Chinese Science Bulletin 2004 Vol. 49 No. 11 1146—1150

Asian origin for *Polystichum* (Dryopteridaceae) based on *rbc*L sequences

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Abstract **Chloroplast *rbc*L sequences of 60 species of *Polystichum* sensu lato (s.l.), including 23 new sequences from southwest China, were used to assess the phylogenetic relationships within the genus. On the basis of estimated evolution rate of *rbc*L gene and the genetic distance data that passed relative-rate tests, we further estimated the divergence times between some clades of the genus. The phylogenetic relationships were inferred using the neighbor-joining and maximum-parsimony methods, both methods producing trees with completely congruent topology. These trees reveal that all species of *Polystichum* s.l. in this study (including *Cyrtomium* and *Cyrtomidictyum*) form a monophyletic group. The basal split in *Polystichum* s.l. separates a clade with all Asian members from a clade containing other species from all over the world. The phylogenetic and divergence time estimation results lead us to suggest that *Polystichum* s.l. originated in Asia in the late Late Cretacous (≈76 Ma) and migrated into other places in the world in early Eocene (≈46 Ma).**

Keywords*: Polystichum*, *rbc*L, phylogeny, origin, divergence time.

DOI: 10.1360/04wc0086

Polystichum Roth is one of the largest genera of ferns, estimated to comprise 300 species. The genus is distributed worldwide with its diversity center located in southwest China and adjacent regions[1]. There is little information on the origin and evolutionary history about *Polystichum*[2]. Little et al.[3] examined *rbc*L sequences and morphological characters of 34 *Polystichum* species and 21 species from other genera in the Dryopteridaceae, which yielded basic insights into the phylogeny of the genus. They found that *Phanerophlebia* and *Polystichopsis* together are the sister group to the monophyletic *Polystichum* s.l. including *Polystichum* s.s., *Cyrtomium* and *Cyrtomidictyum*; they also resolved a tropical American clade and African clade within *Polystichum* s.s., but the species from Asia are not closely related. However, only four species from southwest China (the diversity center of *Polystichum*) were included in their work[3]. As one of the largest fern genera in China, 168 *Polystichum* species are described there[1]. If the genus *Cyrtomium* is also considered, the species in this group would be more abundant. Molecular studies on almost all *Polystichum* and *Cyrtomium* species in their diversity center (southwest China and adjacent regions) have not been reported. In this study, we obtained new *rbc*L sequences from 23 species in *Cyrtomium* in *Polystichum* s.s., which are all from southwest China, in an attempt to investigate the phylogenetic relationships within the group by combining other published sequences (Table 2). Here, we employ relative-rate tests to assess homogeneity of substitution rates between lineages in the constructed phylogenetic tree and estimate the divergence time between the major phylogenetic lineages using estimated molecular evolutionary rate data. We use these new molecular data and divergence time estimations to consider the aspects of the evolutionary history and biogeography of *Polystichum* s.l.

1 Materials and methods

(ⅰ) Plant material. The samples included 23 species in *Polystichum* s.s. and *Cyrtomium*, all from the diversity center of southwest China. Leaf samples for DNA extraction were collected in the field or from the cultivated plants. Voucher and other information are shown in Table 1.

(ⅱ) DNA extraction. Total genomic DNA was extracted from 2 g of fresh or 1 g of silicagel dried leaves using the CTAB procedure[4], and modified according to Shi et al.[5].

(ⅲ) PCR amplification and DNA sequencing. A pair of primers F1 and R1379 (Table 3) were used for *rbc*L gene amplification. The PCR (polymerase chain reaction) was performed in 100-μL volumes with 2.5 units DNA polymerase, 10X buffer, 2.5 mmol/L $MgCl_2$, 0.1 μmol/L dNTP, 0.1 μmol/L primer F1 and R1379, 5% BSA, and ~100 ng sample DNA. The PCR amplification reactions include the following cycles: pre-denaturing at 94℃ for 2 min; followed by 40 cycles of 60 s at 94℃ denaturing, 60 s at 55℃ annealing, and 1 min at 72℃ extension; a final extension for 7 min at 72℃. The amplified products were purified for sequencing by Wizard^R PCR preps DNA Purification System (Promega, USA) following the manufacturer's specifications to remove the redundant small molecular fragments of primers and dNTPs. Purified double-stranded DNA was sequenced on an ABI 377 automated sequencer (Applied Biosystems, CA) with the Bigdye cycle sequencing kit. The sequences were determined in both directions using all the primers listed in Table 2. The primers were designed according to Little et al.[3] with some modifications.

(ⅳ) Sequence analysis. The obtained sequences have been assigned GenBank accession numbers (Table 1). Alignment of the obtained sequences was performed using

Table 1 Samples and locality information for the new *rbc*L sequences in this study

Voucher/No.	Taxa	Origin	Accession no.
Lu SG/H12	*Polystichum acutidens*	Xichou County Yunnan	AY545505
Lu SG/H23	*Cyrtomium aequibasis*	Yanshan County, Kunming	AY545491
Lu SG/A30	*Polystichum attenuatum*	Dawei Mountain, Yunnan	AY545503
Lu SG/H13	*Polystichum christii*	Xichou County, Yunnan	AY545486
Lu SG/B9	*Polystichum brachypterum*	Jingdian Park, Kumming	AY545501
Lu SG/12	*Cyrtomium caryotideum*	Qiongzhu Temple, Kunming	AY545490
Lu SG/17	*Cyrtomium hookerianum*	Xichou County, Yunnan	AY545489
Lu SG/H14	*Polystichum chunii*	Xichou County, Yunnan	AY545498
Lu SG/J25	*Polystichum dielsii*	Xichou County, Yunnan	AY545485
Lu SG/C74	*Polystichum eximium*	Guangnan County, Yunnan	AY545493
Lu SG/I31	*Polystichum grandifrons*	Dawei Mountain, Yunnan	AY545484
Lu SG/28	*Polystichum jizushanense*	Qiongzhu Temple, Kunming	AY545504
Lu SG/C78	*Polystichum longipaleatum*	Guangnan County, Yunnan	AY545495
Lu SG/B15	*Cyrtomium macrophyllum*	Jingdian Park, Kumming	AY545500
Lu SG/C61	*Polystichum makinoi*	Bozhu Mountain, Yunnan	AY545494
Lu SG/C59	*Polystichum nepalense*	Bozhu Mountain, Yunnan	AY545499
Lu SG/H8	*Polystichum nudisorum*	Wenshan County, Yunnan	AY545483
Lu SG/D5	*Polystichum omeiense*	Dawei Mountain, Yunnan	AY545497
Lu SG/B11	*Polystichum pycnopterum*	Jingdian Park, Kumming	AY545502
Lu SG/B32	*Polystichum semifertile*	Jingdian Park, Kumming	AY545496
Lu SG/D8	*Polystichum subacutidens*	Xichou County, Yunnan	AY545488
Lu SG/J28	*Cyrtomium urophyllum*	Xichou County, Yunnan	AY545492
Lu SG/H11	*Polystichum yuanum*	Xichou County, Yunnan	AY545487

Table 2 Specific primers used to amplify and sequence *rbc*L in *Polystichum* s.l.

Name	Direction	5' Position	Primer sequence (5' to 3')
F 1	Forward	1	ATG TCA CCA CAA ACG GAG AC
F 424	Forward	424	CTG CTT ATT CTA AGA CTT TC
F 878	Forward	878	TCA CCG TGC GAT GCA TGC TG
R 1379	Reverse	1379	GC AGC TAA TTC AGG ACT CC
R 940	Reverse	940	CAT GCG TAA TGC TTT GGC

Clustal X[6]. Phylogenetic analyses were performed by the maximum parsimony (MP) method and neighbor-joining (NJ) method using PHYLIP[7] and Mega 2[8] software packages. Parsimony analysis was performed using a branch-and-bound search. The genetic distance used in NJ analysis is calculated by Kimura's two-parameter model[8]. Based on the previous studies of Little et al.[3] we choose *Phanerophlebia umbonata* and *Polystichopsis chaerophylloides* as the outgroups (only one outgroup of *Phanerophlebia umbonata* and *Polystichopsis chaerophylloides* was chosen in PHYLIP software).

(ⅴ) Divergence time estimation. Relative-rate tests were conducted to assess homogeneity of substitution rates among the lineages with RRTree software[9] and Mega 2 software[8]. The genera or clades, which failed to pass the tests, were excluded from divergence time estimation. Because the synonymous substitution rates per site between some *Polystichum* s.l. lineages were saturated and inestimable[9,10], the divergence time was obtained using the nonsynonymous substitution rates per site (Li-Wu-Luo method)[8] and the published *rbc*L nonsynonymous substitution rates $4.57\pm7.3\times10^{-11}$ per site per year in ferns and seed plants[10].

2 Results

(ⅰ) Phylogenetic analysis. The GenBank accession numbers of the new *rbc*L sequences are shown in Table 1. After alignment with the outgroups, there are 1296 base pairs without any insertions or deletions, 224 variable sites and 121 parsimony informative sites in the *rbc*L alignment matrix. The topology of MP tree and NJ tree generally are similar to one another only with different bootstrap values above some branches. In this paper, only the strict consensus tree with maximum parsimony analysis is presented in Fig. 1. Other additional phyloge-

Table 3 Information of published *rbc*L sequences used in this study

Taxa	Location	GenBank Accession No.
Cyrtomidictyum lepidocaulon	Asia	AF537224
Cyrtomium caryotideum	Asia	AF537225
Cyrtomium falcatum	Asia	AF537226
Cyrtomium fortunei	Asia	AF537227
Phanerophlebia umbonata	Mexico	AF537233
Polystichopsis chaerophylloides	Puerto Rico	AF537234
Polystichum acrostichoides	Vermont	AF537235
Polystichum alfarii	Costa Rica	AF537236
Polystichum ammifolium	Madagascar	AF537237
Polystichum craspedosorum	Japan	AF537238
Polystichum cystostegia	New Zealand	AF208392
Polystichum deltodon	Yunnan	AF537239
Polystichum dracomontanum	Africa	AF537240
Polystichum dudleyi	California	AF537241
Polystichum ekmanii	Hispaniola	AF537242
Polystichum fallax	Australia	AY163865
Polystichum fibrillopaleaceum	Japan	AF537243
Polystichum imbricans	Oregon	AF537262
Polystichum lachenense	Szechuan	AF537244
Polystichum lehmannii	Costa Rica	AF537245
Polystichum lentum	Indo-Himalayan	AF537246
Polystichum lonchitis	Alaska	AF537247
Polystichum luctuosum	Africa	AF537248
Polystichum macleae	Africa	AF537249
Polystichum mohrioides	Chile	AF537250
Polystichum munitum	Alaska	AF537261
Polystichum muricatum	Costa Rica	AF537251
Polystichum neolobatum	Japan	AF537252
Polystichum proliferum	Australia	AF208393
Polystichum pungens	Africa	AF537253
Polystichum richardii	New Zealand	AF208394
Polystichum setiferum	Europe	AF537254
Polystichum speciosissimum	Mexico	AF537255
Polystichum stenophyllum	Szechuan	AF537256
Polystichum transkeiense	Africa	AF537257
Polystichum tripteron	Japan	U30832
Polystichum tsus-simense	Yunnan	AF537258
Polystichum turrialbae	Costa Rica	AF537259
Polystichum underwoodii	Hispaniola	AF537260
Polystichum vestitum	New Zealand	AF208395

netic analyses were done using the *rbc*L sequences of *Ctenitis eatonii*, *Ctenitis sinii* (GenBank accession numbers U05614, D43898) and *Dryopteris dickinsii*, *Dryopteris cristata*(GenBank accession numbers U05622, U05923) as outgroup species respectively (data not shown). These analyses produced topologies that are roughly congruent to each other with differences only in some clades with varying bootstrap values. These trees reveal that all species of *Polystichum* s.l. in this study (51 from *Polystichum* s.s., 8 from *Cyrtomium* and 1 from *Cyrtomidictyum*) form a monophyletic group. But *Cyrtomium* and *Polystichum* as commonly recognized are paraphyletic, as is supported by morphological studies [12] and the *trn*L intron sequence analyses (study in preparation). The basal split in *Polystichum* s.l. separates a major clade with all Asian members from another clade containing other species from all over the world. The species constituting the basal clade are *Cyrtomidictyum lepidocaulon*, *Polystichum tripteron*, *Cyrtomium hookerianum*, *Polystichum christii*, *Polystichum omeiense*, *Polystichum deltodon*, *Polystichum yuanum*, *Polystichum subacutidens* and *Polystichum dielsii*, which are all from Asia. The other species analyzed constitute the other monophyletic groups, which in turn comprises two clades——the *Cyrtomium* clade and the *Polystichum* clade (Fig. 1). Included in *Cyrtomium* clade are all *Cyrtomium* species and all from Asia. The *Polystichum* clade comprises most *Polystichum* species from all over the world, among which the species from Middle America form one clade, and some species from Asia and Africa are closely related, indicating that the phylogenetic relationships of *Polysticum* species are correlated with their geographic distributions. The phylogenetic results lead us to suggest that *Polystichum* s.l. originated in Asia and subsequently migrated into other places in the world.

(ⅱ) Divergence time. The relative-rate tests of nonsynonymous nucleotide substitution between lineages of *Polystichum* s.l. in the above phylogenetic tree are conducted (data included in supplementary data). It is found that some clades (such as the *Cyrtomium* clade) or taxa (such as *Polystichum nudisorum*) evolve at a significantly different rate, which were excluded in the estimation of divergence times[10]. The *rbc*L evolutionary rates of some lineages can pass the relative rate test (or are homogeneous based on relative rate test), so the divergence time can be estimated between them.

According to the molecular clock hypothesis, the divergence time between two lineages with a relatively constant rate can be estimated using the sequence divergence (genetic distance) and nucleotide substitutions rate[11]. In ferns and seed plants the published *rbc*L nonsynonymous substitution rate is $4.57\pm7.3\times10^{-11}$ per site per year[10]. Based on the genetic distance among related taxa that passed the relative-rate tests and the *rbc*L molecular evolutionary rate data, the lineages that shared their last common ancestor (divergence time) are estimated as follows: the *Polystichum* s.l. that shared their last common ancestor (the first divergence time) is 75.78 ± 7.89 Ma (Fig. 1, node A); the time when *Polystichum* plants migrated into other places in the world is 46.04 ± 6.67 Ma (Fig. 1, node B).

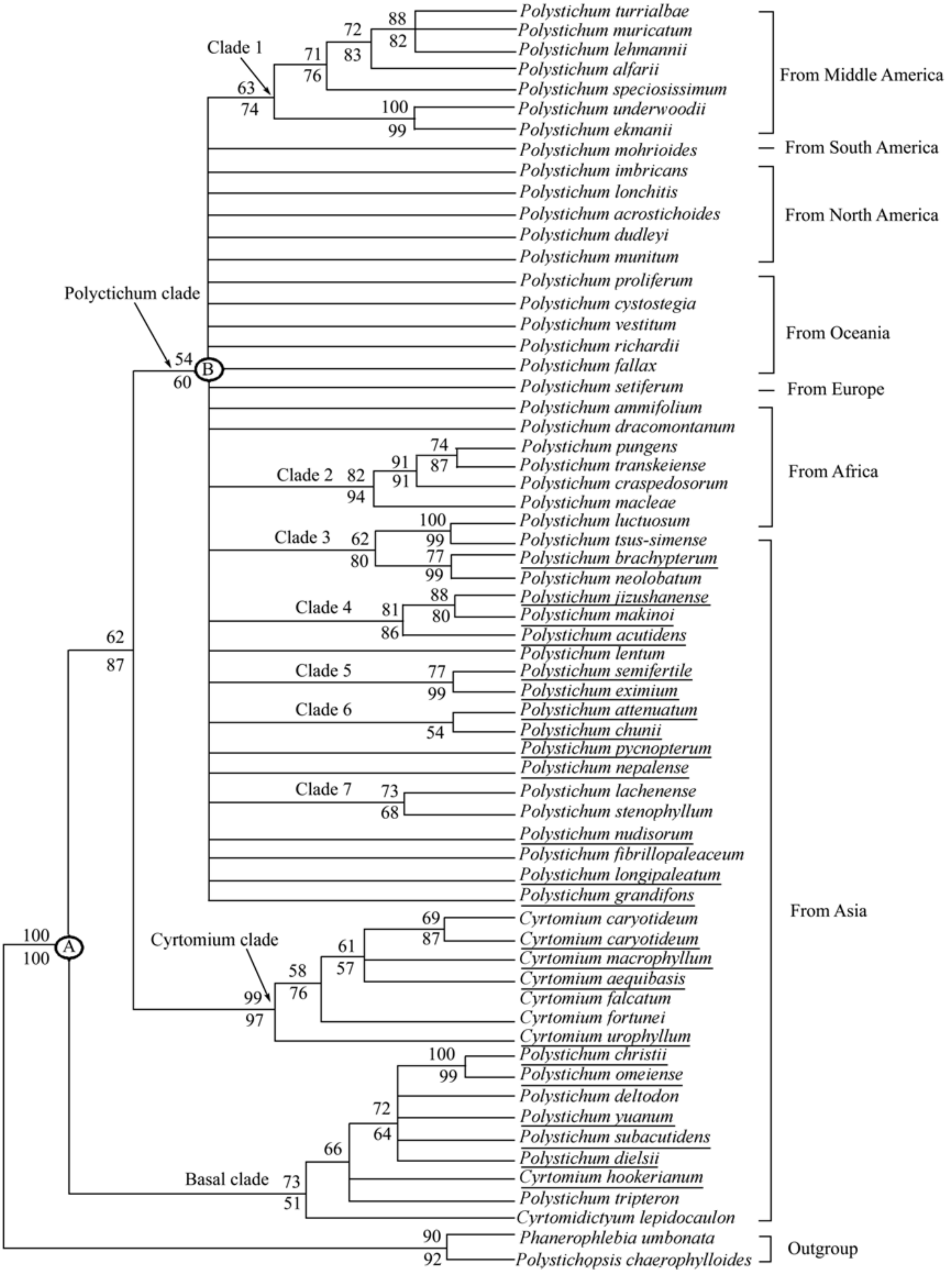

Fig. 1. The strict consensus tree constructed based on *rbc*L sequences of *Polystichum* s.l. using maximum parsimony analysis in Mega2 package. Bootstrap values (1000 replicates) are shown above branches (maximum parsimony analysis) and under branches (neighbor-joining analysis); A and B are the divergent time to be dated; Taxa underlined are those species sequenced in this paper.

3 Discussion

Phylogenetic and biogeographic analyses in the present study suggest a close correlation between relationships and distribution of *Polystichum* plants. This study shows that the most basally positioned species in the genus are all from Asia. Included in the basal clade are *Cyrtomidictyum lepidocaulon*, *Polystichum tripteron*, *Cyrtomium hookerianum*, *Polystichum christii*, *Polystichum omeiense*, *Polystichum deltodon*, *Polystichum yuanum*, *Polystichum subacutidens* and *Polystichum dielsii*. Among them, *Cyrtomidictyum lepidocaulon* is distributed in East Asia only (China, Japan and Korea); *Polystichum tripteron* also only occurs in Eastern Asia (China, Japan, Korea and far east regions of Russia); *Cyrtomium hookerianum* is confined to China, Japan, Northern Viet Nam and Himalayan regions; *Polystichum omeiense* and *Polystichum yuanum* are endemic to China; *Polystichum christii*, *Polystichum dielsii* and *Polystichum subacutidens* are distributed mainly in southwest China, with only few extending into northern Viet Nam; *Polystichum deltodon* occurs in eastern and southern Asia[1]. It is, therefore, suggested that *Polystichum* s.l. firstly originated in Asia, and mainly concentrated in East Asia, especially in southwest China; thus, it is likely that southwest China is the origin center of the genus.

The estimation results of divergence time indicate that the first divergence event (when species of *Polystichum* s.l. started to diverge) is 75.78 ± 7.89 Ma (late Late Cretacous), when climate was very hot and paleogeographic differentiation was accelerated by the active tectonic events in eastern Asia, causing drastic evolutionary changes in flora[13,14], with many modern ferns coming into being[2]; *Polystichum* s.l. may be one lineage among them. The time when *Polystichum* plants moved into other places in the world is 46.04 ± 6.67 Ma (early Eocene), when the global tropical/subtropical warm climate conditions on all continents were suitable for adaptive radiations for *Polystichum* plants.

Acknowledgements We are grateful to Profs. Shi Suhua (Sunyatsen University, Guangzhou), Lü Baozhong (Shanghai Cancer Institute, Shanghai), and Dr. Hao Jiasheng (Nanjing Institute of Geology and Palaeontology, Chinese Academy of Sciences, Nanjing) for discussion and technical assistance. This work was supported by the Ministry of Science and Technology of China (Grant No. 2002CCA03300), the Major Research Project of the Chinese Academy of Sciences (Grant No. KZCX2–SW–130), the National Natural Science Foundation of China (Grant No. 40302003), and the Natural Science Foundation of Yunnan Province (Grant No. 2001C0002M).

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(Received February 7, 2004; accepted April 8, 2004)