

Phylogeny of the fern family Aspleniaceae in Australasia and the south-western Pacific

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Abstract. Aspleniaceae is one of the largest fern families. It is species-rich in Australasia and the south-western Pacific (ASWP), where approximately 115 species occur. In the current study, the chloroplast regions *rbcL*, *trnL–trnF* and *rps4–trnS* were sequenced for 100 Aspleniaceae samples from ASWP. These data were combined with published sequences for species from New Zealand and other regions for phylogenetic analyses. Species of Aspleniaceae from ASWP were placed in six of the eight previously identified inter-continental clades. The majority of species from ASWP were placed in two of these clades, with the remaining four clades each being represented by three or fewer species. Strong biogeographic affinities with South-east Asia were observed and immigration, rather than local radiations of endemic taxa, appears to have made a more important contribution to patterns of diversity in ASWP. This study supports the current taxonomic practice of recognising two genera, *Asplenium* L. and *Hymenasplenium* Hayata, in Aspleniaceae, and identifies future taxonomic work required for the family in this region, including potential synonymising of species, and revision of species complexes or widespread species that are demonstrably non-monophyletic.

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Introduction

Aspleniaceae is one of the largest fern families, including ~700 species (Kramer and Viane 1990; Smith *et al.* 2006). It is diverse in Australasia and the south-western Pacific (here abbreviated to ‘ASWP’ and used to encompass Australia, New Guinea, Vanuatu, Fiji, New Caledonia and New Zealand). Approximately 115 species of Aspleniaceae occur in this region, many of which are local endemics (Table 1).

The family principally comprises a large cosmopolitan genus, *Asplenium* L. (Smith *et al.* 2006). Many taxonomic treatments have also recognised several morphologically identifiable groups as segregate genera or sections (e.g. Copeland 1947; Pichi Sermolli 1977; Tryon and Tryon 1982), some of which occur in ASWP, including *Asplenium* section *Thamnopteris* C.Presl, *Hymenasplenium* Hayata, *Loxoscapha* T.Moore and *Pleurosorus* Fée (Brownsey 1998; Brownsey and Perrie 2011). However, molecular studies are encouraging retention of only one segregate genus, *Hymenasplenium*, which is resolved as sister to all other Aspleniaceae, in contrast to the other segregate genera that are nested within *Asplenium* (Murakami 1995; Murakami *et al.* 1999a; Schneider *et al.* 2004).

Molecular systematic studies have included many species and groups of Aspleniaceae from various parts of the world (e.g. Murakami 1995; Murakami *et al.* 1999a, 1999b; Pinter *et al.* 2002; Van den heede *et al.* 2003; Schneider *et al.* 2004, 2005; Bellefroid *et al.* 2010; Dong *et al.* 2012), including

New Zealand (Perrie and Brownsey 2005; Shepherd *et al.* 2008a, 2008b; Perrie *et al.* 2010). However, the phylogenetic positions of very few Aspleniaceae from other areas of ASWP have been studied through DNA sequencing (Schneider *et al.* 2004; Schneider *et al.* 2005; Yatabe *et al.* 2009).

Morphological classifications (e.g. circumscriptions of segregate genera) and molecular phylogenetic analyses both support the notion that Aspleniaceae in Australia includes several widespread lineages. Schneider *et al.* (2004) established a global Aspleniaceae chloroplast phylogeny incorporating species from most parts of the world, and defined eight major clades. That study included some New Zealand Aspleniaceae, and some widespread species shared with ASWP. Fourteen species (of ~115) that occur in ASWP were included in their study and were placed in four of the major molecular groups. Among these species were *A. nidus* (species authorities provided in Tables 1 and 2) and *A. phyllitidis* of section *Thamnopteris*, and *Hymenasplenium unilaterale* (as *A. unilaterale*). In addition, *Pleurosorus hispanicus* (as *A. hispanicum*) from Europe was placed in a separate group from any species of ASWP included in the study. These findings indicated that at least five of the eight major groups occur in ASWP. However, it remains to be confirmed whether Australian *Pleurosorus* is closely related to the European *Pleurosorus* species, and whether species shared between ASWP and other regions are truly conspecific. With limited sampling from ASWP, it also remains to be determined

Table 1. Aspleniaceae species of Australasia and the south-western Pacific

Distributions are collated from Copeland (1949), Brownlie (1969), Holttum (1974), Salvo *et al.* (1982), Ogle (1987), DuPuy (1993), Green (1994), Jones (1996), Brownsey (1998), Brownsey and Smith-Dodsworth (2000), Short *et al.* (2003), Sasaki (2008) and Brownsey and Perrie (2011). Species analysed for the current study are given in bold. Aus, Australia; NCal, New Caledonia; NZ, New Zealand; PNG, Papua New Guinea; Van, Vanuatu

Aspleniaceae species in Australasia and south-western Pacific	PNG	Aus	Van	Fiji	NCal	NZ
<i>Asplenium acrobryum</i> Christ	Y					
<i>A. aethiopicum</i> (Burm.f.) Bech. ^{A,B}		Y				
<i>A. affine</i> Sw. ^{A,B}	Y					
<i>A. amboinense</i> Willd. ^A	Y		Y	Y		
<i>A. appendiculatum</i> (Labill.) C.Presl		Y				Y
<i>A. athertonense</i> S.B.Andrews		Y				
<i>A. attenuatum</i> R.Br.		Y			Y	
<i>A. australasicum</i> (J.Sm.) Hook. ^B		Y	Y	Y	Y	
<i>A. baileyianum</i> (Domin) Watts		Y				
<i>A. bakeri</i> C.Chr.	Y					
<i>A. bicentenniale</i> D.L.Jones		Y				
<i>A. bipinnatifidum</i> Baker	Y		Y	Y		
<i>A. brassii</i> C.Chr.	Y					
<i>A. bulbiferum</i> G.Forst.						Y
<i>A. capitisyork</i> D.L.Jones		Y				
<i>A. caudatum</i> G.Forst. ^{A,B}	Y	Y	Y	Y		
<i>A. carnarvonense</i> Brownsey		Y				
<i>A. carruthersii</i> Baker			Y	Y		
<i>A. chathamense</i> Brownsey						Y
<i>A. cimmeriorum</i> Brownsey & de Lange						Y
<i>A. clemensiae</i> Copel.	Y					
<i>A. comosum</i> Christ ^A	Y					
<i>A. contiguum</i> Kaulf. ^{A,B}			Y			
<i>A. cromwellianum</i> Rosenst. ^A	Y					
<i>A. cuneatum</i> Lam. ^{A,B}	Y	Y	Y	Y	Y	
<i>A. cymbifolium</i> Christ	Y					
<i>A. decorum</i> Kunze ^A	Y					
<i>A. difforme</i> R.Br.		Y				
<i>A. dimorphum</i> Kunze		Y				
<i>A. dognyense</i> Rosenst.					Y	
<i>A. durum</i> Copel.	Y					
<i>A. filidens</i> Brownlie					Y	
<i>A. filipes</i> Copel.	Y					
<i>A. flabellifolium</i> Cav.		Y				Y
<i>A. flaccidum</i> G.Forst.		Y				Y
<i>A. foersteri</i> Rosenst.	Y					
<i>A. gibberosum</i> (G.Forst.) Mett. ^B			Y	Y		
<i>A. goudeyi</i> D.L.Jones		Y				
<i>A. gracillimum</i> Colenso		Y				Y
<i>A. hapalophyllum</i> Rosenst.	Y					
<i>A. harmanii</i> D.L.Jones		Y				
<i>A. hookerianum</i> Colenso		Y				Y
<i>A. induratum</i> Hook.				Y		
<i>A. insititium</i> Brack. ^B	Y		Y	Y	Y	
<i>A. kelelense</i> Brause	Y					
<i>A. keysserianum</i> Rosenst.	Y					
<i>A. lamprophyllum</i> Carse						Y
<i>A. laserpitifolium</i> Lam. ^{A,B}	Y	Y	Y	Y	Y	
<i>A. laxifolium</i> Alderw.	Y					
<i>A. listeri</i> C.Chr.		Y				
<i>A. lobulatum</i> Mett. ex Kuhn ^{AB}				Y	Y	
<i>A. longissimum</i> Blume = <i>A. acutiusculum</i> Blume ^A	Y	Y				
<i>A. lyallii</i> (Hook.f.) T.Moore						Y
<i>A. macrophyllum</i> Sw. ^{A,B}	Y					
<i>A. marattioides</i> (Brack.) C.Chr. ^B			Y	Y		
<i>A. milnei</i> Carruth.		Y				
<i>A. monotis</i> Christ	Y					

(continued next page)

Table 1. (continued)

Aspleniaceae species in Australasia and south-western Pacific	PNG	Aus	Van	Fiji	NCal	NZ
<i>A. morobense</i> Copel.	Y					
<i>A. musifolium</i> Mett. ^A	Y					
<i>A. nidiforme</i> Alderw. ^A	Y					
<i>A. nidus</i> L. ^{A,B}	Y	Y	Y	Y	Y	
<i>A. nitidum</i> Sw.	Y					
<i>A. normale</i> D.Don ^{AB}	Y	Y				
<i>A. northlandicum</i> (Brownsey) Ogle ^B		Y				Y
<i>A. novae-caledoniae</i> Hook.					Y	
<i>A. novoguineense</i> Rosenst.	Y					
<i>A. nutans</i> Rosenst.	Y					
<i>A. oblongifolium</i> Colenso						Y
<i>A. obtusatum</i> G.Forst. ^B						Y
<i>A. oligolepidum</i> C.Chr.					Y	
<i>A. paleaceum</i> R.Br.		Y				
<i>A. papuanum</i> Copel.	Y					
<i>A. parvum</i> Watts	Y	Y				
<i>A. pauperequitum</i> Brownsey & P.J.Jacks.						Y
<i>A. pellucidum</i> Lam. ^{A,B}	Y	Y				
<i>A. persicifolium</i> J.Sm. ^A	Y					
<i>A. phylitidis</i> D.Don ^A	Y					
<i>A. planicaule</i> Wall. ^{A,B}	Y					
<i>A. polyodon</i> G.Forst. ^{A,B}	Y	Y	Y	Y	Y	Y
<i>A. polyphyleticum</i> Compton					Y	
<i>A. pseudobulbiferum</i> Brownlie					Y	
<i>A. pseudotenerum</i> Brownlie					Y	
<i>A. pteridoides</i> Baker		Y				
<i>A. regis</i> Copel.	Y					
<i>A. remotum</i> T.Moore ^A	Y					
<i>A. richardii</i> (Hook.f.) Hook.f.						Y
<i>A. sancti-christofori</i> Christ in Schum. & Laut.	Y					
<i>A. scandens</i> J.Sm. ^A	Y					
<i>A. schizocarpum</i> (Copel.) Copel. ≡ <i>Diplora schizocarpa</i> (Copel.) C.Chr. ^A	Y					
<i>A. schultzei</i> Brause	Y					
<i>A. scleroprium</i> Hombr.						Y
<i>A. scolopendropsis</i> F.Muell. = <i>D. durvillei</i> (Bory) C.Chr.	Y					
<i>A. setisectum</i> Blume	Y					
<i>A. shawii</i> Copel.	Y					
<i>A. shuttleworthianum</i> Kunze						Y
<i>A. simplicifrons</i> F.Muell.		Y				
<i>A. spirale</i> Holttum	Y					
<i>A. stenolobum</i> C.Chr. ^B				Y		
<i>A. submarginatum</i> Rosenst.	Y					
<i>A. subflexuosum</i> Rosenst.			Y		Y	
<i>A. subglandulosum</i> (Hook. & Grev.) Salvo, Prada & T.E.Diaz = <i>Pleurosorus rutifolius</i> (R.Br.) Fee ≡ <i>P. subglandulosus</i> (Hook. & Grev.) Tindale		Y				Y
<i>A. surrogatum</i> P.S.Green		Y				
<i>A. tafanum</i> C.Chr.	Y					
<i>A. tenerum</i> G.Forst. ^{A,B}	Y	Y		Y		
<i>A. tenuiculum</i> Rosenst.					Y	
<i>A. translucens</i> (Holttum) Viane ≡ <i>Diplora translucens</i> Holttum	Y					
<i>A. trichomanes</i> L. ^{A,B}	Y	Y				Y
<i>A. vieillardii</i> Mett.					Y	
<i>A. vittaeforme</i> Cav. ^A	Y					
<i>A. wernerii</i> Rosenst.	Y					
<i>A. wildii</i> F.M.Bailey		Y				
<i>Hymenasplenium excisum</i> (C.Presl) S.Linds ^{AB}		Y	Y	Y		
<i>Hymenasplenium unilaterale</i> (Lam.) Hayata ^{AB}	Y	Y	Y	Y	Y	
<i>H. subnormale</i> (Copel.) T.Nakaike ^{AB}			Y			

^ASpecies is shared with Indonesia and/or other parts of South-east Asia^BSpecies is shared with other regions of the world.

Table 2. Accessions used in the present study

Details are as follows: taxon name; region of collection, collection identifier (herbarium); *rbcl* GenBank accession number; *trnL-trnF* GenBank accession number; *rps4-trnS* GenBank accession number (— is given here for accessions without *rps4-trnS*). CI, Christmas Island; LHI, Lord Howe Island; NI, Norfolk Island; NP, National Park; NT, Northern Territory; QLD, Queensland; VIC, Victoria; TAS, Tasmania

Asplenium abscissum Willd.; French Guiana, *Boudrie 3278* (BM); AY300102; AY300049; AY549768. *A. aegaeum* Lovis, Reichstein & Zaffran; Crete, *Jermey 9181* (BM); AY300103; AY300050; AY549774. *A. aethiopicum* (Burm.f.) Bech.; Kenya, *Hemp 22* (BM); AF240654; AF525233; AY549823. *A. aethiopicum*; Australia, VIC, Cultivation Gorge, *Ohlsen 228* (MELU); KP774922; KP835385; KP835452. *A. aethiopicum*; Kenya, *Hemp 22* (BM); AF240654; AF525233; —. *A. affine* Sw.; Borneo, *Schneider 954* (SAR); AY300104; AY300051; AY549826. *A. affine*; Papua New Guinea, *Kessler 14066* (UZH); KP774940; KP835388; KP835438. *A. amboinense* Willd.; Papua New Guinea, *Kluge 9014* (UZH); KP774888; KP835372; KP872959. *A. amboinense*; Fiji, *Brownsey & Perrie FLJI 78* (WELT); KP774914; KP835355; KP851899. *A. anceps* Lowe ex Hook & Grev.; Azores, *Vogel 1111* (BM); AY300105; AY300052; AY549795. *A. angustum* Sw.; French Guiana, *Boudrie 3254* (BM); AY300106; AY300053; AY549822. *A. anisophyllum* Kunze; Madagascar, *Kessler 12765* (GOET); AY300107; AY300054; AY549816. *A. antiquum* Makino; cult., *Perrie s.n.* (WELT P022088); EU240033; EU240028; EU240020. *A. appendiculatum* (Labill.) C.Presl; Australia, TAS, Meatus Falls, *Ohlsen 196* (MELU); KP774870; KP851900; KP835458. *A. atheronense* S.B.Andrews; Australia, QLD, South Johnstone River, *Ohlsen 289* (BRI, MELU); KP774875; KP835399; KP835426. *A. attenuatum* R.Br.; Australia, QLD, Carnarvon Gorge, *Ohlsen 317* (BRI, MELU); KP774918; KP218782; KP218768. *A. aureum* Cav.; Canary Islands, *Vogel Cet-116* (BM); AF240642; AF525258; AY549767. *A. australasicum* (J.Sm.) Hook.; Australia, QLD, Lamington NP, *Ohlsen 352* (BRI, MELU); KP774871; KP835357; KP835450. *A. australasicum*; Vanuatu, Efate, *Ohlsen 396* (MELU); KP774851; KP835366; KP835443. *A. australasicum*; Fiji, *Brownsey & Perrie FLJI 74* (WELT); KP774913; KP835368; KP851898. *A. australasicum*; New Caledonia, *Perrie NC 88* (WELT); KP774904; KP835354; KP835432. *A. baileyianum* Watts; Australia, QLD, Bridle Creek, *Ohlsen 267* (BRI, MELU); KP774943; KP639687; KP835408. *A. bicentennale* D.L.Jones; Australia, QLD, Lamb Range, *Ohlsen 273* (MELU); KP774944; KP218787; KP218752. *A. bipinnatifidum* Baker; Papua New Guinea, *Kluge 9037* (UZH); KP851875; KP835369; KP835416. *A. bipinnatifidum* Vanuatu, Tanna, *Ohlsen 394* (MELU); KP774891; KP835370; KP835415. *A. bipinnatifidum*; Fiji, *Brownsey & Perrie FLJI 111* (WELT); KP774915; KP835379; KP872963. *A. bulbiferum* G.Forst.; New Zealand, *Perrie 2159* (WELT P020494); AY283226; AY538182; EF418432. *A. bullatum* Wall. ex Mett.; China, *Chen 0044* (BM); AY300109; AY300056; AY549765. *A. capitisyork* D.L.Jones; Australia, QLD, Tozer Gap, cult. B.Gray, *Ohlsen 277* (MELU); KP774854; KP639686; KP835424. *A. carnarvonense* Brownsey; Australia, QLD, Carnarvon Gorge, *Ohlsen 318* (MELU); KP774855; KP218784; KP218774. *A. carruthersii* Baker; Fiji, *Brownsey & Perrie FLJI 73* (WELT); KP774917; KP835374; KP835417. *A. caudatum* G.Forst.; Borneo, *Schneider 1097* (SAR); AY300110; AY300057; —. *A. caudatum*; Vanuatu, Tanna, *Ohlsen 393* (MELU); KP774879; KP835387; KP835406. *A. chathamense* Brownsey; New Zealand, *Baird s.n.* (WELT P020498); DQ186552; AY283207; —. *A. cordatum* (Thunb.) Sw.; South Africa, *Vogel CET-119* (BM); AF240650; AF525235; AY549778. *A. cristatum* Lam.; Costa Rica, *Cranfill s.n.* (UC); AY549731; AY549834; AF425146. *A. cromwellianum* Rosenst. (UZH); Papua New Guinea, *Kluge 9000* (UZH); KP774933; KP639691; KP835441. *A. cromwellianum* (*A. capitisyork* clade); Papua New Guinea, *Kluge 9171* (UZH); KP774877; KP835404; KP851897. *A. cuneatiforme* H.Christ; Taiwan, *Cranfill TW039* (UC); AY549755; AY549859; AY549827. *A. cuneatum* Lam.; Australia, QLD, The Boulders, *Ohlsen 309* (MELU); KP774945; KP835400; KP835436. *A. cuneatum*; Fiji, *Brownsey & Perrie FLJI 15* (WELT); KP774903; KP835389; KP851896. *A. cuneatum*; New Caledonia, *Perrie NC 134* (WELT); KP774897; KP835390; KP835465. *A. cuspidatum* Lam.; Costa Rica, *Grantham & Parsons 0233090* (UC); AY300111; AY300058; AY549760. *A. dareoides* Desv.; cult. Jessen, *Vogel 351* (BM); AY300112; AY300059; —. *A. decorum* Kunze; Papua New Guinea, *Kluge 9108* (UZH); KP774892; KP835352; KP835429. *A. dielfalcatum* Viane; Hawaii, *Wood 7826* (PTBG); AY549738; AY549841; AY549787. *A. dielmanni* Viane; Hawaii, *Perlman SP18502* (PTBG); AY549739; AY549842; AY549788. *A. dielpallidum* N.Snow; Hawaii, *Wood 7809* (PTBG); AY549740; AY549843; AY549789. *A. difforme* R.Br.; Australia, QLD, Noosa Heads, *Ohlsen 336* (MELU); KP774873; KP835364; KP835454. *A. dimorphum* Kunze; Australia, NI – cult. L.Perrie; *Perrie 3101* (WELT) AY641802; AY641795; —. *A. dognyense* Rosenst.; New Caledonia, *Perrie NC 78* (WELT); KP774895; KP851901; KP835464. *A. elliottii* C.H.Wright; Kenya, *Hemp 4* (BM); AY549753; AY549857; AY549817. *A. erectum* Bory ex Willd.; Kenya, *Hemp 14* (BM); AY300113; AY300060; AY549770. *A. feei* Kunze ex Fee; Cult., *Vogel s.n.* (BM); AF525267; AF525244; AY549818. *A. filidens* Brownlie; New Caledonia, *Perrie NC 85* (WELT); KP774876; KP835396; KP835431. *A. fissum* Kit.; Crete, *Jermey 22816* (BM); AY300114; AY300061; AY549775. *A. flabellifolium* Cav.; Australia, TAS, Snug Falls, *Ohlsen 185* (MELU); KP774850; KP851902; KP835460. *A. flabellifolium*; New Zealand *Perrie et al. 2061* (WELT P020500); AY283227; AY283209; —. *A. flaccidum* G.Forst.; Australia, VIC, Wilson's Promontory NP, *Ohlsen 218* (MELU); KP774910; KP835363; KP835459. *A. flaccidum*; New Zealand, *Perrie 2061* (WELT P020501); AY283228; AY538183; EU250847. *A. fontanum* (L.) Bernh.; Germany, *Vogel F-3-92* (BM); AF525268; AF525239; AY549806. *A. formosum* Willd.; Belize, *Vogel AZO34* (BM); AY300116; AY300063; AY549796. *A. fragile* C.Presl; cult., Bot. Gard. Goett. (GOET); AY549733; AY549836; AY549772. *A. friesiorum* C.Chr.; Kenya, *Hemp 21* (BM); AY549756; AY549860; AY549828. *A. gemmiferum* Schrad.; Kenya, *Hemp 9* (BM); AY300117; AY300064; AY549819. *A. gibberosum* (G.Forst.) Mett.; Fiji, *Brownsey & Perrie 56* (WELT); KP774916; KP835345; KP851876. *A. gibberosum*; Vanuatu, Tanna, *Ohlsen 395* (MELU); KP774924; KP835378; KP851895. *A. goudeyi* D.L.Jones; Australia, LHI, cult. B.Gray, *Ohlsen 280* (MELU); KP774856; KP835371; KP835449. *A. gracillimum* Colenso; Australia, QLD, Lamington NP, *Ohlsen 358* (MELU); KP774908; KP835373; KP835435. *A. hallbergii* Mickel & Beitel; cult., *Vogel 350* (BM); AY300118; AY300065; AY549798. *A. harmanii* D.L.Jones; Australia, QLD, Poonyahra Falls, *Ohlsen 343* (MELU); KP774866; KP835375; KP835457. *A. hemionitis* L.; Azores, *Vogel HEM-9* (BM); AF240648; AF240663; AY549776. *A. heterochroum* Kunze; Belize, *Hughes 42* (BM); AY549745; AY549849; AY549799. *A. hispanicum* (Coss.) Greuter & Burdet (*Pleurosorus hispanicus* (Coss.) Morton); Spain, *Vogel PLE-1* (BM) AY300119; AY300066; AY549764. *A. hobdyi* W.H.Wagner; Hawaii, *Ranker 1806* (COLO); AY549736; AY549839; AY549785. *A. hookerianum* Colenso; Australia, VIC, Bryce Gorge, *Ohlsen 203* (MELU); KP774848; HM543743; KP835425. *A. hookerianum*; New Zealand, *Perrie 2201 & Shepherd* (WELT P020504); AY283229; AY641796; EU250848. *A. incisum* Thunb.; cult., *Jessen s.n.* (BM); AY549748; AY549852; AY549805. *A. insititium* Brack.; Fiji, *Brownsey & Perrie 21* (WELT); KP774864; KP851903; KP835463. *A. jahandiezii* (Litard.) Rouy; France, *Vogel Ja-1-83* (BM); AY300121; AY300068; AY549780. *A. juglandifolium* Lam.; French Guiana, *Boudrie 3249* (BM); AF525269; AF525245; AY459168. *A. kiangsuenae* Ching & Y.X.Jin; China, Mt Lushan, *Chang 102303* (HITBC); JX152738, JQ724224, JQ724309. *A. keyserrianum* Rosenst.; Papua New Guinea, *Kessler 13823* (UZH); KP774936; KP835403; KP835467. *A. laciniatum* L.Don; China, *Cheng s.n.* (BM); AY549747; AY549851; AY549801. *A. lamprophyllum* Carse; New Zealand, *Brownsey s.n.* (WELT P020506); AY283230; AY538185; EU240021. *A. laserpitifolium* Lam.; Papua New Guinea, *Kluge 9074* (UZH); KP774935; KP835405; KP835407. *A. laserpitifolium*; Australia, QLD, Josephine Falls, *Ohlsen 308* (MELU); KP774867; KP835398; KP851894. *A. laserpitifolium*; Vanuatu, Espiritu Santo, *Ohlsen 389* (MELU); KP774882; KP639690; KP835461. *A. laserpitifolium*; Fiji, *Brownsey & Perrie FLJI 72* (WELT); KP774901;

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Table 2. (continued)

KP835381; KP851893. *A. laserpitifolium*; New Caledonia, *Perrie NC 123* (WELT); KP774894; KP835402; KP835462. *A. listeri* C.Chr.; Australia, CI, *Grigg & Moloney s.n.* (MELU); KP774934; KP639696; KP851891. *A. lobulatum* Mett. ex Kuhn; Fiji, *Brownsey & Perrie FIJI 140* (WELT); KP774865; KP835380; KP851890. *A. lobulatum*; New Caledonia, *Perrie NC 94* (WELT); KP774886; KP835394; KP835439. *A. longissimum* Blume; Australia, NT, Wangi Falls, *Ohlsen 381* (MELU); KP774919; KP639699; KP835422. *A. loxoscaphoides* Bak.; Kenya, *Hemp 17* (BM); AY300122; AY300069; —. *A. lunulatum* Sw.; cult., Bot. Gard. Goett. (GOET); AY549732; AY549835; AY549771. *A. marinum* L.; United Kingdom, *rbcL, trnL-trnF, Vogel MAR-5* (BM), *rps4-trnS, Vogel MAR-10a* (BM); AF240647; AF240662; AY549773. *A. maratoides* (Brack.) C.Chr.; Fiji, *Brownsey & Perrie FIJI 187* (WELT); KP774861; KP835365; KP851889. *A. monanthes* L.; Madeira, *Vogel Mona-1* (BM); AY300125; AY300072; AY549797. *A. milnei* Carr.; Australia, LHI – cult. – *Perrie 3605* (WELT P022089); EU240032; EU240030; EU240022. *A. myriophyllum* (Sw.) C.Presl; Cuba, *Morton 25* (UC); AY300127; AY300074; AY549769. *A. nidus* L.; Madagascar, *Fischer T-9* (UC); AF525270; AF525246; AY549807. *A. nidus* (*Thamnopteris* 1); Papua New Guinea, *Kessler 13726* (UZH); KP774889; KP835367; KP835428. *A. nidus* (*Thamnopteris* 2); Papua New Guinea, *Kessler 13789* (UZH); KP774928; KP872961; KP872960. *A. nidus*; Australia, QLD, Clamshell Falls, *Ohlsen 313* (MELU); KP774872; KP835392; KP835455. *A. nidus*; Vanuatu, Espiritu Santo, *Ohlsen 390* (MELU); KP774907; KP835376; KP835411. *A. nitidum* Sw.; Papua New Guinea, *Kessler 13941* (UZH); KP774881; KP835393; KP835468. *A. normale* D.Don; Kenya, *Hemp 5* (BM); AY300128; AY300075; AY549783. *A. normale*; Hawaii, *Ranker 1799* (COLO); AY549735; AY549838; AY549784. *A. normale*; Papua New Guinea, *Kessler 14124* (UZH); KP774925; KP872962; —. *A. normale*; Australia, QLD, Headwaters of the North Beatrice River, *Ohlsen 296* (MELU); KP774926; KP851904; KP835419. *A. northlandicum* (Brownsey) Ogle; Australia, TAS, Remarkable Cave, *Ohlsen 188* (MELU); KP774874; KP835358; KP835423. *A. northlandicum*; New Zealand, *Brownsey s.n.* (WELT P020512); EU240031; AY283218; —. *A. novoguineense* Rosenst.; Papua New Guinea, *Kessler 13945* (UZH); KP774942; KP639689; KP835442. *A. obliquum* G.Forst.; Chile, *Chile 921642* (BM); AY300129; AY300076; AY549812. *A. oblongifolium* Colenso; New Zealand, *Perrie 2019 & Shepherd* (WELT P020508); AY283231; AY538176; EU240026. *A. obtusatum* G.Forst.; New Zealand, *Perrie 2070* (WELT P020510); AY283232; AY538176; EU240025. *A. oligolepidum* C.Chr.; New Caledonia, *Perrie NC 95* (WELT); KP774893; KP835361; KP835445. *A. oligophlebium* Baker; Japan, *Zhang 102404* (HITBC); JX152751; JQ724225; JQ724310. *A. paleaceum* R.Br.; Australia, QLD, Isabella Falls, *Ohlsen 316* (MELU); KP774941; KP218793; KP218779. *A. papaverifolium* (Kunze) Viane; Chile, *PLE CHI*; JX068707; JX068750; JX068790. *A. parvum* Watts; Australia, QLD, Mt Baldy, *Ohlsen 281* (MELU); KP774869; KP835383; KP835451. *A. parvum*; Papua New Guinea, *Kessler 14114* (UZH); KP774863; KP835395; KP835434. *A. pauperequitum* Brownsey et P.J.Jacks; New Zealand, (WELT P20513); AY283233; KP888649; KP888648. *A. pellucidum* Lam.; Australia, QLD, Tchupala Falls, *Ohlsen 299* (MELU); KP774853; KP835386; KP835421. *A. phyllitidis* D.Don; Papua New Guinea, *Kluge 9067* (UZH); KP774906; KP835359; KP851888. *A. polyodon* G.Forst. (*A. listeri* clade); Australia, CI, *Grigg & Moloney s.n.* (MELU); KP774938; KP639698; KP851892. *A. polyodon*; Australia, QLD, Mt Lewis, *Ohlsen 292* (MELU); KP774937; KP218798; KP218778. *A. polyodon*; Vanuatu, Efate, *Ohlsen 387* (MELU); KP851874; KP851905; KP851887. *A. polyodon*; Vanuatu, Espiritu Santo, *Ohlsen 391* (MELU); KP774887; KP851906; KP835418. *A. polyodon*; New Caledonia, *Perrie NC 124* (WELT); KP774900; KP835397; KP835433. *A. polyodon* (*A. listeri* clade); New Caledonia, *Perrie NC 6* (WELT); KP774878; KP835391; KP851886. *A. polyodon*; New Zealand, *Wardlow s.n.* (BM); AY300133; AY300080; AY549829. *A. polyphyleticum* Compton; New Caledonia, *Perrie NC 179* (WELT); KP774852; KP835362; KP835446. *A. praemorsum* Sw.; cult., *Vogel s.n.* (BM); AY549754; AY549858; AY549824. *A. prolongatum* Hook.; cult., NYBG (UC); AY549752; AY549856; AY549813. *A. protensum* Schrad.; Kenya, *Hemp 2* (BM); AY300135; AY300082; AY549825. *A. pteridoides* Baker; Australia, LHI, *Papadopulos 783* (NSW); JF950802; JF950902; —. *A. resiliens* Kunze; USA, *Shaw 19* (ISC); AY549746; AY549850; AY549800. *A. rhizophyllum* L.; USA, *Vogel RHIZO-1* (BM); AY300136; AY300083; AY549781. *A. richardii* (Hook.f.) Hook.f.; New Zealand, (WELT P20515); EF418427; EF418413; EF418431. *A. ruta-muraria* L.; Croatia, *RUT-141*; JX068716; JX068758; JX068801. *A. sandersonii* Hook.; Kenya, *Hemp 12* (BM); AF525274; AF525247; AY549820. *A. scandens* J.Sm.; Papua New Guinea, *Kluge 9024* (UZH); KP774880; KP835350; KP835413. *A. scolopendrium* L.; France, *Vogel SCOL-73* (BM); AF240645; AF525262; AY459169. *A. septentrionale* (L.) Hoffm. subsp. *caucasicum* Hoffm.; Turkey, *Vogel SEPT-17* (BM); AF525275; AF525248; AY549777. *A. setisectum* Blume; Papua New Guinea, *Kessler 14048* (UZH); KP774932; KP639688; KP835466. *A. shuttleworthianum*; New Zealand, Kermadec Islands, (WELT P20517); AY283235; AY283223; EU250849. *A. simplicifrons* F.Muell.; Australia, QLD, Topaz NP, *Ohlsen 251* (MELU); KP774909; KP835349; KP835420. *A. stenolobum* C.Chr.; Fiji, *Brownsey & Perrie FIJI 268* (WELT); KP774859; KP835347; KP851885. *A. submarginatum* Rosenst.; Papua New Guinea, *Kluge 9026* (UZH); KP774923; KP835360; KP835447. *A. subflexuosum* Rosenst.; New Caledonia, *Perrie NC 83* (WELT); KP774905; KP835377; KP835414. *A. subglandulosum* (Hook. & Grev.) Salvo, Prada & T.E.Diaz [*Pleurosorus rutifolius* (R.Br.) Fee]; Australia, VIC, Terrick Terrick NP, *Ohlsen 442* (MELU); KP774929; KP851909; KP835456. *A. subglandulosum* (*Pleurosorus rutifolius*) New Zealand, *Perrie 4625* (WELT); KP774857; KP851910; —. *A. subglandulosum* [*A. subglandulosus* (Hook. & Grev.) Tindale]; Australia, VIC, Wellington River, *Ohlsen 208* (MELU); KP774860; KP851908; KP835453. *A. surrogatum* P.S.Green; Australia, LHI cult. – L.Perrie, (WELT P22090); EU240034; EU240029; EU240024. *A. tenerum* G.Forst.; Borneo, *Schneider 1140* (SAR); AY300145; AY300092; AY549814. *A. tenerum*; Papua New Guinea, *Kluge 9110* (UZH); KP774890; KP835351; KP835430. *A. tenerum*; Australia, QLD, Bridle Creek, *Ohlsen 265* (MELU); KP774858; KP835346; KP835437. *A. tenerum*; Fiji, *Brownsey & Perrie FIJI 14* (WELT); KP774912; KP851911; KP851884. *A. tenuiculum* Rosenst.; New Caledonia, *Perrie NC 220* (WELT); KP774862; KP835356; KP835444. *A. tricholepis* Rosenst.; Bolivia, *Kessler 12603* (); AY549729; AY549832; AY549761. *A. trichomanes* L. 2n (subsp. *trichomanes*); Australia, VIC, Bogong, *Ohlsen 174* (MELU); KP774920; KP851912; KP835412. *A. trichomanes* 2n (subsp. *inexpectatum* Lovis); France, *Vogel I-46-B04*; AY549743; AY549846; AY549793. *A. trichomanes* 4n (subsp. *quadrivalens* D.E.Meyer); Romania, *Vogel Q-272* (BM); AY549744; AY549847; AY549794. *A. trichomanes* 4n (subsp. *quadrivalens*); Australia, VIC, Glenelg River, *Ohlsen 232* (MELU); KP774868; KP851907; KP835410. *A. trichomanes* L. 6n; New Zealand, (WELT P20518); AY283236; AY283224; —. *A. unisorum* Viane; Hawaii, *Wood 7706* (PTBG); AY549741; AY549844; AY549790. *A. varians* Wall. ex Hook. & Grev.; China, *Fraser-Jenkins 10046* (BM); AY300147; AY300094; AY549802. *A. vieillardii*; New Caledonia, *Perrie NC 9* (WELT); KP774911; KP835348; KP835409. *A. viride* Huds.; Austria, *Vogel 1334* (BM); AY549734; AF525238; AY549782. *A. wildii* F.M.Bailey, Australia, QLD, Daintree NP, *Ohlsen 246* (MELU); KP774927; KP851919; KP851877. *A. wrightii* Eaton ex Hook.; Taiwan, *Cranfill TW040* (UC); AY549730; AY549833; AY549766. *A. wrightioides* Christ; AY725031; AY725032; AY725044. *A. yunnanense* Franch.; China, *Fraser-Jenkins 10044* (BM); AY300149; AY300096; AY549803. *Athyrium filix-femina* (L.) Roth.; *rbcL* Sweden, *Larson 7* (UPS); *trnL-F* Canada, *Samoli 541* (TAIF); *rps4* Lehtonen 717 (TUR); JF832056; JF821333; HQ157326. *Diplazopsis calaveriana* (Christ) C.Chr.; *rbcL* China, Yunnan, SG Lu/XCB13; *trnL* China, Jinshoshan Mt., *Wang 704* (WNU); *rps4* China, Yunnan, SG Lu/XCB13 (); JN168015; AF515251; JN168089. *Hymenasplenium excisum* (C.Presl) S.Linds.; Hawaii, *Ranker 1786* (COLO); AY549728; AY549831; AY549758. *H. excisum*; Australia, QLD, Kaaruu Creek, *Ohlsen 305* (MELU); KP774930; KP851913; KP851883. *H. excisum*; Fiji, *Brownsey & Perrie FIJI 190* (WELT); KP774884; KP851914; KP851882. *Hymenasplenium unilaterale* (Lam.) Hayata; Kenya, *Hemp 10* (BM); AF240652;

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Table 2. (continued)

AF525232; AY459170. *H. unilaterale*; Australia, QLD, Topaz NP, Ohlsen 252 (MELU); KP774849; KP851916; KP851879. *H. unilaterale*; Vanuatu, Tanna, Ohlsen 392 (MELU); KP774898; KP851917; KP851881. *H. unilaterale*; Fiji, Brownsey & Perrie FIJI 13 (WELT); KP774885; KP851918; KP851880. *H. unilaterale*; New Caledonia, Perrie NC 177 (WELT); KP774896; KP851915; KP851878. *Rhachidosorus consimilis* Ching; *rbcL* China Yunnan SG Lu/J21; *trnL* China, Maguan SG Lu 2812 (WNU); *rps4* China Yunnan SG Lu/J21; JN168023; AF514837; JN168098.

Accessions unassigned to species

A. sp. 'New Caledonia'; New Caledonia, Perrie NC 74 (WELT); KP774899; KP835401; KP835470. *A. sp.* indet. 'New Guinea 1'; Papua New Guinea, Kessler 14100 (UZH); KP774939; KP639692; KP835440. *A. sp.* indet. 'New Guinea 2'; Papua New Guinea, Karger 1649 (UZH); KP851873; KP835384; KP835469. *A. sp.* 'section *Thamnopteris*'; Papua New Guinea, Kessler 13833 (UZH); KP774921; KP835353; KP835448. *A. sp.* 'Windsor Tableland'; Australia, QLD, Windsor Tableland, Ohlsen 261 (MELU); KP774931; KP835382; KP835427.

whether other clades are represented in this region, and to which clades the majority of local species belong.

The most detailed molecular analysis of Aspleniaceae from ASWP is that of Perrie and Brownsey (2005), which focussed on Aspleniaceae in New Zealand. They sequenced the chloroplast regions *rbcL* and *trnL-trnF* for most Aspleniaceae of New Zealand, confirming relationships established by Schneider *et al.* (2004) and elucidating the close relationship of many previously non-sampled New Zealand species to lineages detected by Schneider *et al.* (2004). New Zealand Aspleniaceae was found to be polyphyletic, with species being placed in four separate parts of the global phylogeny of Schneider *et al.* (2004). The majority were placed in a clade equivalent to the 'Austral' group of regularly hybridising species (Brownsey 1977a), nested within Clade V of Schneider *et al.* (2004). The Austral group has been found to contain four clades, including the 'Obtusatum', 'Bulbiferum' and 'Flaccidum' groups (Perrie and Brownsey 2005; Shepherd *et al.* 2008a), whose species are shared between at least south-eastern Australia and New Zealand, and a further group of two Lord Howe Island species (Shepherd *et al.* 2008a). However, the full geographic extent of the Austral group remains to be determined.

Despite past studies, the relationships of many species of the Aspleniaceae of ASWP, to each other and to those elsewhere, remain to be assessed by molecular phylogenetic analyses. This is a substantial gap in understanding the systematics of Aspleniaceae, given taxa of ASWP are a large proportion of its worldwide diversity. The present study uses sequences of the chloroplast regions *rbcL*, *rps4-trnS* and *trnL-trnF* for all Australian species recognised by Brownsey (1998), and many Aspleniaceae from nearby New Guinea, and the south-western Pacific, with the aim of constructing a well resolved molecular phylogeny of the family in this region. Its goals are to better understand the evolutionary and biogeographic history of this group in ASWP, including the contributions to Aspleniaceae diversity in the ASWP made by *in situ* radiations versus accumulation of lineages through immigration. Additionally, the present study tests the current generic placement of taxa from the region and assesses the delimitation of some species, especially widely distributed species that are disjunct between different parts of ASWP.

Materials and methods

Sampling for molecular study

In total, 296 new DNA sequences were generated from 100 recent collections from wild populations in New Guinea,

Australia, Vanuatu, Fiji and New Caledonia (Table 2). This included all Australian species recognised by Brownsey (1998), and several species from the south-western Pacific and New Guinea. Some collections could not be confidently assigned to a current species and are putatively new; these include Australian *A. sp.* 'Windsor Tableland' and New Caledonian *A. sp.* 'New Caledonia'. In addition to the new sequences generated here, sequences for 70 further accessions were harvested from GenBank. For Aspleniaceae of ASWP this included any accessions sequenced for at least *rbcL* and *trnL-trnF*; for taxa from other parts of the world, this included accessions sequenced for all loci. Care was taken to include the closest sequenced relatives of all ASWP species, on the basis of previous studies and preliminary analyses, as well as a representative sample of Aspleniaceae diversity worldwide.

DNA isolation, amplification, and sequencing

DNA was extracted from 20 mg of silica gel-dried young leaf tissue. For all samples, apart from those from Fiji, leaf tissue was ground using a mortar and pestle with the aid of acid-washed grinding sand (Ajax Finechem, Sydney, NSW, Australia). DNA was isolated from ground samples using a DNeasy Plant Mini Kit (QIAGEN, Melbourne, Vic., Australia), following the manufacturer's instructions and with a final elution volume of 100 µL.

The chloroplast DNA markers *rbcL*, *trnL-trnF* (*trnL* intron and *trnL-trnF* intergenic spacer), and *rps4-trnS* (*rps4* gene and *rps4-trnS* intergenic spacer) were sequenced. These regions were chosen because (1) they are routinely used in fern systematics, enabling comparison of sequences from other studies, including Schneider *et al.* (2004, 2005) and (2) good resolution of both higher- and lower-level relationships can be achieved in Aspleniaceae employing these regions.

Chloroplast DNA markers were amplified by polymerase chain reaction (PCR), performed on a MyCycler thermal cycler (Bio-Rad, Sydney, NSW). Reaction mixtures comprised 5 µL of 5× MyTaq reaction buffer containing 5 mM of each dNTP and 15 mM MgCl₂ (Bioline, Sydney, NSW), 0.125 µL (0.625 units) MyTaq DNA Polymerase (Bioline), 10 pmol of each primer, 2.0 µL of extracted DNA, and ultrapure water added to make a total volume of 25 µL. The *rbcL* gene was amplified using the primers ESRBCL1F (5'-ATG TCA CCA CAA ACG GAG ACT AAA GC-3') and ESRBCL1361R (5'-TCA GGA CTC CAC TTA CTA GCT TCA CG-3') (Schuettpelz and Pryer 2007). *Asplenium pellucidum*, *A. caudatum* and *A. longissimum* were amplified using aF

(5'-ATG TCA CCA CAA ACA GAG ACT AAA GC-3') and cR (5'-GCA GCA GCT AGT TCC GGG CTC CA-3') (Hasebe *et al.* 1994), because of unsuccessful amplification with ESRBCL1F and ESRBCL1361R. The *trnL* intron, *trnL* 3' exon and *trnL-trnF* intergenic spacer were amplified using the primers F (5'-ATT TGA ACT GGT GAC ACG AG-3'; Taberlet *et al.* 1991) and Fern1 (5'-GGC AGC CCC CAR ATT CAG GGR AAC C-3'; Treweek *et al.* 2002). For Australian *A. baileyannum*, *A. polyodon* and *A. tenerum* and New Caledonian *A. polyodon*, amplification with the internal primers D (5'-GGG GAT AGA GGG ACT TGA AC-3') and E (5'-GGT TCA AGT CCC TCT ATC CC-3') (Taberlet *et al.* 1991) was required for sequencing reactions to obtain adequate *trnL-trnF* sequence for analysis. The *rps4* gene and the *rps4-trnS* intergenic spacer were amplified using the forward primer 5'-ATG TCM CGT TAY CGA GGR CCT CGT-3' and the reverse primer 5'-TAC CGA GGG TTC GAA TC-3' (Schneider *et al.* 2005).

Polymerase chain reaction thermocycling conditions for *rbcL* and *trnL-trnF* involved an initial denaturation step of 95°C for 1 min, followed by 33 cycles of 95°C for 1 min, 55°C for 1 min, and 65°C for 4 min, and a final extension of 65°C for 5 min. PCR thermocycling conditions for *rps4-trnS* involved an initial denaturation step of 95°C for 1 min, followed by 37 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, and a final extension of 72°C for 5 min. DNA concentrations were quantified by electrophoresis against Hyperladder I and EasyLadder I (Bioline) and PCR products were purified using illustra ExoSTAR 1-step enzymatic purification (GE Healthcare Life Sciences, Freiburg, Germany). Purified PCR products were then sent to the Australian Genome Research Facility (AGRF), Melbourne Branch, where sequencing reactions and capillary separation, using the 96-capillary analyser AB 3730xl sequencing platform (Applied Biosystems, Foster City, CA, USA), were performed.

For the Fijian samples, genomic DNA was extracted, PCR amplified and the PCR products purified and sequenced following Shepherd *et al.* (2007).

Sequence editing, alignment and analysis

Sequences were edited using Sequencher v. 4.9 (Gene Codes Corporation, Ann Arbor, MI, USA). Sequences were aligned manually in Se-Al Sequence Alignment Editor v. 2.0a11 (Rambaut 2002). Regions of ambiguous alignment were excluded from analyses.

A single *rbcL*, *trnL-trnF* and *rps4-trnS* alignment (submitted to TreeBase) was analysed. Congruence of phylogenetic signal between cpDNA markers was tested by comparing preliminary maximum-parsimony (MP) analyses of individual markers. These analyses showed that any difference in signal among loci affected only some poorly supported nodes that are not shown in the figures of the present study, providing justification for concatenation of loci. The following three analyses were conducted: a MP, a maximum likelihood (ML) and a Bayesian analysis (BA). *Athyrium filix-femina* (Athyriaceae), *Rhachidosorus consimilis* (Rhachidosoraceae) and *Diplaziopsis cavaleriana* (Diplaziopsidaceae) were chosen as outgroups. *Rhachidosorus* and *Diplaziopsis* are the closest

related genera to Aspleniaceae that have all the chloroplast loci employed in the present study publically available (Rothfels *et al.* 2012). *Athyrium* was also included to give a more distant relative in Eupolypods II (Rothfels *et al.* 2012).

Maximum-parsimony analyses were run in PAUP* v 4.0β10 (Swofford 2002). Gap characters in the alignment were treated as a fifth character state. For multiple-base indels, characters were excluded from analyses so that indels were each represented only by a single gap character, when variability did not occur within indels. Question marks, the character recognised in PAUP for missing data, were used to fill gaps, where needed, when indels fell across otherwise variable regions of the alignment. A heuristic tree search was used, with delayed character-state optimisation (DELTRAN) and starting trees were obtained by a closest addition sequence, followed by tree bisection-reconnection (TBR) branch swapping. Bootstrap support for nodes was determined using 1000 'full heuristic' replicates.

MrModel Test v2 (Nylander 2004) was used with the Akaike information criterion (AIC) to assess the most appropriate model of sequence evolution for BA and ML analyses. Separate models of sequence evolution were assessed for the different coding and non-coding for BA and for the entire dataset for ML analyses. BA was performed in MrBayes 3.2.1 (Huelsenbeck and Ronquist 2001). For BA, two concurrent analyses were run, each with four Markov chains of 3 000 000 generations. This number of generations allowed the standard deviation of split frequencies to become less than 0.01. Trees were sampled every 1000 generations. A GTR+I+G substitution model was chosen for *rbcL*, a GTR+G substitution model was chosen for the *trnL* intron, *trnL-trnF* spacer, *rps4*, and the *rps4-trnS* spacer, and a K80+I substitution model was chosen for the *trnL* exon. Default priors were used, and the substitution-model parameters were unlinked across the loci. Tracer v1.5 (Rambaut and Drummond 2009) was used to determine samples to be discarded as 'burn-in'. After the 'burn-in' was discarded, 2700 samples remained for consensus trees to be calculated from. Gap characters were coded in a separate binary dataset as restriction data.

Maximum likelihood (ML) analyses were performed with the PhyML 3.0 web server (<http://www.atgc-montpellier.fr/phyml>, accessed 16 October 2014; Guindon *et al.* 2010). A GTR+I+G substitution model with $\text{nst} = 6$, $\Gamma = 1.3768$ and $\text{pinvar} = 0.2685$ was chosen. ML-optimised equilibrium frequencies, subtree pruning and regrafting (SPR) branch-swapping and 10 random starting trees were applied. Branch support was assessed using an approximate likelihood-ratio test (aLRT, Anisimova and Gascuel 2006). Gap characters were treated as missing data and a single model of sequence evolution was chosen for the entire alignment in ML analyses because the PhyML 3.0 web server cannot process coded indels or

Table 3. Characteristics of sequences used in the present study

Characteristic	<i>rbcL</i>	<i>trnL-trnF</i>	<i>rps4-trnS</i>	Total
Aligned length	1215	1457	1201	3873
Number of variable characters included	458	804	741	2003
Number of parsimony-informative characters	364	625	545	1534

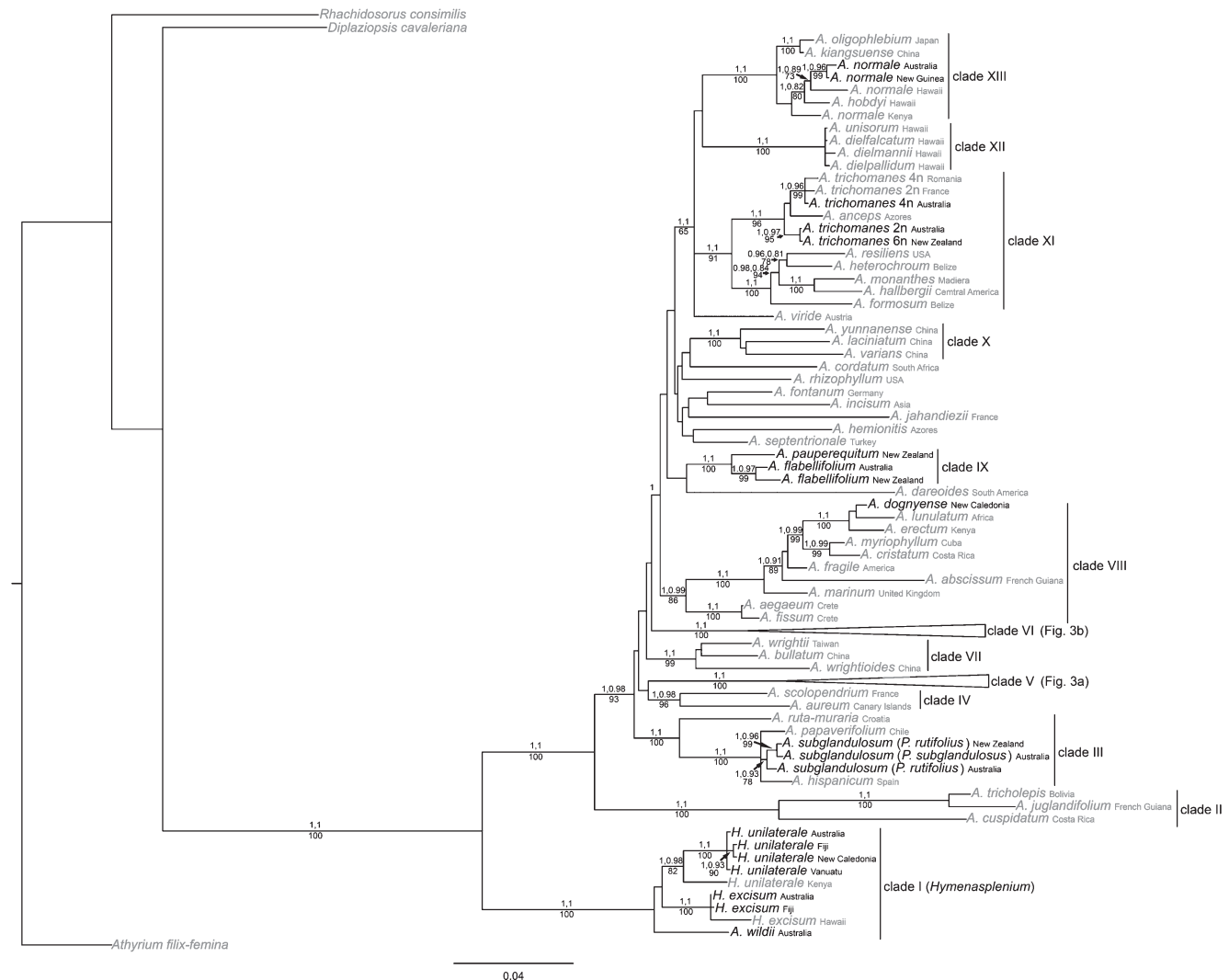


Fig. 1. Bayesian analysis (BA) consensus tree of Aspleniaceae. Posterior probabilities (PP), approximate likelihood ratio-test values (aLRT), and bootstrap support (BS) percentages are given for strongly supported branches (PP and aLRT ≥ 0.95 , BS $\geq 80\%$). Clades V and VI are collapsed (shown as triangles). Relationships within these clades are shown in Fig. 3. Samples external to Australasia and the south-western Pacific (ASWP) are given in grey and ASWP samples are given in black. This tree has a topology similar to those of trees based on analyses using maximum likelihood (ML) (which gave a tree of log likelihood -40536.5) and MP ($>100\,000$ trees of 6655 steps and consistency index of 0.47).

apply separate models to different sections of the dataset in one analysis.

Results

Phylogeny of Aspleniaceae

Sequence characteristics for the alignment are summarised in Table 3. Thirteen strongly supported clades, I–XIII, are here defined within the Aspleniaceae phylogeny. Seven of these clades occur in ASWP. These are Clade I or *Hymenasplenium*, Clade III, Clade V, Clade VI, Clade VIII, Clade IX, Clade XI and Clade XIII (Fig. 1). The distributions of these clades in ASWP are shown in Fig. 2. The majority of ASWP species were placed in Clades V or VI. Topologies of trees generated by the different tree-building methods were very similar and the consensus tree resulting from the BA is presented and described.

Clade I (*Hymenasplenium*) and Clade II were successive sisters to the remaining Aspleniaceae. Relationships among Clades III–XIII were not well resolved, and most nodes among these clades had poor support ($<80\%$ bootstrap support (BS); <0.95 posterior probability (PP) and aLRT support values).

Australasian and south-western Pacific species in Clades I, III, VII, VIII and IX

Clade I (*Hymenasplenium*) is represented in ASWP by the following three species: *H. excisum*, *H. unilaterale* and *A. wildii*. Clade I is absent from temperate Australasia (Fig. 2A).

Clade III (*Pleurosorus* clade) is represented in ASWP by *A. subglandulosum* (which has been previously known as *Pleurosorus rutifolius* and *P. subglandulosus*), and is widespread in temperate and subtropical areas of Australia

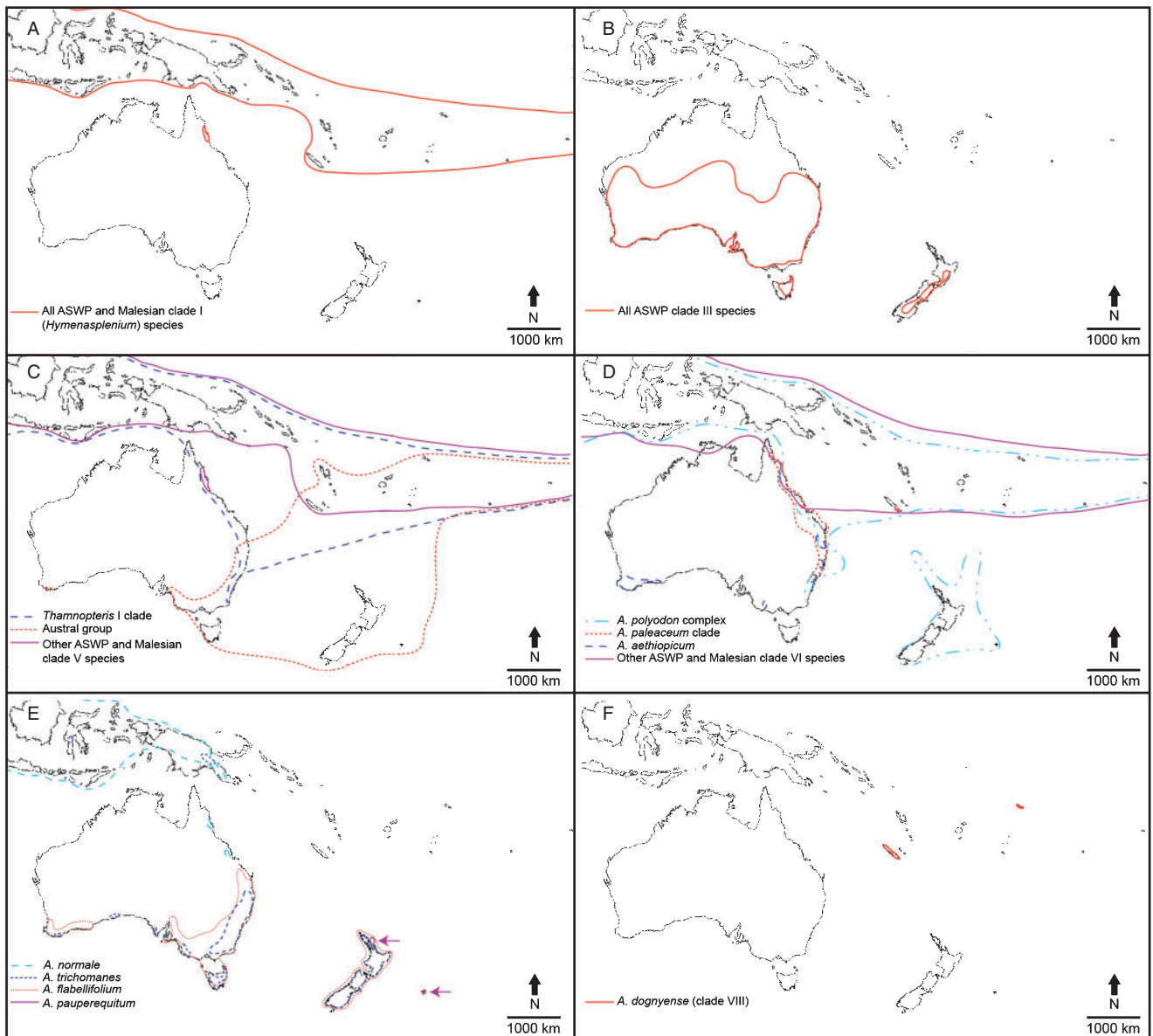


Fig. 2. The distribution in Australasia and the south-western Pacific (ASWP) and surrounding regions of the following: A. *Hymenasplenium* (Clade I); B. Clade III; C. Clade V and some constituent clades; D. Clade VI and some constituent clades and species; E. species in Clades IX, XI, and XIII; F. *Asplenium dognyense* (Clade VIII).

and New Zealand (Fig. 2B). Australasian accessions form a clade with *A. hispanicum* (*P. hispanicus*) from Europe and *A. papaverifolium* (*P. papaverifolius*) from Chile (Fig. 1).

Clade IX is known only from ASWP and comprises *A. flabellifolium* and *A. pauperequitum*. Clades VIII, XI and XIII are represented in ASWP by single species. Clade XI is represented in ASWP by *A. trichomanes* and Clade XIII by *A. normale*. Clades XI and XIII were part of a larger clade that was strongly supported in BA and ML analyses. *Asplenium trichomanes* was not resolved as monophyletic but formed two clades on the basis of the substrate of samples, i.e., those from limestone in one clade (samples labelled 4n Australia, 4n Romania, 2n France; Fig. 1), and those not restricted to

limestone in another (samples labelled 2n Australia, 6n New Zealand). Representatives of ASWP *A. normale* formed a clade and were closely related to *A. normale* and *A. hobdyi* samples from Hawaii. ASWP distributions of species in Clades IX, XI and XIII are shown in Fig. 2E. Clade VIII is represented in the south-western Pacific by a single species, *A. dognyense* from New Caledonia (Figs 1, 2F).

Relationships among species of Clade V

Clade V contained the largest number of species from ASWP sequenced in the present study, with 39 species being represented (Fig. 3A). Several strongly supported clades were

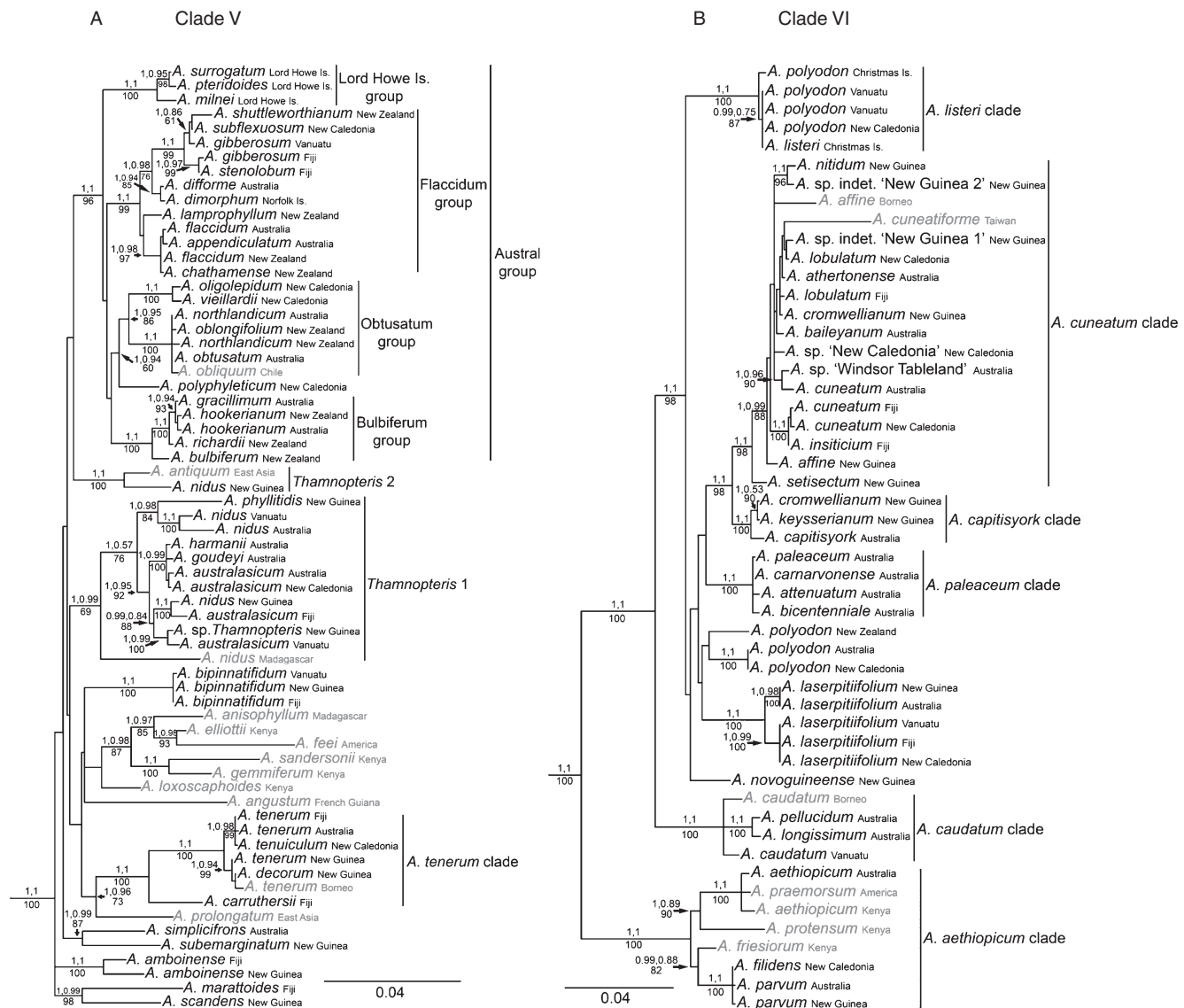


Fig. 3. A. Bayesian analysis (BA) consensus tree of Clade V; B. BA consensus tree of Clade VI. Posterior probabilities (PP), approximate likelihood ratio-test values (aLRT), and bootstrap support (BS) percentages are given for strongly supported branches (PP and aLRT ≥ 0.95 , BS $\geq 80\%$). Samples external to Australasia and the south-western Pacific (ASWP) are given in grey and ASWP samples are given in black.

resolved (Fig. 3A). These include the following: a clade referred to here as *Thamnopteris* 1 (not strongly supported in MP analysis), formed entirely of section *Thamnopteris* collections; the *Thamnopteris* 2 clade, comprising *A. antiquum* and a New Guinean accession labelled as *A. nidus*; the Austral group (sensu Perrie and Brownsey 2005); the *A. tenerum* clade, comprising *A. carruthersii*, *A. decorum*, *A. tenerum* and *A. tenuiculum*; a clade of *A. marattioides* and *A. scandens*; and a clade of *A. simplicifrons* and *A. submarginatum*. Relationships between these clades were not resolved with strong support (Fig. 3A), but relationships within them were mostly congruent between analyses.

The *Thamnopteris* 1 clade included two clades, namely, a clade of mainly *A. australasicum*-like species, and another composed of *A. nidus* and *A. phyllitidis*. Within the latter

clade, a New Guinean population of *A. phyllitidis* was sister to a clade of *A. nidus* samples from Australia and Vanuatu. In the clade comprising mainly *A. australasicum*-like species, the Australian species *A. goudeyi*, *A. harmanii*, and Australian *A. australasicum* and New Caledonian *A. australasicum* formed a clade. This Australian and New Caledonian clade was sister to a clade including *A. australasicum* from Vanuatu and Fiji, a sample not identified to species level from New Guinea, and a New Guinean population identified as *A. nidus*. This is one of four clades in which *A. nidus* was placed by the analyses, making material identified as *A. nidus* markedly polyphyletic. *Asplenium australasicum* was also placed in three separate clades and is polyphyletic.

Within the Austral group, four strongly supported principal clades were recovered. These were a clade of three Lord Howe

Island endemic species (*A. milnei*, *A. surrogatum* and *A. pteridioides*) and the Obtusatum, Flaccidum and Bulbiferum groups. Relationships among these strongly supported clades of the Austral group were not well supported. Within the Obtusatum group, a New Caledonian clade of *A. vieillardii* and *A. oligolepidum* was sister to species that occur in New Zealand. Within the Flaccidum group, Fijian samples of *A. gibberosum* and *A. stenolobum* formed a clade sister to a clade comprising New Zealand *A. shuttleworthianum*, Vanuatu *A. gibberosum* and New Caledonian *A. subflexuosum*.

Some species in Clade V lack close relatives among the sampled species. Samples of both *A. amboinense* and *A. bipinnatifidum* (each strongly supported as monophyletic) do not form well supported clades with any other species. Likewise, *A. scandens* and *A. marattioides*, although shown as sister species, are not closely related to other clades and are highly divergent from each other; i.e. terminal branch lengths in MP analyses of 58 and 65 steps respectively. The same is true of the pair *A. submarginatum* and *A. simplicifrons* (terminal branch lengths 56 and 46).

Relationships among species of Clade VI

Clade VI was the second-most diverse clade in ASWP. At least 25 species of ASWP are placed in this clade (Fig. 3B). Several clades within clade VI were well supported (Fig. 3B). Sister to other groups of Clade VI was the *A. aethiopicum* clade, which included *A. aethiopicum*, Australian and New Guinean *A. parvum* and New Caledonian *A. filidens*. Also well supported was a sister relationship between the *A. caudatum* clade and the rest of Clade VI, excluding the *A. aethiopicum* clade. The *A. caudatum* clade comprised *A. caudatum*, *A. longissimum* (*A. sp.* ‘Litchfield’ Russell-Smith 6470 *sensu* Short *et al.* 2003), and *A. pellucidum*.

A sister relationship between the *A. cuneatum* and *A. capitisyork* clades was well supported. The *A. capitisyork* clade comprised *A. capitisyork* from Cape York, far-northern Australia, which was sister to *A. keyserrianum* and *A. cromwellianum*, both from New Guinea. The *A. cuneatum* clade was a large clade of species from northern Australia, New Guinea, and tropical Pacific Islands, including *A. affine*, *A. athertonense*, *A. baileyum*, *A. cuneatum*, *A. cromwellianum*, *A. insititium*, *A. lobulatum*, *A. nitidum*, *A. setisectum* and New Guinean samples unassigned to species. Samples identified as *A. cuneatum* were not monophyletic: an Australian *A. cuneatum* accession was sister to *A. sp.* ‘Windsor Tableland’ from north-eastern Australia, whereas Fijian and New Caledonian *A. cuneatum* were sister to Fijian *A. insititium*. None of the analyses revealed with certainty the closest relatives of Australian *A. baileyum*, New Guinean *A. affine* and *A. sp.* ‘New Caledonia’. Resolution of the relationships between *A. lobulatum*, *A. sp.* indet. ‘New Guinea 1’ and *A. athertonense* was poor because of low sequence variation among these species (pair-wise differences across all loci no more than five base pairs) and variation among *A. lobulatum* populations.

The rest of Clade VI comprised species or groups of species whose phylogenetic positions were not well resolved. These included *A. laserpitiifolium*, *A. novoguineense*, a clade comprising an Australian and a New Caledonian accession of

A. polyodon, New Zealand *A. polyodon*, the *A. paleaceum* clade, and the *A. listeri* clade. The *A. paleaceum* clade comprised *A. attenuatum*, *A. bicentenniale*, *A. carnarvonense* and *A. paleaceum*. These species were resolved as a polytomy. The *A. listeri* clade comprised epiphytic *A. polyodon* from Christmas Island, north-west of Australia, a coastal limestone-dwelling sample of *A. listeri* from Christmas Island, and coastal limestone-dwelling samples from the Pacific attributed to *A. polyodon*. Consequently, *A. polyodon* is not monophyletic.

Discussion

The Aspleniaceae phylogeny: major clades and generic limits

Results here are congruent with previously published Aspleniaceae phylogenies (Murakami 1995; Murakami *et al.* 1999a; Schneider *et al.* 2004, 2005). All major clades have been recovered in previous studies (Schneider *et al.* 2004, 2005; Chang *et al.* 2013) and the relationships among these clades also match what has been previously found (Schneider *et al.* 2004, 2005). An important congruent finding was the placement of Clade I, *Hymenasplenium*, as sister to other Aspleniaceae. This provides further support for treating the *Hymenasplenium* clade as a separate genus, as has been suggested (Smith *et al.* 2006), and generally followed in recent treatments (e.g. Murakami 1995; Murakami *et al.* 1998, 1999a; Sasaki 2008; Brownsey and Perrie 2011; Gabancho and Prada 2011). The Australian endemic, *A. wildii*, has been previously noted as morphologically similar and most likely related to species of *Hymenasplenium* on the basis of its creeping rhizome and pinnate fronds (Brownsey 1998). This is confirmed by placement of *A. wildii* in the *Hymenasplenium* clade by the chloroplast phylogeny of the present study, and a new combination for *A. wildii* in *Hymenasplenium* will be made elsewhere.

All species of *Pleurosorus* and the section *Thamnopteris* were nested well within *Asplenium* in Clades III and V respectively in agreement with previous molecular phylogenetic studies (Schneider *et al.* 2004, 2005), and supporting inclusion of these taxa in *Asplenium*. Section *Thamnopteris* was previously found not to be monophyletic with predominantly South-east Asian taxon sampling (Murakami *et al.* 1999a; Yatabe *et al.* 2009). In the present study, monophyly of section *Thamnopteris* was not supported. Section *Thamnopteris* accessions form two clades, one including the majority of accessions, including some *A. nidus* samples, and the other with *A. antiquum* and a New Guinean accession supplied to us as *A. nidus*. Placement of South-east Asian *A. nidus* populations in both these clades has previously been demonstrated (Yatabe *et al.* 2001, 2009), and this emphasises that morphological features used for identification in section *Thamnopteris* do not delimit monophyletic species (Murakami *et al.* 1999b; Yatabe *et al.* 2001; Dong *et al.* 2012). Furthermore, the species with entire fronds not belonging to section *Thamnopteris* (i.e. without an anastomosing vein near the margin), *A. simplicifrons* and *A. amboinense*, were placed in two other separate positions in Clade V, indicating that the transition between simple and divided fronds has occurred multiple times even within Clade V.

The present study has shown that the segregate genus *Loxoscapha*, defined by short, submarginal and pouch-like sori (Gastony and Johnson 2001), is also not monophyletic, having arisen on at least two independent occasions. Bellefroid *et al.* (2010) showed that African species of the *Loxoscapha* group were monophyletic. One of these African species, *A. loxoscaphoides*, is included in the present study and does not form a clade with south-western Pacific species, *A. gibberosum* and *A. stenolobum* (Fig. 3A), previously included in *Loxoscapha* (Brownlie 1977). The close relationship of these south-western Pacific species to *A. shuttleworthianum* from New Zealand is supported by the similar 'loxoscaphoid' morphology of *A. shuttleworthianum* (Perrie and Brownsey 2005).

Relationships found here within Clade V are congruent with analyses of Perrie and Brownsey (2005), who focussed on this clade in New Zealand with a smaller sample of species. Among the Austral group are the well supported Obtusatum, Flaccidum and Bulbiferum groups of Perrie and Brownsey (2005). However, the larger number of species sampled in the present study did not resolve uncertainty regarding relationships among these three groups (Perrie and Brownsey 2005; Shepherd *et al.* 2008a).

Several species in Clade V, such as *A. carruthersii*, *A. scandens*, *A. submarginatum*, *A. simplicifrons* and *A. marattioides*, had particularly long branch lengths when compared with other species (Fig. 3A). It is possible that these lengths were artefacts of low taxon sampling in New Guinea, where most of these species occur and possibly many of their close relatives. The morphologically distinct Malesian group of approximately four species sometimes recognised as the segregate genus *Diplora* Baker (see Mickel 1976; Copeland 1913, 1949) likely contributes to this undetected diversity. Partial *rbcL* sequence obtained from herbarium material (WELT P017103) of a putatively new *Diplora* species from the Solomon Islands is placed with the *A. marattioides* + *A. scandens* clade (L. Shepherd, unpub. data).

Biogeography of Aspleniaceae in ASWP

The present study has highlighted that the Aspleniaceae of ASWP is an assemblage of different lineages, some of which are widespread throughout the world. Molecular dating of Aspleniaceae by Perrie and Brownsey (2005), using *rbcL*, supported comparatively recent exchange of ASWP Aspleniaceae with those of other regions. The longest isolation of a clade in and around this region may be that of the Austral group. With the exception of a possibly recently formed circum-antarctic distribution in *A. obtusatum* (Brownlie 1965; Parris 2001), this clade appears to be endemic to southern Australasia and the islands of the south Pacific (Fig. 2C). Otherwise, much more recent exchange with other regions is prevalent, with divergence between the other Aspleniaceae of ASWP and their closest relatives in other regions not nearly as deep as that of the Austral group. This is especially the case for populations of species shared among various regions of the world (see Table 1).

All clades detected within ASWP, including the Austral group, are nested within clades defined by Schneider *et al.* (2004), which are all widespread in other regions. The presence of five of these clades in ASWP had been previously shown by molecular data or suspected on the basis of

morphology (e.g. Clade III – *Pleurosorus*, see Fig. 1). However, our study has extended the known distribution of Clade VIII. Clade VIII was previously known only from Africa, Europe and South America (Schneider *et al.* 2004), but its distribution is here shown to include also the south-western Pacific (Fig. 2F), where its single known representative is the New Caledonian species *A. dognyense*. This species, or a close relative, may also occur in Western Samoa (WELT P0159591). Furthermore, *A. dognyense* is morphologically similar and presumably closely related to the unsequenced *A. macraei* Hook. & Grev., differing mainly by its proliferous fronds (Brownlie 1969; Palmer 2003). *Asplenium macraei* is known from the Austral Islands, and Hawaii (Palmer 2003), suggesting that Clade VIII may be widespread in the Pacific.

Some subclades within the major clades defined by Schneider *et al.* (2004) and the present study are also widespread in several regions of the world. The *A. aethiopicum* clade in Clade VI is likely to be pantropical. This clade contains the *A. aethiopicum*/*A. praemorsum* complex, with Australian and African samples shown to be closely related to each other and to cultivated material of *A. praemorsum*, which is generally considered to be an American species (Fig. 3B). The *A. aethiopicum* clade is also likely to contain Hawaiian *A. contiguum* Kaulf., Asian *A. lepturus* J.Smith ex C.Presl and South American *A. serra* Langsdorff & Fisher. These species have narrow and caudate pinnae, long-creeping rhizomes and reticulate perispores without ridges (Youxing and Viane 2013), a combination of characters so far known only from species of the *A. aethiopicum* clade.

A subclade within Clade III is also widespread in several regions of the world. The subclade of species previously attributed to *Pleurosorus* (*A. hispanicum*, *A. papaverifolium*, *A. subglandulosum*; Fig. 1) positioned within Clade III confirms a particularly remarkable disjunction. No intervening *Pleurosorus* species are known, making this disjunction comparable to that seen in the trans-continental distributions of *A. dalhousiae* Hook. (Van den heede *et al.* 2003), *A. platyneuron* (L.) Britton (Wagner *et al.* 1993; Moran and Smith 2001; Crouch *et al.* 2011) and *A. exiguum* Beddome (Wagner *et al.* 1993).

The greatest sharing of clades between ASWP and another region of the world is with adjacent areas of Malesia and South-east Asia (Fig. 4). Thirty-one species from ASWP also occur in that region (see Table 1). Most of the species shared between ASWP and adjacent Malesia and South-east Asia were placed in Clades V and VI. In addition, several other South-east Asian and Malesian species not sampled in the present study are morphologically similar to some Clade V and Clade VI species and also undoubtedly belong to these clades (e.g. several section *Thamnopteris* species). The high diversity of these clades in this adjacent region helps explain the higher diversity of these clades in ASWP, particularly Clade VI, which predominantly occurs in tropical areas of ASWP adjacent to Indonesia (Fig. 2D). Of the Clade VI species in ASWP, only *A. aethiopicum*, *A. attenuatum* of the *A. paleaceum* clade, and the *A. polyodon* complex occur south of the tropics (Fig. 2D). Apart from the Austral group, other clades in Clade V also predominantly occur in tropical areas of ASWP, with *A. australasicum* of the *Thamnopteris* I clade being the only species that extends into temperate areas (Fig. 2C).

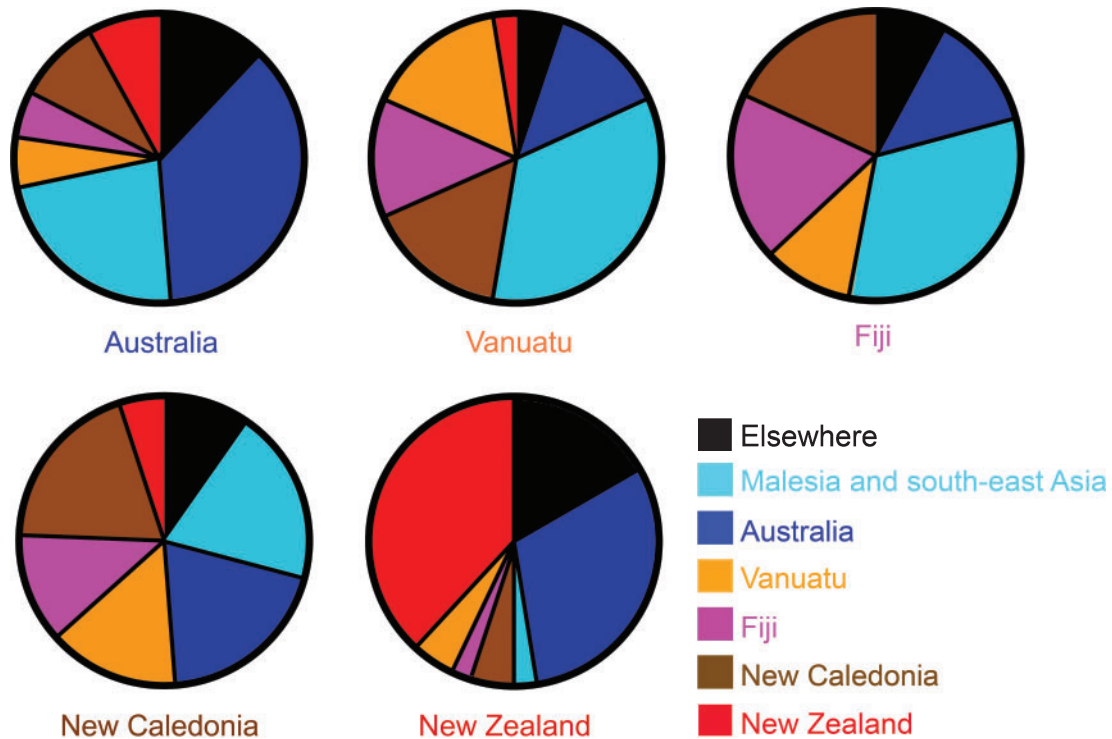


Fig. 4. Summary of geographic affinities of taxa from Australia, Vanuatu, Fiji, New Caledonia and New Zealand. For the taxa of each area, pie graphs depict the geographic distribution of their sister species or sister-clade relationships. For Australian taxa, a large proportion have their sister species or sister clades either in Australia or in Malesia, and a smaller proportion have their closest relatives in New Caledonia, Vanuatu, New Zealand or elsewhere. Sister-species relationships were determined from the phylogenies presented here, previous studies (Perrie and Brownsey 2005; Shepherd *et al.* 2008b), by analyses not shown here that included additional GenBank sequences (e.g. AB014702, AB016188, AB574852, AB574867, AB574886, AB574889, AB574890, AB574891, AB574892, EF463147, JX152738, JX152751), or on the basis of both morphological similarity and the occurrence of hybridisation for unsampled species (Brownlie 1969; Brownlie 1977). The relationships for many New Guinean species are unknown, so data for them are not presented. In instances where species that occur in more than one area are the sister species, or species are sister to a clade that occurs in more than one area, all areas of occurrence of these widespread species or clades are given.

Clades less well represented or absent in ASWP (i.e. Clades II, III, IV, VII, VIII, X, XI, XII and XIII) are predominant in regions more distant from Australia (Fig. 1). Previous studies have also shown several species not included in the present study and that do not occur in ASWP to belong to one of these clades (Van den heede *et al.* 2003; Schneider *et al.* 2004, 2005; Li and Lu 2006; Dyer *et al.* 2012; Chang *et al.* 2013). The influence of surrounding regions on Aspleniaceae diversity in the Pacific is further demonstrated by Hawaii. All the major clades present in ASWP are likely to be present in Hawaii on the basis of previous studies (Schneider *et al.* 2004, 2005; Schuettpelz and Pryer 2007). However, the ‘black-stemmed spleenwort’ clade comprising Clades XI, XII and XIII, well represented in surrounding continents of the northern hemisphere (Schneider *et al.* 2004), contains more species in Hawaii than any of the other major clades with at least 10 species (Schneider *et al.* 2004, 2005), demonstrating the importance of the closer northern hemisphere continents to the fern diversity of Hawaii (also see Geiger *et al.* 2007). In contrast, Clade V, predominant in the southern hemisphere (Schneider *et al.* 2004) and south Pacific, may be represented in Hawaii only by *A. nidus*.

The importance of immigration to patterns of diversity in Aspleniaceae of ASWP and surrounding areas can also be appreciated from the high proportion of species in each area of ASWP whose closest relatives are from other areas (Fig. 4). For instance, among the Vanuatu and Fijian species, more have a sister relationship with species of Malesia than with other local species. Even among taxa from the large continent of Australia, a high proportion have their closest relatives in other areas, especially in Malesia (Fig. 4). The exception to this trend is New Zealand where a high proportion of species had their closest relatives in New Zealand, and more sister relationships were shared with Australia rather than Malesia (Fig. 4).

In general, even when local speciation has apparently occurred in parts of ASWP, it appears to have given rise to only minor species radiations. The largest radiation of species wholly endemic to a single land area comprises only the following three species: *A. milnei*, *A. pteridoides* and *A. surrogatum* endemic to Lord Howe Island, east of mainland Australia. This Lord Howe Island clade supports a previous suggestion that these species have evolved in geographic sympatry (Papadopoulos *et al.* 2011). The New Caledonian clade that includes *A. vieillardii* and *A. oligolepidum* may also

comprise three species, given that partial *rbcL* sequence from New Caledonian *A. novae-caledoniae* is apparently placed with this clade (L. Shepherd, unpub. data). The *A. paleaceum* clade contains four Australian species, but one of its species, *A. attenuatum*, is also known from New Caledonia (not sampled in the present study), making this clade not wholly endemic to Australia.

The majority of island endemic species in ASWP appear to have evolved on the same island where their closest relatives occur. Of the 19 island endemic species whose phylogenetic positions are known, possibly only six (*A. dognyense*, *A. goudeyi*, *A. pauperequitum*, *A. tenuiculum*, and possibly two cryptic species of section *Thamnopteris*) may not have their closest relative also occurring on the same island. These six may be the result of anagenetic speciation, i.e. where an island immigrant evolves into a separate species in isolation from its disjunct progenitor (Stuessy *et al.* 2006; Gehrke and Linder 2011; Patiño *et al.* 2014). This differs from most fern groups in which anagenetic speciation appears to be the prevalent source of island endemics (Patiño *et al.* 2014), including the Blechnaceae ferns of the south-western Pacific (Perrie *et al.* 2014).

Implications for species-level taxonomy

The present study resolves an outstanding taxonomic issue regarding the distinctiveness of *A. carnarvonense* from *A. capitisyork*. Brownsey (1998) considered that these two Queensland endemic species were very similar and, on the basis of spore and overall morphology, placed them together as the only members of the '*Asplenium capitisyork*' group. In contrast, the chloroplast phylogenies presented here placed *A. capitisyork* and *A. carnarvonense* into separate clades with strong support (the *A. capitisyork* and *A. paleaceum* clades respectively). This result provides significant new evidence of the distinctiveness of these taxa, and supports their continued recognition as separate species. However, in Aspleniaceae, hybridisation and allopolyploidy are frequent (Lovis 1973; Reichstein 1981), and it is possible that *A. capitisyork* and *A. carnarvonense* are allopolyploids that share the same parental species but different maternal parents and, hence, different chloroplast haplotypes (Vogel *et al.* 1998). Nuclear DNA markers are required to test this hypothesis.

The present study also identifies the need for taxonomic revision for several members of ASWP Aspleniaceae. In particular, several species as currently circumscribed are not monophyletic (i.e. *A. australasicum*, *A. cromwellianum*, *A. cuneatum*, *A. gibberosum*, *A. nidus*, *A. polyodon* and *A. tenerum*) and potentially contain cryptic species (Paris 1989); taxonomic revision of these groups of species will require molecular study of populations from across their ranges, including outside ASWP. Non-monophyly of these species may be due to these species being allopolyploids with multiple and reciprocal origins (see above). However, a reciprocal polyploidy origin is an unlikely explanation for the *Thamnopteris* species and *A. polyodon*. Multiple chromosome counts have shown only tetraploid counts so far (Manton 1954; Manton and Sledge 1954; Bir 1960; Abraham *et al.* 1962; Kawakami 1970; Brownsey 1977b; Nakato 1987; Yatabe *et al.*

2001, 2002, 2009; Tindale and Roy 2002; Yatabe and Murakami 2003) and diploid species have not yet been recorded in Clade VI and only a few species in Clade V have been reported as diploid (Bir 1960; Mehra 1961; Tindale and Roy 2002). Instead, the prevalent ploidy level in Clades V and VI is tetraploid (Perrie and Brownsey 2005), suggesting that these species are likely derived from tetraploid ancestors and that non-monophyly of these taxa in chloroplast trees is not an artefact of reciprocal allopolyploidy (see Yatabe *et al.* 2009).

Some accessions in the dataset were genetically distinct, and are not easily assigned to a current species; they potentially represent undescribed species. Australian *A. sp.* 'Windsor Tableland' most closely resembles *A. athertonense* morphologically, but its chloroplast sequences were more closely related to Australian *A. cuneatum*. The genetic distinction of *A. sp.* 'Windsor Tableland' from both of these species was greater than that between other pairs of morphologically distinctive species such as *A. athertonense* and *A. baileyianum* (Fig. 3B). Likewise, *A. sp.* 'New Caledonia' was more genetically divergent from its closest relatives and the morphologically similar *A. lobulatum* than were several other well established and morphologically distinctive species pairs (Fig. 3B). This gives support towards treating these accessions as new species.

Conversely, some currently accepted species of Aspleniaceae from ASWP are both genetically (Fig. 3) and morphologically similar and might best be considered conspecific. This includes: *A. parvum* and *A. flidens*; limestone-dwelling *A. polyodon* in the Pacific and limestone-dwelling *A. listeri* from the Australian Territory of Christmas Island in the Indian Ocean; *A. athertonense* and *A. lobulatum*.

For some groups of morphologically distinctive species, only minor chloroplast variation was detected here (e.g. *A. paleaceum* clade, Fig. 3B). These groups potentially represent species complexes (Viane and Reichstein 2003) in which chloroplast similarity has resulted from hybridisation. Study of nuclear genetic variation is needed to more fully understand the relationships of these taxa and assess their taxonomic status.

The present study has clearly highlighted some unresolved taxonomic issues among Aspleniaceae of ASWP. Some of these, including the status of *A. sp.* 'Windsor Tableland', *A. polyodon* and relatives (including *A. listeri*), and the *A. paleaceum* clade are the subjects of more detailed works (Ohlsen 2014; Ohlsen *et al.* 2014a, 2014b) using additional genetic markers or morphological comparisons. The results of those studies, combined with the present one, will form the basis for taxonomic treatment for all Australian Aspleniaceae.

Conclusions

The present study has provided further support for many relationships within the Aspleniaceae recovered from previous studies (Murakami *et al.* 1999a; Schneider *et al.* 2004; Perrie and Brownsey 2005). It has also established relationships among most Aspleniaceae in ASWP, which were previously largely unknown. On comparison with taxa from other regions of the world, it has revealed that ASWP species belong to several large inter-continental clades. The widespread distribution of most species in the region and the close relationship of the

locally endemic species to more widespread species or species endemic elsewhere indicate the importance of immigration as a source of diversity in ASWP. The present study has also exposed taxonomic issues at the genus and species level in Aspleniaceae. Although taxon sampling for some of the ASWP region was comprehensive, many species and groups of Aspleniaceae in Malesia were not sampled in the study. Molecular study involving more taxon sampling in Malesia could provide a focus for future study. Such study will further complete understanding of Aspleniaceae systematics in general, as well as elucidate more detailed biogeographic histories in ASWP and improve taxonomic circumscriptions among species of Aspleniaceae of this region.

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