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Low-copy nuclear DNA sequences reveal a predominance of allopolyploids in a New Zealand *Asplenium* fern complex

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ABSTRACT

Recent generalisations about polyploidy in plants have been largely based on studies of angiosperms. A compelling group to compare with angiosperms is ferns, because of their high polyploidy. The bi-parental inheritance of nuclear DNA sequence markers makes them advantageous for investigating polyploid complexes, but few such markers have been available for ferns. We have used DNA sequences from the low-copy nuclear *LFY* locus to study an *Asplenium* polyploid complex. The New Zealand species of this Austral group comprise seven tetraploids and eight octoploids. *LFY* sequences indicate that allopolyploidy is much more predominant than previously thought, being implicated in the origins of seven of the octoploids. One of the tetraploids has had a central role, being a progenitor for five of the octoploids. All of the octoploids appear to have relatively recent origins, with the dynamic environmental conditions of the Pleistocene possibly playing a role in their formation and/or establishment.

1. Introduction

Many major biotic groups, including vertebrates (Dehal and Boore, 2005) and angiosperms (Bowers et al., 2003; de Bodt et al., 2005), appear to be largely or even entirely palaeopolyploid, with their ancestors having experienced genome duplication(s). There is also much evidence that polyploidy is an ongoing occurrence, being especially frequent in some groups (Ramsey and Schemske, 1998). For instance, polyploidy has been implicated in 2–4% of speciation events within angiosperms, and some 7% amongst ferns (Otto and Whitton, 2000). Despite the pervasiveness of polyploidy, it is unclear if it is simply a relatively common mutation or whether it increases evolutionary rates of diversification and/or adaptation (Otto and Whitton, 2000; Otto, 2007). Other questions include the relative frequencies of auto-polyploidy (genome doubling within one species) and allo-polyploidy (genome doubling in conjunction with hybridisation between two species), what biological and environmental factors promote the formation and establishment of polyploids, and what genetic changes occur post-polyploidy (Soltis et al., 2003; Mable, 2004; Otto, 2007); furthermore, what generalisations can be made across different groups?

Addressing these issues requires additional detailed investigations, and the high incidence of polyploidy in ferns makes them compelling subjects for extending the scope of knowledge beyond angiosperms. *Asplenium* is one of the largest fern genera, with more than 700 species worldwide (Kramer and Viane, 1990). Approximately 74% of species examined cytologically have ploidies higher than diploid (143/192; based on Löve et al., 1977; with additional data from Dawson et al., 2000; Tindale and Roy, 2002; Walker, 1979, gives 77% for 186 unlisted counts). Diploids are generally more frequent amongst northern temperate *Asplenium* floras than elsewhere (Lovis, 1973).

Polyploid *Asplenium* complexes in Europe and North America have been investigated with analyses of chromosome pairing (references in Lovis, 1977; Walker, 1979), and more latterly allozymes (Werth et al., 1985; Vogel unpublished in Vogel et al., 1999; Herrero et al., 2001). Parts of the European complex have also been studied with chloroplast DNA sequences (Vogel et al., 1996; Pinter et al., 2002; Trewick et al., 2002) and nuclear DNA sequences (Van den heede et al., 2003). Of particular interest has been determining the progenitor/s for each polyploid, thereby establishing whether its origin involved auto- or allo-polyploidy.

Another polyploid *Asplenium* complex, termed the 'Austral' group, is present in Australasia, and centred in New Zealand (Brownsey, 1977a; Perrie and Brownsey, 2005a; Shepherd et al., 2008). Analyses using several loci indicate that the chloroplast DNA sequences of the Austral group are monophyletic (Perrie and Brownsey, 2005a; Shepherd et al., 2008). A difference between

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the European and North American *Asplenium* polyploid complexes and the Austral group is that, at least amongst its New Zealand members, it contains no diploids. There are also no diploids amongst the four species of *Asplenium* indigenous to New Zealand that are not part of the Austral group; these species are only distantly related to the Austral group (Dawson et al., 2000; Perrie and Brownsey, 2005a).

In New Zealand the Austral group comprises seven tetraploid and eight octoploid species. The tetraploid Austral species in New Zealand have been partitioned into three subgroups based on chloroplast sequences: the Obtusatum, Flaccidum, and Bulbiferum subgroups (Perrie and Brownsey, 2005a). These subgroups also differ in their frond morphologies: Obtusatum tetraploids are one-pinnate, Flaccidum tetraploids are two- to three-pinnate, and Bulbiferum tetraploids are three-pinnate (Fig. 1). Although each subgroup is well-supported, attempts to resolve their interrelationships using chloroplast sequence data have been unsuccessful because of conflicting signal (Perrie and Brownsey, 2005a; Shepherd et al., 2008).

Four of the New Zealand Austral tetraploids and four of the octoploids are endemic to New Zealand (Perrie and Brownsey, 2005a); of the remainder, the only species extending beyond Australasia are *A. northlandicum*, *A. shuttleworthianum* (both south Pacific), and *Asplenium obtusatum* (circum-Antarctic). Several species not found in New Zealand have been attributed to the Austral group on account of their chloroplast DNA sequences, (Shepherd et al., 2008; also see Perrie et al., 2005), but, based on these data and their morphology, none of these species are likely to have been involved in the origins of the Austral octoploids in New Zealand, except for a couple of possibilities (see Section 4).

Both auto- and allo-polyploidy have been implicated in the origins of the Austral octoploids in New Zealand. Inferences for the origin of each octoploid were initially based on morphology, and in particular through comparison with known sterile hybrids between tetraploid species (Brownsey, 1977b; Brownsey and de Lange, 1997). More recently, sequencing of the *trnL-trnF* intergenic spacer established the tetraploid chloroplast parent for some of the octoploids (Perrie and Brownsey, 2005a). For instance, *Asplenium hookerianum* was determined to be the chloroplast parent of the octoploid *A. gracillimum*, previously considered to be a putative autopolyploid of *A. bulbiferum* (Brownsey, 1977b). However, these data, along with additional *trnL* intron sequences (Perrie and Brownsey, 2004), were unable to sufficiently distinguish between a number of the tetraploid taxa; in particular, *A. obtusatum* and *A. oblongifolium*, and *A. chathamense*, *A. flaccidum* subsp. *flaccidum* and *A. flaccidum* subsp. *haurakiense*. This precluded precise determination of the chloroplast parent for many of the octoploids.

In any case, the uniparental inheritance of cpDNA means it can track only one parental line (thought to be maternal in *Asplenium*; Vogel et al., 1998), even if the subject has a hybrid (including allopolyploid) origin. In contrast, the bi-parental inheritance of nuclear loci allows the detection of both parental lines, if they are differentiated. Nuclear loci can therefore potentially distinguish auto- from allo-polyploid origins, where sequences from one or two progenitor taxa are expected in the polyploid respectively. There has been little development of nuclear DNA sequence markers in ferns (but see Van den heede et al., 2003; Ishikawa et al., 2002; Ebihara et al., 2005; Adjie et al., 2007). The only previous such investigation in *Asplenium* was by Van den heede et al. (2003), who used nuclear ribosomal internal transcribed spacer DNA sequences to investigate a largely European group centred on *A. ceterach*. Attempts to produce sequences from this locus in New Zealand *Asplenium* have not been successful (Shepherd and Perrie, unpublished data). An alternative promising locus is the homologue of the floral-development gene known as *LEAFY* (*LFY*) in *Arabidopsis* and *FLORICAULA* in *Antirrhinum* (Frohlich and Meyerowitz, 1997). In this study we sequenced the second intron of *LFY* from members of the New Zealand Austral group of *Asplenium* with the main aim of

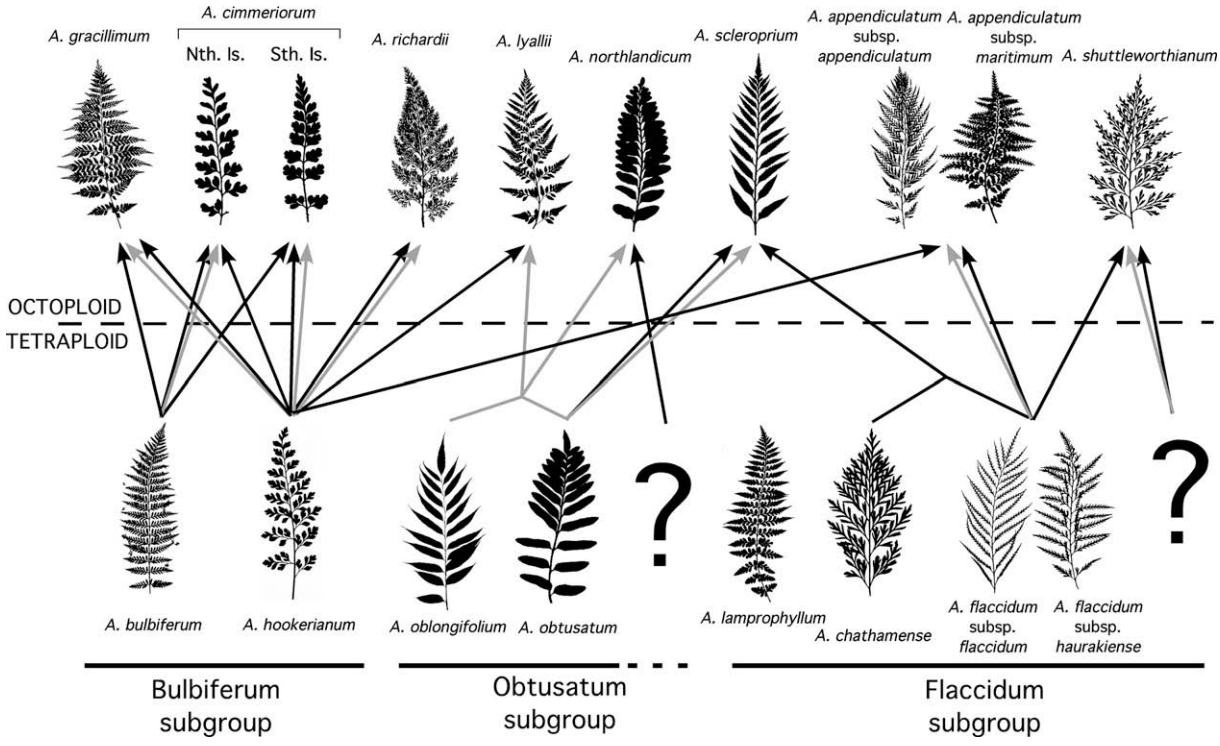

Fig. 1. Inferred origins of the New Zealand octoploids in the Austral *Asplenium* group. Black arrows indicate the contribution of a *LFY* sequence from a tetraploid to an octoploid. Grey arrows represent the inheritance of the chloroplast genome (chloroplast data from Perrie and Brownsey, 2005a; Perrie et al., 2005; Shepherd et al., 2008; Perrie and Shepherd unpublished data). Inferred unidentified tetraploids are represented by '?'.

determining the progenitors of the octoploid taxa. A secondary objective was to explore the utility of *LFY* for resolving the relationships between the Bulbiferum, Obtusatum, and Flaccidum subgroups.

2. Materials and methods

2.1. Sampling and DNA extraction

Our sampling included all 17 New Zealand species and subspecies in the Austral group of *Asplenium*, as well as an outgroup, *A. antiquum* (Table 1). *Asplenium antiquum* is the closest known relative of the Austral group according to chloroplast DNA sequences (Shepherd et al., 2008). We failed to obtain *LFY* PCR products from *A. australasicum*, the only other species from the larger clade within which the Austral group is nested (see Shepherd et al., 2008) for which we had material. *Asplenium northlandicum* is often treated as a subspecies of *A. obtusatum* (Brownsey and Smith-Dodsworth, 2000), but we refer to it here at the species level because it appears not to be especially closely related to *A. obtusatum s.s.* At least two samples were analysed for each taxon, except for the outgroup *A. antiquum* and the largely subantarctic *A. scleroprium*. Four samples of *A. hookerianum* were sequenced owing to the high level of variation detected at the chloroplast *trnL-trnF* locus for this species (Shepherd et al., 2007). The *A. hookerianum* samples selected exhibited different *trnL-trnF* haplotypes (haplotypes A, H, Q, and U from Fig. 2 of Shepherd et al., 2007). Five *A. gracillimum* samples were included because cpDNA and AFLP indicate that this octoploid has had multiple origins (Perrie et al. unpublished data) and samples were selected to represent this diversity. DNA was extracted from silica gel dried frond tissue using a modified CTAB method (Doyle and Doyle, 1990).

2.2. PCR amplification and sequencing

LFY DNA sequences were amplified using unpublished primers designed for *Polystichum* by Heather Driscoll (leafyF 5′-TGA AGT GCA GCA GAT GTC AA and leafyR 5′-GCT AGC ACC TTT CAG CTT GG; http://www.uvm.edu/%7Edbarring/barrprotos.html, accessed July 2005). PCR amplification was performed in 20 μl volumes containing 1× PCR buffer (10 mM Tris–HCl, 50 mM KCl, pH 8.3; Roche Applied Science, Auckland), 1.5 mM $MgCl_2$, 1 M betaine, 250 μmol dNTPs, 10 ρmol of each primer, 1 U Taq polymerase (Roche Applied Science, Auckland) and approximately 50 ng of template DNA. PCR

Table 1
Asplenium samples used in this study

Taxon	Sample identifier	Collection location	WELT voucher	GenBank Nos.
Tetraploids				
A. antiquum		Cultivated	P022088	EU560987
A. bulbiferum	A	Palmerston North	P020494	EU561003
	B	Mount Taranaki	P022110	EU561004
A. chathamense	A	Chatham Island	P022114	EU560996
	B	Chatham Island	P020498	EU560997
A. flaccidum subsp. *flaccidum*	A	Dunedin	P020501	EU560993
	B	Paihia	P020502	EU560992
A. flaccidum subsp. *haurakiense*	A	Cultivated	P020503	EU560998
	B	Karikari Peninsula	P022117	
A. hookerianum	A	Dannevirke	P020504	EU560988
	B	Arthur's Pass	P021863	EU560989
	C	Kaeo	P021834	EU560990
	D	Puhoi	P021840	EU560991
A. lamprophyllum	A	Auckland	P020506	EU560994
	B	Matamata	P022137	EU560995
A. oblongifolium	A	Wellington	P022093	EU561001
	B	Palmerston North	P020509	EU561002
A. obtusatum	A	Bluff	P020510	EU560999
	B	Jacksons Bay	P022111	EU561000
Octoploids				
A. appendiculatum subsp. *appendiculatum*	A	Mount Cook	P020492	
	B	Ruahine Ranges	P022119	
A. appendiculatum subsp. *maritimum*	A	Wellington	P020493	
	B	Wellington	P022092	
	C	Cape Farewell	P022115	
A. cimmeriorum	A	Punakaiki	P020499	
	B	Punakaiki	P022103	
	C	Waitomo	P022104	
	D	Waitomo	P021117	
A. gracillimum	A	Wellington	P022105	
	B	Urewera Ranges	P022106	
	C	Hokitika	P022109	
	D	Hamilton	P022108	
	E	Warkworth	P022107	
A. lyallii	A	Ruahine Ranges	P020552	EU560984
	B	Castlepoint	P020507	EU560983
A. northlandicum	A	Karikari Peninsula	P022116	EU560985
	B	Auckland	P020512	EU560986
A. richardii	A	Lake Hawea	P022112	EU560981
	B	Mount Cook	P020515	EU560982
A. scleroprium		Invercargill	P020516	
A. shuttleworthianum	A	Cultivated	P020517	
	B	Cultivated	P022098	

GenBank Nos. were not obtained for samples with indels on different alleles.

thermocycling conditions involved an initial denaturation step of 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min, and extension at 72 °C for 1 min, with a final extension time of 72 °C for 5 min.

PCR products were purified by digestion with 1 U shrimp alkaline phosphatase (SAP, USB Corp., Cleveland, USA) and 5 U exonuclease I (Exo I, USB Corp., Cleveland, USA) at 37 °C for 30 min, followed by inactivation of the enzymes at 80 °C for 15 min. All PCR products were sequenced in both directions with the ABI Prism Big Dye Terminator cycle sequencing kit version 3.1 and run on an ABI 3730 DNA sequencer (Allan Wilson Centre Genome Service, Palmerston North, New Zealand).

A single band was obtained for all species except *Asplenium oblongifolium* and *A. obtusatum*. For these two species a second, larger fragment co-amplified. The two bands were sequenced in *A. oblongifolium* by separation on 1.5% low electrophoresis agarose gel, followed by a band-stab (Bjourson and Cooper, 1992) and re-amplification using the original PCR conditions. The sequence of the shorter fragment was alignable to *LFY* sequences obtained from the other Austral *Asplenium* species, whereas the longer sequence was unable to be aligned and showed no homology to *LFY* sequences deposited on GenBank. An internal primer, leafyshortR 5′-TAA ACC AAA GCA CCC AGC T, was designed that was specific to the shorter sequence. This primer was used in combination with the leafyF primer to amplify and sequence the *LFY* locus in *A. oblongifolium* and *A. obtusatum* using the original PCR conditions. This primer pair was also used to amplify *LFY* sequence from octoploids suspected of being related to the Obtusatum subgroup based on morphology; i.e., *A. northlandicum*, *A. scleroprium* and *A. lyallii*.

The *Asplenium scleroprium LFY* sequence, amplified using the leafyF and leafyR primer pair, contained a number of indels which resulted in large tracts of double sequence in both sequencing directions. Initial analysis indicated that two different *LFY* sequences occurred in *A. scleroprium*, one each from the Obtusatum and Flaccidum subgroups. In an attempt to amplify each sequence independently, alternative reverse primers were designed from the tetraploid sequences we had generated to amplify only the sequence from either the Obtusatum subgroup (leafyOBTR 5′-CAG GCT TGA CAT GAT ACC CCA) or the Flaccidum subgroup (leafynonOBTR 5′-CAG GCT TCA CAT GAT AGC CAT). Amplification was performed using the leafyF primer with either leafyOBTR or leafynonOBTR, and the original PCR conditions, except for an annealing temperature of 50 °C.

2.3. Cloning of LFY sequences

All genetic engineering in New Zealand, including the cloning of DNA into *Escherichia coli* bacteria, legally requires approval from the Environmental Risk Management Authority. For indigenous organisms, this involves consultation with representatives from the iwi (Maori tribe) with jurisdiction for (1) where the work is carried out and (2) the collection location of every sample whose DNA is to be cloned. This is an extensive process if samples are sourced from many different regions. Therefore, to demonstrate that additive *LFY* sites reflected the presence of two different sequences (see below), we cloned *LFY* sequences from two octoploids, *Asplenium gracillimum* and *A. appendiculatum* subsp. *maritimum*, that occur around Wellington, an area for which we had the necessary approvals.

LFY sequences were cloned using the pGEM-T Easy Vector System (Promega), following the manufacturer's instructions. Colonies containing inserts were transferred to 50 μl Tris pH 8.0 and denatured at 95 °C for 10 min. Clones of *Asplenium gracillimum* (sample A) and *A. appendiculatum* subsp. *maritimum* (sample B) were amplified using M13 primers and sequenced as above until two different sequence types were recovered more than once (a total of four clones for *A. gracillimum* and seven clones for *A. appendiculatum* subsp. *maritimum* were sequenced). One of the clones from the *A. appendiculatum* subsp. *maritimum* sample differed from the clone type 2 sequence (Table 2. that was recovered four times by a single base-pair (bp): a C at alignment position 136. This appeared to be a polymerase-induced PCR error, as there was no sign of a C at this position in the direct sequence.

2.4. Data analysis

Sites were designated additive only when there were peaks of two different nucleotides, with each clearly distinguishable from background noise and occurring on both DNA strand directions. A BLASTn search for short, nearly exact matches (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) was performed for several of the tetraploid *Asplenium LFY* sequences using the GenBank database and the default algorithm parameters.

LFY sequences were aligned by eye. The relationships of the tetraploid taxa were constructed in PAUP* 4.0b10 (Swofford, 2002) using maximum parsimony (MP) and maximum likelihood (ML). A heuristic search algorithm was used for the MP and ML analyses with 100 random addition replicates and tree-bisection-reconnection branch-swapping. The most appropriate model of sequence evolution was determined using the Akaike information criterion (AIC) in Modeltest 3.7 (Posada and Buckley, 2004): model K81uf (base frequencies of $A = 0.2543$, $C = 0.1709$, $G = 0.2202$, $T = 0.3546$; rate matrix = [1.0000 3.1967 2.0811 2.0811 3.1967]. Nodal support was assessed using 1000 (MP) or 100 (ML) bootstrap replicates (BS). Phylogenies were also constructed with MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001), with nst = 6, rates = equal, and the default priors. Two concurrent analyses were run, each with four Markov chains of 10,000,000 generations. Chains were sampled every 1000 generations, and the first 50% of these samples were discarded as 'burn-in'. At this point, the standard deviation of split frequencies was less than 0.01, indicating convergence to a stationary distribution had been achieved. MP, ML and Bayesian analyses were repeated with either the exclusion or retention of the section of the alignment affected by missing data because of primer set differences.

3. Results

3.1. LFY sequence variation and phylogenetic relationships within tetraploid Austral Asplenium

A BLASTn search of the *Asplenium LFY* sequences revealed that short regions were similar to *LFY* exon 2 from other fern species (AF105109 from *Matteuccia*; Frohlich and Parker, 2000; DQ490954–DQ490963 from *Ceratopteris*; Adjie et al., 2007) and the lycophyte *Isoetes* (AY541764–AY541808; Hoot et al., 2004). The sequence amplified in the Austral *Asplenium* comprised 29 bp of *LFY* exon 2 followed by a region of intron 2, with the exon/intron boundary determined from comparison with *Isoetes LFY* sequences (Hoot et al., 2004). The alignment of sequences from the tetraploid species was 364 bp in length (available from the corresponding author upon request and summarised in Table 2). No additivity occurred across all samples of a subgroup. Additivity was observed within some of the tetraploid samples, but only at a few sites in each case.

Each tetraploid species was distinguished by its *LFY* sequence, although *A. oblongifolium* has no autapomorphies. The three Austral *Asplenium* subgroups, previously recognised from morphology and

Table 2
Variable nucleotide sites in the Austral *Asplenium LFY* sequences in comparison with the outgroup *A. antiquum*

	0000000000000000111111111111111111111111112222222222222222233333
	3566777777899999001112333333445666677777899912333334455558991122
	5867012389013589161290234567673178901237101418145795623456245673 4
TETRAPLOIDS	
A. antiquum	GTAGTAGG:GCAGGCCTTCTGCTRGGTGTCAC:ATGCCTAGCTTAATCTTAAT:::::GGTTAGG
BULBIFERUM SUBGROUP	
A. hookerianum A	.:.....TA....TT...TC.T::::::C.......G..........A...T.....A.......
A. hookerianum B	.:.....TA....TT...TC.T.A...CC.......G..........AA..T.....A.......
A. hookerianum C,D	.:.....TA....TT...TC.T.A...CC.......G..........A...T.....A.......
A. bulbiferum A,B	.:..........TT...TC.T.A............G..........A...T.....A....G..
FLACCIDUM SUBGROUP	
A. lamprophyllum A	A:.............T..T.A...CCT..T...G......GC..A...T.T..........
A. lamprophyllum B	A:.............T..T.A...SCT..T...G......GC..A...T.T..........
A. chathamense A,B	A:............T..T..T.A....CT.....G......G...A...C.TT.....C....
A. flaccidum subsp. *flaccidum* A	A:.............T..T.A....CT....C.G......R...A.A.C.T..........
A. flaccidum subsp. *flaccidum* B	A:.............T..T.A....CT....C.G......G...A.A.C.T..........
A. flaccidum subsp. *haurakiense* A	A:.............T..T.A....CT....C.G......G...A.A.C.T..........
A. flaccidum subsp. *haurakiense* B	A:.....2.......T..T.A....CT....Y.G......G...A.W.C.T..........
OBTUSATUM SUBGROUP	
A. oblongifolium A,B	......T..TT.....A.T.T..A...AC..T.::::::.C......A...TATTTTA???????
A. obtusatum A	..TCCTT..TT.T...A.T.T..A...AC..T.::::::.C....T.A...TA????????????
A. obtusatum B	..TCCTT..TT.T...A.T.T..A...AC..T.::::::RC....T.A...TATTT.A???????
OCTOPLOIDS	
A. richardii A,B	.:.....TA....TT...TC.T.A...CC.......G..........A...T.....A.......
A. lyallii A,B	.:.....TA....TT...TC.T.A...CC.......G..........A...T.....A.......
A. cimmeriorum (South Island) A,B	.:.....K1....TT...TC.T.A...SY.......G..........A...T.....A....R..
A. cimmeriorum (North Island) C	.:...M.K1....TT...TC.T.A...SY.......G..........A...T.....A....R..
A. cimmeriorum (North Island) D	.:.....K1....TT...TC.T.A...SY.......G..........A...T.....A....R..
A. gracillimum A,B,C,D	.:.....K1....TT...TC.T.A...SY.......G..........A...T.....A....R..
A. gracillimum A clone type 1	.:..........TT...TC.T.A............G..........A...T.....A....G..
A. gracillimum A clone type 2	.:.....TA....TT...TC.T.A...CC.......G..........A...T.....A.......
A. gracillimum E	.:.....K1..R.TT...TC.T.A...SY.......G..........A...T.....A....R..
A. app. subsp. *maritimum* A	R:.....K1....KY...TY.T.A...SCY....Y.G......K...A.W.Y.4...1.......
A. app. subsp. *maritimum* B	R:.....K1....KY...TY.T.A...SCY....Y.G......K...AWW.Y.4...1.......
A. app. subsp. *maritimum* B clone type 1	.:.....TA....TT...TG.T.A...CC.......G..........AA..T.....A.......
A. app. subsp. *maritimum* B clone type 2	A:.............T..T.A....CT....C.G......G...A.A.C.T..........
A. app. subsp. *maritimum* C	R:.....K1....KY...TY.T.A...SCY....Y.G......K...A.W.Y.4...1R.....
A. app. subsp. *appendiculatum* A	R:.....K1....KY...TY.T.A...SCY....Y.G......K...A.WRY.4...1.......
A. app. subsp. *appendiculatum* B	R:.....K1....KY...TY.T.A...SCY....Y.G......K...A.W.Y.4...1.......
A. shuttleworthianum A,B	A:..............T..T.A....CT....Y.G......G..YM.W.Y.T1..1.......
A. scleroprium	R4WSYWK2.KY.K...W.T.KY.A...RCY.Y.142234.S..K.W.A...YWT???????????
A. northlandicum A,B	.:.......T.......GA.T..A....G.......G....TG....A...T.T.....A.C.TA

Singletons for *A. antiquum* are not shown.: = gap, ? = sequence not recovered, because of the use of the leafyshortR primer in the Obtusatum subgroup. Symbols for additive sites are as follows: M = A + C, R = A + G, W = A + T, S = C + G, Y = C + T, K = G + T, K = G + T, 1 = : + A, 2 = : + G, 3 = : + C, 4 = : + T. Shaded bases represent apomorphic character states for tetraploids that also occur in octoploids. Subgroup synapomorphies: red, Bulbiferum subgroup; yellow, Flaccidum subgroup; grey, Obtusatum subgroup. Tetraploid autapomorphies: blue, *A. hookerianum*; purple, *A. bulbiferum*; black, *A. flaccidum*; green, *A. obtusatum*. (For interpretation of the references to colour in this legend, the reader is referred to the web version of this article.)

chloroplast sequences, were supported by *LFY* sequences. The Bulbiferum subgroup shared three synapomorphies (shown in red in Table 2), the Obtusatum subgroup was supported by nine substitution synapomorphies and a 6 bp synapomorphic deletion (grey in Table 2) and the Flaccidum subgroup shared 2 synapomorphies (yellow in Table 2).

With all data included, analyses of the *LFY* sequences from the tetraploid taxa recovered six MP trees (score = 61) and a single ML tree (score = 839.78951). In all tree-building analyses the three subgroups were recovered with high support: Bulbiferum subgroup, 85% MP bootstrap (BS), 94% ML BS, 1.00 posterior probability (PP); Flaccidum subgroup, 88% MP BS, 92% ML BS, 1.00 PP; and Obtusatum subgroup: 100% MP BS, 100% ML BS, 1.00 PP. Results were little changed when sites with missing data were excluded from analyses (not shown).

The Bulbiferum and Flaccidum subgroups were supported as sister taxa with low to moderate support (68% BS MP, 62% BS ML, and 0.72 PP). This relationship was supported by one transition at alignment position 120 and a one bp deletion at alignment position 58 in these subgroups.

3.2. LFY sequence variation in octoploid Austral Asplenium

Amplification and sequencing with the leafyF and leafyR primers produced clean sequence with no additive sites for three octoploid species: *Asplenium richardii*, *A. lyallii*, and *A. northlandicum* (Table 2). Identical *LFY* sequences were recovered from *A. richardii* and *A. lyallii*; these sequences were the same as that obtained from *A. hookerianum* samples C and D. In contrast, *A. northlandicum* had a very different sequence from any of the tetraploids and possessed nine autapomorphies. However, it did share two synapomorphies with the tetraploids of the Obtusatum subgroup (alignment positions 79 and 119). For both *A. northlandicum* and *A. lyallii*, amplification with the leafyF and leafyshortR primers produced the same sequence as obtained with the leafyF and leafyR primers.

The *LFY* sequences from the remaining five octoploid species demonstrated additive nucleotide sites (Table 2). Furthermore, direct sequencing of several of the octoploids resulted in a region of clean sequence followed by sequence with many overlaid peaks. In all cases the start of the mixed sequence corresponded to the posi-

tion of an insertion/deletion (indel) event in one or more of the tetraploids, indicating the presence of different length *LFY* sequences. Even though in these regions the direct sequences were mixed, it was straightforward to determine the different contributing sequences because they were consistently displaced by the size of the indel (almost always 1 bp).

A comparison of synapomorphic and autapomorphic characters in the tetraploid taxa with the corresponding sites in the octoploid taxa allowed the determination of putative parental species, or at least subgroup, for the octoploids. All of the samples of *Asplenium cimmeriorum* and *A. gracillimum* exhibited the two autapomorphies for *A. hookerianum* (alignment positions 73 and 78) and the autapomorphy for *A. bulbiferum* (alignment position 317). The cloning of the *LFY* locus in *A. gracillimum* sample A confirmed the presence of two different sequences: one identical to that found in *A. bulbiferum* and the other identical to that found in *A. hookerianum* samples C and D.

The two autapomorphies for *Asplenium hookerianum* also occurred in *A. appendiculatum* subsp. *maritimum* and *A. appendiculatum* subsp. *appendiculatum*. These two taxa also demonstrated additivity at alignment positions 35 and 147, both of which are synapomorphies for the Flaccidum subgroup. They also show additivity at positions 169 and 237. At both of these sites all of the *A. flaccidum* samples exhibit an apomorphic state, although it is not fixed (i.e., *A. flaccidum* subsp. *haurakiense* sample B is heterozygous at both sites). Cloning of *A. appendiculatum* subsp. *maritimum* sample B revealed the presence of one sequence identical to *A. flaccidum* subsp. *flaccidum* sample B (clone type 2; Table 2) and a second identical to *A. hookerianum* sample B (clone type 1; Table 2).

The additive sites in the *Asplenium shuttleworthianum LFY* sequence suggest the presence of a sequence from *A. flaccidum*, as indicated by positions 169 and 237, as discussed above. The other sequence appears to derive from an unsampled member of the Flaccidum subgroup because *A. shuttleworthianum* is fixed for the Flaccidum subgroup synapomorphies at alignment positions 35 and 147.

For *Asplenium scleroprium*, amplification and sequencing with the leafynonOBTR and leafyF primers produced a single clean sequence. The final, superimposed sequence presented in Table 2 was determined by comparison of this sequence with the partially mixed sequence produced by the leafyF and leafyR primers. No product was amplified with the leafyOBTR and leafyF primers. The six autapomorphies for *A. obtusatum* were present in the *A. scleroprium LFY* sequence (alignment positions 66, 67, 70, 71, 93, and 228). The two synapomorphies for the Flaccidum subgroup (positions 35 and 147) were present, as was a synapomorphy uniting *A. flaccidum* with *A. chathamense* (position 245; to the exclusion of *A. lamprophyllum*) and a 1bp deletion that otherwise occurred in one of the different sequences in *A. flaccidum* subsp. *haurakiense* sample B (position 73).

4. Discussion

4.1. Tetraploid LFY diversity and relationships

Tetraploidy has been proposed to be the ancestral state for the Austral *Asplenium* group (Perrie and Brownsey, 2005a; although see Shepherd et al., 2008). However, the absence of fixed additivity across the tetraploid species is consistent with *LFY* acting as a single-copy locus in this group, and it sheds no light on the ultimate polyploid origins of this group, particularly because of the possibility of sequence loss (see also Section 4.2). Although peripheral to the objectives of our study, future investigations using *LFY* to examine the evolution of *Asplenium* would likely benefit from a determination of how many homoeologous *LFY* loci are present amongst the different ploidies.

The *LFY* sequences exhibited relatively more variation within the Austral *Asplenium* than previously published cpDNA sequences. Each tetraploid species was able to be distinguished with *LFY*, although neither the *A. oblongifolium* or *A. flaccidum* samples had respective synapomorphies (*A. flaccidum* did have two nucleotide sites that were fixed for derived states except for additivity in one sample). The six substitutions between *A. oblongifolium* and *A. obtusatum* across the approximately 260 bp of *LFY* sequence contrasts markedly with the four substitutions distinguishing these species in 3700 bp of chloroplast sequence (Shepherd et al., 2008). Despite limited infra-specific sampling, *LFY* sequence variation was even detected within some of the tetraploid species, with, for example, three different sequences being found in the four *A. hookerianum* samples.

The three Austral *Asplenium* subgroups previously identified with cpDNA sequences were also supported by nuclear *LFY* sequences, with each subgroup exhibiting two to ten synapomorphies. Despite considerable support for the subgroups, the relationships between them were not resolved with any confidence.

4.2. Polyploidy in the Austral group octoploids

The inferred origin for each of the Austral group octoploids in New Zealand is summarised in Fig. 1. Only single *LFY* sequences were amplified from each of the octoploids *Asplenium lyallii*, *A. northlandicum* and *A. richardii*. Two hypotheses may explain these results: (1) these octoploids are autopolyploids and inherited two identical copies of *LFY*; or (2) these octoploids are allopolyploids that originally had *LFY* sequences from two different tetraploids, but the locus contributed by one parent has been subsequently lost or become non-amplifiable because of mutation in the primer site(s). Allopolyploids frequently lose redundant DNA sequences (reviewed in Paun et al., 2007).

The second hypothesis provides the most plausible explanation for *Asplenium lyallii*, given its *LFY* and cpDNA sequences. The *LFY* sequence from this species was identical to one obtained from *A. hookerianum*. However, cpDNA sequence from the same *A. lyallii* samples indicated that this species' chloroplast parent was a member of the Obtusatum subgroup (either *A. oblongifolium*, *A. obtusatum*, or some close, unidentified relative; Perrie and Brownsey, 2004, 2005a). The *LFY* sequence type of the Obtusatum subgroup has seemingly been lost from *A. lyallii*. *Asplenium lyallii* has long been regarded on morphological grounds as a probable allopolyploid (Brownsey, 1977b), but determining which species from the Obtusatum subgroup was involved will require additional data. The (assumedly tetraploid) sterile hybrid *A. hookerianum* × *oblongifolium* has been putatively recorded once (Perrie and Brownsey, 2004), but *A. hookerianum* × *obtusatum* is not known (Brownsey, 1977b).

The second hypothesis, of allopolyploidy with subsequent loss of one of the parental sequences, may also apply to *Asplenium northlandicum*. This octoploid used to be regarded as a subspecies of *A. obtusatum* (i.e., *A. obtusatum* subsp. *northlandicum*) as it can only be morphologically distinguished by the size of its stipe scales and spores (Brownsey, 1977a). However, *A. northlandicum* does not appear to be an autopolyploid of either *A. obtusatum* or *A. oblongifolium*. Not only did *A. northlandicum* lack a number of *LFY* synapomorphies for these two tetraploids, it also displayed nine autapomorphies. Either these tetraploids are not involved in the parentage of *A. northlandicum*, or evidence of their involvement has been lost. The latter seems more likely given the much closer relationship between the cpDNA sequences of *A. obtusatum*, *A. oblongifolium* and *A. northlandicum* (Shepherd et al., 2008).

Taken together, the *LFY* and cpDNA sequences suggest that *A. northlandicum* is an allopolyploid, with *A. obtusatum*, *A. oblongifolium*, or a close relative as the chloroplast parent, and an unknown tetraploid (the source of the very different *LFY* sequence of *A. northlandicum*) as the other parent. This supports the taxonomic recognition of *A. northlandicum* as a distinct species (Ogle, 1987), rather than including it as a subspecies of *A. obtusatum*. Expanded sampling may identify extant tetraploids with *LFY* sequence identical, or at least more similar, to that in *A. northlandicum*. Of particular interest are the circum-Antarctic and Pacific populations outside New Zealand that have been referred to *A. obtusatum* (Brownsey, 1998), as well as other one-pinnate species from the South Pacific, such as the New Caledonian *A. oligolepidum*, which may be allied to the Obtusatum subgroup (Shepherd et al., 2008).

Asplenium richardii provides the only example of probable autopolyploidy amongst the New Zealand Austral octoploids. Both *A. richardii* samples had a *LFY* sequence identical to one of the variants obtained from *A. hookerianum*. Although it is possible that a contribution from another parent may have been lost, as outlined above, the morphology, cpDNA sequences and AFLP DNA-fingerprinting of *A. richardii* provides no evidence for the involvement of any species in addition to *A. hookerianum* (Brownsey, 1977b; Perrie and Brownsey, 2005b).

LFY sequences from the remaining octoploids indicated that they are all allopolyploids. Both *Asplenium cimmeriorum* and *A. gracillimum* are allopolyploids of *A. bulbiferum* and *A. hookerianum*, whose sterile hybrid has been recorded (Brownsey, 1977b). *A. gracillimum* was previously considered a putative autopolyploid, and subspecies, of *A. bulbiferum* (Brownsey, 1977a,b). However, *A. gracillimum* was found to have the cpDNA of *A. hookerianum* (Perrie and Brownsey, 2005a). This led to the suggestion of it being an allopolyploid, with the revival of its recognition as a separate species (Perrie et al., 2005), although the involvement of *A. bulbiferum* rested solely on morphological resemblance. *Asplenium cimmeriorum* was suggested to be an allopolyploid at its initial description (Brownsey and de Lange, 1997), although its parentage was not clear. However, *LFY* sequences now provide molecular data that clearly implicates allopolyploid origins from *A. bulbiferum* and *A. hookerianum* for both *A. cimmeriorum* and *A. gracillimum*. The *LFY* sequences from *A. cimmeriorum* samples A, B, and D, and *A. gracillimum* samples A, B, C, and D were identical and consistent with derivation from the *LFY* sequences found in *A. bulbiferum* samples A and B, and *A. hookerianum* samples C and D (this is the plesiomorphic, or ancestral, sequence in *A. hookerianum*). The other *A. cimmeriorum* and *A. gracillimum* samples differed only in each having a single additive site with an autapomorphy.

Asplenium appendiculatum subsp. *appendiculatum* and *A. appendiculatum* subsp. *maritimum* are both allopolyploids of *A. hookerianum* and *A. flaccidum*. The latter's involvement is not surprising. *Asplenium appendiculatum* (as *A. terrestre*) was not segregated from *A. flaccidum* until recognised by Brownsey (1977a), and origins via autopolyploidy from *A. flaccidum* (Brownsey, 1977b) or *A. chathamense* (for *A. appendiculatum* subsp. *maritimum*; Brownsey, 1985) have been proposed. An allopolyploid origin including *A. hookerianum* from the Bulbiferum subgroup is, therefore, a novel and unexpected result, although the sterile hybrid *A. flaccidum* subsp. *flaccidum* × *hookerianum* has been recorded (Brownsey, 1977b). The subspecies of *A. appendiculatum* are primarily differentiated ecologically, although the morphological extremes are quite distinctive (Fig. 1). It is unclear whether they have diverged subsequent to a single shared allopolyploid event, or if they have originated independently; there is weak cpDNA sequence data, comprising a single substitution prone to homoplasy within the Austral group, consistent with the latter hypothesis (Perrie and Brownsey, 2005a). However, the cloned *LFY* sequence from *A. appendiculatum* subsp. *maritimum* sample B shared a synapomorphy (an A at alignment position 235) with *A. hookerianum* sample B, suggesting *A. appendiculatum* subsp. *maritimum* may have had more than one origin. Alternative explanations for this shared polymorphism include homoplasy, tetraploid-to-octoploid gene-flow subsequent to the initial octoploid origin, or a single polyploid origin involving an unreduced gamete from an individual of *A. hookerianum* that was polymorphic for the *LFY* sequences detected in samples B and C/D. Determining the precise relationships of *A. flaccidum*, *A. appendiculatum*, their subspecies, and *A. chathamense* requires further data.

Asplenium scleroprium has long been considered on morphological grounds a probable allopolyploid, with parents from the Obtusatum and Flaccidum subgroups (probably *A. obtusatum* and *A. flaccidum*; Brownsey, 1977b). Chloroplast DNA sequence data indicated the chloroplast had been inherited from the Obtusatum subgroup, but could not establish the exact species (Perrie and Brownsey, 2004, 2005a). However, *LFY* sequences clearly identified *A. obtusatum* as one of the parents, and one synapomorphy in additional cpDNA sequence data from the *rps4* locus confirmed this result (comparison between *A. scleroprium* GenBank Accession EU560980, and data from Shepherd et al., 2008). The other parent is from the *A. chathamense* and *A. flaccidum* lineage, as indicated by a synapomorphy (alignment position 245) that is additive in *A. scleroprium*. *Asplenium scleroprium* is, however, not additive for the *A. chathamense* autapomorphies (alignment positions 99, 253 and 395), or for the derived states present in *A. flaccidum* (alignment positions 169 and 237), which are fixed except for being heterozygous in *A. flaccidum* subsp. *haurakiense* sample B. Furthermore, *Asplenium scleroprium* is additive for an apomorphic deletion (alignment position 73) otherwise found only in *A. flaccidum* subsp. *haurakiense* sample B. This suggests a derivation from the latter taxon, although this seems anomalous given their present non-overlapping distributions: *A. flaccidum* subsp. *haurakiense* occurs coastally around the northern North Island, while *A. scleroprium* is distributed southward from the southern tip of the South Island (*A. obtusatum* overlaps with *A. scleroprium*, as does *A. flaccidum* subsp. *flaccidum*, and the sterile hybrid *A. flaccidum* subsp. *flaccidum* × *obtusatum* has been recorded; Brownsey, 1977b). Further sampling and data would be desirable to confirm this result.

The relationships and origins of *Asplenium shuttleworthianum* have been somewhat obscure (Brownsey, 1977a; Perrie and Brownsey, 2005a). *LFY* sequences indicate that it is an allopolyploid, with *A. flaccidum* as one parent. The other, and apparent chloroplast, parent is also from the Flaccidum subgroup. However, it is not closely related to any of the sampled tetraploids, since *A. shuttleworthianum* has *LFY* sequences additive for several apomorphic substitutions not found elsewhere, and its cpDNA is also distinctive (Perrie et al., 2005). Candidates for the second parent of *A. shuttleworthianum* include the morphologically-similar complex of Pacific ferns that encompasses *A. gibberosum*.

These inferences made about the origins of the New Zealand Austral octoploids have a couple of caveats. The first is that the polarities of the mutations observed within the *LFY* sequences of the Austral group were inferred with a single outgroup. While additional outgroups should lessen the prospect of getting the polarity wrong because of homoplasy, most of the progenitors were inferred using multiple variable nucleotide sites, and in these cases all of the relevant sites are unlikely to be simultaneously affected by homoplasy. Furthermore, the polarities we have inferred for the individual tetraploid species are consistent with the morphological and cpDNA sequence data. For instance, the polarity for the single inferred synapomorphy of *A. bulbiferum* (alignment position 317) is unlikely to be wrong, given the strong morphological and cpDNA sequence data indicating *A. bulbiferum* is sister to *A. hookerianum* rather than to a clade comprising the Flaccidum and Obtusatum groups plus *A. hookerianum*. The second caveat is

that we only sampled Austral tetraploids from New Zealand, and that some of the New Zealand Austral octoploids may have been derived from tetraploids not found in New Zealand. However, we know of no likely candidates on the basis of morphology and available cpDNA sequences, other than those already mentioned above for *A. northlandicum* and *A. shuttleworthianum*.

4.3. The predominance of allopolyploidy in the Austral Asplenium

The biparentally-inherited *LFY* locus revealed a number of previously unrecognised relationships between the tetraploid and octoploid New Zealand species of the Austral *Asplenium* group. In particular, allopolyploidy is far more predominant in this group than previously suggested from morphology and cpDNA sequence data (Brownsey, 1977b; Perrie and Brownsey, 2005a). *Asplenium appendiculatum* and *A. northlandicum* were previously considered possible autopolyploids (Brownsey, 1977b), as was *A. gracillimum* until recently (Perrie and Brownsey, 2005a), while the origin of *A. shuttleworthianum* has not been explicitly contemplated. However, *LFY* sequences, sometimes in conjunction with cpDNA sequence data, indicate that all of these are allo-octoploids, along with *A. lyallii* and *A. scleroprium*. Allo-octoploids have formed both from tetraploids within the same Austral subgroup, as well as every possible combination of the three subgroups. *Asplenium richardii* is apparently the only auto-octoploid.

The high level of allopolyploid events within the New Zealand Austral *Asplenium* is consistent with overseas studies of the genus. Two thirds of the polyploids in European *Asplenium* (Lovis, 1977; Reichstein, 1981) and all of the *Asplenium* polyploids in North America are allopolyploids (Wagner et al., 1993). Furthermore, spore-size data from the New Zealand Austral group (Shepherd et al., 2007; Perrie, unpublished data) has provided no evidence for cryptic auto-octoploids amongst the tetraploid species. Known *Asplenium* autopolyploids appear to be largely confined to Europe (see Braithwaite, 1986, for a South African example). Collectively these data emphasise the importance of allopolyploidy in the generation of diversity in *Asplenium*. This contrasts with recent suggestions that autopolyploidy has had a more significant role in angiosperm speciation than previously thought (Soltis et al., 2007). Extending investigations beyond angiosperms is required to assess the extent and relative importance of autopolyploidy in species diversification and evolution in general. A high rate of allopolyploidy appears to be typical of *Asplenium* worldwide, but it may also be a feature of the wider New Zealand vascular plant flora (Hair, 1966).

The New Zealand Austral *Asplenium* tetraploids differ markedly in their involvement in forming octoploids. There is no evidence for *A. lamprophyllum* contributing to any of the octoploids, while *A. hookerianum* appears to have had a central role, being a progenitor for five of the octoploid species. There is also evidence that some of the octoploid 'species' derived from *A. hookerianum* have formed multiple times (i.e., *A. cimmeriorum* and *A. gracillimum*; Perrie et al., unpublished).

Asplenium hookerianum has extensive cpDNA variation (Shepherd et al., 2007) suggesting it is an old lineage. The phylogeography of its cpDNA indicates it survived at least the last glacial period in widespread and possibly large populations (Shepherd et al., 2007), therefore possibly giving it more opportunities to be involved in polyploid formation. Furthermore, the preference of *A. hookerianum* for dry habitats (Shepherd et al., 2007), which were a feature of the Pleistocene Ice Age in New Zealand (McGlone et al., 1993), may not only explain its own success, but have conferred an advantage for its polyploid derivatives, all of which occur in drier and/or limestone habitats. Preliminary molecular dating of cpDNA sequences supports this hypothesis that the octoploids arose during the Pleistocene, with the tetraploids within each Austral subgroup only diverging from one another within the last several million years (Perrie et al., unpublished; divergences within the Obtusatum subgroup beginning at 1.3 million years ago, Bulbiferum 2.6, Flaccidum 5.6; molecular dating using the *rbcL* and *rps4* chloroplast gene DNA sequences from Shepherd et al., 2008, and the methods of Perrie and Brownsey, 2007). The tetraploid subgroups themselves diverged from one another approximately ten million years ago (Perrie et al., unpublished), and it is perhaps striking that the extant Austral octoploids are much more recent. Indeed, most of the New Zealand Austral octoploids have identical or very similar cpDNA to their progenitor tetraploids (Perrie and Brownsey, 2005a).

Pleistocene origins have also been postulated for many Northern Hemisphere polyploids (Abbott and Brochmann, 2003), with the harsh and variable environmental conditions of the Ice Age possibly favouring polyploid formation. Environmental disturbance seems to increase the incidence of hybridisation (Soltis et al., 2003) while variation in temperature and water stress have both been linked to an increased production of unreduced gametes (Ramsey and Schemske, 1998; Mable, 2004). Glacial periods in New Zealand were also characterised by lowered annual temperatures in addition to drought, and with periodic freezing air masses (McGlone et al., 1993).

Selection for niche partitioning in newly formed polyploids has been proposed to reduce both competition and back-crossing with their progenitors (Otto and Whitton, 2000). However, none of the octoploids within the Austral *Asplenium* have markedly transgressive (i.e., novel) niches, with all except *A. northlandicum* frequently occurring with one or even both progenitor tetraploids, although their relative competitive abilities across different habitats have not been experimentally studied. With no obvious niche differences now, the octoploids may have initially escaped ecological and reproductive competition with their tetraploid progenitors through allopatry brought about by the cycles of fragmenting and expanding forests of the dynamic environment during the Pleistocene. Interestingly, and perhaps because of niche constraints, there is no extant allo-octoploid of one of today's most common tetraploid (sterile) hybrids, *A. bulbiferum* × *flaccidum*, which is frequently found as a low epiphyte in wet forest. The situation in New Zealand, where many of the *Asplenium* octoploids are widespread and common, contrasts with Europe where many of the allotetraploids have restricted distributions (Lovis, 1977) and it is the autotetraploids that are widespread (Vogel et al., 1999).

4.4. Future directions for LFY sequences in ferns

The infra-specific variation detected at the *LFY* locus for some tetraploids may be of use for phylogeographic studies. For instance, *LFY* may be useful for determining whether the pronounced east-west cpDNA phylogeography found in *A. hookerianum* (Shepherd et al., 2007) is also reflected in the nuclear genome, or if it is merely chloroplast lineage sorting.

The biparental inheritance of the *LFY* locus means it is likely to be extremely useful for testing and refining hypotheses of relationships and origins within polyploid complexes in *Asplenium* and other fern groups. Its value is heightened by the present lack of nuclear DNA sequence markers for ferns. However, the potential for losing one parental copy (e.g., *A. lyallii* above) means it is likely that multiple nuclear (plus chloroplast) sequence markers will be necessary for complete resolution of a complex's infra-relationships. Continued investigations in ferns and other groups will be crucial for testing the generality of specific polyploid processes, given that much of the present understanding is based on observations made with angiosperms (e.g., Soltis et al., 2007).

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