intron sequences to those of *A. obtusatum* subsp. *obtusatum*, while the other *A. lyallii* sample exhibits a one base-pair difference. The *trn*L intron sequence of the octoploid *A. obtusatum* subsp. *northlandicum* Brownsey is identical to that of *A. obtusatum* subsp. *obtusatum*, but as noted by Perrie & Brownsey (2004), the former exhibits two autapomorphies in the *trn*L-*trn*F intergenic spacer. The sequence polymorphisms within the *trn*L intron and *trn*L-*trn*F intergenic spacer for the Obtusatum Chloroplast Group taxa are listed in Table 2.

Discussion

The two DNA sequence base-pair differences in the *trn*L intron reported here are the first detected between *Asplenium oblongifolium* and *A. obtusatum* subsp. *obtusatum*, and follow the investigation of three other chloroplast DNA sequence data sets (Perrie & Brownsey 2004). Indeed, the two taxa (or, at least, their chloroplasts) appear very closely related, with only two differences found across 2443 base-pairs. Investigating this *trn*L intron genetic polymorphism in conjunction with variation in spore ornamentation and stipe scale size may help to clarify the boundary between *A. oblongifolium* and *A. obtusatum* subsp. *obtusatum* in the northwest of the South Island, where their usual morphological and ecological distinctiveness appears to breakdown.

The two base-pair differences between the *Asplenium oblongifolium* and *A. obtusatum* subsp. *obtusatum* samples are reconstructed as autapomorphic in the former (*see* Fig. 1). These base-pair differences occur in two geographically distant samples, which suggests that they may be widespread in *A. oblongifolium* rather than representing only geographically local variation. One of the changes, an A to C transversion at alignment position 56, has been independently acquired by *A. flaccidum* subsp. *flaccidum* (*see* Outgroup, Table 2).

Unfortunately, the *trn*L intron data are unable to resolve which of *Asplenium oblongifolium* and *A. obtusatum* subsp. *obtusatum* were the chloroplast parents in the allopolyploid events producing *A. lyallii* and *A. scleroprium*. The chloroplast *trn*L intron sequences of *A. lyallii* and *A. scleroprium* are identical to those of *A. obtusatum* subsp. *obtusatum*, except for an autapomorphy exhibited by one of the *A. lyallii* specimens, but the resemblance is sympleisomorphic, being based on shared, ancestral character states. The data are therefore unable to discriminate between *A. obtusatum* subsp. *obtusatum*, *A. oblongifolium* (e.g. ancestral *A. oblongifolium* before the base-pair

autapomorphies evolved, or, if these differences are not fixed in *A. oblongifolium*, individuals that retain the pleisomorphic states), or their common ancestor, being the chloroplast parent in the allopolyploid events producing *A. lyallii* and *A. scleroprium*.

Given the seemingly very high similarity of the chloroplast DNA of *Asplenium oblongifolium* and *A. obtusatum* subsp. *obtusatum*, how best to identify the chloroplast parents of *Asplenium lyallii* and *A. scleroprium* is uncertain. Already four chloroplast DNA loci, encompassing 2443 base-pairs, have been investigated. Possibly, sequencing of additional chloroplast DNA loci may find synapomorphies between the octoploids and one or other of the tetraploids, thereby identifying which of *A. oblongifolium* and *A. obtusatum* subsp. *obtusatum* was involved in each instance. Alternatively, methods that assess variation in biparentally inherited genetic markers from the nuclear genome (e.g. DNA fingerprinting, Perrie *et al.* 2003; DNA sequencing of the nuclear ITS locus, Van den heede *et al.* 2003) may provide sufficient resolution to address this question.

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