JSE Journal of Systematics and Evolution

Journal of Systematics and Evolution 51 (2): 154–163 (2013) doi: 10.1111/j.1759-6831.2012.00230.x

Research Article

Phylogenetic position of *Kontumia* (Polypodiaceae) inferred from four chloroplast DNA regions

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Abstract The phylogenetic position of the monotypic genus *Kontumia* (Polypodiaceae) is contentious. It has been suggested that *Kontumia* is related to *Gymnogrammitis dareiformis* (Drynarioideae) based on spore ornamentation and blade dissection, or it has been assigned as a member of microsoroid ferns (Microsoroideae) based on the shape of rhizome scales. In the present study, we determined the phylogenetic position of *K. heterophylla* using four chloroplast DNA regions (*rbcL*, *atpB*, *rps4* + *rps4-trnS* intergenic spacer [IGS], and *trnL-F* IGS). A parsimony consensus tree indicated that *K. heterophylla* is not related to *G. dareiformis*, but is nested within the *Leptochilus* lineage of Microsoroideae. This relationship is also supported by sharing of clathrate scales on the rhizome, which is not found in *G. dareiformis*. Although marked morphological disparities are found between *K. heterophylla* and the *Leptochilus* lineage in terms of leaf dissection, fertile-sterile leaf differentiation, and sori arrangement, our results indicate that these characters have evolved independently several times in Polypodiaceae.

Key words fern, *Kontumia*, *Leptochilus* lineage, Microsoroideae, molecular phylogeny, Polypodiaceae.

The phylogenetic positions of monotypic genera have been studied because they are presumed to be distinct from other previously recognized genera and/or because their phylogenetic relationships are uncertain (e.g. Li et al., 2011, 2012; Tate, 2011). In several recent studies, the evolution of monotypic fern genera has been explored using phylogenetic approaches based on nucleotide sequence data (Schneider et al., 2002; Lu & Li, 2006; Kreier et al., 2007). Although these studies have revealed considerable conflicts between existing classifications and inferred phylogenetic relationships, molecular phylogenetic approaches have provided new insights into the systematic position and relationships of these ferns and the evolution of morphological characters (Pryer et al., 1995; Schneider et al., 2006, 2009; Sundue et al., 2010).

Kontumia S. K. Wu & L. K. Phan, a monotypic genus of Polypodiaceae, is found in the closed evergreen tropical seasonal broad-leaved submontane forests of Kon Tum province, Vietnam (Wu et al., 2005). The genus is characterized by a creeping habit, dimorphic leaves, clathrate scales on the rhizome and petiole, narrow wings on the petiole of sterile leaves, and orbicular sori positioned distally on the veinlets. The phylogenetic relationships of *K. heterophylla* S. K. Wu & L. K. Phan are contentious (Wu et al., 2005; Christenhusz et al., 2011). Based on similar spore morphology (i.e. monolete with the perispore beset irregularly with globules and spines) and highly divided leaves, Wu et al. (2005) suggested that it was most closely related to *Gymnogrammitis dareiformis* Ching ex Tardieu & C. Chr. (Drynarioideae). More recently, Christenhusz et al. (2011) indicated possible relationships between *K. heterophylla* and the microsoroid ferns (Microsoroideae) based on observations of rhizome scales. However, the systematic position of *K. heterophylla* within Polypodiaceae has not been addressed using molecular data.

At least two hypotheses can be invoked based on the controversy surrounding the taxonomic position of *Kontumia*: (i) *K. heterophylla* is related to *G. dareiformis* but the presence of clathrate scales on the rhizome and petiole is a distinct characteristic that is found in many microsoroideae species; and (ii) *K. heterophylla* is nested within the Microsoroideae clade and its morphological characteristics, such as spore ornamentation with spine-like structures and highly dissected leaves, are homoplastic features shared with *G. dareiformis*.

In the present study we explored the phylogenetic position of the monotypic genus *Kontumia* using data from four chloroplast (cp) DNA regions. Variations in cpDNA sequences have been useful when investigating

Received: 21 June 2012 Accepted: 21 September 2012

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evolutionary relationships and classification in many fern species (e.g. Hasebe et al., 1995; Liu et al., 2008). Based on the inferred phylogeny, we also discuss the evolution of diagnostic morphological traits of this monotypic genus.

1 Material and methods

1.1 Taxon sampling

In order to determine phylogenetic relationships, in addition to three accessions of *Kontumia heterophylla*, we sampled 33 taxa (37 accessions) from three subfamilies of Polypodiaceae (Drynarioideae, Microsoroideae, and Platycerioideae). We included four species (*Campyloneurum angustifolium* Fée, *Polypodium vulgare* L., *Dictymia mckeei* Tindale, and *Loxogramme abyssinica* M. G. Price) belonging to Loxogrammoideae and Polypodioideae from GenBank. Therefore, our sampling included species currently recognized in all five subfamilies of Polypodiaceae (Christenhusz et al., 2011). We also downloaded 12 sequences for *rbcL*, seven for *rps4*, and six for *trnL-F* from GenBank for individual analyses of *Leptochilus* lineage species (Tables 1, 2). Based on previous phylogenetic studies, we included three species (*Araiostegia yunnanensis* Tagawa & K. Iwats, *Davallia mariesii* H. J. Veitch, and *D. tyermanni* T. Moore) belonging to the family Davalliaceae to function as outgroups (Hasebe et al., 1995; Smith et al., 2006; Liu et al., 2007; Schuettpelz & Pryer, 2007). Voucher information and GenBank accession numbers of all species sampled are listed in Table 1.

1.2 DNA isolation, amplification, and sequencing

Genomic DNA was isolated from either field-collected, silica gel-dried fronds or well identified living plants cultivated in Kunming Botanical Garden (KBG), Yunnan, China. Genomic DNA was extracted using a Universal Genomic DNA Extraction Kit (Takara, Dalian, China) and the DNA concentration of each sample was determined using a spectrophotometer.

In all, four chloroplast regions (*rbcL*, *atpB*, *rps4* + *rps4-trnS* intergenic spacer [IGS], and *trnL-F* IGS) were amplified and sequenced for this study. For simplicity, the *rps4* + *rps4-trnS* IGS is hereafter referred to as *rps4*. Primer sets used for the polymerase chain reaction (PCR) were as follows: *rbcL*: ESRBCL1F and ESRBCL1361R (Schuettpelz & Pryer, 2007); *atpB*: ESATPB172F (Schuettpelz & Pryer, 2007) and ATPB910R (Pryer et al., 2004); *rps4*: rps4F (Nadot et al., 1995) and trnSR (Smith & Cranfill, 2002); and *trnL-F* IGS: e and f (Taberlet et al., 1991). The PCR amplification was performed using a PTC 200 thermocycler (MJ Research, Watertown, MA, USA) as follows: initial denaturation for 4 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 50 °C (*atpB*) or 55 °C (*rbcL*, *rps4*, and *trnL-F*), and 1 min 30 s at 72 °C, ending with a 10 min extension step at 72 °C.

Amplified DNA samples were analyzed by electrophoresis on a 1.5% agarose gel run in 1× Tris-acetate-EDTA (TAE) buffer and detected by ethidium bromide staining. The PCR products were then purified using a QiaQuick gel extraction kit (Qiagen, Valencia, CA, USA) and directly sequenced in both directions using the amplification primers with an ABI Prism™ BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, Norwalk, CT, USA) and an ABI 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA). All sequences were deposited in GenBank (Table 1).

1.3 Phylogenetic analyses

Consensus sequences were assembled for each individual using DNA Baser v.2.75 (http://www.DnaBaser.com, accessed 5 April 2012). Multiple-sequence alignment was undertaken using Clustal X v.1.81 (Thompson et al., 1997) with the default alignment parameters and then adjusted manually using the alignment criterion presented by Zurawski & Clegg (1987), in which gaps are considered as characters and the number of evolutionary events is minimized as much as possible. To minimize the effect of possible misaligned segments of the *trnL-F* sequences, 34 nucleotide columns were deleted prior to analysis. The incongruence length difference (ILD) test was conducted to assess data congruency (Farris et al., 1995); this was performed using PAUP* v.4.0b10 (Swofford, 2003) with 1000 heuristic search replications. We assessed the significance of incongruence at $P < 0.05$, although this threshold is too conservative for the homogeneity test (Cunningham, 1997). Combined data matrices for this study were deposited in TreeBASE (http://www.treebase.org/, study accession number S12772; accessed 28 May 2012).

Phylogenetic analyses were performed using maximum parsimony (MP) and Bayesian inference (BI) of phylogeny. Each data set was analyzed separately and then a combined analysis was performed with all regions. The MP analyses were performed in PAUP* v.4.0b10. Gaps were treated as missing data. All characters and character states were equally

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Table 1 Species names, collection localities, voucher deposition, and GenBank accession numbers for sequences included in the phylogenetic analyses

Family	Subfamily	Taxon[†]	Locality, voucher	*rbcL*	*atpB*	*rps4*	*trnL-F*
Ingroups							
Polypodiaceae	Drynarioideae	*Aglaomorpha coronans* Copel.	Cult. KBG, China; Kim 2012-11 (KUN)	JX103723	JX103681	JX103765	JX103807
		Drynaria bonii Christ	Laos; Wu 239 (KUN)	JX103690	JX103648	JX103732	JX103774
		Drynaria quercifolia J. Sm.	Laos; Wu 2396 (KUN)	JX103692	JX103650	JX103734	JX103776
		Drynaria rigidula Bedd.	Laos; Wu 2305 (KUN)	JX103691	JX103649	JX103733	JX103775
		Gymnogrammitis dareiformis Ching	China; Shui et al. 92335 (KUN)	JX103689	JX103647	JX103731	JX103773
		Selliguea rhynchophylla Fraser-Jenk.	Laos; Wu 2453 (KUN)	JX103693	JX103651	JX103735	JX103777
	Loxogrammoideae	*Dictymia mckeei* Tindale	—	DQ164441*	EF463492*	DQ164472*	DQ164504*
		Loxogramme abyssinica M. G. Price	—	DQ164443*	EF463498*	DQ164474*	DQ164506*
	Microsoroideae	*Goniophlebium argutum* J. Sm._a	Laos; Wu 2440 (KUN)	JX103709	JX103667	JX103751	JX103793
		Goniophlebium argutum J. Sm._b	Cult. KBG, China; Kim 2012-9 (KUN)	JX103721	JX103679	JX103763	JX103805
		Kontumia heterophylla S. K. Wu & P. K. Lôc_a	Vietnam; WP-135 (KUN)	JX103688	JX103646	JX103730	JX103772
		Kontumia heterophylla S. K. Wu & P. K. Lôc_b	Vietnam; WP-136 (KUN)	JX520933	JX520931	JX520935	JX520937
		Kontumia heterophylla S. K. Wu & P. K. Lôc_c	Vietnam; WP-201 (KUN)	JX520934	JX520932	JX520936	JX520938
		Lepisorus macrosphaerus Ching	Cult. KBG, China; Kim 2012-3 (KUN)	JX103697	JX103655	JX103739	JX103781
		Lepisorus scolopendrius Mehra & Bir	Laos; Wu 2441 (KUN)	JX103698	JX103656	JX103740	JX103782
		Leptochilus axillaris Kaulf._a	Laos; Wu 2344 (KUN)	JX103699	JX103657	JX103741	JX103783
		Leptochilus axillaris Kaulf._b	Laos; Wu 2439 (KUN)	JX103700	JX103658	JX103742	JX103784
		Leptochilus axillaris Kaulf._c	Laos; Wu 2462 (KUN)	JX103701	JX103659	JX103743	JX103785
		Leptochilus cantoniensis Ching_a	—	EU482946*	—	EU482996*	EU483042*
		Leptochilus cantoniensis Ching_b	—	EU482945*	—	EU482995*	EU483041*
		Leptochilus decurrens Blume	Cult. KBG, China; Kim 2012-12 (KUN)	JX103724	JX103682	JX103766	JX103808
		Leptochilus digitatus Noot.	Laos; Wu 2515 (KUN)	JX103695	JX103653	JX103737	JX103779
		Leptochilus elegans Kurata	—	AB575242*	—	—	—
		Leptochilus ellipticus Noot.	—	EU482949*	—	EU482999*	EU483045*
		Leptochilus hemionitideus Noot.	Laos; Wu 2437 (KUN)	JX103694	JX103652	JX103736	JX103778
		Leptochilus hemitomus Noot.	—	EU482951*	—	EU483001*	EU483047*
		Leptochilus henryi Ching	—	GQ256254*	—	EU483002*	EU483048*
		Leptochilus intermedia Ching & Chu H. Wang	—	EU363239*	—	—	—
		Leptochilus leveillei Ching	—	EU363240*	—	—	—
		Leptochilus longipes Ching	—	EU363241*	—	—	—
		Leptochilus pothifolius Fraser-Jenk.	Laos; Wu 2712 (KUN)	JX103696	JX103654	JX103738	JX103780
		Leptochilus pteropus Fraser-Jenk.	—	EU482965*	—	—	—
		Leptochilus simplicifrons Tagawa	—	EU482953*	—	EU483003*	EU483049*
		Leptochilus triphylla Ching	—	EU363244*	—	EU363257*	—
		Leptochilus wrightii Ching	Cult. KBG, China; Kim 2012-15 (KUN)	JX103727	JX103685	JX103769	JX103811
		Microsorum cuspidatum Tagawa	Cult. KBG, China; Kim 2012-6 (KUN)	JX103707	JX103665	JX103749	JX103791
		Microsorum insigne Copel.	Laos; Wu 2435 (KUN)	JX103703	JX103661	JX103745	JX103787
		Microsorum lucidum Copel.	Cult. KBG, China; Kim 2012-14 (KUN)	JX103726	JX103684	JX103768	JX103810
		Microsorum membranaceum Ching	Cult. KBG, China; Kim 2012-2 (KUN)	JX103704	JX103662	JX103746	JX103788
		Microsorum punctatum Copel.	Laos; Wu 2506 (KUN)	JX103705	JX103663	JX103747	JX103789
		Microsorum sp.	Laos; Wu 2367 (KUN)	JX103708	JX103666	JX103750	JX103792
		Neocheiropteris palmatopedata Christ	Cult. KBG, China; Kim 2012-1 (KUN)	JX103706	JX103664	JX103748	JX103790
		Neolepisorus fortunei Li Wang	Cult. KBG, China; Kim 2012-5 (KUN)	JX103702	JX103660	JX103744	JX103786
		Neolepisorus ovatus Ching	Cult. KBG, China; Kim 2012-8 (KUN)	JX103720	JX103678	JX103762	JX103804
		Tricholepidium maculosum Ching	Cult. KBG, China; Kim 2012-4 (KUN)	JX103710	JX103668	JX103752	JX103794
	Platycerioideae	*Platycerium coronarium* Desv.	Laos; Wu 2597 (KUN)	JX103711	JX103669	JX103753	JX103795
		Platycerium wallichii Hook.	Cult. KBG, China; Kim 2012-16 (KUN)	JX103728	JX103686	JX103770	JX103812
		Pyrrosia flocculosa Ching	Laos; Wu 2310 (KUN)	JX103712	JX103670	JX103754	JX103796
		Pyrrosia lingua Farw.	Cult. KBG, China; Kim 2012-13 (KUN)	JX103725	JX103683	JX103767	JX103809

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Family	Subfamily	Taxon†	Locality, voucher	*rbcL*	*atpB*	*rps4*	*trnL-F*
		Pyrrosia mannii Ching	Laos; Wu 2571 (KUN)	JX103715	JX103673	JX103757	JX103799
		Pyrrosia nummulariifolia Ching_a	Laos; Wu 2570 (KUN)	JX103713	JX103671	JX103755	JX103797
		Pyrrosia nummulariifolia Ching_b	Laos; Wu 2574 (KUN)	JX103714	JX103672	JX103756	JX103798
		Pyrrosia stigmosa Ching	Laos; Wu 2586 (KUN)	JX103716	JX103674	JX103758	JX103800
		Pyrrosia subfurfuracea Ching	Cult. KBG, China; Kim 2011-2 (KUN)	JX103729	JX103687*	JX103771	JX103813
	Polypodioideae	*Campyloneurum angustifolium* Fée	—	AF470344*	AY459515*	AY362645*	AY083647*
		Polypodium vulgare L.	—	EF551065*	EF463510*	EF551081*	EF551119*
Outgroups							
Davalliaceae		*Araiostegia yunnanensis* Tagawa & K. Iwats.	Laos; Wu 2458 (KUN)	JX103718	JX103676	JX103760	JX103802
		Davallia mariesii H. J. Veitch	Laos; Wu 2463 (KUN)	JX103717	JX103675	JX103759	JX103801
		Davallia tyermanni T. Moore	Laos; Wu 2567 (KUN)	JX103719	JX103677	JX103761	JX103803

Material was obtained from either existing collections or from plants cultivated in the Kunming Botanical Garden (KBG).
Abbreviations for herbaria follow *Index Herbariorum* (KUN = Kunming Institute of Botany).
†Lowercase letters following the name of a taxon indicate that multiple accessions of that taxon are included.
*Accessions obtained from GenBank.
—, missing data.

weighted and unordered. Searches were conducted over 100 random taxon addition replicates with tree bisection-reconnection (TBR), branch swapping, and MulTrees in effect. Bootstrap analyses (BP; 1000 pseudoreplicates) were conducted to examine the relative level of support for clades on the cladograms (Felsenstein, 1985). The consistency index (CI) and retention index (RI) were calculated to measure the level of homoplasy in the data set.

The BI phylogeny for the four cpDNA regions was constructed using MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003). Based on the Akaike information criterion, jModelTest v.0.1.1 (Posada, 2008) assigned the GTR + G model of molecular evolution to the *trnL-F* region and the GTR + I + G model to the *rbcL*, *atpB*, and *rps4* regions and to the combined data set. Four Markov Chain Monte Carlo simulations were run simultaneously and sampled every 100 generations for a total of 1 000 000 generations. The first 2500 trees (25%) of the sample trees from each run were discarded (they represented the burn-in) as determined by Tracer v.1.5 (Rambaut & Drummond, 2009). Bayesian consensus tree was constructed from the remaining trees, yielding the posterior probability (PP) values for each clade.

1.4 Morphological character evolution

Three characters (leaf dissection, fertile-sterile leaf differentiation, and sori arrangement) were included in the morphological character evolution analysis. All three characters were selected because they were used as diagnostic features for *K. heterophylla* (Wu et al., 2005). The leaf dissection was coded as follows: 0, bi- to quadripinnate; 1, pinnate/pinnatifid; or 2, simple/palmatifid. Fertile-sterile leaf differentiation was coded as: 0, monomorphic; or 1, dimorphic. Sori arrangement was coded as: 0, separate; 1, linear (coenosori); or 2, sorus absent (acrostichoid). Morphological data were obtained from herbarium specimens and floras (Ching, 1966; Shi & Zhang, 1999; Wu & Wang, 1999; Lin et al., 2000; Wu et al., 2005; Wang et al., 2010b).

To infer patterns of character evolution, we used one of two most parsimonious trees for 41 accessions from the analysis of the combined molecular data. In the data set we included one accession per species because all species sampled were monophyletic (see Results) and exhibited identical states for all three morphological characters. Character reconstructions were performed under the assumption of unordered and unweighted character states with the Ancestral State Reconstruction Package in Mesquite v.2.75 (Maddison & Maddison, 2011).

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Table 2 Summary of the molecular data sets used in the present study, with tree statistics corresponding to the maximum parsimony analyses

Parameters	*rbcL*	*atpB*	*rps4*	*trnL-F*	Combined
No. accessions (ingroup/outgroup)	59 (56/3)	47 (44/3)	54 (51/3)	53 (50/3)	47 (44/3)
Alignment length (bp)	1178	654	1071	249	3111
No. variable characters (%)	310 (26.3)	164 (25.1)	530 (49.5)	164 (65.9)	1136 (36.5)
No. parsimony-informative characters (%)	235 (19.9)	119 (18.2)	383 (35.8)	115 (46.2)	818 (26.3)
No. MPT	295	164	4	240	2
Length of MPT	731	338	1205	449	2458
CI	0.420	0.474	0.513	0.482	0.495
RI	0.758	0.781	0.805	0.786	0.779
Evolutionary models (AIC)	GTR + I + G	GTR + I + G	GTR + I + G	GTR + G	GTR + I + G

The CI values are calculated without considering constant characters.
MPT, most parsimonious trees; CI, consistency index; RI, retention index; AIC, Akaike information criterion; GTR, general time reversible; I, proportion invariant; G, gamma.

2 Results

The cpDNA *rbcL*, *atpB*, *rps4*, and *trnL-F* regions included 59, 47, 54, and 53 accessions corresponding to 1178, 614, 1071, and 249 characters, respectively. The *trnL-F* sequence produced the greatest proportion of variable and parsimony-informative characters (Table 2). The MP and BI analyses revealed the same topology for individual data sets. Although MP analyses of each individual chloroplast region provided low resolution within Microsoroideae, *Kontumia heterophylla* formed a clade with the members of the *Leptochilus* lineage (data not shown).

The ILD test indicated that the four cpDNA sequence data partitions were not significantly incongruent ($P = 0.053$). The combined data set had 3111 aligned positions, with 1136 (36.5%) variable sites, 818 (26.3%) of which were parsimoniously informative (Table 2). Maximum parsimony analysis performed on the combined data set for the four cpDNA regions resulted in two equally parsimonious trees, each of 2458 steps (CI = 0.495; RI = 0.779). The BI phylogram was identical in topology to the strict consensus tree sampled by the MP analysis, except that three taxa of *Microsorum* (i.e. *M. cuspidatum*, *M. incidum*, and *Microsorum* sp.) were a sister group to the clade including *K. heterophylla*, *Leptochilus* species, and *M. insigne* (Fig. 1). Monophyly of Polypodiaceae was strongly supported with the maximal value (BP = 100%; PP = 1.00; Fig. 1). Within Polypodiaceae, five clades were well supported and corresponded to the subfamilies Microsoroideae (BP = 100%; PP = 1.00), Playtcerioideae (BP = 100%; PP = 1.00), Drynarioideae (BP = 99%; PP = 1.00), Polypoidioideae (BP = 66%; PP = 1.00), and Loxogrammoideae (BP = 100%; PP = 1.00). As for individual results, three accessions of *K. heterophylla* were found to be nested in the *Leptochilus* lineage (BP = 100%; PP = 1.00; Fig. 1) as the putative sister of a clade comprising *L. digitatus*, *L. pothifolius*, and *L. wrightii*. The type of the former genus *Colysis*, *L. hemionitideus*, was found to be sister to the type of *Leptochilus*, *L. axillaris* (BP = 97%; PP = 1.00).

Three morphological characters (leaf dissection, leaf differentiation, and sori arrangement) were mapped on one of two most parsimonious trees constructed from combined cpDNA sequence data (Fig. 2; Table 3). The morphological character states of blade dissection, leaf differentiation, and sori arrangement evolved at least nine, four, and five times, respectively. No apomorphies for the clade including *K. heterophylla* and *Leptochilus* were identified. Moreover, the diagnostic morphological features of *K. heterphylla*, which are highly dissected blades (bi- to tripinnatifid), dimorphism between sterile and fertile leaves, and a separated sorus, were distributed diffusely across the cladogram (Fig. 2).

3 Discussion

As in most current studies, we used variations in cpDNA sequences (*rbcL*, *atpB*, *rps4*, and *trnL-F*) to assess the phylogenetic position of monotypic *Kontumia* within Polypodiaceae (Haufler et al., 2003; Kreier et al., 2008; Wang et al., 2010a). We consistently identified five major clades within Polypodiaceae (Fig. 1) and this results is in general agreement with the subfamilial concept proposed by Christenhusz et al. (2011) and hypotheses suggested by other authors on the basis of molecular DNA sequence data (Ranker et al., 2004; Schneider et al., 2004; Kreier & Schneider, 2006).

Our molecular phylogenetic analyses based on both individual and combined data sets do not support *K. heterophylla* as being closely related to *Gymnogrammitis dareiformis*; this relationship was originally suggested primarily on the basis of apparently

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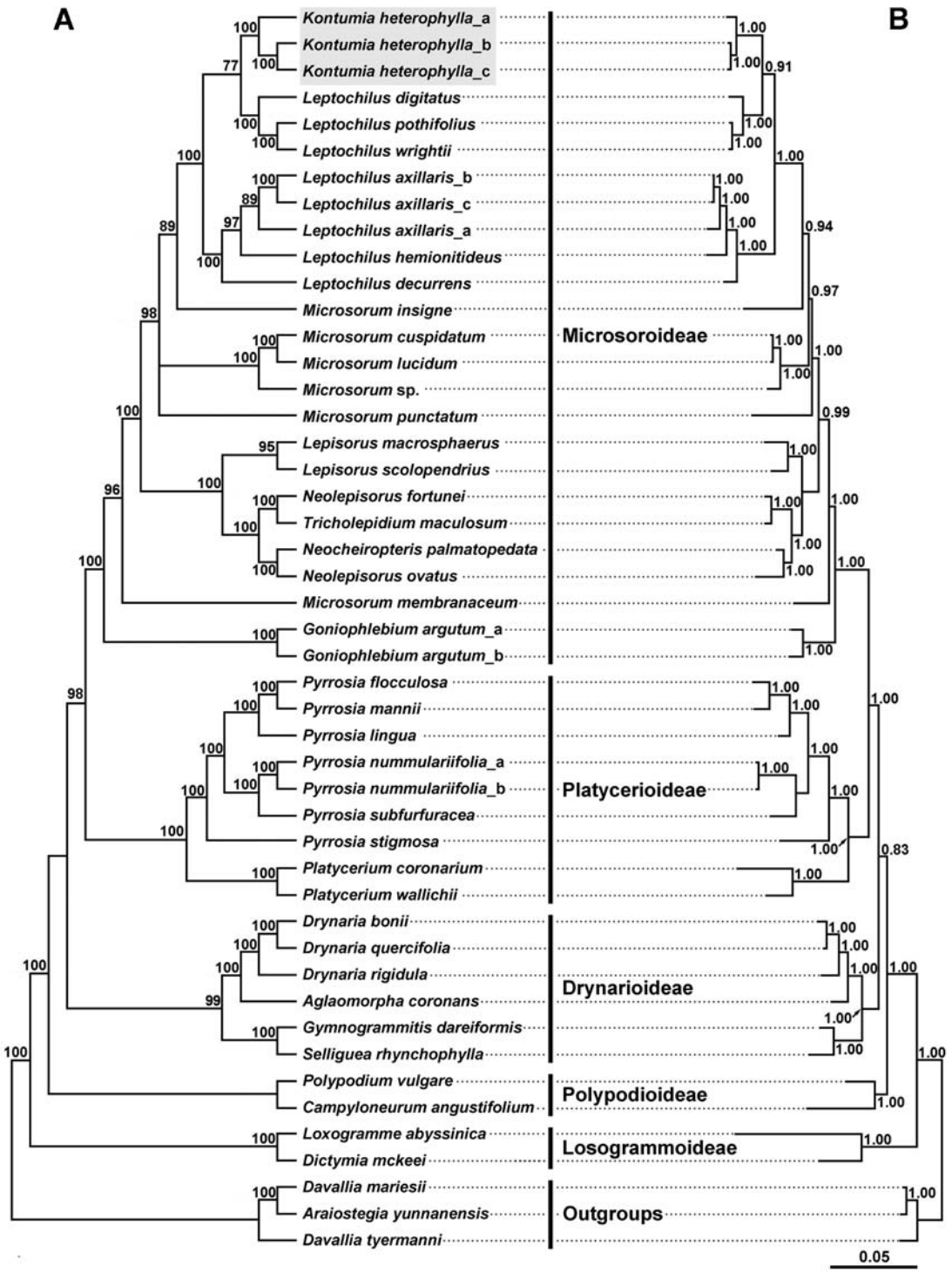

Fig. 1. **A**, Strict consensus tree based on the two most parsimonious trees obtained by maximum parsimonious (MP) analysis of the combined data set. Numbers near nodes indicate bootstrap values (>75%) for MP analysis. **B**, Bayesian consensus phylogram obtained using the Treeanotater from the Bayesian analyses of the combined data set. Numbers near nodes indicate support values (Bayesian posterior probability [PP]). The bar represents 0.05 nucleotide substitutions per site. Lowercase letters (a, b, or c) following the name of a taxon indicate that multiple accessions of that taxon are included. Three accessions of *Kontumia heterophylla* are highlighted in gray. Subfamily classification follows Christenhusz et al. (2011).

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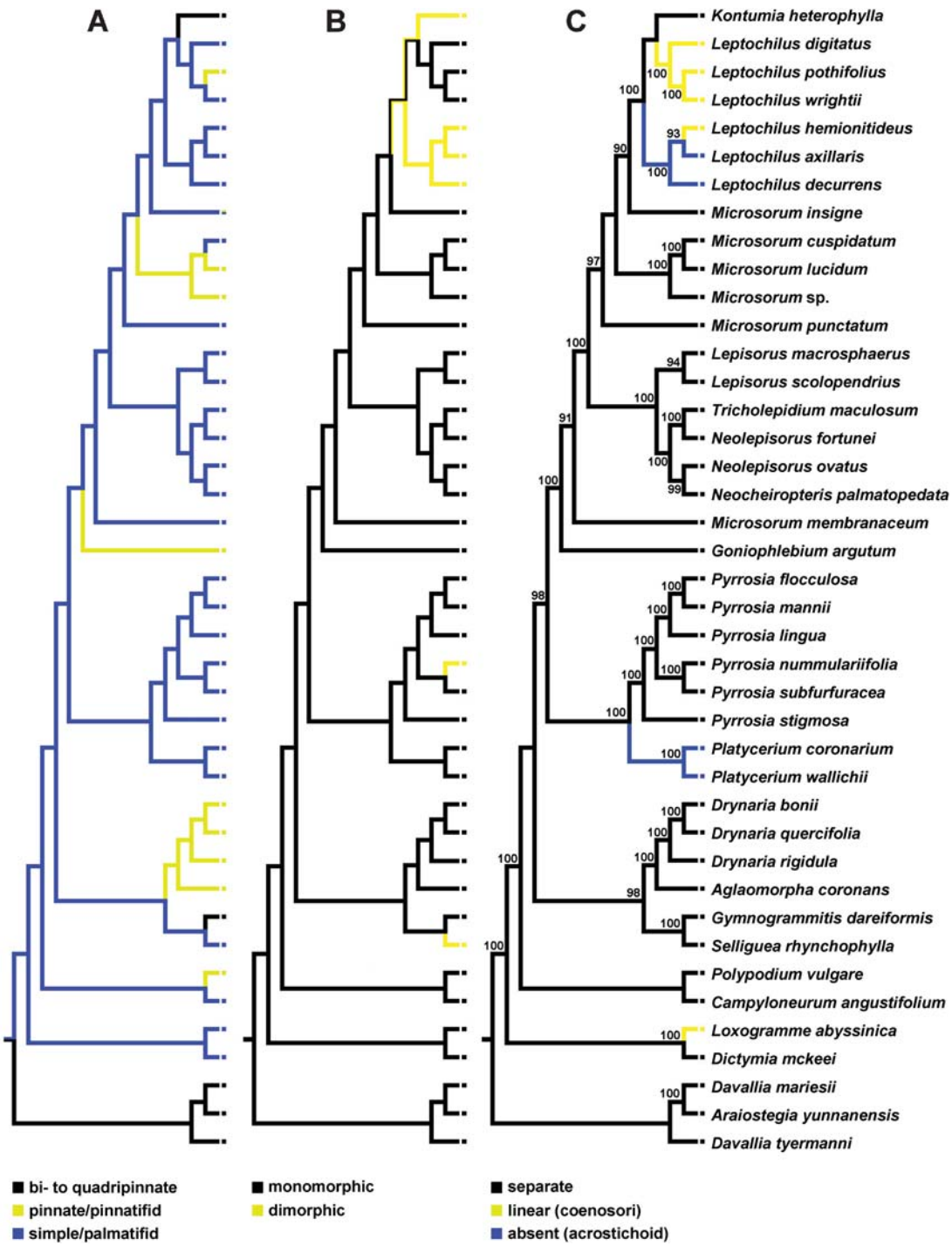

Fig. 2. Reconstruction of the evolution of selected morphological characters based on most parsimonious tree topology from combined molecular data. **A**, Leaf dissection. **B**, Fertile-sterile leaf differentiation. **C**, Sori arrangement. Numbers near nodes indicate bootstrap values (>75%) for maximum parsimony (MP) analysis. For descriptions of the character states, see Table 3.

superficial similarities in spore ornamentation and blade dissection (Wu et al., 2005). Although the spore ornamentation of *K. heterophylla*, with its spine-like structures, is similar to that of *G. dareiformis*, the spores of *K. heterophylla* differ from those of *G. dareiformis* in having a thin perine layer (Schneider et al., 2002; Wu et al., 2005). The blade dissection feature does not appear to have phylogenetic significance in uniting

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Table 3 Matrix of morphological character states used to reconstruct character evolution by parsimony reconstructions over a chloroplast DNA-based phylogenetic hypothesis

Taxon	Characters		
	1	2	3
Aglaomorpha coronans	1	0	0
Campyloneurum angustifolium	2	0	0
Dictymia mckeei	2	0	0
Drynaria bonii	1	0	0
Drynaria quercifolia	1	0	0
Drynaria rigidula	1	0	0
Goniophlebium argutum	1	0	0
Gymnogrammitis dareiformis	0	0	0
Kontumia heterophylla	0	1	0
Lepisorus macrosphaerus	2	0	0
Lepisorus scolopendrius	2	0	0
Leptochilus axillaris	2	1	2
Leptochilus decurrens	2	1	2
Leptochilus digitatus	2	0	1
Leptochilus hemionitideus	2	1	1
Leptochilus pothifolius	1	0	1
Leptochilus wrightii	2	0	1
Loxogramme abyssinica	2	0	1
Microsorum cuspidatum	2	0	0
Microsorum insigne	1/2	0	0
Microsorum lucidum	1	0	0
Microsorum membranaceum	2	0	0
Microsorum punctatum	2	0	0
Microsorum sp.	1	0	0
Neocheiropteris palmatopedata	2	0	0
Neolepisorus fortunei	2	0	0
Neolepisorus ovatus	2	0	0
Platycerium coronarium	2	0	2
Platycerium wallichii	2	0	2
Polypodium vulgare	1	0	0
Pyrrosia flocculosa	2	0	0
Pyrrosia lingua	2	0	0
Pyrrosia mannii	2	0	0
Pyrrosia nummulariifolia	2	1	0
Pyrrosia stigmosa	2	0	0
Pyrrosia subfurfuracea	2	0	0
Selliguea rhynchophylla	2	1	0
Tricholepidium maculosum	2	0	0
Outgroups			
Araiostegia yunnanensis	0	0	0
Davallia mariesii	0	0	0
Davallia tyermanni	0	0	0

Character 1: leaf dissection. This was coded as 0 (bi- to quadripinnate), 1 (pinnatifid/pinnate), or 2 (simple and palmatifid).
Character 2: fertile-sterile leaf differentiation. This was coded as 0 (monomorphic) or 1 (dimorphic).
Character 3: sori arrangement. This was coded as 0 (separated), 1 (linear [coenosori]), or 2 (sorus absent [acrostichoid]).

related species within Polypodiaceae. That is, highly divided blades (bi- to quadripinnate) have evolved independently in *Kontumia* and *Gymnogrammitis* (Fig. 2).

Nor do the results indicate that *K. heterophylla* occupies an intermediate position between *Gymnogrammitis* and the microsoroid ferns. Instead, our studies placed *K. heterophylla* as a member of Microsoroideae, including *Goniophlebium*, *Lepisorus*, *Leptochilus*, *Microsorum*, *Neocheiropteris*, *Neolepisous*, and *Tricholepidium* (Fig. 1). Such a relationship was suggested by Christenhusz et al. (2011) based on observations of the rhizome scales. The rhizome scales may be associated with the biological functions (e.g. storage and transportation of water in plants exposed to high irradiance and desiccation) of indumenta in the epiphytic, hemiepiphytic, or climbing ferns (Tsutsumi & Kato, 2008). Moreover, rhizome scales are useful when inferring evolutionary relationships for microsoroid ferns (Wang et al., 2010a). The morphological feature "clathrate rhizome scales" is shared by *K. heterophylla* and most species of the Microsoroideae, whereas it is absent in some Old World polypods, such as *Gymnogrammitis*, *Selliguea*, and *Drynaria* (Schneider et al., 2002). Although currently accessible evidence is insufficient to reconstruct the evolution of rhizome scales, clathrate scales on the rhizome support the close relationship between *K. heterophylla* and the microsoroid ferns.

Within the Microsoroideae clade, the *Leptochilus* lineage was recovered in previous analyses to include species previously assigned to various genera, such as *Colysis*, *Leptochilus*, *Paraleptochilus*, *Dendroglossa*, and *Nistarika* (Nooteboom, 1997; Shi & Zhang, 1999; Kreier et al., 2008; Christenhusz et al., 2011). Our molecular phylogeny also indicated that the type species of the genus *Colysis*, *L. hemionitideus*, was sister to the type of *Leptochilus*, *L. axillaris* (Fig. 1). Thus, *Colysis* species are undoubtedly members of the *Leptochilus* lineage. *Kontumia heterophylla* is nested in the *Leptochilus* lineage with maximal support (BP = 100%; PP = 1.00). When a traditionally recognized genus nests within another, one solution is integration of the genera that cause the paraphyly into the progenitor genus (Kreier et al., 2007; Li et al., 2011). To separate *K. heterophylla* generically renders the *Leptochilus* lineage paraphyletic. Thus, the phylogenetic position of *K. heterophylla* is obviously in the *Leptochilus* lineage based on individual and combined cpDNA evidence presented here (Fig. 1).

The *Leptochilus* clade including *Kontumia* and *Leptochilus* shows marked morphological disparity, especially concerning blade dissection, fertile–sterile leaf differentiation, and sori arrangement. Based on these characters, *K. heterophylla* has been characterized separate to *Leptochilus* species (Shi & Zhang, 1999; Lin et al., 2000; Wu et al., 2005). For example, the blades of *K. heterophylla* are more highly dissected (bi- to tripinnatifid) than those of *Leptochilus*. The leaves of most *Leptochilus* species are simple or palmatifid (Shi & Zhang, 1999; Lin et al., 2000). The sorus of *K. heterophylla* is orbicular and positioned terminally on the veinlets, whereas those of *Leptochilus* are

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elongate to linear and between adjacent veinlets or form an acrostichoid pattern (Table 3). Although these characters have previously been considered to be useful for the classification of fern plants (Shi & Zhang, 1999; Wang et al., 2010b), they have evolved independently several times in Polypodiaceae (Fig. 2). Moreover, these morphological characters are uninformative in many lineages of Polypodiaceae (Schneider et al., 2002; Haufler et al., 2003; Otto et al., 2009). Otto et al. (2009) proposed that the leaf shape in the *Pleopeltis* clade (Polypodiaceae) is a response to non-biological stresses, such as exposure to sunlight or limited access to water.

In conclusion, the present study represents the first examination of the molecular phylogenetic position of *K. heterophylla*. The analyses strongly support *K. heterophylla* having a close relationship with the *Leptochilus* lineage of Microsoroideae. The results of the present study also suggest that blade dissection, differentiation between sterile and fertile leaves, and sori arrangement, which are diagnostic morphological characters of *K. heterphylla*, are uninformative in Polypodiaceae because they appear in different clades in the strict consensus tree.

Acknowledgements This work was supported by grants from the research program for Postdoctoral Scholar, Key Laboratory of Biodiversity and Biogeography, Kunming Institute of Botany, CAS (Grant No. Y0205111L1) to CK, West Light Foundation of the CAS to H-GZ, Hundred Talents Program of the Chinese Academy of Sciences (Grant No. 2011312D11022) and the National Natural Science Foundation of China (Grant No. 31061160184) to HS, and the National Geographic Society of the United States (Grant Nos. 6300-98 and 7312-13) to S-GW.

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