

Phylogeny of cryptogrammoid ferns and related taxa based on *rbcL* sequences

Gangmin Zhang, Xianchun Zhang and Zhiduan Chen

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Cryptogrammoid ferns were often classified into the cheilanthoid group (of Pteridaceae sensu Tryon et al.) or Sinopteridaceae sensu Ching. In order to elucidate the phylogenetic relationships of cryptogrammoid ferns and their related taxa, we sequenced the chloroplast *rbcL* gene for 11 Asian species representing *Cryptogramma*, *Onychium*, *Aleuritopteris*, *Leptolepidium*, *Cheilosoria*, and *Doryopteris*. Sequences of other related groups were obtained from GenBank. Phylogenetic analysis of a data matrix including 32 taxa was conducted using the maximum-parsimony (MP) and neighbor-joining (NJ) methods. The results were as follows: (1) *Cryptogramma*, *Coniogramme*, and *Llavea* form a moderately supported clade and constitute a cryptogrammoid group that is distantly related to the cheilanthoid ferns. (2) *Onychium* is rejected from both the cheilanthoid and the cryptogrammoid group, but closely related to *Actiniopteris* and *Pteris*. (3) The cheilanthoid group is monophyletic, which is supported by a high bootstrap value. It comprises most of the genera in Sinopteridaceae sensu Ching and some genera in Hemionitidaceae sensu Pichi Sermolli. (4) In the cheilanthoid group, several genera from the Old World (including *Aleuritopteris*, *Leptolepidium*, *Cheilosoria*, and *Sinopteris*), which have been subsumed into *Cheilanthes* by some authors, are grouped together and distantly related to the American taxa of *Cheilanthes*. The present study supports the treatment of the cryptogrammoids as either a separate family or subfamily of a broadly circumscribed Pteridaceae, but Pichi Sermolli's circumscription of Cryptogrammeaceae should be emended to include *Coniogramme* and to exclude *Onychium*.

G. Zhang, X. Zhang and Z. Chen, Laboratory of Systematic and Evolutionary Botany & Herbarium, Institute of Botany, Chinese Academy of Sciences, Xiangshan, Nanxincun 20#, Beijing 100093, China. E-mail: zhangxc@ns.ibcas.ac.cn.

Introduction

The family Cryptogrammeaceae was proposed by Pichi Sermolli (1963) for the traditional concept of cryptogrammoid ferns that includes three genera: *Cryptogramma*, *Llavea*, and *Onychium*. It differs from other related taxa in its green or stramineous stipe and narrow fertile segments with continuous indusia. The phylogeny of cryptogrammoids has

long been in dispute. Ching (1978) did not accept Cryptogrammeaceae and put the above three genera into his Sinopteridaceae. This treatment (Ching 1978) has been followed by some pteridologists (Kung 1988; Shing & Wu 1990; Wang & Wang 2001). Based on a morphometric analysis, Guo et al. (1992) concluded that *Onychium* was distantly related to *Cryptogramma* and the cheilanthoid ferns, and suggested that *Onychium* should be excluded

from Sinopteridaceae. Tryon & Tryon (1982) included *Cryptogramma*, *Llavea*, and *Onychium* as part of tribe Cheilantheae in a broadly circumscribed family Pteridaceae. In their subsequent taxonomic treatment (Tryon et al. 1990), *Onychium* was transferred into Pteridaceae subfamily Taenitidoideae, whereas *Cryptogramma* and *Llavea* remained in subfamily Cheilantheoideae.

The phylogeny of the three genera (*Cryptogramma*, *Llavea*, and *Onychium*) discussed above and their related groups has not been resolved satisfactorily based solely on studies involving sporophytic morphological characters. Recent analyses have shown that sequences of the chloroplast gene *rbcL* are useful to help elucidate the relationships at higher taxonomical levels in various groups of Pteridophyta and specifically in Pteridaceae sensu lato (Hasebe et al. 1995; Gastony & Rollo 1995, 1998; Gastony & Johnson 2001). For example, *Llavea*, *Coniogramme*, and *Onychium* should be removed from the cheilanthoid ferns (Gastony & Rollo 1995, 1998; Gastony & Johnson 2001), and the former two formed a clade supported by a high bootstrap value (Gastony & Rollo 1995, 1998). However, only one species of *Onychium* and two Asian species of cheilanthoid ferns were sampled, and *Cryptogramma* was not included in the previous molecular phylogenetic analyses.

In this study, we sequenced the chloroplast *rbcL* gene for 11 Asian species representing *Cryptogramma*, *Onychium*, *Aleuritopteris*, *Leptolepidium*, *Cheilosoria*, and *Doryopteris*, and conducted a molecular phylogenetic analysis in order to improve our understanding of the relationships among these ferns. The aim is to address the following questions:

(1) determine whether the family Cryptogrammaceae sensu Pichi Sermolli is monophyletic; (2) elucidate the phylogenetic relationships among *Cryptogramma*, *Llavea*, *Onychium* and their related groups, such as cheilanthoid and pteroid ferns; (3) evaluate previous treatment of cryptogrammoid ferns.

Materials and methods

Samplings of taxa

The origin of materials is listed in Table 1. All vouchers are deposited in the National Herbarium (PE), Institute of Botany, the Chinese Academy of Sciences. The taxonomy of 11 newly sequenced taxa provisionally follows Ching's (1978) system: one species of *Cryptogramma* (*C. brunoniana*); three species of *Onychium* (*O. plumosum*, *O. tenuifrons*, and *O. contiguum*); seven species represent *Aleuritopteris*, *Leptolepidium*, *Cheilosoria*, and *Doryopteris*. These sequences were analyzed together with those of 21 additional related species that were obtained from GenBank and an as-yet unpublished sequence (*Pellaea calomelanos*) generated by professor Gerald J. Gastony of Indiana University.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from silica-gel-dried leaves following the CTAB method of Doyle & Doyle (1987). About 50 mg leaf material from each of the specimens was used.

The *rbcL* gene was amplified with primers 1F [5'-

Table 1. Origin of materials.

Taxa	Origin	Voucher	GenBank accession number
<i>Onychium contiguum</i> Hope	Simla, India	G. M. Zhang 459	AY266416
<i>O. plumosum</i> Ching	Muli, Sichuan, China	G. M. Zhang 280	AY266408
<i>O. tenuifrons</i> Ching	Muli, Sichuan, China	G. M. Zhang 281	AY266415
<i>Cryptogramma brunoniana</i> Wall. ex Hook. et Grev.	Bomi, Xizang, China	S. Y. Dong S9	AY266407
<i>Aleuritopteris niphobola</i> (C. Chr.) Ching	Kangding, Sichuan, China	G. M. Zhang 258	AY266409
<i>A. argentea</i> (Gmél.) Fée	Donglingshan Mt., Beijing, China	G. M. Zhang 460	AY266410
<i>A. albomarginata</i> (C. B. Clarke) Ching	Simla, India	G. M. Zhang 457	AY266411
<i>A. grisea</i> (Blanford) Panigrahi	Zhongdian, Yunnan, China	G. M. Zhang 326	AY299653
<i>Leptolepidium kuhnii</i> (Milde) K. H. Shing et S. K. Wu	Donglingshan Mt., Beijing, China	G. M. Zhang 461	AY266412
<i>Cheilosoria insignis</i> (Ching) Ching et K. H. Shing	Gyaca, Xizang, China	S. Y. Dong S32	AY266413
<i>Doryopteris concolor</i> (Langsd. et Fisch.) Kuhn	Changjiang, Hainan, China	S. Y. Dong 178	AY266414

ATG TCA CCA CAA ACA GAA ACT-3'] (Chen et al. 1998) and 1351R [5'-CTT CAC AAG CAG CAG CTA GTT CAG GAC TCC-3'] (Gastony & Rollo 1995). PCR was carried out in a MJ Research PTC-200 machine with a 25 μ L volume, containing about 10-40 ng plant DNA, 1.5 mM MgCl₂, 250 μ g BSA, 0.5 mM dNTP, 2 μ M of each primer, and 1 unit of Taq polymerase. PCR procedures were as follows: Two cycles of 1 min at 94! for template denaturation, 20 s at 48! for primer annealing, and 1 min at 72! for primer extension. Then 35 cycles of 20 s at 94!, 20 s at 48!, and 1 min at 72!, followed by a final extension of 7 min at 72 !. All PCR products were purified using a 1.5 % agarose gel (1 \times TAE) following the protocol of Pharmacia's GFX™ PCR DNA and Gel Band Purification Kit prior to being sequenced.

The internal primer 440F [5'-GGT AAT GTT TTT GGA TTT AAG GC-3'] designed by Gastony & Rollo (1995) was also used for sequencing *rbcL* in this study. The sequencing reactions were carried out using the dideoxy chain termination method with ABI PRISM™ BigDye Terminator Cycle Sequencing Ready Reaction Kit. Sequencing products were analyzed on an ABI 377 Automated DNA Sequencer (PE Applied Biosystems).

Phylogenetic analysis

The 1219-bp region between base pairs 51 and 1269 of *rbcL* gene, numbered from the initial codon of cheilanthoid ferns (Gastony & Rollo 1995), was used for the phylogenetic analysis. Sequences were aligned with the CLUSTAL W package (Higgins 1994) without any manual adjustment. Phylogenetic analyses were performed with PAUP* 4.0b4a (Swofford 1998) using the maximum parsimony (MP) method. All character states were specified as unordered and equally weighted. Heuristic parsimony searches were conducted with 1000 replicates of random addition of sequences, in combination with ACCTRAN character optimization and MULPARS + TBR branch swapping and STEEPEST DESCENT options set on to search for multiple islands of the most parsimonious trees (Maddison 1991). Bootstrap values were calculated from 1000 replicates with a single random addition of sequences. In addition, a distance tree was constructed with the neighbor-joining (NJ) method. Two species of Dennstaedtiaceae were selected as the outgroup, based on the previously published broad-scale analysis (Hasebe et al. 1995).

Results

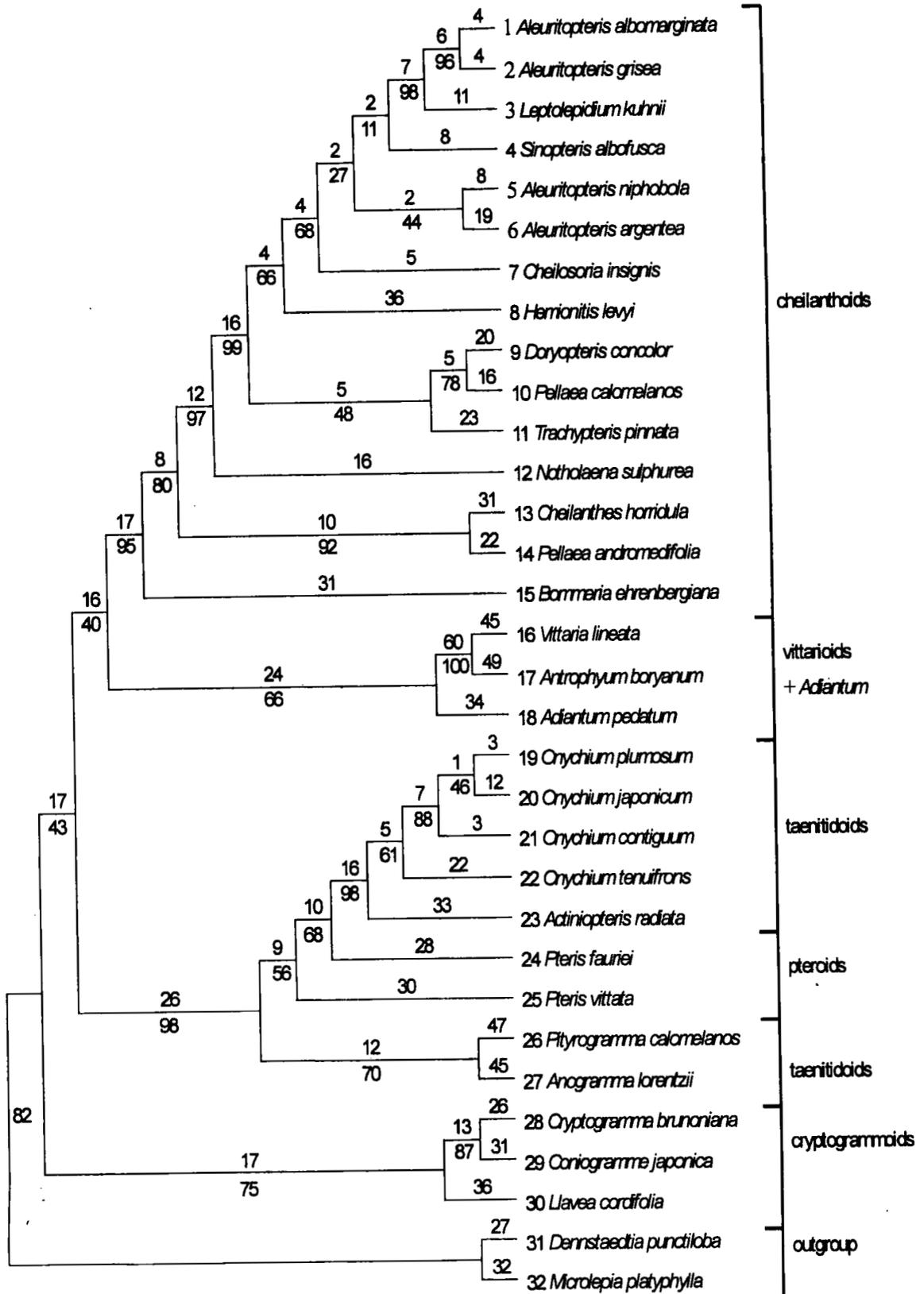
The data matrix for the 32 taxa contained 411 variable sites (33.7 %), of which 304 were phylogenetically informative. During the first random addition sequence replicate, PAUP* found two equally most parsimonious trees of 1172 steps with a consistency index (CI) of 0.4881, and a retention index (RI) of 0.5119. No new minimal trees were found during the next 999 random replicates, suggesting that all of the most parsimonious trees have been found. The two equally most parsimonious trees varied only by swapping the relative positions of *Onychium japonicum* and *O. contiguum*.

One of the two equally most parsimonious trees is depicted as Fig. 1. There are four main clades within ingroup, i. e., cheilanthoids (taxa from 1 to 15), vittarioids + *Adiantum* (taxa from 16 to 18), taenitidoids + pteroids (taxa from 19 to 27), and cryptogramroids (taxa from 28 to 30), but the relationships among these major groups were not resolved satisfactorily because of low bootstrap values (<50%) for some clades. All of the above constituted the main part of Pteridaceae sensu Tryon et al. plus Vittariaceae. Their monophyly was strongly supported by a bootstrap value of 100%.

Cryptogramma, *Coniogramme*, and *Llavea* formed a moderately supported clade. These three genera constituted the cryptogramroid group, which was sister to all other members of the ingroup in MP tree (Fig. 1).

Four species of *Onychium* were grouped together and sister to *Actiniopteris* in a clade supported by a high bootstrap value (98%). The *Onychium* + *Actiniopteris* clade was united with two species of *Pteris*, which did not form a separate clade. *Pityrogramma* and *Anogramma* (members of the subfamily Taenitidoideae sensu Tryon et al.) were grouped together and sister to the above taxa. All of them (19-27) then formed a clade supported by a bootstrap value of 98%.

At the top of MP tree, all Asian species representing *Aleuritopteris*, *Leptolepidium*, *Sinopteris*, and *Cheilosoria*, which have been subsumed into *Cheilanthes* by some authors (Tryon & Tryon 1982; Tryon et al. 1990), formed a clade with a bootstrap value of 68%. They were quite deeply separated from American *Cheilanthes* represented by *C. horridula*. Within the Asian group, some subclades collapsed in the bootstrap test, and the intergeneric relationships were not resolved satisfactorily. *Doryopteris concolor*, *Pellaea calomelanos*, and *Trachypteris pinnata* formed a clade that was sister to the above group. All of the above taxa were robustly embedded within the cheilanthoid group



and far separated from the cryptogrammoid ferns.

The NJ tree obtained using Kimura's two-parameter model (Kimura 1980) is shown in Fig. 2. Its topology is basically consistent with that of MP tree. The main difference between the two analyses is in the position of vittarioid + *Adiantum* group. In NJ tree it was basal to the rest of the ingroup, but this structure has a low bootstrap value (57%). In addition, some clades were with higher bootstrap support in NJ tree. For example, *Cryptogramma brunoniana* and *Coniogramme japonica* were grouped together with a bootstrap value of 97%, and these two genera were sister to *Llavea* with strong support (BS=99%). Just as in the MP tree, intergeneric relationships among *Aleuritopteris*, *Cheilosoria*, and *Sinopteris* were poorly resolved because of low bootstrap support.

Discussion

Composition of cryptogrammoid ferns

Cryptogrammaceae sensu Pichi Sermolli comprises three genera: *Cryptogramma*, *Llavea*, and *Onychium*. This is the traditional concept of cryptogrammoid ferns. Based on *rbcl* gene sequence analyses, *Onychium* is robustly rejected from the cryptogrammoid group, but *Coniogramme* has a close relationship with *Cryptogramma* and *Llavea*. The latter three genera form a clade (with a high bootstrap value in the NJ tree of Fig. 2) and constitute the cryptogrammoid group.

Cryptogramma is a small genus distributed in the temperate and boreal regions of the Northern Hemisphere and South America, while *Llavea* is a monotypic genus of Mexico and Guatemala. These two genera are often placed together in spite of their different distributions (Ching 1940; Pichi Sermolli 1963, 1977; Tryon & Tryon 1982; Tryon et al. 1990). Although the contours of the tuberculate spore morphology of *Llavea* are formed by perispore, unlike the verrucate contours of *Cryptogramma* that are formed by exospore (Tryon & Lugardon 1991), the common characters such as dimorphic leaves, sporangia borne in soral lines along lateral veins, and tetrahedral-globose spores with prominent angles indicate the alliance of the two genera.

Coniogramme is a medium-size genus mainly distributed in subtropical and tropical regions of the

Old World. Based on the character of exindusiate sori spread along with veins, Ching (1940, 1978) and Pichi Sermolli (1977) placed it in Hemionitidaceae. Tryon & Tryon (1982) listed the genus in Pteridaceae tribe Taenitideae. Later, they transferred *Coniogramme* into the subfamily Cheilantheoideae (Tryon et al. 1990).

In the first molecular phylogenetic analysis on cheilanthoid ferns (Gastony & Rollo 1995), *Coniogramme* was strongly grouped with *Llavea*, and the two genera were far removed from the cheilanthoid group as they were in Gastony & Rollo (1998). In our study, *Coniogramme* and *Cryptogramma* form a clade sister to *Llavea*. All of them constitute a cryptogrammoid group. This is consistent with previous results (Gastony & Rollo 1995, 1998) which did not include *Cryptogramma*. Although *Coniogramme* is very different from *Cryptogramma* and *Llavea* in sporophytic features, its tetrahedral-globose spores with slightly tuberculate or verrucate ornamentation (Tryon & Lugardon 1991) suggest an affinity with them.

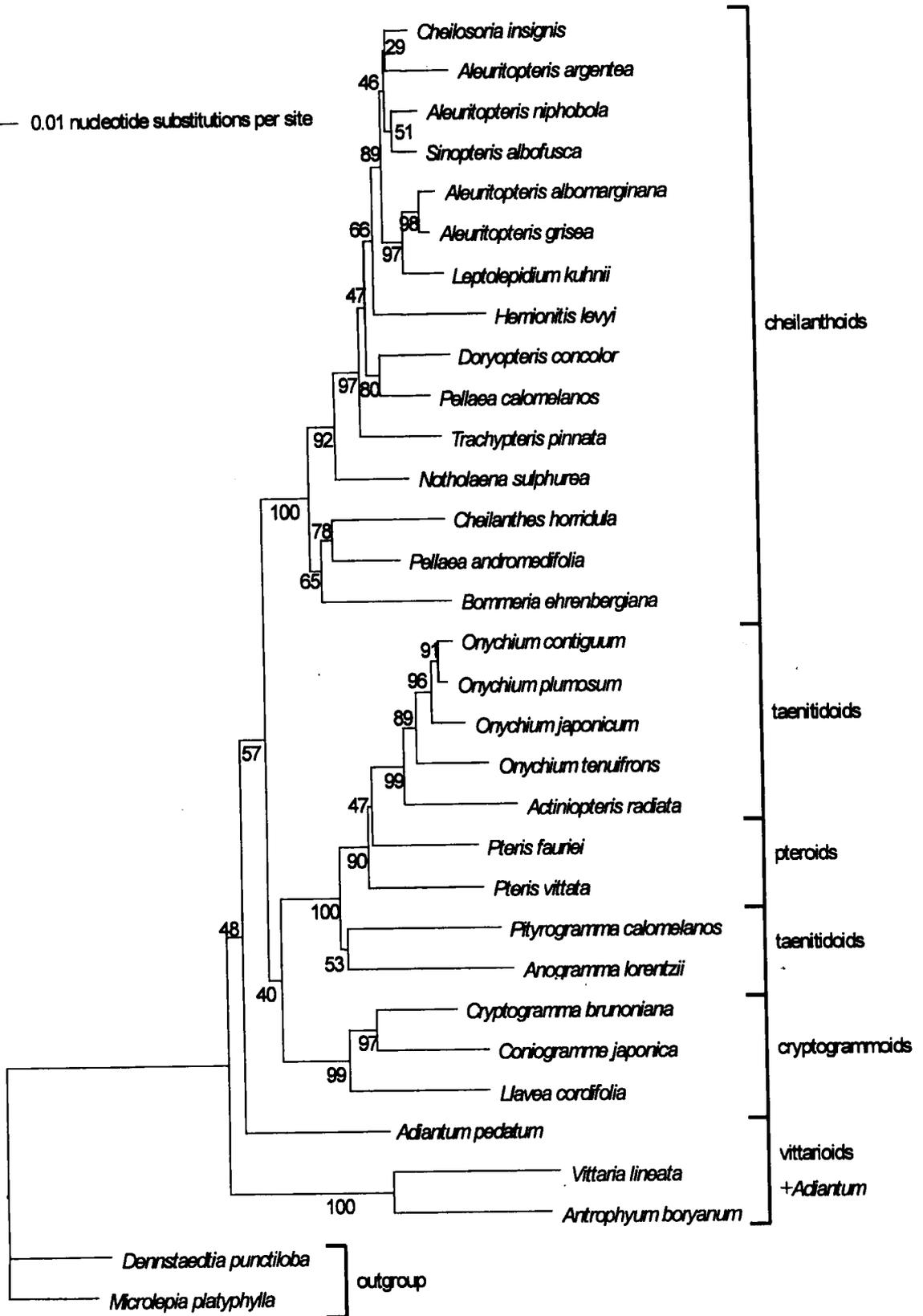
Phylogenetic placement of *Onychium*

The genus *Onychium* was once merged into *Cryptogramma* (Diels 1902). Later, Christensen (1906) recognized it as an independent genus. From then on, it has long been allied with *Cryptogramma* and *Llavea* in various fern classifications (Christensen 1938; Ching 1940, 1978; Pichi Sermolli 1963, 1977; Tryon & Tryon 1982). Based on the evidence from morphology, anatomy, and palynology, Nayar et al. (1972) considered it more suitable to put *Onychium* into the pteroid rather than the cheilanthoid group, and transferred *Onychium* into the family Pteridaceae sensu Nayar (Nayar & Kaur 1974). Tryon et al. (1990) revised their previous opinion (Tryon & Tryon 1982) and put *Onychium* into subfamily Taenitidoideae of Pteridaceae sensu lato.

In the present study, the four species of *Onychium* sampled form a clade with a high bootstrap value, suggesting that the genus is monophyletic and distinctive from *Cryptogramma*. The genus *Onychium* is allied with *Actinopteris*, *Pteris*, *Pityrogramma*, and *Anogramma* and far separated from the cryptogrammoid and cheilanthoid groups. This is also in agreement with the result of Gastony & Rollo (1995).

Fig. 1. One of the two equally most parsimonious trees based on *rbcl* gene sequences (Length = 1172 steps, CI = 0.4881, RI = 0.5119). Branch lengths are indicated above the branches. Bootstrap percentages based on 1000 replicates are provided below the branches. Two species of Dennstaedtiaceae are designated as the outgroup.

— 0.01 nucleotide substitutions per site



In determining the phylogenetic placement of *Actiniopteris radiata*, Gastony & Johnson (2001) found that it was strongly related to *Onychium* (BS=99%), but the relationships between *Actiniopteris* + *Onychium* clade and the other taenitidoid genera (including *Pityrogramma*, *Anogramma*, and *Taenitis*) and *Platyzoma* were only weakly resolved, with some bootstrap values as low as 22% to 53%. All of those genera formed a clade with *Pteris* supported by a bootstrap value of 95%. Recently, Nakazato & Gastony (2003) used maximum likelihood analysis of a larger selection of taenitidoid genera (*Taenitis*, *Eriosorus*, *Jamesonia*, *Anogramma*, *Pityrogramma*, and *Cosentina*) and found that these formed a strongly supported taenitidoid clade. *Actiniopteris* + *Onychium* formed a clade sister to those taenitidoids, and *Pteris* species formed a clade that was sister to the two preceding clades. This topology has only moderate bootstrap support but very strong support from Bayesian posterior probability, suggesting either that the taenitidoids consist of two major subgroups or subclades (one = *Taenitis* through *Cosentina* in the preceding sentence, and the other = *Actiniopteris* + *Onychium*), the total clade of these two elements being sister to *Pteris*, or that *Actiniopteris* + *Onychium* are intermediate between the taenitidoids and pteroids. In this study, *Onychium* and *Actiniopteris* were united with *Pteris* in a clade that was sister to clade formed by *Pityrogramma* and *Anogramma*, but this topology was supported by only a bootstrap value of 56% in Fig. 1. As shown in previous studies (Gastony & Johnson 1995; Nakazato & Gastony 2003) and the present study, the precise positioning of *Onychium* based on *rbcl* is still problematical.

Sporangia of *Onychium* are borne along a marginal commissure, and the spores have prominent equatorial flanges (Tryon & Lugardon 1991; Yu et al. 2001a, b). These character states are similar to those of *Actiniopteris* and *Pteris* and different from those of cryptogrammoid ferns, providing morphological support for the exclusion of *Onychium* from the cryptogrammoid group.

The relationship between the cryptogrammoid and cheilanthoid groups

Sinopteris, *Aleuritopteris*, *Cheilosoria*, *Leptolepidium*, *Doryopteris*, *Pellaea*, etc. are embedded in the

cheilanthoid group, in agreement with earlier studies that included fewer Asian species (Gastony & Rollo 1995, 1998). Tryon & Tryon (1982) and Tryon et al. (1990) did not accept the first four of these genera, instead placing them into a large and admittedly unnatural genus *Cheilanthes*. In this study, *Sinopteris*, *Aleuritopteris*, *Cheilosoria*, and *Leptolepidium* form a clade in both Fig. 1 and Fig. 2, but the relationships among these genera are not well resolved because of low bootstrap support (BS<50%) for some subclades. As was previously shown for *Sinopteris* and *Aleuritopteris* by Gastony & Rollo (1995, 1998), they are far separated from the American *Cheilanthes*, and should belong to a different group. Further investigation is needed to clarify the relationships among them. In the MP tree (Fig. 1), the cryptogrammoid group was at the base of the ingroup, whereas cheilanthoid ferns were more derived. They are only distantly related and should not be classified together. This was also shown for *Coniogramme* and *Llavea* of the cryptogrammoid group by Gastony & Rollo (1995, 1998) and for *Coniogramme* alone by Gastony & Johnson (2001) and by Nakazato & Gastony (2003).

Evaluation of the previous treatment of cryptogrammoid ferns

As shown here, cryptogrammoid ferns are composed of three genera: *Cryptogramma*, *Llavea*, and *Coniogramme*, and this group is far separated from cheilanthoid ferns in both Fig. 1 and Fig. 2. While in Ching's (1940, 1978) system, *Cryptogramma*, *Llavea*, and *Onychium* were placed in the family Sinopteridaceae along with other cheilanthoid genera, including among others *Sinopteris*, *Aleuritopteris*, *Cheilanthes*, *Pellaea*, etc. Other genera, like *Coniogramme*, *Hemionitis*, *Bommeria*, *Pityrogramma*, etc., were classified into a different family, Hemionitidaceae, similar in circumscription to Pichi Sermolli's (1977) concept of that group. Moreover, our study supports earlier hypotheses based upon both morphological and molecular data (Tryon & Tryon 1982; Tryon et al. 1990; Hasebe et al. 1995; Gastony & Rollo 1995, 1998) that the circumscription of cheilanthoid ferns should include not only *Sinopteris*, *Aleuritopteris*, *Cheilanthes*, *Pellaea*, etc., but also such genera as *Hemionitis*, *Bommeria*, etc., which were segregated by Pichi Sermolli (1977) and Ching (1978) as Hemionitidaceae.

Fig. 2. Tree obtained using the neighbor-joining method. The horizontal branch lengths are proportional to the estimated number of nucleotide substitutions per site. Bootstrap values are percentages of 1000 bootstrap replicates.

Tryon et al. (1990) recognized the differences between *Onychium* and the cryptogrammoid ferns and transferred *Onychium* into subfamily Taenitidoideae of Pteridaceae (Tryon et al. 1990). This conclusion is largely congruent with the placement of the genus in the molecular analyses of Gastony & Rollo (1995) and Gastony & Johnson (2001), and also with our results, but as noted above, further molecular data will be necessary to resolve relationships within the pteroid/taenitoid clade adequately. Tryon et al. (1990) included the three genera of cryptogrammoid ferns into their subfamily Cheilantheoideae of Pteridaceae, a position that our data and those of the molecular studies mentioned above do not support.

Comparing the available systems of fern classification, we believe that the cryptogrammoid ferns are best treated as a separate family. However, Pichi Sermolli's (1963) circumscription of Cryptogrammeaceae should be emended to include *Coniogramme* and to exclude *Onychium*. Some pteridologists may prefer to recognize a broadly circumscribed Pteridaceae (sensu Tryon et al. 1990) comprising several subfamilies, but to date no subfamilial combination has been published to segregate specifically the cryptogrammoid ferns from the cheilanthoid groups.

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