Phylogeny, historical biogeography and characters evolution of the drought resistant fern *Pyrrosia* Mirbel (Polypodiaceae) inferred from plastid and nuclear markers

Xueping Wei¹, Yaodong Qi¹, Xianchun Zhang³, Li Luo¹, Hui Shang³, Ran Wei², Haitao Liu¹ & Bengang Zhang¹

*Pyrrosia* s.l. comprises ca. 60 species with a disjunct Africa/Asia and Australia distribution. The infrageneric classification of *Pyrrosia* s.l. is controversial based on the phylogenetic analyses of chloroplast markers and morphology. Based on the expanded taxon sampling of *Pyrrosia* s.l. (51 species), we investigated its phylogeny, biogeography, character evolution and environmental adaptation by employing five chloroplastid markers (*rbcL*, *matK*, *psbA-trnH*, and *rps4 + rps4-trnS*) and one single (low)-copy nuclear gene, *LEAFY*. *Pyrrosia* s.l. was divided into six major clades and eight subclades. Reticulate evolution was revealed both among clades and among species in *Pyrrosia* s.l. Ancestral character state optimization revealed high levels of homoplasy evolution of the diagnostic characters in *Pyrrosia* s.l., while the crassulacean acid metabolism pathway seems to have an independent origin. Molecular dating and biogeographic diversification analyses suggested that *Pyrrosia* s.l. originated no later than the Oligocene and the main clades diversified during the Oligocene and Miocene, with southern Asia, the Indo-China Peninsula and southwestern and southern China as the most likely ancestral areas. Transoceanic long-distance dispersal, rather than vicariance, contributed to the intercontinental disjunction. Diversification scenarios of *Pyrrosia* s.l. under geological movements and climate fluctuation are also discussed.

*Pyrrosia* Mirbel (Polypodiaceae) is a terrestrial fern genus that constitutes subfamily Platycerioideae B.K. Nayar along with *Platycerium* Desv¹. This genus contains ca. 51–100 species and is widely distributed in Asia, ranging from Australia and New Zealand to Siberia and from Africa to various south Pacific islands²–⁷. *Pyrrosia* is well circumscribed by stellate hairs and characteristic connective venation pattern, which are two key characters to understanding the evolution of Polypodiaceae⁴,⁸–¹⁰. Most species of *Pyrrosia* are drought tolerant, and five species have been reported to use crassulacean acid metabolism (CAM) pathway¹¹–¹⁵. The occurrence of CAM is considered an effective adaptation to arid environments, although CAM in lycophytes and ferns is rare¹⁶–¹⁸.

The uniform appearance of species of *Pyrrosia* leads to difficulties in species-level classification. Several authors have conducted taxonomic revisions on regional or worldwide scales²–⁴,⁸,¹⁹–²⁶. Giesenhagen²⁷ was the first to systematically describe the morphology and classification of 50 species. Ching¹ considered species from mainland Asia and neighboring islands and treated *Pyrrosia* as a natural genus; he transferred 49 species into *Pyrrosia* and described five new species of this genus. Shing¹, Shing and Iwatsuki²,²⁰ considered more than 100 species in *Pyrrosia* and recognized 64 species in Asia and the adjacent Oceania. Hovenkamp⁴ completed a monograph of *Pyrrosia* from a global perspective and recognized ca. 51 species with a wide species concept. Finally,

¹Key Laboratory of Bioactive Substances and Resources Utilization of Chinese Herbal Medicine, Ministry of Education, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing, China. ²State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Beijing, 100093, China. ³Shanghai Chenshan Plant Science Research Center, Chinese Academy of Sciences; Shanghai Chenshan Botanical Garden, Shanghai, 201602, China. Correspondence and requests for materials should be addressed to B.Z. (email: bgzhang@implad.ac.cn)
it is widely accepted that Pyroídia contains ca. 60 species. However, the infrageneric classification of Pyroídia is controversial (Supplementary Table S1). Nayar and Chandra divided 14 species from India into six groups. Shing divided Pyroídia, excluding Drymoglossum C. Presl and Saxiglossum C. Presl, into two subgenera: subg. Pyroídia and subg. Niphopsis. subg. Niphopsis includes only P. samarensis and P. angustata, and subg. Pyroídia was divided into two sections and five series. Hovenkamp recognized ten groups based on cladistics analyses using 24 morphological characters, while seven species were not included in any groups. Furthermore, he considered five groups therein were not well-established monophyletic group due to the lack of autapomorphies. This consideration maybe related to the absence of trait evaluation based on a robust phylogenetic tree. Nearly all the groups established by Hovenkamp are irrecconcilable with those recognized in Shing’s classification, except that the P. angustata-group of Hovenkamp is equivalent to the subg. Niphopsis of Shing. Yang completed a taxonomic revision of Pyroídia, excluding Drymoglossum C. Presl and Saxiglossum C. Presl, in China and proposed two subgenera and six sections. Unlike Hovenkamp, Yang grouped P. stigmata, which previously belonged to the P. costata-group, with sect. Drakeanae, which was equivalent to the P. sheareri-group of Hovenkamp based on the fronds shape and indumenta characters. Yang also grouped P. subfurfuracea and P. calvata, belonging to the P. sheareri-group of Hovenkamp, with sect. Costatae, which is equivalent to the P. costata-group, based on the scales-bearing types. In addition, infrageneric classification of Pyroídia was always closely linked to the recognition of the segregate genera Drymoglossum and Saxiglossum. Drymoglossum and Saxiglossum are currently accepted as members of Pyroídia, which has been confirmed by phylogenetic work.

Several molecular phylogenetic studies involved Pyroídia, some of which revealed that Pyroídia was monophyletic and Platycerium was a sister group. In the most recently study, Vasques et al. established a subgeneric classification of Pyroídia based on three chloroplast (cp) markers of 38 species. Six subgenera were proposed: subg. Lune, subg. Neoniphopsis, subg. Niphobolus, subg. Niphopsis, subg. Pyroídia and subg. Solis. Testo and Sundue studied the evolution of ferns based on six chloroplast markers of a 4000-species dataset suggested that Pyroídia was paraphyletic and that P. liebuschi (Hieron) Schelpe was nested in Platycerium. Zhou et al. segregated the P. africana-group as a new genus Hovenkampia Li Bing Zhang & X.M. Zhou, and recognized four clades in Pyroídia based on five cp markers in the recently study. Zhao preliminarily studied the infrageneric relationship of Pyroídia based on 26 species, mainly in Asia, by using one cpDNA, rps4-trnS, and one nrDNA, LEAFY. Four main clades were recognized in Pyroídia by rps4-trnS and a potential hybrid origin of P. piloselloides was suggested. Nevertheless, the sampling is far from completion, and the P. africana-group was not included. Evidences from both single parent genetic chloroplast markers and parental genetic nuclear gene are urgent needed to test the monophyletic of Pyroídia and further understand the phylogenetic relationship within Pyroídia based on more taxa.

The integration of phylogenetics, historical biogeography, paleogeography and climatology has provided a new perspective to understand the origin and diversification of biotas, which are of great interest to evolutionary biologists. Pyroídia is exclusively distributed in paleotropic regions, while Platycerium, the mostly related group of Pyroídia, is distributed in paleotropic regions and South America. Based on morphological analyses, Hovenkamp proposed that Pyroídia originated from Africa in the Jurassic and that the present distribution of the genus was reached via ‘rafting’ to the Indian subcontinent and Australia. Schneider et al. and Schuettelpelz and Pryer dated the original time of Pyroídia to the late Eocene (ca. 35 Ma) based on molecular dating, while Testo and Sundue established the original time of the main lineage of Pyroídia in early Paleocene (ca. 63.4 Ma). Kreier and Schneider discussed the phylogeny and biogeography of Platycerium and reestablished two lineages (Africa + Madagascar + South America and Australia + Asia) in Platycerium. However, the ancestral distributions for the basal nodes are poorly resolved. Therefore, elucidating the historical biogeography of Pyroídia is perplexing due to differences in the distribution and species diversity centre of Pyroídia from those of Platycerium.

The present study, based on comprehensive taxa sampling, employs nucleotide sequences of five chloroplast DNA markers (rbcL, matK, psbA-trnH, and rps4 + rps4-trnS) and one single (low)-copy nuclear gene, LEAFY, to reconstruct the phylogeny of Pyroídia and to explore its historical biogeography. In addition, evolution of the morphological diagnostic characters and environmental adaptations related to drought resistance are investigated.

**Results**

**Sequence characteristics.** Five cpDNA gene regions—matK, rbcL, psbA-trnH, and rps4 + rps4-trnS—were amplified in all 109 Pyroídia individuals and the related taxa in Polypodiaceae and Davalliaceae. The sequence characters and parsimony-informative sites for individual gene markers are summarized in Table 1. The combined data matrix of the cpDNA fragments included up to 3760 nucleotides, of which 1325 (32.58%) were

| Gene    | Length (bp) | Alignment length (bp) | Number of variable characters (%) | Number of parsimonious informative characters (%) |
|---------|-------------|-----------------------|-------------------------------|-----------------------------------------------|
| matK    | 820         | 820                   | 510 (61.20)                    | 425 (51.83)                                   |
| rbcL    | 1277        | 1277                  | 306 (23.96)                    | 244 (1)                                       |
| rps4 + rps4-trnS | 927–987   | 1042                  | 399 (38.11)                    | 306 (29.23)                                   |
| psbA-trnH | 303–496   | 597                   | 110 (18.43)                    | 79 (13.23)                                    |
| combined cpDNA | 3353–3560 | 3760                  | 1325 (32.58)                   | 1054 (28.03)                                  |
| LEAFY   | 955–1009    | 1081                  | 606 (56.06)                    | 515 (47.64)                                   |

Table 1. Descriptive statistics of analyzed DNA sequence used in the present study.
A unique insertion/deletion (up to 169 bp at site 131 to 300 bp) was present in the \textit{psb}A-\textit{trn}H gene of \textit{Pyrrosia}. Maximum parsimony (MP) analyses of the combined cpDNA data set resulted in 14,809 equally most parsimonious trees with a length of 4,007 steps. The consistency and retention index were relatively high (CI = 0.60, RI = 0.86). The optimal maximum likelihood (ML) phylogram for the combined cpDNA data was $-\text{InL} = 26,674.4552$.

Polymerase chain reaction (PCR) amplification of \textit{LEAFY} was successfully performed in 81 individuals representing 39 species of \textit{Pyrrosia} and the related taxa in Polypodiaceae and Davalliaceae. The amplified \textit{LEAFY} gene fragment included intron 1, exon 2, intron 2 and the flanking exon 1 and exon 3 sequences. The aligned data matrix had 1081 characters, including 606 variable characters and 515 potentially parsimony-informative characters (Table 1). The MP analyses were stopped with 13,200 equally most parsimonious trees of 1539 steps sampled (CI = 0.61, RI = 0.88). The optimal ML phylogram had an $-\text{InL}$ value of 9,969.4164.

**Phylogenetic analyses.** \textit{cpDNA}. The three phylogenetic analyses (MP, ML and Bayesian inference (BI)) revealed congruent topologies based on the combined data set of five cpDNA markers. Platycternioideae was recognized as a well-supported monophyly. The traditional \textit{Pyrrosia} was paraphyletic, six main monophyletic clades (labelled as clades I–VI) were resolved (Fig. 1). Clade I clustered with \textit{Platycerium} with weak support, and this clade was the endemic African species, \textit{P. schimperiana} (Mett. ex Kuhn) Alston (=\textit{Hovenkampia schimperiana} (Mett. ex Kuhn) Li Bing Zhang & X. M. Zhou) which belonged to the \textit{P. africana}-group of Hovenkamp. Herein, we use \textit{Pyrrosia} s.l. to represent \textit{Pyrrosia} including \textit{P. africana}-group, and \textit{Pyrrosia} s.s. to represent \textit{Pyrrosia} without \textit{P. africana}-group. Clade II comprised \textit{P. costata} and \textit{P. stigmosa}, this clade corresponds to the \textit{P. costata}-group.
described by Hovenkamp4, and it was resolved as the basal clade of Pyrrosia s.s. Clades III only contained P. niphoboloides. Clades IV to VI consisted of species distributed in Asia and Oceania, except the widespread P. lan-
celata, which occupied Asia, Oceania and Africa. Clade IV contained four subclades (subclades A, B, C and D): P. angustissima, a species that previously belonged to the separated monotype genera Saxiglossum, was resolved as subclade A; P. mammularifolia, P. rasamalae and P. kinabaluensis comprised subclade B with high support; subclade C consisted of species of the P. angustata-group; subclade D included the P. lingua-group and two unde-
cided species, P. laevis and P. ensata. Clade V was strongly supported as the sister to clade VI, and both contained two subclades (subclades E and F; subclades G and H). Clade V contained P. piloselloides (subclade E) and species from the P. confluens-group, P. lanceolata-group and P. foveolata (subclade F). Clade VI was separated into two well-supported subclades: subclade G contained some of the species of the P. sheareri-group, and subclade H consisted of some of the species of the P. sheareri-group and species of P. porosa-group.

LEAFY. Clade I, II, V and VI were recognized as monophyletic groups. Clade III was the basal clade of Pyrrosia s.l. Phylogenetic topologies of cpDNA and LEAFY showed significant conflicts of the relationship among clades I, IV and V, and subclades within clade IV (Fig. 2). (1) Clade I and V embed in clade IV, and resulted the mono-
phly clade IV in cpDNA trees split into three parts in the tree generated from LEAFY: the first part, P. angus-
tata (subclade C), was resolved as the basal group of clade IV; the second included subclades A, B and a species,
P. laevis, which belonged to subclade D; the third included species from subclade D. (2) The phylogenetic position of P. schimperiana (clade I, = H. schimperiana, P. africana-group) was contradictory between datasets: P. schimperiana was resolved as an independent clade in the cpDNA trees (Fig. 1), while in the tree generated from LEAFY, P. schimperiana was nested in the second part of clade IV. (3) Clade V was the sister group of clade VI in the cpDNA trees, while it nested into clade IV and clustered with subclade D in the phylogenetic tree of LEAFY.

Furthermore, an individual of P. lingua had two sequence types of LEAFY: the sequences of one type clustered with other samples of P. lingua, and the sequences of another one clustered with P. petiolaris. Similarly, one individual of P. porosa also had two sequence types, one type clustered with P. nudicaulis, and the sequences of another type nested with P. assimilis.

**Molecular dating.** The mean ages and 95% highest posterior density (HPD) intervals of all labelled nodes within Platycterioidae were indicated in the chronogram (Fig. 3b) and in Table 2. The crown age of Platycterioidae was in the late Eocene (node 1, 37.98 Ma, HPD = 25.75–46.71 Ma). Clade I was separated from Platycterus at ca. 26.33 Ma (node 5, HPD = 16.78–39.39). The estimated age of the Pyrrosia s.s. (clades II–VI) was approximately the boundary of the Eocene and Oligocene (node 2, 33.71 Ma, HPD = 22.64–42.43). The most common ancestor (MRCA) of the main Asian and Oceanian species was dated back 30.07 Ma (node 3, HPD = 20.80–38.75); Clade IV separated from clades V and VI at ca. 27.40 Ma (node 4, HPD = 19.56–35.90), and the latter two clades separated at ca. 24.19 Ma (node 6, HPD = 16.71–31.16). The crown node of clade IV was dated back to 22.82 Ma (node 7, HPD = 14.06–30.73). Estimated crown ages for clades V and VI are very similar, at 17.98 Ma and 18.14 Ma, respectively (nodes 11 and 10, Fig. 3b and Table 2).

**Ancestral area reconstruction.** The ancestral distributions obtained from the dispersal-extinction-cladogenesis (DEC) model for the major clades are shown in Fig. 3b and Table 2. The DEC model suggested that Platycterioidae most likely originated in areas E (Fig. 3b, node 1). The origin for the MRCA of Pyrrosia s.s. was unclear; two areas including southern Asia, Indo-China Peninsula and southwestern and southern China (area B), and Africa (area E), were all the supposed origin. The MRCA of the most Asian and Oceanian species originated in areas B (node 4). Clade IV may have originated in areas B and expanded towards Eastern Asia (area A) and Malesia (area C). The sister clades V and VI may have originated in area B and then expanded towards Eastern Asia (area A) and Australasia (area D), respectively. Clade I and Platycterus were suggested that originated in areas E. Several historical dispersal events were inferred, including the dispersal from area B to A (26.5 times), C (26.5 times), D (17.5 times) and E (17 time), and from area E to C (4.5 times), B (4 times), A (0.5 time), D (0.5 time) and D to B (3 time), C (3 times). The split of Pyrrosia s.s. from clade I and Platycterus and the split of clades II, V, VI, the diversification of the crown group of clade VI, V and VI and the infraspecific range expansions were suggested as dispersal events. The split of clade III from clades IV–VI was estimated as vicariance (node 3). Several independent dispersal events were detected within clades IV and V, respectively.

**Discussion**

**Infrageneric relationships within Pyrrosia s.l.** Six main clades and eight subclades were recognized according to the cpDNA phylogenetic reconstructions of Pyrrosia s.l. in this study. We suggested 11 groups for the infrageneric delimitation of Pyrrosia s.l.

P. schimperiana was resolved as clade I which clustered with Platycterus. This results was similar to the studies of Testo and Sundue47 and Zhou et al., but different from the most studies that treat P. schimperiana as the basal group of Pyrrosia.44,46, Zhou et al. established a new genus Hovenkampia, and described its diagnostic characters different from Pyrrosia s.s. as: rhizomes completely parenchymatous; stomata polytopic; perispore thin, tightly adhering to the exosporous surfaces. As Hovenkamp, Hennipman10 and Ufffen and Hennipman24 pointed that the characters above are all not synapomorphy of P. africana-group. Rhizomes of the P. rhodesiana (P. porosa-group) are parenchymatous. Stomata of P. marnii and P. penangiana are polytopic. P. africana-group shared the finely granulose epispore ornamentation with the species in clade VI of our study. Furthermore, scales of this species are pseudopeltate; the fronds are monomorphic, oblanceolate and estipitate; the indumenta are monomorphic and indumenta rays are narrow boat-shaped, these characters are all shared by more than one group of Pyrrosia s.s.

Clade II is monophyletic and as the basal group of Pyrrosia s.s. lineage. Species of this clade are all monomorphic fronds, short rhizome, basifixed scales. Smooth spores are the homogeneous characters of this clade. This clade is in the equivalent of P. costata-group of Hovenkamp, Pyrrosia Clade of Zhou et al., and subg. Pyrrosia of Vasques et al.26.

Clade III contained only P. niphoboloides, which was assigned to the P. piloselloides-group by Hovenkamp1 (or members of previous segregated genus Drynopoglossum) along with P. piloselloides. However, these species did not cluster together in the present cpDNA phylogenetic trees, with P. piloselloides clustered in clade V, which indicates that the P. piloselloides-group described by Hovenkamp is polyphyletic, although the members have similar morphological characters (e.g. dimorphic fronds, venation, and sori arrangement). While, P. niphoboloides was included in Galeoglossa subclade, which is in the equivalent of subclade B of clade IV of our study in Zhou et al.35 Vasques et al.46 treated this species in subg. Solis, which was the basal group of Pyrrosia except subg. Lunae. The sequences of P. niphoboloides we used were downloaded from GenBank. The phylogenetic position of P. niphoboloides still need more evidences.

Members of clade IV are characterized by long-creeping rhizomes, and peltate scales, but they have various frond shapes, indumenta and venation types, and spore ornamentation. This clade has four relatively independent subclades. Species of this clade are widely distributed in East and South Asia, and extend to the Indonesia Archipelago. The basal subclade A, which includes only P. angustissima, had been previously described as the segregated genus Saxiglossum46. We recognized that P. angustissima belonged to the basal member of clade IV.
Figure 3. Global biogeographical patterns of *Pyrrosia* s.l. (a) Map showing five biogeographical areas in colors as defined in this study. (b) Schematic chronograms (maximum clade credibility topology) based on cpDNA data using uniform priors obtained from BEAST. The geological time scale (60 Ma–present) is shown at the bottom. Node numbers and mean ages refer to Table 2. Blue bars represent 95% highest posterior density of node age. Color-coded pie diagrams represent the probabilities of different states of ancestral area reconstruction (AAR) based on the dispersal–extinction–cladogenesis (DEC) model in RASP. Arrowheads represent the possible split events inferred in RASP. Biogeographical area abbreviations: A, eastern Asia (including central, eastern and northeastern China, Korea Peninsula, southern Japan and Far East); B, southern Asia, the Indo-China Peninsula and southwestern and southern China; C, Malesia (including Malaysia, Indonesia, and the Philippines); D, Australasia (including Australia, New Guinea, New Zealand, and the South Pacific islands); E, Africa (including Madagascar). Geological epoch abbreviations: Pl., Pliocene; Ple., Pleistocene. The spatial data of Fig. 3a was freely downloaded from http://www.diva-gis.org/Data, the base map was generated by ArcGIS v.9.3 (http://www.esri.com/software/arcgis/arcgis-for-desktop), and Fig. 3a was drawn by CorelDRAW v.x 8 (http://www.coreldraw.com/en/product/graphic-design-software/).
Table 2. Mean age and 95% highest posterior density (HPD) values of each node. Ancestral areas with Akaike weight revealed by the dispersal–extinction–cladogenesis (DEC) model in RASP. Node numbers and area abbreviations refer to Fig. 3.

| Node | Mean (Ma) | 95% HPD (Ma) | DEC model (Akaike weights) |
|------|-----------|--------------|---------------------------|
| 1    | 37.98     | 25.75–46.71  | E (0.52)                  |
| 2    | 33.71     | 22.64–42.43  | BE (0.57)                 |
| 3    | 30.07     | 20.80–38.75  | BE (0.67)                 |
| 4    | 27.40     | 19.56–35.90  | B (0.69)                  |
| 5    | 26.33     | 16.78–39.39  | E (0.78)                  |
| 6    | 24.19     | 16.71–31.16  | B (0.65)                  |
| 7    | 22.82     | 14.06–30.73  | B (0.47)                  |
| 8    | 19.96     | 13.09–28.57  | B (0.59)                  |
| 9    | 18.67     | 11.95–27.36  | B (0.61)                  |
| 10   | 18.14     | 12.36–27.31  | B (1.00)                  |
| 11   | 17.98     | 12.31–24.19  | BE (0.75)                 |
| 12   | 15.35     | 8.50–21.95   | BC (0.55)                 |
| 13   | 15.27     | 9.44–23.36   | B (0.59)                  |
| 14   | 14.15     | 8.34–20.15   | B (0.39)                  |
| 15   | 13.80     | 10.04–21.79  | B (1.00)                  |
| 16   | 2.76      | 1.22–12.22   | B (0.87)                  |

*P. angustissima* has some autapomorphy characters, including linear-triangular rhizome scales, linear laminae with involute margins and a special type of drynarioid venation. These features of *P. angustissima* clearly distinguish it from other members of clade IV, which makes it a separate subgroup. Subclade B contains three species, *P. nummularisfolia*, *P. kinabaluensis* and *P. rasamalae*, all of which belonged to the *P. albicans*-group of Hovenkamp. These species have succulent lamina with distinct water-tissues, dimorphic indumenta (acicular and woolly rays), and spores with longitudinal ridges and finely granule, but they lack hydathodes. Subclade C contains *P. angustata* and *P. samarenseis*, which belong to *P. angustata*-group of Hovenkamp. This subclade has similar morphology, including dimorphic fronds, entire margin scales, pseudo-drynarioid venation, and spores with longitudinal ridges (which occurs in only this subgroup). Subclade D, which was recognized as monophyletic, includes *P. laevis*, *P. ensata* and the *P. lingua*-group. The close relationship between the *P. lingua*-group and *P. laevis* revealed here agrees with the findings of Yang, who assigned them to section *Pyroleia*. Hovenkamp did not consider the *P. lingua*-group to be a well-established monophyletic group because the morphology of the spore was heterogeneous and there are no autapomorphies in this group. We found that species of subclade D all featured long-creeping rhizomes and fronds from monomorphic to moderately dimorphic with distinct stipitate and lamina ovate to lanceolate. In this subclade, persistent boat-shaped ray hairs exist in all species and coarsely sparse tubercules are the main spore ornamentation. The presence of common features in subclade D suggests that this group is monophyletic. Clade IV was mostly similar to subg. *Niphobolus* clade of Zhou et al. and Niphopsis clade of Vasques et al. with *P. niphoboloides* an exception. Nevertheless, the phylogenetic relationships among species, especially the species of subclade D were still uncertain.

Clade V includes subclades E and F. Subclade E contains only *P. piloselloides*, which is a member of the *P. piloselloides*-group. Subclade F contains species of the *P. confluens*-group, the *P. lanceolata*-group and an undescribed species, *P. foveolata*. Both of the former two groups were considered as monophyletic by Hovenkamp, on the basis of restricted hydathodes, monomorphic indumenta, large sori with short paraphyses (in the *P. confluens*-group), and sunken sori with centrally situated paraphyses (in the *P. lanceolata*-group). Nearly all species of these two groups and *P. foveolata* have common features, such as: long-creeping rhizomes, fronds that are dimorphic in various ways (with the exception of *P. longifolia*), lamina that are elliptic or elongated to strap-shaped with decurrent base and indistinct stipes, sori that are sunken with distinct stellate paraphyses, indumenta that are monomorphic and persistent, indumenta rays that are short boat-shaped, and perispore that are bilateral with tuberculate and irregularly verrucated protuberances. Evidence was sufficient to support the *P. confluens*-group, *P. lanceolata*-group and *P. foveolata* as monophyletic. Clade V of our study was equivalent to subg. *Niphobolus* of Vasques et al. and Niphobolus clade of Zhou et al. *P. rasamalae* was included in Niphobolus clade inexplicably in Zhou et al., while, this species was treated as a member of Clade IV in our study with highly support.

Clade VI occupies the most obvious common characters, including thick and short rhizomes, monomorphic fronds, and distinct hydathodes. Subclades G and H are resolved as separate monophilies with highly supported values. Subclade G contains *P. bonii*, *P. calvata*, *P. subtruncata*, *P. fengiana*, *P. shearerii*, *P. drakeana* and *P. subfurfuracea*. Three species of the *P. shearerii*-group together with *P. polydactyla*, *P. hastata*, and *P. flocculosa* are nested in subclade H, which contains most species of the *P. porosa*-group. Our results are quite different from Yang's treatment, in which *P. subfurfuracea* and *P. calvata* were added to the *P. costata*-group based on similar thick and short rhizomes, un-peltate (basifixed and pseudopeltate) scales and other features. Subclade G shares the
following common features: pseudopeltate or basifixed scales, monomorphic fronds with a distinct stipe, densely granulated perispores, and dimorphic indumenta with aciculate rays appressed to a layer with woolly rays (indumenta of *P. sheareri* are appressed and boat-shaped). Subclade H contains species of the *P. porosa*-group, three species belonging to the *P. sheareri*-group and three undecided species—*P. transmorrisonensis*, *P. mannii*, and *P. penangiana*. Two separate species, *P. mannii* and *P. penangiana*, were resolved as the basal lineages of subclade H. *P. polydactyla* and *P. hastata* formed the sister group of the lineages including the *P. porosa*-group and *P. flocculosa*. The frond shape of *P. hastata* and *P. polydactyla* is most unique in *Pyrrhoa* s.l., and both are pedately divided to 4/5 depth of the frond into 3–5 to 6–8 divisions with distinct stipes. The indumenta are boat-shaped and persistent. The other species of subclade H share many common features, for example, linear to narrow lanceolate and oblongate fronds with gradually narrowed and decurrent fronds base, and fronds stipitiate; the indumenta are persistent and dense with aciculate rays, and in most species, aciculate rays are appressed to a layer with mainly woolly rays (woolly ray stellate hairs are not obvious in *P. gralla, P. assimilis*, and *P. penangiana*). Although *P. polydactyla* and *P. hastata* shared some morphological characters with subclade G, they were polyphyletic in our study, and paraphyly in Vasques et al. and Zhou et al. Zhou *et al.* combined them as a *P. sheareri* group.

**Incongruence between the cpDNA and LEAFY phylogenetic trees and potential reticulate evolution.** The chloroplast genome is maternally inherited in ferns, and the nuclear genome exhibits amphilepis. Consequently, comparative studies of these two genomes might uncover potential reticulate evolution, including introgression and/or hybrid speciation. LEAFY is a well-studied single-copy gene in ferns, and it has been successfully used to resolve reticulate evolution at low taxonomic levels. Considering the high proportion of hybridization in ferns and the advantages of LEAFY, both cpDNA and LEAFY were included in the phylogenetic analyses. Each of four clades I, II, V and VI was resolved as monophyletic with high support values, both in the cpDNA and LEAFY gene trees. CpDNA and LEAFY gene trees showed incongruent phylogenetic positions for clade I and clade V. Both of clade I and V nested in clade IV in the nuclear tree. Hybridization and incomplete lineage sorting (ILS) are two important factors that lead to phylogenetic incongruence. According to the result of ancestral area reconstruction, the potential ancestral area of clade I was area E, ancestral areas of clade V are area B or E, and the ancestral area of clades IV and VI was B. Dispersal events have been inferred between area B and E. By contrast, ILS is resulted from the ancestral alleles being sorted into some lineages randomly. In our study, both alleles of clade I and V nested in clade IV, thus, hypothesis of ILS seems less plausible although it is difficult to distinguish from ancient hybridization, especially without genomic data.

We suggested that clade V might be an ancient hybrid origin and reached the current distribution areas during species dispersal in history. Ancestral species of clade IV might be the male parent of clade V and ancestral species of clade VI might be the female parent of clade V. We suggested three potential scenarios of the origin of clade I: (1) the new established genus *Hovenkampia* was an ancient hybrid origin, ancestral species of *Platycerium* were the female parent and *Pyrrhoa* s.s. the male parent; (2) *P. schimperiana* hybrid species with other species in *Hovenkampia* and species in clade IV as its parents; (3) three individuals of *P. schimperiana* used in this study are hybrid individuals.

In subclade H, *P. porosa* had two divergent homoeologous copies of LEAFY, one copy of which clustered with *P. nudicaulis*, while another copy clustered with *P. assimilis* and then nested with *P. tonkinensis*. In the cpDNA tree, individuals of *P. porosa, P. nudicaulis*, and *P. assimilis* were clustered together and were distinctly separated from *P. tonkinensis*. In addition, the basal chromosome number within *Pyrrhoa* s.l. was 36 or 37; most of the reported data of *P. porosa* are tetraploid and hexaploid, only one diploids of *P. porosa* have been reported in India. It revealed that some plants of the *P. porosa* might be allopolyploid. Furthermore, one individual of *P. gralla* clustered with *P. davidii*, and the copies of another individual nested in *P. tonkinensis* in the phylogenetic tree of LEAFY, while it was distinctly separated from *P. tonkinensis* in the cpDNA tree. The short branches of cpDNA trees (Fig. 1) and molecule dating (Fig. 3) showed that *P. porosa, P. nudicaulis, P. assimilis, P. tonkinensis*, and *P. gralla* were differentiated recently. Based on the results of cpDNA and nrDNA, a recent hybrid or reticulate evolution were revealed in *P. porosa*-group. In subclade D, *P. lingua* also had two divergent homoeologous copies of LEAFY, a copy of which clustered with *P. martini*, while another one clustered with *P. petiolosa* in the phylogenetic tree of LEAFY. However, individuals of *P. lingua* and *P. petiolosa* were distinctly separated in the cpDNA tree. Only diploids were reported in *P. lingua* and *P. petiolosa*. Incongruence between the cpDNA and LEAFY trees suggested a recent hybrid might exist in *P. lingua*-group. Nevertheless, owing to the limited information on chromosome numbers of *Pyrrhoa* s.l., the acquisition of more exact results will require more samples and further comprehensive cytological studies.

**Morphological characters assessment and evolution.** Several features, including the presence of specialized sterile fronds, rhizome growth-form, the distance between adjacent phylloidea, the insertion type of rhizome scales, scale margin morphology, stomata type, venation type, sori arrangement, indumenta shape and epispore ornamentation, were all treated as diagnostic characters for infragenetic classification and species delimitation of *Pyrrhoa* s.l. in previous studies. If we only use morphologic characters to evaluate the infragenetic classification in *Pyrrhoa* s.l., most groups or sections are not monophyletic. Ancestral character state optimization based on a stable molecular phylogenetic tree in this study enabled a synthetic evaluation of all diagnostic characters (Fig. 4).

(1) The presence of specialized fertile fronds is essentially different from monomorphic and moderately dimorphic fronds, in which fertile fronds are the same or longer and narrower than sterile fronds. Dimorphic fronds appeared in clade III and scattered in clades IV and V. Hovenkamp considered that monomorphic fronds transformed in the presence of specialized sterile fronds; thus, it can be observed that dimorphic fronds have evolved multiple, independent times in *Pyrrhoa* s.l. (Fig. 4a). (2) Rhizome growth-forms and the distance
between adjacent phyllopodia are relevant characters. Short and thick rhizomes correspond with contiguous phyllopodia and long-creeping rhizomes correspond with separate phyllopodia. Long-creeping rhizomes are homoplastic characters, occurring in clades III, IV and V (Fig. 4b). (3) Regarding the insertion type of rhizome scales, basifixed scales are considered ancestral, and they occur in clades II and VI. Peltate scales are the predominant type in \textit{Pyrrosia} s.l., and both basifixed scales and pseudopeltate scales appear to have evolved at least five times (Fig. 4c). (4) Scales with ciliate-dentate margins are the mostly ancestral types, and scales with long and curly cilia margins are apomorphic within clade VI, while scales with entire margins independently evolving
at least five times (Fig. 4d). (5) The dominant pericytic stomata type of *Pyrospia* s.l. is a deuterogenic feature in Polypodiaceae. Three species of *Pyrospia* s.l. (*P. schimperiana*, *P. manni* and *P. penangiana*) still have polycytic stomata, which are common in other genera of Polypodiaceae. Polycytic stomata in *Pyrospia* might be the result of reverson or secondary development (Fig. 4e). (6) Pseudo-drynarioid and campyloneuroid venation types of *Pyrospia* s.l. are deuterogenic features in Polypodiaceae, and drynarioid venation is the ancestral feature. Species occupying pseudo-drynarioid and campyloneuroid venation were both polyphylectic, and they interspersed in clade II, IV, V and VI. A high frequency of homoplasies of venation types appears within *Pyrospia* s.l. (Fig. 4f). (7) Sori arrangement is an important diagnostic character in *Pyrospia* s.l. and the other ferns. Confluent sori and orbicular and discrete sori have been treated as the diagnostic character even at the species and genus level in Polypodiaceae46–72. Coenosori may be considered as one of the main characters to separate the previous genus Drymoglossum from *Pyrospia* s.l., although some authors consider this classification “purely artificial”89. The homoplasies of confluent sori in *Pyrospia* s.l. was confirmed in this study. Meanwhile, the other three types of sori arrangement were also homoplasies (Fig. 4g). (8) There are four main shapes of stellate hairs in *Pyrospia* s.l.: hairs with straight acicular rays, those with straight boat-shaped rays, those with curly wooly rays, and those with straight boat-shaped and curly wooly rays at one axis. The indumenta of each species may be monomorphic or dimorphic. Monomorphic indumenta contain only one type of stellate hair above, while dimorphic indumenta contain two types of stellate hairs. We recognized five types of indumenta in *Pyrospia* s.l. (Fig. 4h) and found that both monomorphic and dimorphic indumenta are homoplastic. Dimorphic indumenta occurred in clades II, IV and VI, while monomorphic indumenta occurred in all clades. In addition, clades I, II and V, and subclades D all have boat-shape or lanceolate hairs, and the other clades (subclades) except *P. hastata*, *P. polyaductyla*, and *P. sheareyi* have acicular hairs. Overall, indumenta of *Pyrospia* s.l. are homoplasies (Fig. 4h). (9) Epispore ornamentation appears relatively unique. Clades II and subclade C show that each clade or subclade is obvious bispiculate spores with dense, small granulate and warty or spire protuberances, and clades I and VI have spores with more or less dense finely granulose protuberances. Smooth spores appear only in clade II, and spores with longitudinal ridges appear only in subclade C. These two types of epispore ornamentation can be treated as synapomorphies of clade II and subclade C, respectively. Epispore ornamentation of subclades A and D appears sparsely granulose and irregularly ridged (with the exception of *P. abbreviata*), and those of subclade B and *P. abbreviata* of subclade D are longitudinally ridged with finely granulose protuberances (Fig. 4i).

Ancestral character state optimization revealed high levels of homoplastic evolution in *Pyrospia* s.l. Only the epispore ornamentation is synapomorphic in clade II and subclade C, respectively. There is no single character that can be used as an apomorphy to distinguish groups (clades and subclades) in *Pyrospia* s.l. from each other; thus, the combination of two or more characters are necessary to identify all groups. For instance, given that rhizome growth-forms are similar between subclades G and H and clade II, combining this character with the indumenta morphology and the fronds shape can identify clade II, while combining the frond shape and insertion type of rhizome scales can identify subclades G and H. Furthermore, some of the anatomical characters, such as the distribution pattern of collenchyme, sclerenchyma and parenchymatous cells in rhizomes, might be helpful in defining traits for monophyletic group recognition and species identification in *Pyrospia* s.l.42,52.

**Ecological adaptations.** Most species of *Pyrospia* s.l. are extremely drought tolerant. The morphological characters such as the texture of the lamina that is coriaceous, thick-leathery, succulent, or peronate; the presence of a thick cuticle on the epidermis; sunken stomata; sunken sori; or distinct hydathodes are all xerophytic adaptations. Regarding drought resistance, poikilohydrous and succulent forms are considered two different growth-forms4. Poikilohydrous plants of *Pyrospia* s.l. can roll and stretch their fronds in response to drought resistance, which are similar to resurrection plants. Most of these species, such as *P. porosa*, *P. schimperiana*, are short rhizomes, the anticlinal walls of the adaxial epidermis appear thin or slightly thickened, hypodermis and water tissue are absent or indistinct, and the indumenta form dense mat. Poikilohydrous plants are adaptable to seasonal climates with long dry periods. By contrast, succulent plants of *Pyrospia* s.l., namely, *P. abbreviata*, *P. angustissima*, *P. longifolia* and *P. mannii*, can survive by storing water during short periods of drought. These species are long-creeping rhizome, the adaxial epidermis are longitudinally ridged with finely granulose protuberances and VI, while succulent form species nested in clades III, IV and V. These two growth-forms adapting to drought resistance are all independently polyphylectic in *Pyrospia* s.l.

In addition to morphological specializations, another key innovation associated with the success of *Pyrospia* s.l. in more arid habitats is the form of photosynthesis known as crassulacean acid metabolism (CAM). The CAM cycle has been reported in five species of *Pyrrosia* s.l. (*P. schimperiana*, *P. manni* and *P. penangiana*) still have polycytic stomata, which are common in other genera of Polypodiaceae. Polycytic stomata in *Pyrospia* might be the result of reverson or secondary development (Fig. 4e). (6) Pseudo-drynarioid and campyloneuroid venation types of *Pyrospia* s.l. are deuterogenic features in Polypodiaceae, and drynarioid venation is the ancestral feature. Species occupying pseudo-drynarioid and campyloneuroid venation were both polyphylectic, and they interspersed in clade II, IV, V and VI. A high frequency of homoplasies of venation types appears within *Pyrospia* s.l. (Fig. 4f). (7) Sori arrangement is an important diagnostic character in *Pyrospia* s.l. and the other ferns. Confluent sori and orbicular and discrete sori have been treated as the diagnostic character even at the species and genus level in Polypodiaceae46–72. Coenosori may be considered as one of the main characters to separate the previous genus Drymoglossum from *Pyrospia* s.l., although some authors consider this classification “purely artificial”89. The homoplasies of confluent sori in *Pyrospia* s.l. was confirmed in this study. Meanwhile, the other three types of sori arrangement were also homoplasies (Fig. 4g). (8) There are four main shapes of stellate hairs in *Pyrospia* s.l.: hairs with straight acicular rays, those with straight boat-shaped rays, those with curly wooly rays, and those with straight boat-shaped and curly wooly rays one axis. The indumenta of each species may be monomorphic or dimorphic. Monomorphic indumenta contain only one type of stellate hair above, while dimorphic indumenta contain two types of stellate hairs. We recognized five types of indumenta in *Pyrospia* s.l. (Fig. 4h) and found that both monomorphic and dimorphic indumenta are homoplastic. Dimorphic indumenta occurred in clades II, IV and VI, while monomorphic indumenta occurred in all clades. In addition, clades I, II and V, and subclades D all have boat-shape or lanceolate hairs, and the other clades (subclades) except *P. hastata*, *P. polyaductyla*, and *P. sheareyi* have acicular hairs. Overall, indumenta of *Pyrospia* s.l. are homoplasies (Fig. 4h). (9) Epispore ornamentation appears relatively unique. Clades II and subclade C show that each clade or subclade is obvious bispiculate spores with dense, small granulate and warty or spire protuberances, and clades I and VI have spores with more or less dense finely granulose protuberances. Smooth spores appear only in clade II, and spores with longitudinal ridges appear only in subclade C. These two types of epispore ornamentations can be treated as synapomorphies of clade II and subclade C, respectively. Epispore ornamentation of subclades A and D appears sparsely granulose and irregularly ridged (with the exception of *P. abbreviata*), and those of subclade B and *P. abbreviata* of subclade D are longitudinally ridged with finely granulose protuberances (Fig. 4i).
have a single origin in Pyrrosia s.l. The results of our molecular dating indicate that the divergence time of clade V was dated to ca. 17.98 Ma (node 11), which revealed an early Miocene origin of the CAM pathway in Pyrrosia s.l.

The emergence of CAM photosynthesis in different taxa may be driven by the same external driving forces. Just as the occurrence of CAM pathways in Bromeliaceae was driven by the increasing aridity and declining CO₂ concentrations during the Cretaceous and Miocene, the diversification of the clades of Pyrrosia s.s. in Testo and Sundue's study were also dated back to the Oligocene, with a steady period of ca. 20 My from the early Eocene to the early Oligocene. Therefore, we believe Pyrrosia s.l. originated much earlier than Oligocene and underwent diversification during the Oligocene and Miocene.

Diversification of Pyrrosia s.l. The poor fossil record of polygrammoid ferns discourages estimations of exact differentiation times and recognition the distribution area of ancestor clades. Molecular dating by Schuettpelz and Pryer 60 showed that Pyrrosia s.l. originated in Oligocene (ca. 35.1 Ma). Testo and Sundue 62 estimated the origination time between Pyrrosia s.s. and Platypterium to be 63.41 Ma. The diversification of most of the main clades of Pyrrosia s.s. in Testo and Sundue's study were also dated back to the Oligocene, with a steady period of ca. 20 My from the early Eocene to the early Oligocene. Therefore, we believe Pyrrosia s.l. originated much earlier than Oligocene and underwent diversification during the Oligocene and Miocene.

Ancestral area reconstruction based on the DEC model in RASP 3.2 revealed that Southern Asia, the Indo-China Peninsula and southwestern and southern China (area B); and Africa (area E) are the probable ancestral areas of Pyrrosia s.l. Janssen et al. 44 suggested that Pyrrosia s.l. might be of African origin because the African species P. liebuschii was the basal clade of Pyrrosia s.l. Holtum 63 suggested that the African species may have dispersed from Africa because P. shearei is closer to the primitive conditions, and this species predominantly lives in China with a centre of distribution located in Southeast Asia. Although P. shearei is neither the ancestor nor the basal species of Pyrrosia s.l. based on our phylogenetic analyses, area B, particularly the Himalayan region, has a considerably higher species diversity. Furthermore, the phylogenetic analyses based on LEAFY determined that P. costata (representing clade II), was the basal clade, and the Africa species P. schimperiana (clade I) was nested into clade IV. The ancestral area of clade II was assumed to be area B or areas B and C, which further demonstrated that area B is most likely the original area of Pyrrosia s.l. Moreover, this area also displays high species diversity in nearly all subfamilies in Polypodiaceae, and it is another diversity center outside of the tropical Americas. Southeast Asia is the origin area of Thylacopteris 40, Microsorum 78, Lepisorus 79 and drynarioid ferns 45; as well as some taxa in Eupolypods II Deparia 80 and some angiosperms 49,80. Similar patterns were also found in the closely related microsoroids, which have a diversity centre in southeastern Asia and colonized to African regions several times 44.

The distribution of Pyrrosia s.l. presents an Africa-Asia disjunction, which is common in plants and has recently attracted much attention 42,44,49,54,57,80–83. Four main driving mechanisms for the disjunctive distribution have been summarized: (1) transoceanic long-distance dispersal, (2) overland migration via land bridges, (3) boreotropical dispersal via Eocene forests, and (4) rafting of India 49,54,82,83. Hovenkamp 68 proposed that the disjunctive distribution pattern of Pyrrosia s.l. resulted from vicariance, such as the breakup of Africa from Gondwana (120–140 Ma) and the separation of Australia from India (125 Ma), rather than dispersal. The Indian plate became progressively more isolated from eastern Gondwana in the Cretaceous and Paleocene, and it moved northward towards Asia with glancing contact to Asia at ca. 57 Ma 85. In our analyses, the divergence time of Africa lineages and Asia-Australasian lineages was estimated to be much younger (Fig. 3b); thus, the split could not be vicariance resulting from the breakup of Gondwana and Laurasia, and the Indian plate as either a raft or stepping stone is therefore too old for the origin of Pyrrosia s.l. In addition, the “boreotropical” floristic connection hypothesis, in which the plants moved across the North Atlantic during late Paleocene to the middle Eocene, presents a time frame that is too early to explain the Africa-Asia disjunction of Pyrrosia s.l. 85,86. The closure of the Tethys Sea led to the direct connection between mainland Africa and western Asia during the early Miocene 87,88, and the split time of the African lineages and Asia-Australasia lineages seems to fit the time frame of the overland migration hypothesis (Fig. 3b). However, no species or fossil records of Pyrrosia s.l. have been reported in northern Africa or the adjacent Asian area, which weaken the support for this hypothesis. Overland migration is therefore an alternative dispersal scenario. Transoceanic long-distance dispersal has been used extensively to explain the intercontinental disjunction of plants 49,52–54,57,82,83,89,90. Fern spores are minute, and like wind-dispersed seeds and pollen, they may have been transported transoceanic by prevailing monsoon winds or ocean currents between Africa and northern India 46,53,57,91. Transoceanic long-distance dispersal might be the most plausible hypothesis to clarify the Africa-Asia disjunction of Pyrrosia s.l.

Within Pyrrosia s.s., multiple dispersal events from area B to C and sequentially to area D, and from area B to A can be inferred. Southwest Asia suffered frequent orogenesis, particularly the Himalayan regions, after experiencing many rapid uplifts and unroofing 82. Meanwhile, Southeast Asia also suffered a complex interplay of plate movements and grew incrementally through the addition of continental fragments. A collision with Southeast Asia ca. 25 Ma years ago 83. Frequent and severe geological movements during the Miocene provided the possibility for the dispersal between areas. The global climate fluctuated dramatically from the late Oligocene, as the monsoon system was established and subsequently strengthened by the late Oligocene warming, mid-Miocene climatic optimum and persistent Miocene cooling 86,84,95. Dramatic climate fluctuation may have triggered the speciation and diversification of Pyrrosia s.l., and frequent habitat fragmentations and range transition may have led to the accumulation of species diversity.

We infer the following diversification scenario for Pyrrosia s.l.: Pyrrosia s.l. originated from Southern Asia, the Indo-China Peninsula and southwestern and southern China (area B) before the Oligocene, and the global climate subsequently underwent a rapid cooling at the late Eocene and the early Oligocene with the temperature declining ca. 5°C 86. Contemporaneously, Southeast Asia, particularly the Himalaya regions, experienced aridification. The sudden cooling and aridification may have been a devastating blow for Pyrrosia s.l. Some of the ancestral species may have migrated or dispersed to the much warmer southern areas and finally reached Africa. It is undisputed that climate cataclysm and long-distance dispersal can cause species extinction. The species of
of Polypodiaceae: Platycerioideae, Loxogrammoideae, Drynarioideae, Microsoroideae, Polypodioideae and Grammitidoideae and its sister group Davalliaceae based on the latest classification for extant lycophytes and ferns, PPGL[1]. In Platycerioideae, all groups of Pyrosia s.l. proposed by Hovenkamp[2] as well as the two previously segregated genera Drynoglossum and Saxiglossum were included. In total, 112 individuals representing 51 species of Pyrosia s.l. were sampled (Supplementary Table S2). One to five individuals from the different regions were collected for each species. 19 individuals were used besides Pyrosia s.l.: Platycerium bifurcatum (Cav.) C. Chr., Pl. coronarium (O.F. Müll.) Desv. and Pl. wallichii Hook were sampled in Platycerium. Samples of Loxogrammoideae, Drynarioideae, Microsoroideae, Polypodioideae and Grammitidoideae and its sister group Davalliaceae were chosen as outgroups for the phylogenetic analyses.

Our taxon sampling strategy was designed to include all six subfamilies of Polypodiaceae: Platycerioideae, Loxogrammoideae, Drynarioideae, Microsoroideae, Polypodioideae and Grammitidoideae and its sister group Davalliaceae based on the latest classification for extant lycophytes and ferns, PPGL[1]. In Platycerioideae, all groups of Pyrosia s.l. proposed by Hovenkamp[2] as well as the two previously segregated genera Drynoglossum and Saxiglossum were included. In total, 112 individuals representing 51 species of Pyrosia s.l. were sampled (Supplementary Table S2). One to five individuals from the different regions were collected for each species. 19 individuals were used besides Pyrosia s.l.: Platycerium bifurcatum (Cav.) C. Chr., Pl. coronarium (O.F. Müll.) Desv. and Pl. wallichii Hook were sampled in Platycerium. Samples of Loxogrammoideae, Drynarioideae, Microsoroideae, Polypodioideae and Grammitidoideae and its sister group Davalliaceae were chosen as outgroups for the phylogenetic analyses.

Materials and Methods
Sample collection. Our taxon sampling strategy was designed to include all six subfamilies of Polypodiaceae: Platycerioideae, Loxogrammoideae, Drynarioideae, Microsoroideae, Polypodioideae and Grammitidoideae and its sister group Davalliaceae based on the latest classification for extant lycophytes and ferns, PPGL[1]. In Platycerioideae, all groups of Pyrosia s.l. proposed by Hovenkamp[2] as well as the two previously segregated genera Drynoglossum and Saxiglossum were included. In total, 112 individuals representing 51 species of Pyrosia s.l. were sampled (Supplementary Table S2). One to five individuals from the different regions were collected for each species. 19 individuals were used besides Pyrosia s.l.: Platycerium bifurcatum (Cav.) C. Chr., Pl. coronarium (O.F. Müll.) Desv. and Pl. wallichii Hook were sampled in Platycerium. Samples of Loxogrammoideae, Drynarioideae, Microsoroideae, Polypodioideae and Grammitidoideae and its sister group Davalliaceae were chosen as outgroups for the phylogenetic analyses.

DNA extraction, PCR amplification, cloning, and sequencing. Total genomic DNA was extracted from silica gel-dried leaves or herbarium material using the Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China) following the manufacturer’s instructions.

For each individual, five cpDNA regions (matK, rbcL, psbA–trnH and rps4 + rps4–trnS) and the nuclear gene region (LEAFY) were separately amplified with standard PCR. The matK region was amplified using primers and PCR protocols introduced by the CBoL Plant Barcoding Working Group (http://www.barcodinglife.org/index.php/Public_Primer_PrimerSearch). The rbcL region was amplified using primers 1F29 and 1351R29, following the PCR protocol described by Hasebe et al.100. The psbA–trnH region was amplified using primers psbAF and trnHR according to the protocol outlined by Chen et al.101. Amplification primers and protocols to amplify rps4 + rps4–trnS were those described by Nadot et al. Smith and Cranfill102,103. LEAFY was amplified using primers 1dF and 3dR, which were designed by Zhao48.

The PCR products were purified using PEG 8000 or the TIANgel Midi Purification Kit (Tiangen Biotech, Beijing, China) following the manufacturer’s protocol. Then they were directly sequenced in both directions using the amplification primers with an ABI PrismTM BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, Norwalk, CT, USA). Sequences were analysed using the ABI 3730XL automated sequencer (Applied Biosystems, Foster City, CA, USA). Cloning of samples with allelic variation in LEAFY was conducted with a pEASY-T3 Cloning Kit according to the manufactures’ protocols (Transgen Biotech), and 6 to 12 clones were sequenced for each sample. GenBank accession numbers are listed in Supplementary Table S2.

Phylogenetic analysis. Sequences were assembled in the ContigExpress program of the Vector NTI Suite v.6.0 (Informax, North Bethesda, Maryland, U.S.A.). New combined sequences were assembled in single-region datasets that were aligned using CLUSTAL X v.18,104 and then manually adjusted in BioEdit v.7.1.1105. Phylogenetic analyses of the combined cpDNA data set and LEAFY were performed using MP, ML and BI in PAUP * 4.0b106, RAxMLv7.0.4107, and MrBayes v3.2.5108, respectively.

In MP analyses, all characters were equally weighted and gaps were treated as missing data. A heuristic search was performed with 1000 random addition replicates, tree bisection-reconnection (TBR) swapping and the MulTrees option in the analysis program. Bootstrap support values (BSmp) based on 1000 replicates with 10 random additions per replicate, and were used to estimate the confidence of the clades. We employed jModelTest v.2.1.7109 to identify the best fitting models for ML and BI analyses. In the ML analyses, GTR + G was determined to be the best-fit model according to the Akaike information criterion (AIC) implemented in jModelTest and the BI analyses used the TIM1 + G model determined by the Bayesian information criterion (BIC) for cpDNA datasets. TrN + 1 + G was determined to be the best model by AIC and BIC for LEAFY. RAxML was conducted with the fast bootstrap option, using 1000 replicates. For BI analyses, four Markov chain Monte Carlo (MCMC) chains were run for 1,000,000 generations each, and were sampled every 1000 generations, starting with a random tree. The convergence of runs and estimation of burn-in were assessed using Tracer v.1.6110 and Bayesian posterior probabilities (PPB) were calculated for the majority consensus tree of all sampled trees after discarding those sampled within the burn-in phase in MrBayes.
Divergence time estimation. Bayesian approaches were employed of cpDNA data set to estimate the divergence times of Pyrrosia s.l. in BEAST v 1.8.011 with an uncorrelated lognormal distributed (UCLD) relaxed clock model, the GTR+G substitution model and a Yule process tree prior. The MCMC chains were run for 100,000,000 generations with sampling every 10000 generations and at least 10% burn-in phase. The tree was calibrat ed at the most basal node (Polypodiaceae and Davalliacae, 60.4 Ma) obtained from divergence time estimate carried out with leptosporangiate ferns46. The effective sample size (ESS) was estimated in Tracer v.1.6 to be >200 for each parameter. The maximum clade credibility tree with median branch lengths and a 95% highest HPD interval on nodes was compiled using TreeAnnotator v.1.8.0.

Ancestral area reconstruction. Five biogeographical regions were defined: (A) eastern Asia (including central, eastern and northeastern China, Korea Peninsula, southern Japan and Far East); (B) southern Asia, the Indo-China Peninsula and southwestern and southern China; (C) Malesia (including Malaysia, Indonesia, and the Philippines); (D) Australasia (including Australia, New Guinea, New Zealand, and the South Pacific islands); and (E) Africa (including Madagascar) (Fig. 3a). The geographical distribution of Pyrrosia s.l. mostly followed Hovenkamp4 and referred to other literatures3,8,32. We carried out biogeography analyses using the DEC model12,13 implemented in RASP 3.214. We inferred possible biogeographical scenarios across 1000 trees obtained from the BEAST analysis in the DEC analysis. A composite Akaikes weight was used to summarize biogeographic reconstructions across trees15. The maximum area number was set to four because only P. lanceolata occupied four biogeographical regions.

Morphological character evolution. The data employed for the reconstruction of the evolution of morphological characters were obtained and our own observations of morphological character variation using herbarium specimens (Supplementary Table S3) and referred to the previous publications3-8,19-26. In addition, we considered observations made during fieldwork.

The evolution of morphological characters was reconstructed with likelihood using Mesquite v.3.0416. We input the tree set obtained from the ML analysis based on a simple cpDNA data set (supplementary Fig. S1). In order to exhibit the morphological characters clearly in the phylogenetic tree at species level, this data set includes one individual for each species. Due to the intraspecific variation of the sequences are small, we chose one individual randomly for each species. All characters were scored as discrete binary or multistate characters and treated as unordered and equally weighted (Supplementary Table S4).

References
1. The Pteridophyte Phylogeny Group. A community-derived classification for extant lycophytes and ferns. J. Syst. Evol. 54, 563–603 (2016).
2. Shing, K. H. & Iwatsuki, K. On the fern genus Pyrrosia Mirbel (Polypodiaceae) in Asia and adjacent Oceania: 2. J. Jpn. Bot. 72, 72–88 (1997).
3. Shing, K. H. A Reclassification of the FernGenus Pyrrosia. Am. Fern J. 73, 73–78 (1983).
4. Hovenkamp, P. H. A Monograph of the Fern Genus Pyrrosia (Polypodiaceae). (E. J. Brill/Leiden University Press, 1986).
5. Holtum, R. E. Flora of Malaya. Vol. II. Ferns of Malaya. (ed. Holtum, R. E.) (Government Printing Office, Singapore, 1954).
6. Nayar, B. K. & Chandra, S. Morphological Series within the Genus Pyrrosia, and Their Phylogenetic Interpretation. Can. J. Bot. 45, 615–634 (1967).
7. Nayar, B. K. Studies in Polypodiaceae IV. Drymoglossum Presl. J. India Bot. Soc. 66, 169–179 (1957).
8. Lin, Y. X., Chang, X. C. & Hovenkamp, P. H. In Flora of China Vol. 3 (eds Wu, Z.Y., Raven, P. H. & Hong, D. Y.) 786–786 (Science Press; Missouri Botanical Garden Press, 2013).
9. Wu S. H. & Ching R. C. Fern families and genera of China. 485–549 (Science Press, 1991).
10. Hennipman, E. The systematics of the Polypodiaceae. (eds Baas, P., Kalkman, K. & Geesink, R.) 105–120 (Springer Netherlands, 1990).
11. Winter, K., Osmond, C. & Hubick, K. Crassulacean acid metabolism in the shade. Studies on an epiphytic fern, Pyrrosia longifolia, and other rainforest species from Australia. Oecologia 68, 224–230 (1986).
12. Winter, K., Osmond, C. & Hubick, K. Krassulacean acid metabolism in the shade. Studies on an epiphytic fern, Pyrrosia longifolia, and other rainforest species from Australia. Oecologia 68, 224–230 (1986).
13. Young, S. C. & Hew, C. S. Diffusive Resistance, Titratable Acidity, and CO2 Fixation in Two Tropical Epiphytic Ferns. Am. Fern J. 66, 121–124 (1976).
14. Winter, K., Osmond, C. & Hubick, K. Crassulacean acid metabolism in the shade. Studies on an epiphytic fern, Pyrrosia longifolia, and other rainforest species from Australia. Oecologia 68, 224–230 (1986).
15. Griffiths, H., Ong, B., Avadhani, P. & Goh, C. Recycling of respiratory CO2 during crassulacean acid metabolism: alleviation of photoinhibition in Pyrrosia piloselloides. Plants 179, 115–122 (1989).
16. Shing, K. H. & Iwatsuki, K. On the fern genus Pyrrosia Mirbel (Polypodiaceae) in Asia and adjacent Oceania: 2. J. Jpn. Bot. 72, 19–35 (1997).
17. Shing, K. H. & Iwatsuki, K. On the fern genus Pyrrosia Mirbel (Polypodiaceae) in Asia and adjacent Oceania: 2. J. Jpn. Bot. 72, 19–35 (1997).
18. Nayar, B. K. & Chandra, S. Morphological Series within the Genus Pyrrosia, and Their Phylogenetic Interpretation. Can. J. Bot. 45, 615–634 (1967).
19. Nayar, B. K. Studies in Polypodiaceae IV. Drymoglossum Presl. J. India Bot. Soc. 66, 169–179 (1957).
20. Minardi, B. D., Voytena, A. P. & Randi, A. M. The epiphytic fern
21. Nayar, B. K. Morphology of spores and prothalli of some species of Polypodiaceae. Botanical Gazette 123, 223–232 (1962).
22. Kottman, K., Thammahaworn, A. & Chantaranothai, P. Comparative Anatomy of the Genus Pyrrosia Mirbel (Polypodiaceae) in Thailand. Nat. Hist. J. Chulalongkorn Univ. 7, 75–85 (2007).
23. Uffele, G. A. Synapsotroposy in the fern genus Pyrrosia (Polypodiaceae). Blumea 31, 57–64 (1985).
24. Uffele, G. A. V. & Hennipman, E. The Spores of Pyrrosia Mirbel (Polypodiaceae), a SEM study. Pollen Spores 27, 155–198 (1985).
25. Yang, L. H. A Systematic Study on the Fern Genus Pyrrosia Mirbel. Ph. D. thesis, (Yunnan University, 2012).
26. Ching, R. C. On the fern genus Pyrrosia Mirbel from the Mainland of Asia including Japan and Formosa. Acta Phytotax. Geobot. 4, 180–181 (1935).
27. Giesenhan, K. F. G. Die FarnGattung Niphobolus (Verlag von Gustav Fischer 1901).
28. Nayar, B. K. & Chandra, S. Ferns of India, XV, Pyrrosia Mirbel. Bull. Natl. Bot. Gard. Lucknow 117, 1–98 (1965).
29. Copeland, E. Genera Filicum-the Genera of Ferns., 189 (The Chronica Botanica Co., Waltham, Mass., 1947).
30. Pichi-Sermolli, R. E. G. Tentamen Pteridophytorum genera in taxonomic ordinem redigendi. Webbia 31, 313–512 (1977).
31. Ravensberg, W. & Hennipman, E. The Pyrrosia species formerly referred to Drymogllossum and Saxiglossum (Filicales, Polypodiaceae). Leiden Botanical Series 9, 281–310 (E.J. Brill/Leiden University Press, 1986).
32. Lin, Y. X. In Flora Reipublicae Popularis Sinicae Vol. 62(2) (ed. Delectas Flora Reipublicae Popularis Sinicae Academiae Sinicae Edita) 116–155 (Science Press, 2000).
33. Liu, H. M. Embracing the pteridophyte classification of Ren-Chang Ching using a generic phylogeny of Chinese ferns and lycophytes. J. Syst. Evol. 54, 307–335 (2016).
34. Smith, A. R. et al. A classification for extant ferns. Taxon 55, 705–731 (2006).
35. Zhou, X. M. et al. A plastid phylogeny and character evolution of the Old and New world fern genus Pyrrosia (Polypodiaceae) with the description of a new genus: Hovenkampia (Polypodiaceae). Mol. Phylogenet. Evol. 114, 271–294 (2017).
36. Christenhusz, M. J. & Chase, M. W. Trends and concepts in fern classification. Mol. Phylogenet. Evol. 81, 309–314 (2016).
37. Xu, B., Wu, N., Gao, X. F. & Zhang, L. B. Analysis of DNA sequences of six chloroplast and nuclear genes suggests incongruence, introgression, and incomplete lineage sorting in the evolution of Lespedeza (Fabaceae). Mol. Phylogenet. Evol. 62, 346–358 (2012).
38. Vijayakanth, P. & Sahaya, S. S. Pyrrosia porosa (C. Presl) Hovenkamp – A new diploid cytotype of south India from Kollil Hills of eastern Ghats, Tamil Nadu, India. Int. J. Plant. Biol. Res. 5, 1057–1058 (2017).
39. Pal, S. & Pal, N. Spore morphology and taxonomy of Polypodiaceae. Grana 10, 141–148 (2009).
40. Christensen, C. Revision of the polypodi genera with longitudinal coenosori (Cochlidiinae and "Drymoglossinae"); with a discussion of their phylogeny. Dansk Botanisk Arkiv 6, 3–93 (1929).
41. Wang, L. et al. A molecular phylogeny and a revised classification of tribe Lepiosoreae (Polypodiaceae) based on an analysis of four plastid DNA regions. Bot. J. Linn. Soc. 162, 28–38 (2010).
Acknowledgements
The authors gratefully acknowledge Dr. Bing Liu (IBCAS), Dr. Yea-Chen Liu (NCYU), Dr. Zhong-Yang Li (GNNU) and Mr. Qi Wei (Roy Garden) for providing biological materials. We are grateful to Ms. Hui-Juan Zhao (IBCAS) for providing two sequences of Platycerium bifurcatum (Cav.) C. Chr. (MF450485, MF450486). We are grateful to Dr. Yea-Chen Liu (NCYU) for his helpful suggestion. We are grateful to the editor Dr. J. Heinrichs and Dr. Xinwei Xu and three anonymous reviewers for their very valuable suggestion in improve our manuscript. This study was financially supported by the National Natural Science Foundation of China (Grant No. 81573531) and the CAMS Initiative for Innovative Medicine (CAMS-12M) (Grant No. 2016-12M-2-003).

Author Contributions
X.W., Y.Q., X.Z., H.L., and B.Z. conceived and designed the study. X.W., Y.Q. and X.Z. performed the research. X.W. and L.L. performed the experiments. X.W., Y.Q., H.S. and R.W. analyzed the data. X.W. and Y.Q. wrote the manuscript. All authors revised the manuscript.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-017-12839-w.

Competing Interests: The authors declare that they have no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2017