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Phylogeny of the paleotropical fern genus *Lepisorus* (Polypodiaceae, Polypodiopsida) inferred from four chloroplast DNA regions

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ABSTRACT

Phylogenetic relationships within the paleotropical genus *Lepisorus* (Polypodiaceae) were investigated using plastid DNA sequences from four regions: *rbcL*, *rps4* and *rps4-trnS* IGS, *trnL* intron plus *trnL-F* IGS, *rbcL-atpB* IGS. Over 4000 nucleotides were sequenced for 77 specimens belonging to 54 species. Each cpDNA region was analyzed separately and combined into a single dataset. All phylogenetic analyses, maximum parsimony, maximum likelihood and Bayesian Inference of phylogeny, revealed the paraphyly of *Lepisorus* with the monotypic *Drymotaenium miyoshianum* and of the paleotropical genus *Belvisia* nested within the *Lepisorus* clade. Nine well-supported major clades were found. The phylogenetic results provided new evidence for the sectional classification of *Lepisorus*. The evolution of three morphological characters, clathrateness of rhizome scales, margin of rhizome scales and defoliated leaves, and the evolution of the karyotype, were reconstructed to identify lineage specific phenotypic character states or combination of characters. Unique character combinations, rather than synapomorphies, were found to be of systematic value in sectional delimitation. The variation of chromosome numbers is largely due to a single aneuploidy event instead of a stepwise reduction during the evolutionary history of this genus.

1. Introduction

The fern genus *Lepisorus* (J. Sm.) Ching comprises about 40 species according to some researchers (Hennipman et al., 1990; Zink, 1993), but more than 68 are recognized alone from China (Lin, 2000). The genus is distributed throughout the Old World tropics and subtropics with a diversity center in the Sino-Himalayan region, the proposed area of origin (Zink, 1993), and a second smaller diversity center in the Afromadagascan region (Zink, 1993). The genus *Lepisorus* is commonly found in tropical to temperate areas of eastern Asia, with a few species extending to the Malaysian region including New Guinea and with one species reaching the Hawaiian Islands. *Lepisorus* members are common epiphytes or lithophytes (Zink, 1993), from very low altitudes in eastern Asia up to 5000 m in the arid Himalayas. Some species of *Lepisorus* even have medicinal properties (Abla and Ababakri, 2007).

Based on morphological distinctness, Ching (1933) raised *Lepisorus* to generic rank for the first time, although Smith (1846) recognized already the group as a distinct entity. Ching (1933) pointed out remarkable difference from the Neotropical genus *Pleopeltis* Humb. and Bonpl. ex Willd., with which *Lepisorus* was frequently confused in the past. Despite of many voices of disapproval (Copeland, 1947; Pichi-Sermolli, 1977), many Asian pteridologists accepted *Lepisorus* as a distinct genus. At the end of the 20th century, many pteridologists approved Ching's classification (Hennipman et al., 1990; Zink, 1993) and finally phylogenetic studies based on DNA sequence data proved the distant relationship between *Lepisorus* and *Pleopeltis* (Schneider et al., 2004b,c).

Although there is strong support for the independent generic status, relationships among *Lepisorus* and supposedly affine species are thus far inconclusive. Ching and Wu (1980) segregated a putative genus *Platygyria* Ching from *Lepisorus* and Hennipman et al. (1990) assigned *Paragramma* (Blume) T. Moore to be a synonym of *Lepisorus*. Recently, molecular phylogenetic results revealed that *Belvisia* Mirbel and *Drymotaenium* Makino were embedded within *Lepisorus* (Kreier et al., 2008; Schneider et al., 2004c). These analyses indicated *Lepisorus* to be paraphyletic and demonstrated the need of an exhaustive study on the intrageneric and intergeneric relationships.

At a local scale, Zink (1993) published a comprehensive revision of Afromadascan *Lepisorus*, in which nine species were recognized. Whereafter, Yu and Lin (1996, 1997) made a

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comprehensive revision of Chinese species and delimited six sections (*Lepisorus*, *Pleioomma* S.L. Yu, *Sclerophyllon* S.L. Yu, *Macrophyllon* S.L. Yu, *Pachyphyllon* S.L. Yu and *Hymenophyton* Ching ex. S.L. Yu and Y.X. Lin). Finally, Lin (2000) modified this classification in the context of Flora of China, in which only two sections (*Lepisorus* and *Hymenophyton*) were kept. Recently, Liu et al. (2008) made a taxonomic revision of section *Hymenophyton*, in which they reduced the number of accepted species from 16 to 5. However, up to the present, neither a taxonomic revision nor a molecular phylogeny focused on *Lepisorus* on a worldwide scale has been released.

Species delimitation and intrageneric classification within *Lepisorus* has been subject of controversial discussion, because many of the taxonomically informative characters reflect quantitative rather than qualitative variation. In addition, characters used to differentiate species show variation within some species. The intrageneric classification is often founded on the shape and structure of the rhizome scales and paraphyses (Lin, 2000; Liu et al., 2008; Yu and Lin, 1996; Zink, 1993). Rhizome scales are always considered to be the most important character for the classification of *Lepisorus*: shape of rhizome scales varies from lanceolate (Fig. 1F), ovate-lanceolate (Fig. 1D) to ovate-orbicular; most species own completely clathrate rhizome scales (Fig. 1D and E), and a small number of species exhibit partly clathrate rhizome scales (Fig. 1A and B) and completely non-clathrate rhizome scales (Fig. 1C); margins of rhizome scales are also variable in different species, entire (Fig. 1D) or toothed (Fig. 1E).

Given the morphological uniformity of the genus, molecular data are required to depict phylogenetic relationships among these species. However, *Lepisorus* has received scant phylogenetic attention. To date, the best sampling of *Lepisorus* can be ascribed to Kreier et al.'s study (2008), even in which only a few species were included. The study, however, recovered evidence for the paraphyly of *Lepisorus* in respect to *Belvisia*, *Drymotaenium* and *Platygyria*. To address the question of the monophyly of *Lepisorus*, we need a more comprehensive taxon sampling, which is provided in this study. This study presents for the first time a dense taxon sampling of *Lepisorus* species throughout its distribution range. With this extensive sampling, we intend to: (1) reconstruct the phylogeny of the genus *Lepisorus*, (2) explore the relationships of *Lepisorus* and its affinities such as *Belvisia, Drymotaenium* and *Platygyria*, (3) evaluate the evolution of selected morphological characters, and (4) to explore the evolution of the karyotype. The last question is of particular interest because Lovis (1977) mentioned this genus to be of great significance in relation to our understanding of cytological evolution of ferns.

2. Materials and methods

2.1. Taxonomic sampling

Our taxon sampling was designed in consideration of the results of previous morphology-based studies (Ching, 1978a,b; Ching et al., 1983; Hovenkamp, 1998; Lin, 2000; Liu et al., 2008; Yu and Lin, 1996, 1997; Zhang et al., 2003; Zink, 1993) and DNA-based studies (Kreier et al., 2008; Schneider et al., 2004c). We took special attention to represent all sections proposed by Yu and Lin (1997) and to include all morphologically distinct putative lineages within this genus. We sampled also carefully the three genera found to be nested within *Lepisorus* as well as the sometimes-accepted segregate genus *Paragramma*. In total 70 individuals, respectively, 54 species of *Lepisorus*, including representatives of all sections of *Lepisorus*, were sampled to obtain a comprehensive taxonomic coverage of the paleotropical fern genus *Lepisorus*. The sampling included 54 species out of about 70 species currently known for *Lepisorus* and represented all currently recognized sections (Lin, 2000; Yu and Lin, 1997). In addition, five representatives from the genera *Belvisia*, *Drymotaenium* and *Paragramma* were also included since they were most closely related to *Lepisorus* (Kreier et al., 2008; Schneider et al., 2004c). Multiple DNA accessions from different distributional regions were sampled for some species that are taxonomically difficult to circumscribe. Four species representing the genera *Lemmaphyllum* C. Presl, *Neocheiropteris* Christ, *Neolepisorus* Ching and *Tricholepidium* Ching were added as outgroup taxa based on previous molecular results (Kreier et al., 2008;

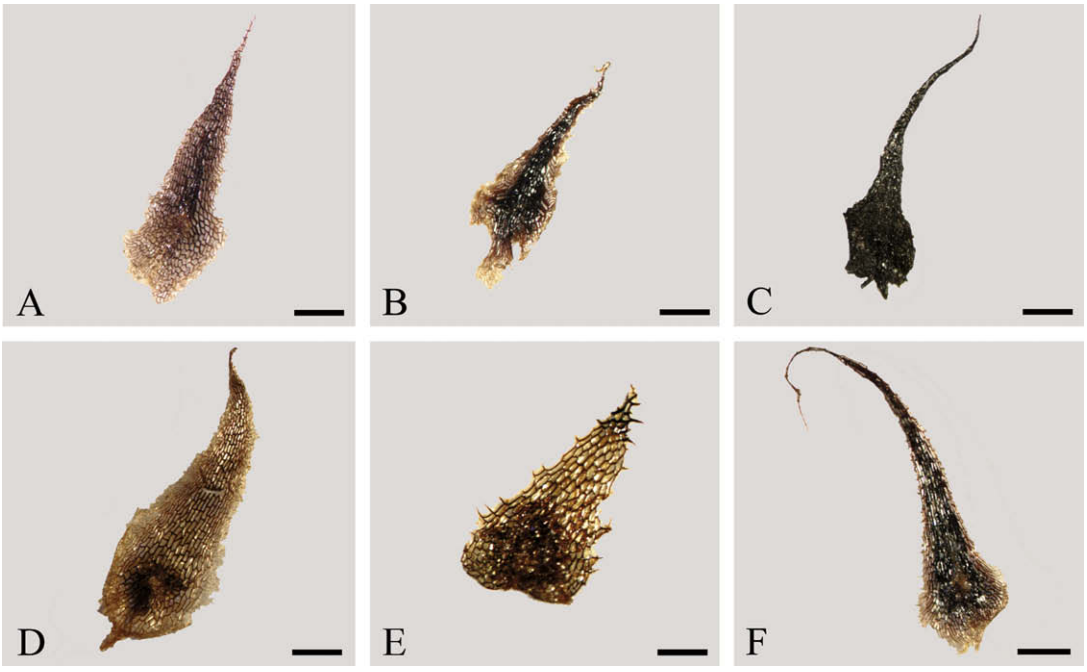

Fig. 1. Rhizome scales of selected *Lepisorus* species. (A) *L. contortus*. Type I non-clathrate. (B) *L. thunbergianus*. Type II non-clathrate (C) *L. sordidus*. Type III non-clathrate (D) *L. scolopendrium*. (E) *L. likiangensis*. (F) *L. tibeticus*. Scale bars indicate 500 μm. Photographs: Qi, X.-P., Sun, J.-Q.

Table 1
Information regarding taxon names, collecting localities, collector, voucher deposition and GenBank accession numbers for sequences included in the phylogenetic analyses. *Abbreviations:* BGZ, Botanical Garden Zurich; TBG, Tuebingen Botanic Garden. Herbaria abbreviation follows Index Herbariorum (http://sciweb.nybg.org/science2/IndexHerbarirum.asp).

Taxa	Locality, voucher	*rbcL*	*rbcL-atpB*	*trnL-F*	*rps4-trnS*
Belvisia annamensis (C. Chr.) Tagawa	Hainan; D Li 873 (PE)	GQ256252	GQ256079	GQ256166	GQ256324
Belvisia henryi (Hieron) Tagawa	Yunnan; Shui 80679 (PE)	GQ256253	GQ256080	GQ256167	GQ256325
Belvisia mucronata (Fée) Copel.	Malaysia, Jaman 5891 (UC)	AY362562	GQ256081	GQ256168	AY362629
Belvisia platyrhynchos (Kunze) Copel.	Cult. BGZ; Kreier s.n.	DQ642152	GQ256082	GQ256169	DQ642190
Belvisia spicata (L.) Copel.	French Polynesia; Ranker 1915 (COLO)	EF463244	GQ256083	GQ256170	DQ642191
Drymotaenium miyoshianum Makino	Sichuan; C.C. Liu DB06104 (PE)	GQ256255	GQ256085	GQ256172	GQ256327
Lemmaphyllum microphyllum C. Presl	Guangxi; X.C. Deng 31753 (PE)	GQ256314	GQ256154		GQ256390
Lepisorus affinis Ching	Cult. SZBG; Zhang 4219 (PE)	GQ256256	GQ256086	GQ256173	GQ256328
Lepisorus albertii (Regel) Ching	Xinjiang; Zhang 4325 (PE)	GQ256257	GQ256087	GQ256174	GQ256329
Lepisorus angustus Ching	Tibet; Z.H. Shen S25 (PE)	GQ256290	GQ256127	GQ256214	GQ256364
Lepisorus angustus Chiing	Sichuan; Zhang 0645 (PE)		GQ256088	GQ256175	GQ256330
Lepisorus annuifrons Ching	Japan; Kyoto Kokubo s.n. (TI)	GQ256258	GQ256089	GQ256176	GQ256331
Lepisorus asterolepis (Baker) Ching	Sichuan; Zhang 5171 (PE)	GQ256259	GQ256090	GQ256177	GQ256332
Lepisorus bampsii (Pichi Serm.) M.J. Zink	Africa; R. Viane 11233 (PE)	GQ256260	GQ256091	GQ256178	GQ256333
Lepisorus bicolor Ching	Tibet; Zhang 5157 (PE)	GQ256261	GQ256092	GQ256179	GQ256334
Lepisorus boninensis Ching	Japan; TBG acc.54022	GQ256262	GQ256093	GQ256180	GQ256335
Lepisorus clathratus (C.B. Clarke) Ching	Yunnan; Zhang 4533 (PE)	GQ256275	GQ256110	GQ256197	GQ256349
	Yunnan; Zhang 4515 (PE)	GQ256263	GQ256094	GQ256181	GQ256336
Lepisorus confluens W.M. Chu	Yunnan; C.D. Xu s.n. (PE)	GQ256264	GQ256095	GQ256182	GQ256337
Lepisorus contortus (Christ) Ching	Chongqing; Zhang 5204 (PE)	GQ256265	GQ256096	GQ256183	GQ256338
	Shanxi; Q.R. Liu (PE)	GQ256266	GQ256097	GQ256184	GQ256339
	Sichuan; Zhang 5187 (PE)		GQ256098	GQ256185	GQ256340
	Tibet; Zhang 4699 (PE)	GQ256308	GQ256148	GQ256235	GQ256384
Lepisorus elegans Ching et W.M. Chu	Yunnan; Zhang 4444 (PE)	GQ256268	GQ256100	GQ256187	GQ256342
Lepisorus excavatus (Bory ex Willd.) Ching	Grande Comore; Rakotondrainibe 6785 (P)	DQ642156	GQ256101	GQ256188	DQ642194
	Tanzania; Hemp 3561 (DSM)	DQ642155	GQ256102	GQ256189	DQ642193
Lepisorus hachijoensis Kurata	Japan; Zhang 4358 (PE)	GQ256269	GQ256103	GQ256190	GQ256343
Lepisorus heterolepis (Rosenst.) Ching	Tibet; Zhang 5064 (PE)	GQ256270	GQ256104	GQ256191	GQ256344
Lepisorus hsiawutaiensis Ching et S.K.Wu	Hebei; Q.R. Liu (PE)	GQ256271	GQ256105	GQ256192	GQ256345
Lepisorus kawakamii (Hayata) Tagawa	Taiwan; Ranker 2051 (COLO)	EU482940	GQ256106	GQ256193	DQ482990
Lepisorus kuchenensis (Y.C. Wu) Ching	Guangxi; J.M. Xi 08188 (PE)	GQ256272	GQ256107	GQ256194	GQ256346
Lepisorus lewissi (Baker) Ching	Anhui; Y Liu 05620 (PE)	GQ256273	GQ256108	GQ256195	GQ256347
Lepisorus likiangensis Ching et S.K. Wu	Tibet; Zhang 5117 (PE)	GQ256274	GQ256109	GQ256196	GQ256348
	Yunnan; Zhang 4468 (PE)	GQ256303	GQ256143	GQ256230	GQ256379
	Yunnan; Zhang 4488 (PE)	GQ256267	GQ256099	GQ256186	GQ256341
Lepisorus lineariformis Ching et S.K. Wu	Tibet; Zhang 4771 (PE)	GQ256276	GQ256111	GQ256198	GQ256350
	Yunnan; Zhang 4437 (PE)	GQ256277	GQ256112	GQ256199	GQ256351
Lepisorus longifolius (Blume) Holttum	Malay Peninsula; Cranfill BF012 (UC)	DQ642157	GQ256113	GQ256200	DQ642195
Lepisorus loriformis (Wall.) Ching	Yunnan; Zhang 4440 (PE)	GQ256278	GQ256114	GQ256201	GQ256353
Lepisorus macrosphaerus (Baker) Ching	Tibet; Zhang 4794 (PE)	GQ256280	GQ256116	GQ256203	GQ256354
Lepisorus marginatus Ching	Hubei; Zhang 3360 (PE)	GQ256281	GQ256117	GQ256204	GQ256355
Lepisorus medogensis Ching et Y.X. Lin	Tibet; Z.D. Famg XZ-266 (PE)	GQ256282	GQ256118	GQ256205	GQ256356
Lepisorus megasorus (C. Chr.) Ching	Taiwan; Cranfill TW069 (UC)	DQ642158	GQ256119	GQ256206	DQ642196
Lepisorus monilisorus (Hayata) Tagawa	Taiwan; H.M. Zhang 20050117 (PE)	GQ256283	GQ256120	GQ256207	GQ256357
Lepisorus morrisonensis (Hayata) H. Ito	Tibet; Zhang 5113 (PE)	GQ256284	GQ256123	GQ256208	GQ256358
	Tibet; Zhang 4736 (PE)	GQ256285	GQ256124	GQ256209	GQ256359
Lepisorus obscure-venulosus (Hayata) Ching	Guangxi; Zhang 4151 (PE)	GQ256286	GQ256125	GQ256210	GQ256360
Lepisorus oligolepidus (Baker) Ching	Tibet; Zhang 5082 (PE)	GQ256287	GQ256126	GQ256211	GQ256361
Lepisorus onoei Ching	Japan; Zhang 4352 (PE)	GQ256288	GQ256127	GQ256212	GQ256362
Lepisorus patungensis Ching et S.K. Wu	Hubei; Zhang 3413 (PE)	GQ256289	GQ256128	GQ256213	GQ256363
Lepisorus pseudonudus Ching	Sichuan; Zhang 4249 (PE)	GQ256291	GQ256130	GQ256215	GQ256365
Lepisorus pseudo-ussuriensis Tagawa	Taiwan; Cranfill TW093 (UC)	EU482943	GQ256131	GQ256216	EU482993
Lepisorus pumilus Ching and S.K. Wu	Gansu; M.Z. Wang 60667 (PE)	GQ256292	GQ256132	GQ256217	GQ256366
Lepisorus scolopendrium (Buch.-Ham. ex D. Don.) Menhra	India; Zhang 2295 (PE)	GQ256293	GQ256133	GQ256218	GQ256367
	Tibet; Zhang 4659 (PE)	GQ256294	GQ256134	GQ256219	GQ256368
	Tibet; Y.D. Tang YD-076 (PE)	GQ256295	GQ256135	GQ256220	GQ256369
Lepisorus sinensis (C. Chr.) Ching	Yunnan; Shui 81069 (PE)	GQ256296	xxxxxxxx	GQ256221	GQ256370
Lepisorus sordidus (C. Chr.) Ching	Sichuan; Zhang 0612(PE)	GQ256297	GQ256136	GQ256222	GQ256371
	Yunnan; Zhang 3218 (PE)	GQ256298	GQ256137	GQ256223	GQ256372
Lepisorus stenistus (C.B. Clarke) Y.X. Lin	Tibet; Z.D. Fang XZ-412 (PE)	GQ256279	GQ256115	GQ256202	GQ256353
Lepisorus subconfluence Ching	Yunnan; Zhang 4518 (PE)	GQ256299	GQ256138	GQ256224	GQ256373
Lepisorus sublinearis (Baker) Ching	Yunnan; Shui 80595 (PE)	GQ256300	GQ256139	GQ256225	GQ256374
	Yunnan; Shui 81060 (PE)	GQ256301	GQ256140	GQ256226	GQ256375
Lepisorus suboligolepidus Ching	Yunnan; Zhang 4537 (PE)		GQ256140	GQ256227	GQ256376
Lepisorus subsessilis Ching et Y.X. Lin	Guangxi; Zhang 1075 (PE)		GQ256141	GQ256228	GQ256377
Lepisorus thaipaiensis Ching et S.K. Wu	Shanxi; G.Y. Rao 2005-045A (PE)	GQ256302	GQ256142	GQ256239	GQ256378
Lepisorus thunbergianus (Kaulf.) Ching	Chongqing; Zhang 5205 (PE)	GQ256305	GQ256145	GQ256232	GQ256381
	Japan; Koichi Ohora 2005042404 (TI)	GQ256304	GQ256144	GQ256231	GQ256380
	Yunnan; Zhang 4544 (PE)	GQ256306	GQ256146	GQ256233	GQ256382
Lepisorus tibeticus Ching et S.K. Wu	Tibet;Zhang 4694 (PE)	GQ256307	GQ256147	GQ256234	GQ256383
Lepisorus tosaensis (Makino) H. Ito	Japan; Sayumi Fujimoto 2005042904 (TI)	GQ256309	GQ256149	GQ256236	GQ256385
Lepisorus uchiyamae (Makino) H. Ito	Japan; Sayumi Fujimoto 2005042902 (TI)	GQ256310	GQ256150	GQ256237	GQ256386

(*continued on next page*)

Table 1 (*continued*)

Taxa	Locality, voucher	*rbcL*	*rbcL-atpB*	*trnL-F*	*rps4-trnS*
Lepisorus ussuriensis (Regel and Maack) Ching var. *distans* (Makino) Tagawa	Japan; Sayumi Fujimoto SF05051602 (TI)	GQ256316	GQ256152	GQ256239	GQ256388
Lepisorus ussuriensis (Regel et Maack) Ching	Heilongjiang; B.D. Liu s.n. (PE)	GQ256315	GQ256151	GQ256238	GQ256387
Lepisorus xiphiopteris (Baker) W.M. Chu ex Y.X. Lin	Yunnan; C.D. Xu A0303 (PE)	GQ256317	GQ256153	GQ256240	GQ256389
Neocheiropteris palmatopedata (Baker) Christ	Yunnan; Zhang 4482 (PE)	GQ256318	GQ256160	GQ256246	GQ256396
Neolepisorus ensatus (Thunb.) Ching	Korea; Zhang 3611 (PE)	GQ256319	GQ256161	GQ256247	GQ256397
Platygyria inaequibasis Ching and S.K. Wu	Tibet; Zhang 4615 (PE)	GQ256320	GQ256162	GQ256248	GQ256398
Platygyria soulinena (Christ) X.C. Zhang and Q.R. Liu	Sichuan; Zhang 5168 (PE)	GQ256321	GQ256163	GQ256249	GQ256399
Platygyria waltonii (Ching) Ching and S.K. Wu	Tibet; Zhang 4639 (PE)	GQ256322	GQ256164	GQ256250	GQ256400
Tricholepidium maculosum (Christ) Ching	Yunnan; Shui 80596 (PE)	GQ256323	GQ256165	GQ256251	GQ256401

Schneider et al., 2004c). Voucher information and GenBank numbers of all species sampled for DNA are listed in Table 1.

2.2. DNA isolation, amplification and sequencing

Total genomic DNA was extracted from silica gel dried leaves using the modified CTAB procedure of Doyle and Doyle (1987). For each taxon, four plastid genome regions (*rbcL*, *rbcL-atpB*, *rps4* + *rps4-trnS*, *trnL-trnF*) were amplified separately with standard polymerase chain reaction (PCR). *RbcL-atpB* region including *rbcL-atpB* intergenic spacer (IGS) and part of *atpB* coding region was amplified with primers: *rbcL*-r49R forward (5′-CAC CAG CTT TGA ATC CAA CAC TTG C-3′) and *atpB*-r609R reverse (5′-TCR TTD CCT TCR CGT GTA CGT TC-3′) (http://www.pryerlab.net/). The *rbcL* gene, *rps4* + *rps4-trnS* IGS and *trnL-trnF* region including the *trnL* intron and the *trnL-trnF* IGS were amplified using primers 1F (Olmstead et al., 1992) and 1351R (Gastony and Rollo, 1995), *rps4*-F (Nadot et al., 1995) and *rps4*-R (Smith and Cranfill, 2002), and fern1 (Trewick et al., 2002) and f (Tarberlet et al., 1991), respectively. For simplification, the *trnL-trnF* region will be called *trnL-F* throughout this paper.

The PCR products were purified using a GFX™ PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA), and were then directly sequenced. Sequencing reactions were conducted using the DYEnamic™ ETDye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech). Sequences were analyzed using MegaBACE™1000 DNA Analysis Systems, following the manufacture's protocols. Sequence data were edited and assembled in ContigExpress program from the Vector NTI Suite 6.0 (Informax Inc., North Bethesda, MD). All sequences have been deposited at GenBank (see Table 1 for accession numbers). The resulting sequences were aligned using CLUSTAL X (Thompson et al., 1997), and further adjusted manually in BioEdit (Hall, 1999) and MacClade 4.0 (Maddison and Maddison, 2002). Ambiguous positions were detected visually and excluded from all phylogenetic analyses.

2.3. Phylogenetic analysis

Maximum parsimony (MP) analyses of the four plastid DNA regions were conducted separately with the same settings as for the combined data matrix analysis (see below). The four majority-rule consensus topologies were inspected for topological conflicts using a threshold of 90% bootstrap value or higher values (Johnson and Soltis, 1998). We observed no topological conflict among data sets and hence all four regions were combined into a single data set.

MP analyses of the combined data set were run using PAUP 4.0b10 (Swofford, 2002). All characters were weighted equally and gaps were treated as missing data. The most parsimonious trees were obtained with heuristic searches of 1000 replicates with random stepwise sequence addition, tree bisection–reconnection (TBR) branch swapping, and saving 10 trees from each random sequence addition. Bootstrap support values (BS) were calculated with 1000 simple stepwise addition replicates with TBR branch swapping, and 10 trees saved per replicate.

Mrmtgui (http://genedrift.org/mtgui.php) was used to determine the appropriate DNA substitution model and gamma rate heterogeneity using the Akaike Information Criterion (AIC). Maximum likelihood (ML) trees were generated using the program GARLI (Zwickl, 2006) with the GTR model plus GAMMA and Invariant site variable implemented. All parameters were estimated simultaneously for the tree search. All analyses were preformed with the default settings and several times repeated. The default setting of this software was also employed to calculated bootstrap values for ML analyses based on 100 bootstrap replicates. Bayesian inference of phylogeny (BI) was performed using MrBayes ver. 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) using a single model for all regions and separate models for coding versus non-coding partitions. Four chains were run, each for 2,000,000 generations, and were sampled every 1000 generations, starting with a random tree. The convergence of runs and estimation of burn-in were checked using Tracer ver. 1.4 (Rambaut and Drummond, 2007). Bayesian posterior probabilities (PP) were calculated as majority consensus of all sampled trees after discarding the trees sampled within the burn-in phase.

2.4. Morphological and cytological character evolution within *Lepisorus*

The data for morphological reconstructions were obtained (1) from the literature (Hovenkamp and Franken, 1993; Takamiya, 1996; Yu and Lin, 1996; Zink, 1993), (2) by our observations during field trips, and (3) by examination of herbarium specimens (PE). Character definitions and the data matrix used were presented as Table 2. All characters were scored as discrete either binary or multistate characters. These morphological characters were selected because they were either used as diagnostic features in taxonomic treatments or discussed as potential synapomorphic character states (Lin, 2000; Liu et al., 2008; Yu and Lin, 1996, 1997; Zhang et al., 2003; Zink, 1993). Particular care was given to observation of rhizome scale characters because scale structures may be correlated with the age of scales. Considerable differences are often due to different stages in the maturation of scales, rather than expressing taxonomic distinctness. Therefore, all information on scales given in present study was taken from presumably mature scales.

In the past over fifty years, researchers reported a considerable amount of chromosome data concerning *Lepisorus*. Summaries of existing counts were given in Zink (1993) and Takamiya (1996). Additional information was obtained from the IPCN database (http://mobot.mobot.org/W3T/Search/ipcn.html) and Tindale and Roy (2002). An unexpectedly high variability of chromosome numbers has been found for *Lepisorus* compared with other genera in

Table 2
Matrix of morphological character states used to reconstruction the evolution of these characters. Morphological characters: (1) clathrateness of rhizome scales: 0 = completely calthrate; 1 = completely non-clathrate; 2 = type II partly clathrate; 3 = type I partly clathrate. (2) Margin of rhizome scales: 0 = entire; 1 = toothed. (3) Defoliating leaves: 0 = summer-green; 1 = evergreen. (4) Basic chromosome number: (0) $n = 23$, 25, 26; (1) $n = 35$, 36, 37. Missing data were codes as "?".

Taxon	Characters			
	1	2	3	4
Belvisia annamensis	0	1	1	?
Belvisia henryi	0	1	1	?
Belvisia mucronata	0	1	1	1
Belvisia platyrhynchos	0	1	1	?
Belvisia spicata	0	1	1	?
Drymotaenium miyoshianum	0	1	1	0
Lepiosrus affinis	0	0	1	?
Lepiosrus albertii	0	1	0	?
Lepiosrus angustus	2	1	1	0
Lepiosrus annuifrons	0	1	0	1
Lepiosrus bampsii	0	0	0	?
Lepiosrus bicolor	0	0	0	1
Lepiosrus boninensis	3	1	1	0
Lepiosrus clathratus	0	1	0	1
Lepiosrus confluens	0	1	1	?
Lepiosrus contortus	3	1	1	0
Lepiosrus elegans	0	1	1	?
Lepiosrus excavatus	0	0	0	1
Lepiosrus hachijoensis	3	1	1	0
Lepiosrus heterolepis	2	1	1	?
Lepiosrus hsiawutaiensis	0	1	0	?
Lepiosrus kawakamii	0	0	1	?
Lepiosrus kuchenensis	0	0	1	1
Lepiosrus lewissi	2	1	1	?
Lepiosrus likiangensis	0	1	0	?
Lepiosrus lineariformis	2	1	1	?
Lepiosrus longifolius	0	1	1	1
Lepiosrus loriformis	0	1	1	0
Lepiosrus macrosphaerus	0	0	1	1
Lepiosrus marginatus	0	0	1	?
Lepiosrus medogense	1	1	1	?
Lepiosrus megasorus	0	0	1	?
Lepiosrus monilisorus	3	1	1	?
Lepiosrus morrisonensis	0	0	0	1
Lepiosrus obscure-venulosus	3	1	1	0
Lepiosrus oligolepidus	2	1	1	0
Lepiosrus onoei	3	1	1	0
Lepiosrus patungensis	0	1	0	?
Lepiosrus pseudonudus	0	1	1	?
Lepiosrus pseudo-ussuriensis	0	1	1	?
Lepiosrus pumilus	0	1	0	?
Lepiosrus scolopendrium	0	0	0	1
Lepiosrus sinensis	2	1	1	?
Lepiosrus sordidus	1	1	1	?
Lepiosrus stenistus	0	1	1	?
Lepiosrus subconfluence	2	1	1	0
Lepiosrus sublinearis	0	1	1	?
Lepiosrus thaipaiensis	0	1	0	?
Lepiosrus thunbergianus	2	1	1	0
Lepiosrus tibeticus	2	1	1	?
Lepiosrus tosaensis	2	1	1	0
Lepiosrus uchiyamae	0	1	1	1
Lepiosrus ussuriensis	0	1	1	1
Lepiosrus ussuriensis var. distans	0	1	1	1
Lepiosrus xiphiopteris	0	1	1	?
Platygyria inaequibasis	0	1	0	?
Platygyria souliena	0	1	0	?
Platygyria waltonii	0	1	0	?

Polypodiaceae. In order to explore the underlying evolutionary pattern of chromosome numbers in this genus, we estimated their basic chromosome numbers (BCMs). By doing so, we circumvent the problem of polyploidy. Most often $x = 35$ is reported, with related numbers of $x = 36$ or 37. For simplification, we name this serial BCM as type I. Another serial BCM is based on $x = 25$, with aneuploid number transformation of $x = 23$ and 26. Similarly, we call them as type II.

BCMs were calculated with consideration of polyploidy chromosome numbers. For example, Mitui (1971) reported the chromosome number of *L. tosaensis* as $2n = 150$. We treated the plant as hexaploid and estimated $x = 25$. Furthermore, *L. thunbergianus* is an outstanding example for polyploidy in this fern genus. Its chromosome number appears to range from $2n = 50$, $2n = 75$ to $2n = 100$. With the same rule, its BCM was regarded as $x = 25$. We mapped the polyploid level of *Lepisorus* on the phylogenetic trees (not shown), and it turned out that polyploidy taxa were randomly dispersed among the terminal taxa.

We used the reported chromosome data with necessary caution. On one hand, we excluded some reports from our analysis based on doubt concerning the taxonomic identity of the material counted and/or the quality of preparations used to generate the counts. For instance, we ignored a chromosome count of $2n = 39$ for *L. pseudonudus*. We suspect that it is an incorrect count created by a low quality of the chromosome squash or misidentified plant material. On the other hand, we made our choice between different reported chromosome numbers for a single species. Both $n = 31$ and $n = 36$ were published for *L. scolopendrium*. In this case, we took its affinities into account. $N = 31$ really added odds to the reported chromosome number in this clade. Therefore, we discarded $n = 31$ and chose $n = 36$ as its BCM. Besides, an irregular chromosome number ($2n = 94$) was found for *L. subconfluence*, whose BCM we treated as type II for the reason that we interpreted the count as a polyploid resulting from a hybridization of diploids with type II BCM.

The evolution of morphological and cytological characters was reconstructed using the software Mesquite ver. 2.5 (http://mesquiteproject.org/mesquite/mesquite.html).

All the characters were unordered and equally weighted. Missing data were coded as "?". The data matrix was reduced to a single accession for each included species and *L. longifolius* was assigned as the outgroup after pruning the original outgroups. The phylogeny was reconstructed as described above for the complete dataset. Maximum likelihood reconstruction was used to recover the phylogenetic hypothesis that fits best to the generated DNA sequence data. Maximum parsimony and maximum likelihood reconstruction of character evolution were performed for the phylogenetic hypothesis found using ML analyses of the reduced dataset. The ML character evolution was performed using the Markov k-state 1 parameter model (MK 1) as described by Lewis (2001). Phylogeny (Pagel and Lutzoni, 2002) was taken into account by reconstructing the character evolution over 900 phylogenetic trees generated in a Bayesian inference of phylogeny using 1,000,000 generations with a sample frequency one sample out of 1000 generation, and a burn-in sampling phase exclusion of 101 trees. Trace-characters-over-trees command was used to reconstruct the characters for the 900 assembled trees and the results were summarized as percentage of changes of character states on a given branch among all 900 trees.

3. Results

3.1. Independent phylogenetic analysis of each of the four cpDNA regions

The characteristics and statistics of individual plastid DNA regions from the MP analyses are presented in Table 3. The *trnL-F* region produced the most parsimony-informative characters per sequence length (13.7%). The maximum parsimony analyses of each individual plastid DNA region provided very low resolution within *Lepisorus* (results not shown). ML and BI analyses were also

Table 3
Statistics of the results obtained in maximum parsimony results of *rbcL*, *rbcL-atp*B IGS, *rps*4 plus *rps4-trn*S IGS, *trnL-F* IGS and the combined dataset including: length of aligned sequences (AL), number of variable characters (NVCh), number of parsimonious informative characters (NPICh), number of most parsimonious trees (NMPT), length of most parsimonious trees (LMPT); consistency index (CI), retention index (RI) rescaled consistency index (RC). CI is given without the proportion of invariant sites.

Region	ALen (bp)	NVCh (%)	NPICh (%)	NMPT	LMPT	CI	RI	RC
rbcL	1267	170 (13.4)	92 (7.3)	7910	278	0.647482	0.880633	0.570194
rbcL-atpB	1285	235 (18.3)	128 (10.0)	1000	307	0.817590	0.945841	0.773310
rps4-trnS	1121	281 (25.1)	153 (13.6)	7901	455	0.696703	0.912548	0.635775
trnL-F	953	234 (24.6)	131 (13.7)	531	372	0.739247	0.924689	0.683574
Combined	4626	920 (19.9)	504 (10.9)	9990	1470	0.684354	0.899480	0.615563

Table 4
Best-fitting models and parameter values for separate maximum likelihood analyses of the *rbcL*, *rbcL-atpB* IGS, *rps4* plus *rps4-trnS* IGS and *trnL-F* IGS and combined dataset as inferred using Mrmtgui. I, proportion of invariable sites; G, gamma distribution.

Region	AIC selected model	Base frequencies				−ln *L*	I	G
		A	C	G	T			
rbcL	GTR+I+G	0.2607	0.2201	0.2527	0.2665	3624.1470	0.7011	1.0381
rbcL-atpB	GTR+G	0.3054	0.1949	0.2181	0.2816	3797.5557	0	0.5063
rps4-trnS	K81uf+I+G	0.3225	0.1735	0.1875	0.3165	4311.2983	0.3153	0.8328
trnL-F	GTR+I+G	0.3129	0.1953	0.1849	0.3069	3603.6270	0.2995	1.0881
Combined	TVM+I+G	0.2978	0.1978	0.2097	0.2947	16102.0996	0.5165	1.1013

performed for individual DNA regions; the best-fitting models and parameter values are shown in Table 4. The results (trees not shown) were similar to those obtained using MP.

3.2. Analysis of combined data

The combined data matrix consisted of 4626 nucleotides (Table 3). All phylogenetic analyses consistently identified nine major clades, labeled as clades I–IX, albeit minor differences exist concerning the relationships of these clade among the best topologies found with MP (Fig. 2), ML (Fig. 3) and BI (Fig. 4) procedures. Five hundred and four out of 4626 nucleotide positions (10.9%) were found to be parsimony informative (Table 3). The MP analyses were stopped with 9990 equally most parsimonious trees of 1470 steps sampled. Consistency and retention indices were high for the size of the dataset indicating a low level of homoplasy with the dataset (CI = 0.684354, RI = 0.899480). Despite a lack of statistical support for many internal nodes, the MP strict consensus tree topology was largely consistent with the ML and BI analyses (described below), with nine main clades evident (marked with I–IX in Fig. 2). Noticeably, the relationship among clades I, II and III was unresolved in the MP analysis, resulting in the ambiguity of the most basal clade. All the nine main clades are well-supported (Fig. 2). The ML tree (−ln *L* = 16102.0996) is shown as Fig. 3. Noticeably, relationships among basal clades I, II and III shifted slightly with a reduced sampling, in which we included only one accession for each species and excluded two species with incomplete sequences for all four regions. BI analyses resulted in a majority-rule consensus tree (Fig. 4) with robust posterior support for each clade.

The three phylogenetic reconstruction approaches found a monophylum (MPBS = 66, MLBS = 66 and PP = 0.99) consisting of the genus *Lepisorus* plus the monophyletic palaeotropical genus *Belvisia*, the monotypic genus *Drymotaenium*, and the alpine genus *Platygyria* (Figs. 2–4). For convenient narration, we marked this monophyletic lineage as *Lepisorus* sensu lato (Figs. 2–4). The segregation of these three genera as independent genera results into a parahpyletic genus *Lepisorus*. *Lepisorus (Paragramma) longifolius* is resolved as the sister to all *Lepisorus* s.l. species in MP, ML and BI (with separate models for coding versus non-coding regions) trees (MPBS = 98, MLBS = 100 and PP = 1.00) (Figs. 2–4). The sister relationship between *Paragramma/Lepisorus longifolius* and the *Lepisorus* s.l. is not found in Bayesian analyses employing a single model of substitutions for all four regions. Within the "*Lepisorus* s.l." lineage, nine clades are consistently and highly supported (Figs. 2–4).

We identified thirteen indels that may represent synapomorphies for some clades (Table 5). Clades I and II were simultaneously supported by indel "b" in the *rbcL-atpB* alignment. The monophyly of *Platygyria* was reinforced by the synapomorphic indel "a" from *rbcL-atpB*. Indel "k" in the *rps4-trnS* alignment was synapomorphy for other clades except I, II and III. In addition, clades IV and VII received support from indels "h" and "e", both from *trnL-F*, representing. The close relationship between clades VI and VII was enhanced by the indel "m" from *rps4-trnS*. The poorly resolved subclade A in clade IX was supported by indel "c" from *rbcL-atpB*.

Seven out of 11 species sampled with multiple individuals were found to be monophyletic. However, the other four were recovered as non-monophyletic. Among them, *L. angustus*, *L. clathratus* and *L. scolopendrium* are likely caused by insufficient variation, while *L. thunbergianus* might need to be redefined.

4. Discussions

4.1. Monophyly of Lepisorus s.l

Lepisorus longifolius, the type of the genus *Paragramma*, is resolved as the sister to the *Lepisorus* s.l. lineage in MP, ML and BI trees employing heterogeneous model for coding versus non-coding partitions (MPBS = 98, MLBS = 100 and PP = 1.00), but this relationship is not resolved in BI trees employing homogenous model for all regions. *Lepisorus longifolius* has experienced several nomenclatural changes since its introduction by Blume (1828) as *Grammitis longifolius*. Hooker (1854) assigned the species to *Polypodium* L., while T. Moore (1857) and Copeland (1947) placed it in *Paragramma*. The generic status of *Paragramma* was rejected by Hennipman et al. (1990) who assigned it to be a synonym of *Lepisorus*. Our molecular phylogenetic results support the acceptance of *Paragramma* as an independent genus. These results are congruent with the previous reports on the phylogeny of microsoroids including a much smaller species sampling of lepisoroids (Kreier et al., 2008). Morphology and geological distribution of *Paragramma* and *Lepisorus* are different. The former is endemic to tropical Southeast Asia while the latter is mainly centered in subtropical and temperate

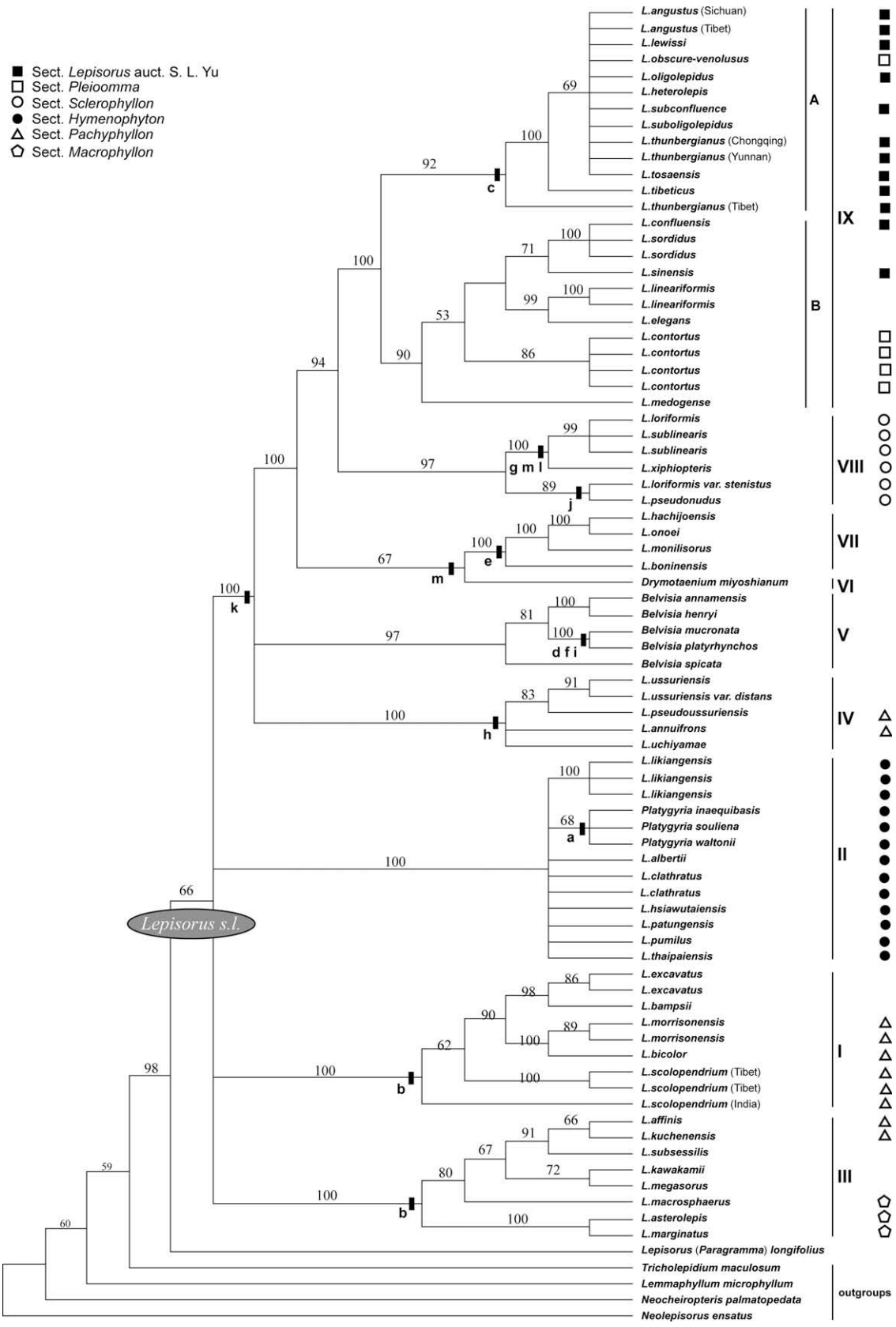

Fig. 2. Strict consensus tree based on 9990 maximum parsimonious trees obtained by a maximum parsimony analysis of combined data set. Numbers above branches correspond to maximum parsimony bootstrap values over 50%. Single letters above dark squares on branches refer to synapomorphic indels in *rbcL-atpB*, *trnL-F* and *rps4-trnS* alignments. Main clades discussed in the text are indicated by roman numbers. Symbols on the right side indicate assignments to sections as in Yu and Lin (1997).

East Asia with a sub-center in Africa and Madagascar. *Paragramma/Lepisorus longifolius* has its distinctive paraphyses, consisting of intermediate forms ranging from peltate scales to simple hairs (Copeland, 1947). In contrast, species of *Lepisorus* have only peltate

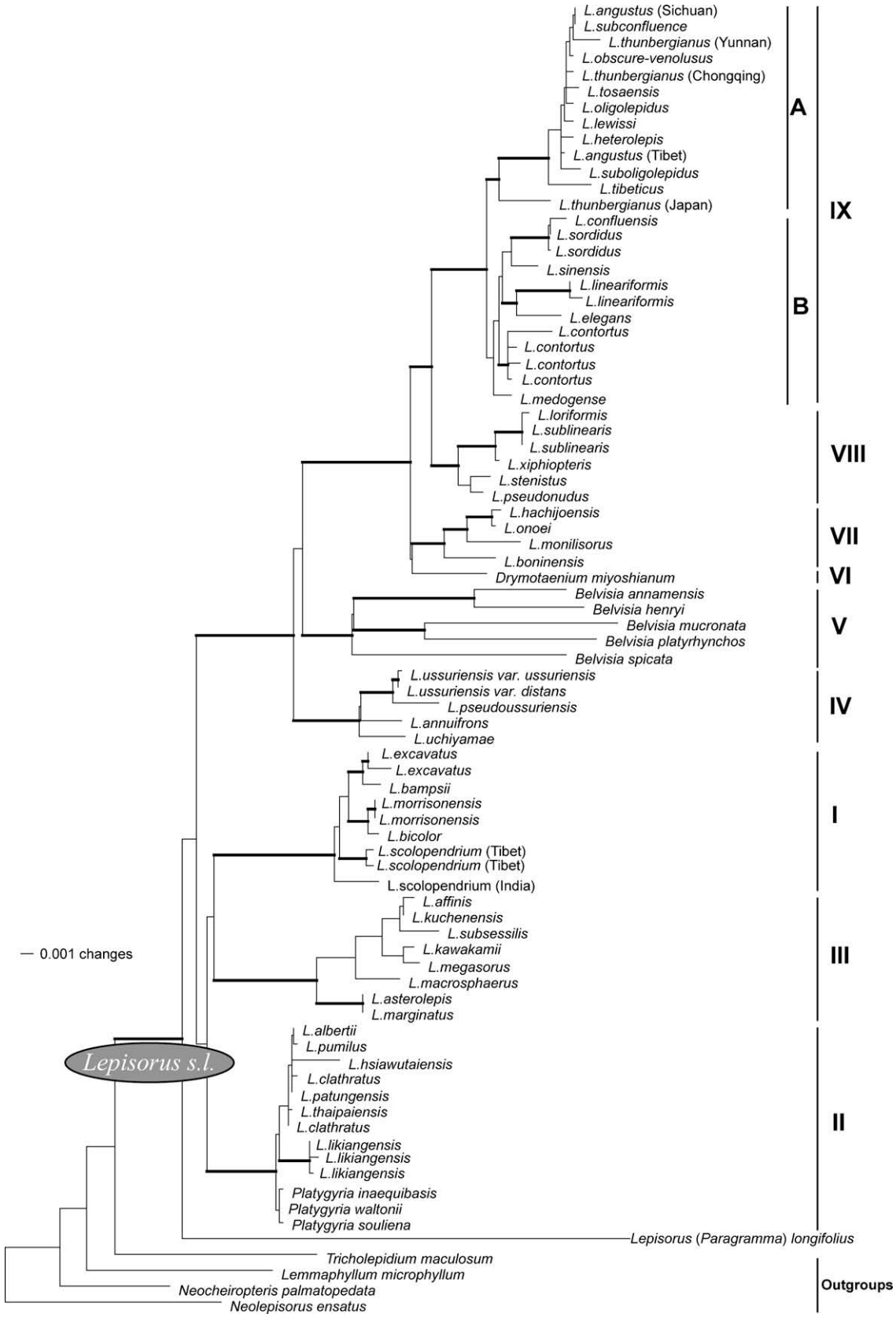

Fig. 3. Phylogram generated in a maximum likelihood analysis of the combined dataset. Thickened branches indicate bootstrap support values ⩾95%.

paraphyses. Future studies need to provide evidence for the relationships of the other member of the genus *Paragramma*, the New Guinea endemic *Paragramma*/*Lepisorus balteiformis*. The position of *L. longifolius* will need to be investigated in a dataset comprising a more comprehensive sampling of both lepisoroid and microsoroid ferns. The present study focused mainly on the clade

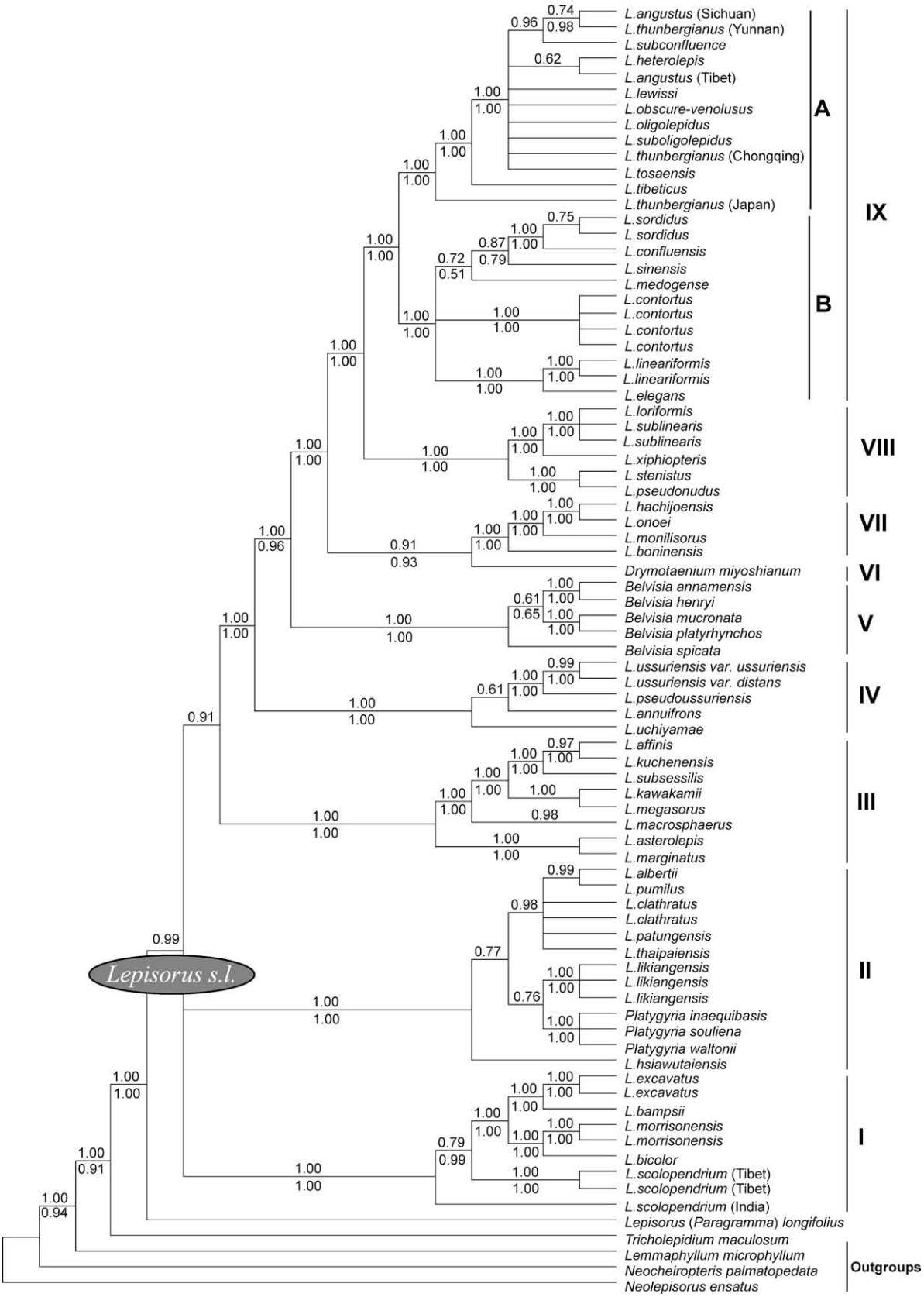

Fig. 4. Majority Consensus tree based on 2,000,000 generations without the burn-in phase of about 200,000 generations obtained using MrBayes. Posterior support values generated with separate models for coding versus non-coding partitions are given above branches. Posterior support values generated with a single model for all regions are shown below branches. The sister relationship between *Paragramma*/*Lepisorus longifolius* and the *Lepisorus* s.l. is not found in analysis employing a single model of substitutions for all regions.

Table 5
Synapomorphic indels revealed by the alignment of intergenic spacers *rbcL-atpB* IGS, *trnL-F* region and *rps4-trnS* IGS sequences.

Indel designation	Position	Source of indels
a	348–366	*rbcL-atpB*
b	421–427	*rbcL-atpB*
c	440–444	*rbcL-atpB*
d	487–508	*rbcL-atpB*
e	26–30	*trnL-F*
f	37–40	*trnL-F*
g	313–316	*trnL-F*
h	714–718	*trnL-F*
i	857–861	*trnL-F*
j	903–910	*trnL-F*
k	667–671	*rps4-trnS*
l	707–714	*rps4-trnS*
m	758–772	*rps4-trnS*

including all other species of *Lepisorus*, *Drymotaenium*, *Belvisia* and *Platygyria*.

Our results clearly demonstrate that *Lepisorus* is paraphyletic with three previously defined genera *Belvisia*, *Drymotaenium* and *Platygyria* nested within the *Lepisorus* clade. This result was already reported in Kreier et al. (2008), but with sparse sampling *of Lepisorus*. Plants of *Belvisia* are common epiphytes in tropical Africa, Australia and Southeast Asia including southern China. The genus circumscription refers to the coenosori restricted to the strongly narrowed tip of the leaf (Hovenkamp and Franken, 1993). Our research rejects the suggestion of putative close relationships of *Lemmaphyllum* and *Belvisia* (Hovenkamp and Franken, 1993). However, *Lepisorus* and *Belvisia* share the "Belvisia" type of exospore and an inconspicuous perispore. The *Belvisia* type of exospore is 3–8.5 μm thick and distinctly verrucate, fissured, or cerebriform (Hennipman, 1990; van Uffelen, 1993, 1997; Zink, 1989). The clathrate rhizome scales and the similar basic chromosome number also support close relationships between *Lepisorus* and *Belvisia* (Hennipman et al., 1990).

The monotypic genus *Drymotaenium* is traditionally distinguished from *Lepisorus* at the generic rank based on the occurrence of a coenosorus and very narrow leaves. However, coenosori are also found in two species of *Lepisorus*, *L. sinensis* and *L. confluens*. In addition, *L. subconfluens* shows an intermediate sori form between separate and fused sori. Coenosori are known to evolve independently in many lineages of Polypodiaceae and thus the character is highly homoplastic. Rhizome scales of *Drymotaenium* are clathrate and the cell lumina are almost rectangular and relatively small. Similar scales are found in *L. loriformis* and its affinities. These similarities support a close relationship between these two genera.

We hesitated to suggest the inclusion of a morphological distinctive taxonomic unit, such as *Belvisia*, into a redefined genus *Lepisorus*, but this may be the only option to achieve a natural classification without breaking-up the genus *Lepisorus* into several smaller but morphologically poorly distinctive genera. The need to redefine accepted genus delimitation has been now frequently demonstrated for various groups of ferns including Aspleniaceae (Schneider et al., 2004a), Davalliaceae (Tsutsumi and Kato, 2006), Hymenophyllaceae (Ebihara et al., 2006) and Polypodiaceae (Schneider et al., 2004c, 2006). In several cases, the redefinition of the genera resulted in the establishment of new genera, e.g., *Serpocaulon* A.R. Smith (Smith et al., 2006a), or the re-establishment of existing genera, e.g., *Synammia* C. Presl (Schneider et al., 2006). In other cases, the circumscription of existing genera had to be modified to include newly described species such as *Microgramma microsoroides* (Salino et al., 2008) or to integrate small traditionally recognized genera into larger genera to avoid paraphyletic genera, e.g. the redefinition of *Pleopeltis* (Otto et al., 2009). As mentioned above, *Belvisia* is distinct from *Lepisorus* by the synapomorphy of the formation of coenosori and the location at the elongated tip of leaves. In some species, this tip is well differentiated into a narrow fertile spike (Hovenkamp and Franken, 1993). Coenosori evolved frequently and independently in Polypodiaceae by merging sori and can not been seen as an indicator of deep taxonomic relationships.

One other putative segregate of *Lepisorus*, *Platygyria*, forms a monophyletic group nested deeply within *Lepisorus clathratus* assemblage. *Platygyria* has been reported as restricted to eastern Himalayas and Hengduan Mountains (Ching, 1979). The genus is identified by its very broad annulus of non-thickened cell-walls and indistinct stomium (Ching, 1979). Sporangia without a differentiated annulus are also known from other fern species nested in other large genera such as *Asplenium* (Brownsey, 1976). Thus, the character is likely a poor indicator of a generic delimitation of a lineage. The morphological resemblence of the scales and paraphyses supports close relationships among *Platygyria* and *L. clathratus* complex. Since all the species of *Platygyria* were nested deeply within *Lepisorus clathratus* complex, we see no clue to regard *Platygyria* as an independent genus. But, the diversification of these plants in the high altitude of the Himalayas requires extensive researches with population sampling.

4.2. Phylogeny within Lepisorus

All MP, ML and Bayesian analyses of the combined data consistently recognized nine well-supported clades within a broad *Lepisorus,* including "*Belvisia*" clade V and "*Drymotaenium*" clade VI (Figs. 2–4). Clade I includes species occurring from the eastern Himalayan regions (SW China, India and Nepal) to Taiwan and Africa. Species in this clade share the synapomorphic characters of clathrate rhizome scales, summer-green fronds and nearly entire margins of rhizome scales. It is noteworthy that this clade is home to the two African species included in our sampling, *L. excavatus* and *L. bampsii*. Our phylogenetic results corroborate a close relationship between African and Himalayan species, because all other representatives of this clade are distributed in the eastern Himalayan regions. This pattern was also reported for other genera of Polypodiaceae (Janssen et al., 2007; Kreier et al., 2008). *Lepisorus scolopendrium* from India is resolved as the sister of the remaining members within this clade, isolated from two conspecific specimens from Tibet. The relationships within the clade are weakly-supported and additional evidence is required to obtain a robust phylogeny of this clade.

The distribution of species constituent to clade II is centered in Sino-Himalayan regions with extension towards north and central China. All species show a preference to grow in alpine habitats. For example, members of the putative segregate genus *Platygyria*, endemic to Hengduan–Himalaya Mountains, are predominant at high altitude from 3000 m to about 5000 m, and it is suspected to be the champion in ferns from the point of elevation occurrences. All representatives possess the synapomorphies of thin herbaceous summer-green fronds, clathrate rhizome scales consisting of large clear lumina with long-protruding teeth on the margin, and irregular or even ovate-lanceolate paraphyses. The shed of leaves in the fall may be an adaptation to the climate in the high altitudes of the Himalayas to withstand the water stress during the winter months. The group recognized in our phylogenetic trees corresponds to section *Hymenophyton* (Yu and Lin, 1997). Species in this clade show a high morphological similarity but the phylogenetic trees are poorly resolved. The extremely short branch lengths (Fig. 3), coupled with an earlier contention that the group was considered as representing a single species, *L. clathratus*, suggest that the group is in need of critical taxonomic reevaluation based on an exhaustive study of

the genetic differentiation combined with exhaustive reexamination of herbarium specimens (Liu et al., 2008).

Species of clade III are mainly distributed in southeast China including Taiwan, with the exception of *L. marginatus*, which extends to central and north China (Gansu, Hebei and Shanxi provinces of China). Species of clade III typically have relatively large size of fronds. They are further distinctly characterized by more or less orbicular to ovate-orbicular rhizome scales that are usually falling off when mature. All species possess a tuft of hairs in the center region of scales. This character is found only in species belonging to clade III, but it is reported from other Polypodiaceae belonging either to *Tricholepidium* (Ching, 1978c), *Goniophlebium* (Blume) C. Presl s.l. (Rödl-Linder, 1990), *Microsorum* Link (Bosman, 1991), *Neocheiropteris* Christ (Bosman, 1991) and *Pleopeltis* (Mickel and Smith, 2004). Some authors previously treated *L. asterolepis* as a variety of *L. macrosphaerus*. The accessions of the two species were nested in different positions within the clade and thus we inclined to recognize it at the specific rank.

Clades IV and VII are firstly recognized in our phylogenetic analysis. Species in these two clades are mostly endemic in Japan, with some exceptions such as the Taiwan endemics *L. monilisorus* and *L. pseudo-ussuriensis* as well as *L. ussuriensis* distributed in Northeast China. However, these two clades are distinguished in their indiments and chromosome numbers. Representatives of clade IV possess concolored rhizome scales with small transparent lumina, while members of clade VII have bicolored rhizome scales with a central band of dark, thick-walled cells. Furthermore, members of clade IV share a basic chromosomal number of about 35, while derived species of clade VII have a haploid chromosomal number of about 25.

Clades V and VI represent two monophyletic genera. Their recognition renders *Lepisorus* paraphyletic. The basal species of clade V, *Belvisia spicata*, is quite different from the others. *Belvisia spicata* is the only intercontinental species from tropical Africa to Asia and extending to Australia, contrasting to others that are usually restricted in tropical Asia. In addition, different from round or peltate paraphyses in other species, *B. spicata* possesses paraphyses with irregularly branched and lobed blades. Furthermore, in *B. spicata*, rhizome scales usually have thick-walled central cells and thin-walled marginal cells, contrary to all thick-walled scale cells in other species belonging to the *Belvisia* clade (Hovenkamp and Franken, 1993). Clade VI is consisted of only one species, *Drymotaenium miyoshianum*, which is distributed in eastern Asia (China and Japan). Its phylogenetic position, deeply within *Lepisorus*, is not surprising given the morphological and cytological similarities to species of *Lepisorus*. These relationships were suspected in the past by several authors (Hovenkamp and Franken, 1993; Zink, 1993).

Distribution of members in clade VIII is centered in eastern Himalayas, only *L. loriformis* occurs outside of this region by extending its range to central China, e.g., Hubei, Shaanxi and Gansu provinces. Clade VIII corresponds to section *Sclerophyllon* (Yu and Lin, 1997). Plants of this group have coriaceous leaves, and clathrate rhizome scales consisting of more or less isodiametric, small cells and with sparsely toothed margins. *Lepisorus stenistus* was considered to be a variety of *L. loriformis* (Ching, 1933), which is rejected by our molecular analysis for the reason that the two species are nested within different well-supported subclades of clade VIII. This conclusion is further substantiated by morphological characters. *Lepisorus stenistus* is distinguished from *L. loriformis* by longer fronds, evident stipes and marginal sori.

Clade IX is split into two subclades (Figs. 2–4). With the exception of *L. heterolepis*, species of subclade A typically have bicolored rhizome scales with wide band of dark central cells and narrow band of transparent marginal cells. Although there is no apparent synapomorphy for subclade B, the clade is biogeographically consistent as all members occur in the eastern Himalayas. Clade IX shows a more extensive variability in its morphology than other clades. For example, *L. medogense* and *L. sordidus* bear exclusively non-clathrate rhizome scales; rhizome scales of *L. tosaensis*, *L. angustus* and *L. thunbergianus* with a large percent of dark central cells and only 1–2 rows of transparent marginal cells; rhizome scales of *L. contortus* is mostly composed of transparent cells only with a narrow band of dark cells in the center; and *L. elegans* possesses clathrate rhizome scales. Another similar example lies in the arrangement of sori: *L. sinensis* and *L. confluens* exhibit coenosori, and *L. subconfluence* has an intermediate sori form between fused and separate sori, while others bear separate sori. *Lepisorus thunbergianus* is an especially interesting case, because here morphological data are clearly inadequate for the identification of genetically monophyletic lineages. Because the Japanese *L. thunbergianus* is sister to a subclade including two Chinese *L. thunbergianus*, our phylogenetic analyses suggest that *L. thunbergianus* is not monophyletic in its current circumscription. The result is also supported by chromosomal numbers. Takamiya (1996) reported diploid, triploid and tetraploid for *L. thunbergianus*. The occurrence of tetraploids and triploids indicates frequent reticulate hybridization within this clade or members of this clade contribute to the formation of hybrids. The uniparental inherited cpDNA will not provide sufficient evidence to explore this pattern and nuclear markers will be required to study this clade (Gastony and Yatskievych, 1992; Vogel et al., 1998). This clade includes *L. contortus* and *L. obscure-venulosus*, which were previously recognized as members of section *Pleioomma*. It is understandable since *L. contortus*, *L. angustus* and *L. tibeticus* are frequently confused with *L. thunbergianus* and species boundaries among them are totally obscure.

Yu and Lin (1997) recognized six sections in *Lepisorus*: sect. *Lepisorus*, sect. *Pleioomma*, sect. *Sclerophyllon*, sect. *Macrophyllon*, sect. *Pachyphyllon* and sect. *Hymenophyton*. Section *Hymenophyton* and sect. *Sclerophyllon* are further corroborated by our results as natural groups corresponding to clade III and clade VIII. Section *Macrophyllon* was typified with *L. macrosphaerus* (Yu and Lin, 1997) and therefore corresponds to clade I. However, the species assigned by Yu and Lin (1997) to sect. *Pachyphyllon* are dispersed across clades I, II, IV. Clade II corresponds to sect. *Pachyphyllon* considering the position of the type of this section, *L. bicolor*. Clades IV and VII are both newly proposed monophyletic groups, and no current section names are applicable for them. Yu's sect. *Lepisorus* is based on *L. thunbergianus* and thus the section corresponds to clade IX. However, the section name is based on a putative misinterpretation of the type of *Lepisorus*. *L. nudus* is the type according to Zink (1993), but this species was found to be not closely related to clade IX (Kreier et al., 2008). We accept the arguments provided by Zink and the clade IX will need a new section name.

4.3. Exploring the evolution of Lepisorus phenotypes

The evolution of several phenotypic characters, e.g., chromosome numbers, clathrateness of rhizome scales, margins of rhizome scales and seasonal defoliation, were reconstructed by mapping them on the phylogenetic hypothesis found by the maximum likelihood analyses of the reduced combined dataset. In addition, we used a Bayesian approach to take phylogenetic uncertainty into consideration. The two characters of the rhizome indumenta were chosen based on their importance to the taxonomy of *Lepisorus* and their putative functions. The dormancy of the sporophyte, defined here by the obligate shed of all leaves in the fall, is a putative adaptive character allowing these ferns to successfully colonize high altitudes or cold temperate regions. The evolution of chromosome numbers did not get much attention in the recent years but *Lepisorus* displays a notable variation of the chromosome numbers (Manton, 1950).

4.3.1. Rhizome scales

As mentioned above, rhizome scales play an extraordinary important role in species delimitation of *Lepisorus*. Their value for the taxonomy of these ferns may be correlated with the biological importance of indumentum structures in epiphytic ferns. Several functions were proposed for rhizome scales in epiphytic ferns (Müller et al., 1981; Tsutsumi and Kato, 2008): (1) reflection of light resulting in lower surface temperature, (2) reduction of evaporation, (3) water absorption through the cell-walls, and (4) water transportation in capillary spaces between closely appressed scales and the rhizome surface.

The two selected characters, clathrateness and margin of rhizome scales exhibit some phylogenetic information (Fig. 5). Clathrate scales (Fig. 1D and E) are plesiomorphic in the lepisoroid clade and are conserved in clades I, II, III, IV and V (Fig. 5). Species with completely non-clathrate scales (Fig. 1C) are rare in *Lepisorus*. Partly clathrate rhizome scales are presumably differentiated into two types in *Lepisorus*: type I (Fig. 1A), scales with only a narrow band of dark cells in the center and a broad band of transparent cells on the margins; type II (Fig. 1B), scales with a broad band of dark cells in the center and 1–2 rows of transparent cells on the margin. The sequence of the transformation of the scale structure from clathrate to non-clathrate scales is reconstructed using the generated phylogenetic framework. The expected evolutionary pathway follows the sequence of transformation from completely clathrate scales to type I, to type II partly clathrate scales, and finally to completely non-clathrate scales, ordered as sequence of reduction of the proportion of clathrateness within a scale. However, our phylogenetic reconstruction provides evidence for an alternative pathway. Clathrate rhizome scales evolve into type I scales in clade VII and type II scales as prevalent in clade IX in parallel. Since the ancestral state of clade IX is ambiguous in our reconstruction, it is still inconclusive as to whether completely non-clathrate scales, occurring in *L. medogense* and *L. sordidus*, are indirectly derived from type II partly clathrate scales or directly evolved from clathrate scales. However, several reversal events occur in clade IX, such as in *L. obscure-venolusus* from type II to type I partly clathrate scales. The reversals include also the return to fully clathrate scales. Table 6 gives detailed information of six nodes, in which character state changes. Except for nodes 2 and 6, other nodes occupy either a high percentage of equivocal changes or a low percentage of present nodes.

Clathrate rhizome scales can be divided into two varieties. One variation has relatively large cell lumina (Fig. 1E), whereas the second variation has relatively small cell lumina (Fig. 1D). The first variation is restricted to clade II which includes members occurring preferably in alpine habitats, especially in eastern Himalayas. The large cell lumina may generate the iridescence of the rhizome scales which is found common in species belonging to clade II. The large cell lumina and iridescence of rhizome scales are putative adaptations to alpine environments.

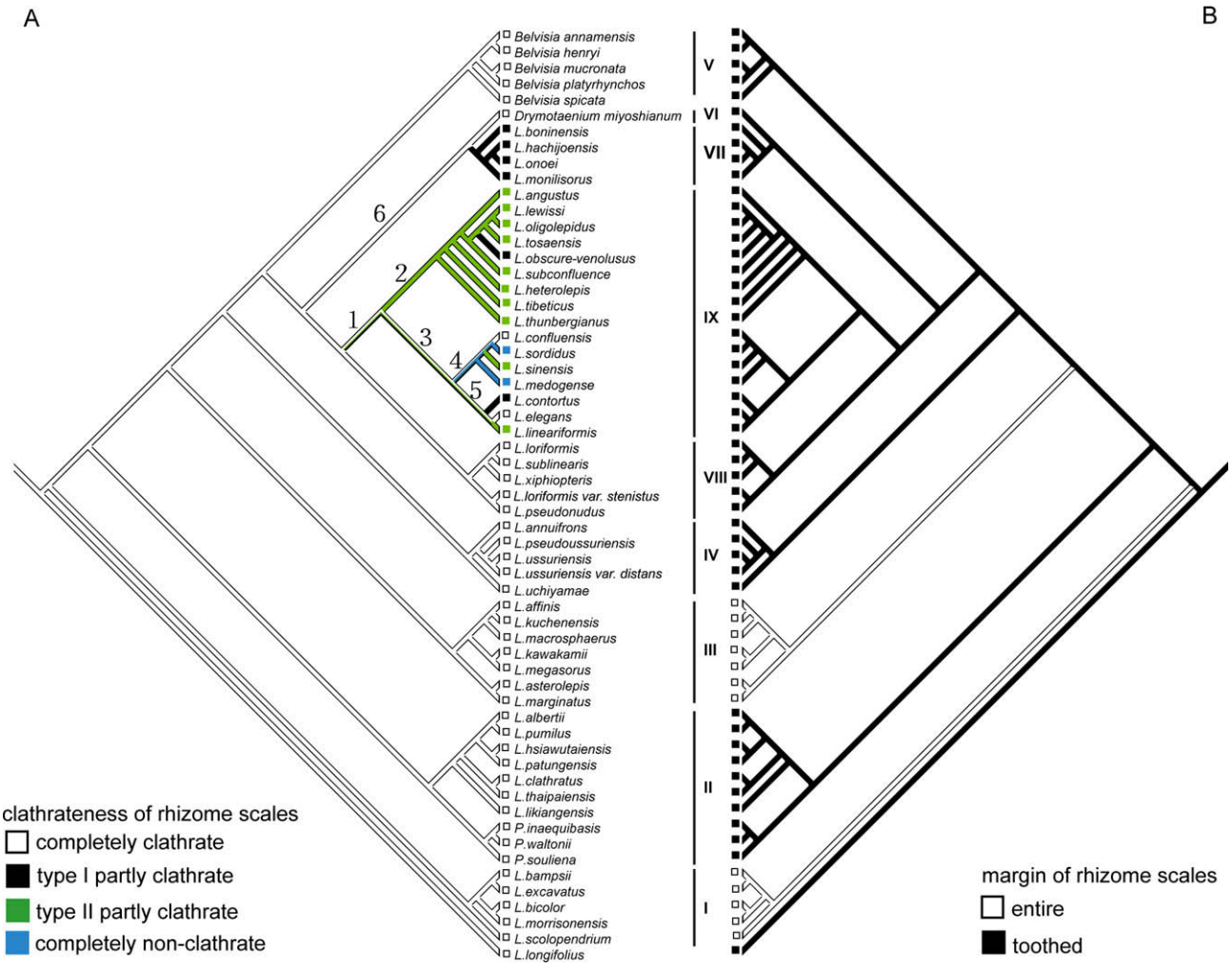

Fig. 5. Evolution of selected characters mapped on the best tree obtained in the maximum likelihood analysis of combined dataset. (A) Clathrateness of rhizome scales. Nodes important for character changes were marked as 1–6. (B) Margin of rhizome scales.

Table 6
Results of trace-character "clathretness of rhizome scales" over the 900 Bayesian trees. *Abbreviations:* NPR, present node; EQU, equivocal node; CS0, character state 0; CS1, character state 1; CS2, character state 2; CS3, character state 3; MLCS0, maximum likelihood value of character state 0; MLCS1, maximum likelihood value of character state 1; MLCS2, maximum likelihood value of character state 2; MLCS3, maximum likelihood value of character state 3.

	1	2	3	4	5	6
NPR%	100	100	100	72	38	91
EQU%	99	0	99	71	37	0
CS0%	0	0	0	0	0	91
CS1%	0	0	0	0	0	0
CS2%	0.8	100	0.8	0.8	0.2	0
CS3%	0	0	0	0	0	0
MLCS0	0.482	0.013	0.451	0.341	0.427	0.971
MLCS1	0.022	<0.001	0.063	0.311	0.025	<0.001
MLCS2	0.484	0.986	0.453	0.342	0.429	0.001
MLCH3	0.012	<0.001	0.033	0.007	0.119	0.027

The last selected character concerning rhizome scales reflects the variation of their margins that vary from almost entire (Fig. 1D) to bearing teeth (Fig. 1E). We ignore here the variation of the teeth length and density. Species with margins bearing teeth are the most common state in most clades (Fig. 5), and this state is inferred as plesiomorphy for *Lepisorus* s.l. Species with entire margins of scales are restricted to clade I and III (Fig. 5). Mapping this character onto the phylogeny is equivocal with regard to whether the evolution of scales with entire margins occurred independently twice or not, since relationships among basal clades are unresolved. Entire scale margins evolve only once in phylogenetic scenarios in which clades I and III are grouped as sisters, but twice in scenarios in which clades I and III are not sister clade to each other.

4.3.2. Leaf characters

We did not attempt to reconstruct the evolution of highly variable characters such as the length of the stipe, shape, size and texture of lamina, but focused instead on the contrast of evergreen versus summer-green species. Seasonal defoliation is a rare character in ferns but known to occur in some temperate ferns. The potential to shed leaves via decision mechanisms at the base of the stipe is the plesiomorphic character state of Polypodiaceae, but this is usually a facultative response to water stress. Seasonal reduction of the leaf mass in adaptation to local climates has been reported for *Drynaria fortunei* (Christ, 1910). Seasonal abortion of leaves is found in clades I and II, whereas all other species of *Lepisorus* s.l. are evergreen (Fig. 6). The evolution of the character is unresolved because the reconstructions of the character are equivocal in 99% of the Bayesian trees (node 1 in Fig. 6). The poor resolution at the base of the phylogeny restricts our capability to reconstruct

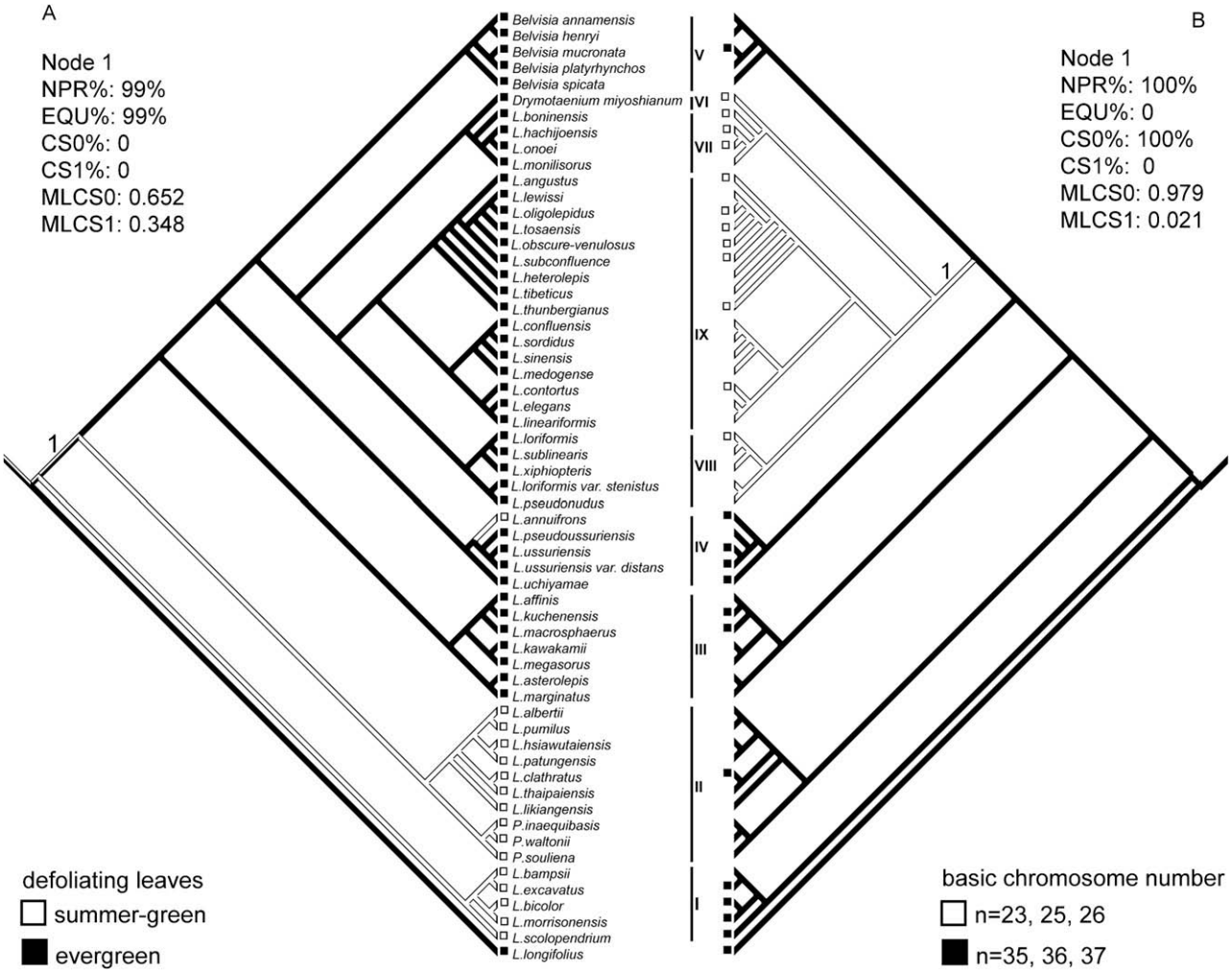

Fig. 6. Evolution of selected characters mapped on the best tree obtained in the maximum likelihood analysis of combined dataset. (A) Defoliating leaves. (B) Basic chromosome number. *Abbreviations:* NPR, present node; EQU, equivocal node; CS0, character state 0; CS1, character state 1; MLCS0, maximum likelihood value of character state 0; MLCS1, maximum likelihood value of character state 1.

the evolution of this character and thus we do not know if seasonal defoliation evolved independently in clades I and II or may be plesiomorphic for *Lepisorus* s.l. Based on the given topology, sporophyte dormancy has a single origin with a reversal event happening to *L. annuifrons* in clade IV. However, we could not disregard the potential of alternative relationships among the major clades.

The seasonal defoliation is conserved within clades I and II. Representatives of these two clades occur mainly in eastern Himalayas, Hengduan Mountains and other high mountains in north China, where water insufficiency is a common stress in the long winter. Therefore, defoliation may be an adaptive survival strategy for these species by keeping themselves dormant, actually alive in winter through abortion of leaves. This interpretation is supported by our experiments. Defoliated individuals *Platygyria waltonii* and its affinities were collected in the field, and cultivated in our conservatory. New leaves unfolded within the next week.

4.3.3. Basic chromosome number (BCM)

The study of karyotypes especially the number of chromosomes was a prolific research topic in the second half of the 20th century. The importance of cytology has fully been recognized at least since the publication of Manton's "Problems of Cytology and Evolution in the Pteridophyta" in 1950. In the recent years, the study of chromosome numbers has lost its appeal to the wide community of botanists, but the integration of cytology and phylogeny will likely revitalize this research field (Lysak et al., 2006; Peruzzi et al., 2009).

Based on the selected data and tentative scoring, our reconstruction reveals that type I ($x = 35, 36, 37$) BCM is the plesiomorphic chromosome number within *Lepisorus*. This inference is supported by the fact that most genera of Polypodiaceae possess BCM between 35 and 37 chromosomes (Tindale and Roy, 2002). BCM equal with $x = 35$ was reported for *Neocheiropteris palmatopedata* (Gibby, 1985), $x = 36$ for *Neolepisorus ensatus* (Mitui, 1968) and $x = 36$ for *Lemmaphyllum microphyllum* (Mitui, 1968). Type II BCM is derived from type I secondarily by decreasing chromosome numbers and it is suggested as the ancestral state of clades VI, VII, VIII and IX. We reconstructed the transition as a single event located at node 1 (Fig. 6B), where type I BCM changes to type II BCM. The maximum likelihood reconstruction supported the transition with the maximum likelihood value of character state 0 ($x = 23, 25, 26$) totally prevails against that of character state 1 ($x = 35, 36, 37$). We did not find evidence for a sequence of gradual reduction as expected in the past (Lovis, 1977), but it appeared to be a single aneuploid event. However, more counts for species belonging to the clades VI to IX are required to confirm our inference. This study contains one of the first attempts to explore the evolution of fern chromosome numbers by using a phylogenetic framework, whereas previous studies relied mainly on assumed relationships.

4.3.4. Perspectives

We studied also other phenotypic characters that may contain phylogenetic information, although many of them show trends of convergent evolution. As an example, ovate-orbicular rhizome scales are restricted to clade III. Paraphyses are also a character of particular interest in *Lepisorus* because these structures are found in all species. Lanceolate to ovate-lanceolate paraphyses are characteristics for species in clade II, distinguished from orbicular paraphyses found in most of other species. The exploration of these characters requires exhaustive investigation of specimens and a strong-supported phylogenetic frame with a dense taxon sampling.

The results of this study provide many new insights to the evolution of this mainly East Asian genus. The first obvious impact is the proposal of new species combinations to transfer species of the former segregated *Belvisia* and *Drymotaenium* to *Lepisorus*. The second obvious result is the foundation to further studies on the biogeographic history of the genus especially in the context of the evolution of the Tibetan plateau. Several other avenues for future research on *Lepisorus* can be easily proposed such as to utilize this genus to study genome evolution in ferns.

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