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Hymenasplenium inthanonense (Aspleniaceae), a New Fern Species from Doi Inthanon, and its Phylogenetic Status

KUNIO IWATSUKI*, JUN YOKOYAMA** & NORIAKI MURAKAMI***

ABSTRACT

A new species of Aspleniaceae, *Hymenasplenium inthanonense* N. Murak. et J. Yokoy. was described from Doi Inthanon, Thailand. This species is closely related to *H. cheilosorum*, but can be distinguished by it more medial sorus position and less dimidiate pinnae. It is readily recognizable even in mixed populations by its morphological, cytological, ecological and molecular characters. The relatively large (about 10 base pairs) difference in the slowly evolving *rbcL* gene between *H. inthanonense and H. cheilosorum* indicated that they differentiated into two different biological species long ago.

INTRODUCTION

The genus *Hymenasplenium* was first proposed by Hayata (1927) for *Asplenium unilaterale* Lam. due to the peculiar vascular system of its dorsiventral long creeping rhizomes. Most of the other members of Aspleniaceae have short radial rhizomes. It was later reduced to a section of *Aslenium* by Iwastsuki (1975), who recognized five closely related species (*A. unilaterale* Lam. *A. subnormale* Copel., *A. excisum* Presl, *A. obscurum* Blume and *A. cheilosorum* Kunze) and a variety (*A. unilaterale* var. *udum* Atkins.). Recently, molecular phylogenetic analyses using *rbc*Lsequences clearly showed that *Hymenasplenium* is the most basally diverged group in Aspleniaceae and is not closely related to any of the other members of Aspleniaceae (Murakami 1995). Thus, it was recommended that the genus *Hymenasplenium* should be revived instead of *Asplenium* sect. *Hymenasplenium*. Hence, the generic name, *Hymenasplenium* is followed in this paper.

* Faculty of Science, Rikkyo University, Nishi-Ikebukuro, Toshima-ku, Tokyo 171-0021 JAPAN.
** Biological Institute, Graduate School of Science, Tohoku University Aoba-ku, Sendai 980-0845 JAPAN.
*** Department of Botany, Graduate School of Science, Kyoto University, Kitashirakawa-Oiwake-cho, Sakyo-ku, Kyoto 606–8502. JAPAN.

During our 1993–1994 botanical expedition to Vietnam, Laos and Thailand in cooperation with the Forest Herbarium, Royal Forest Department, Thailand, we found a new species, *Hymenasplenium inthanonense*. We first collected this species in a mountain forest at 1735m alt. in a valley of Doi Inthanon, the highest mountain in Thailand. Later, we confirmed its occurrence up to 1895 m alt. in the same valley. This species is endemic to Doi Inthanon and has not been collected from anywhere else.

This new species is similar in gross morphology to *H. cheilosorum*, which co-exists in the mid elevations of Doi Inthanon, but is easily distinguished from it by its more medial sori and not so deeply lobed pinnae. Careful comparison of the morphology, cytology and ecology of these two species in mixed populations was made to examine the validity of creating a new species and found many differences between *H. inthanonense* and *H. cheilosorum*.

The phylogenetic status of the new species was examined by determining the nucleotide sequence of its *rbc*L gene and comparing it with those of possibly related species. The molecular phylogenetic method has been shown to be very powerful in elucidating the phylogenetic relationships of plants (Chase et al 1993; Hasebe et al 1995). The phylogenetic affinity of *H. inthanonense* especially to *H. cheilosorum* was supported by the molecular analyses. Molecular data such as *rbc*L sequences are also useful for finding and identifying intra-morphospecific biological units (Murakami et al 1998). The *rbc*L sequence of *H. inthanonese* was about 10 base pairs different from that of *H. cheilosorum* from Thailand and China. This supports the fact that *H. inthanonense* differentiated from *H. cheilosorum* long ago, and thus should be treated as a different species.

The discovery of this rare endemic species was the result of the botanical inventories for the "Flora of Thailand" and "Flora of Indochina" projects. Recent construction of paved roads and rapid development in Doi Inthanon have threatened the montane forest habitat and survival of this species. The conservation measure of this endangered species should be employed urgently.

MATERIAL AND METHODS

All plant specimens in this study were collected from mountain forests at 1735–1895 m alt. of Doi Inthanon. Two mixed populations of *H. inthanonense* and *H. cheilosorum* at 1735 m were carefully examined. All individuals were mapped, and one fertile leaf from each individual was collected for morphological and cytological investigation. For morphological comparison, pinnae shape and soral position were observed. Spore number per sporangium was counted for each specimen in order to determine their reproductive mode 64 spores are contained in one sporangium of a sexually reproducing fern and 32 in that of an apogamous reproducing one. The validity of this method was confirmed for *Hymenasplenium* by careful cytological observation of spore numbers per sporangium and chromosome numbers of both meiosis and mitosis by Mitui et al (1989).

Plant materials for molecular phylogenetic experiments were collected from the same population at the same time. Thus, the voucher should be the type itself. The methods of extracting DNA from the plant materials and determining the 1,162 nucleotides of the *rbc*L sequence were the same as described in Hasebe et al (1994). For comparison, plant materials of *H. cheilosorum* from the mixed population of Doi Inthanon (voucher: Murakami et al 93–T603) were used. Both sexual and apogamous forms *of H. cheilosorum* can be found in Yunnan, China. Plant materials of *H. cheilosorum* from *Xishuangbanna*, Yunnan Prov., China (voucher: sexual form, Murakami and Cheng 93–C358; apogamous form, Murakami and Cheng 93-C291) were also molecularly analyzed. The *rbc*L sequences of species possibly related to *Hymenasplenium* and Aspleniaceae, which were determined in our previous studies (Murakami 1995), were also used for comparison. For phylogenetic analyses, the computer program PAUP version 3.1.1 (Swofford 1993) was used. Most parsimonious trees were searched by the branch and bound procedure. In order to evaluate monophyly robustness of the trees, bootstrap analyses (Felsenstein 1985) were conducted.

RESULTS AND DISCUSSION

Morphological, cytological and ecological comparison with the most similar species, *H. cheilosorum*

The easiest and most reliable way to recognize a new species is to carefully examine the area where the new species and its closest relatives grow together or nearby. If the new species is recognizable even in mixed populations, it can be a good species in biological meaning.

Fortunately, the new species *H. inthanonense* (Fig. 1a) and its closest relative *H. cheilosorum* (Fig. 1b) grow nearby or sometimes even in mixed populations on Doi Inthanon. We drew distribution maps of two mixed populations and collected leaf specimens of both species (*H. inthanonense*, 33 individuals and *H. cheilosorum*, 23 individuals). We also counted the spore numbers per sporangium to determine the reproductive types of each specimen.

Cytologically, all individuals identified as *H. inthanonense* by their sorus position and pinnae shape had 64 spores per sporangium, indicating sexual reproduction. In contrast, all those identified as *H. cheilosorum* had 32 spores and were determined to be apogamous. The validity of spore counting to estimate cytotypes in *Hymenasplentium* was confirmed by Mitui et al. (1989). Apogamous types of *H. cheilosorum* were reported from India, Ceylon, South-western China, Taiwan and Japan. Therefore, the geographical distribution of apogamous forms is quite wide, in contrast to that of the sexual type *of H. cheilosorum*, which is confined to Xishuangbanna, Yunnan, China (Cheng and Murakami 1998)

Distribution maps of the two species are shown in Fig. 2a and 2b. Though they grow nearby, their preferred habitats are well differentiated. *Hymenasplenium inthanonense* usually grows on steep soil slopes along streams, whereas *H. cheilosorum* tends to grow on stream side rocks. The latter species also grows sometimes in soil, but still closer to the stream than the former species. *Hymenasplenium cheilosorum* may require a wetter

habitat than *H. inthanonense*. Apogamous species often grow in drier habitats than sexual species (Bierzychudek 1985), but this is not the case for these *Hymenasplenium* species.

Phylogenetic status of *H. inthanonense* inferred from *rbc*L sequences

The similarities in gross morphology strongly suggested the phylogenetic affinity between the new species and *H. cheilosorum*. We performed molecular phylogenetic analyses using nucleotide sequencing of the *rbc*L gene in order to examine the phylogenetic status of the new species, *H. inthanonense*. The obtained molecular tree (Fig. 3) clearly showed that *H. inthanonense* is most closely related *to H. cheilosorum*, although there were about 10 base pair differences out of 1,162 (Fig. 4). Such a large difference in the slow evolving *rbc*L gene also supports its validity as a new species. These molecular characters are also useful in identifying morphologically similar species.

Hymenasplenium inthanonense N. Murak. et J. Yokoy., **sp. nov.** (Fig. 1a)

Thailand, Chiang Mai Prov., Doi Inthanon, valley near the trail at the Check Point, at 1735m alt., on soil cliff along stream, in half-deep shade, 12 January, 1994, N. Murakami and J. Yokoyama 93-T602 KYO-holotype; BKF, TI, KUN-isotypes).

Haec species *H. cheilosori* (Kunze) Tagawa similis, sed ab eo differt soris interioribus et pinnis plerumque minus profunde lobatis quam id.

This species is similar to *H. cheilosorum* (Kunze) Tagawa, but different from it by the following characters: its sori are not confined to the pinnae lobes, but are found somewhat further inside from the bottom of the lobes toward costa, and its pinnae are usually not so deeply lobed as those *H. cheilosorum*. Its *rbc*L sequence is about 10 base pairs different from that of *H. cheilosorum* as shown in Fig. 4.

Its *rbc*L sequence is about 10 base pairs different from that of *H. cheilosorum* as shown in Fig. 4.

Rhizome long-creeping, 3–6.5 mm diameter, bearing two rows of alternate fronds closely (2–5 mm apart) on dorsal surface, scaly near apex; scales dark in color, gradually narrowing towards apex, up to 5.5 by 1 mm, clathrate, the cell-walls very thick, Stipe 15–22 cm long, dark purple, polished but dirty on the lower portion bearing scales. Rachis similar colored to stipe, glabrous, grooved above; Frond 30–40 cm long, 4–7 cm wide, pinnate, narrowly lanceolate in outline, subtruncate at base, attenuately acuminate at apex; pinnae 2.0–3.0 cm long, 0.7–1.1 cm wide, 30–40 pairs, subquadrangular, dimidiate, the lower half very narrow, the midrib close to entire lower margin, rounded at apex, truncate at acroscopic base, lobed to 1/7–1/5 way on upper margin, lobes rounded or forked at apex, 1.5–2 mm broad, lower pinnae shortly stalked, thin, pale green; veins all free, distinct and slightly raised on both surfaces, the lateral veins once forked, the two branches passing to each tooth of a lobe; Sori supramedial, not confined to lobes, 2–4 mm long; indusia thin, opening outwardly. Reproduction sexual, with 64 spores per sporangium.

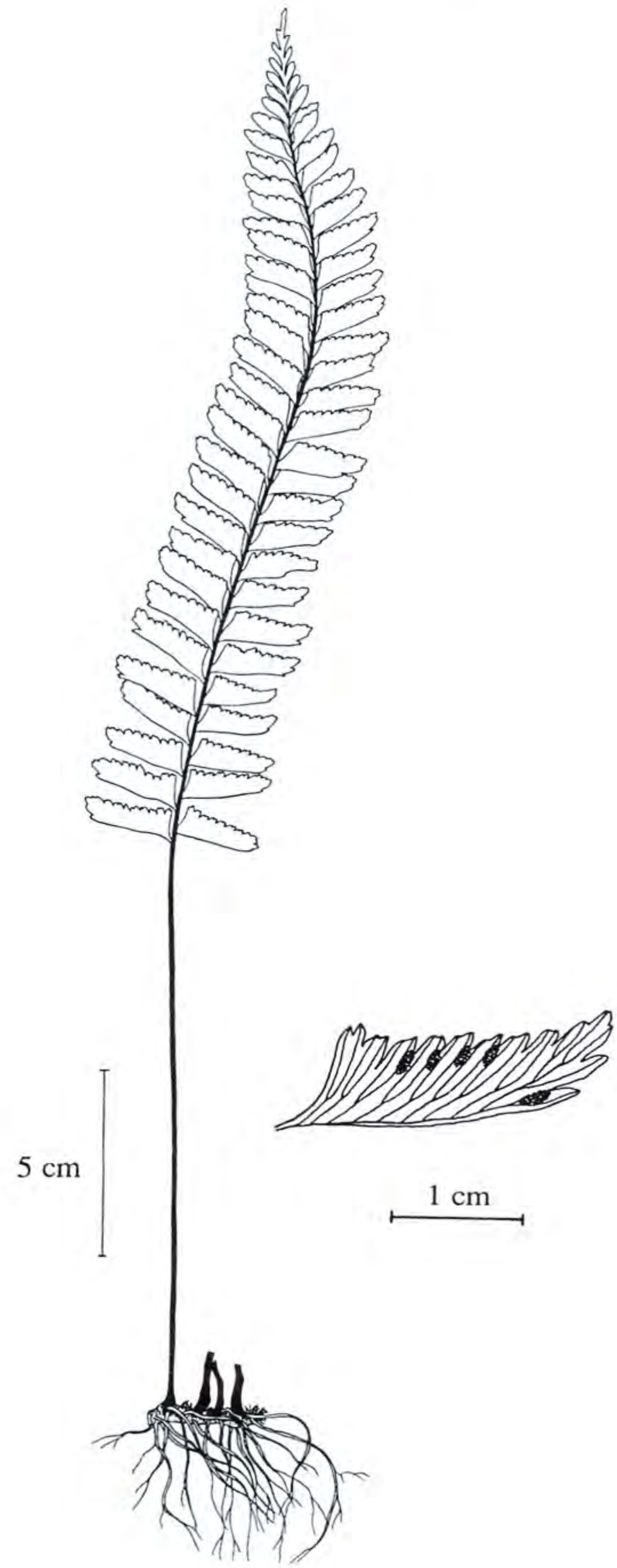


Figure 1a. *Hymenasplenium inthanonense* N. Murak. et J. Yokoy. sp. nov.

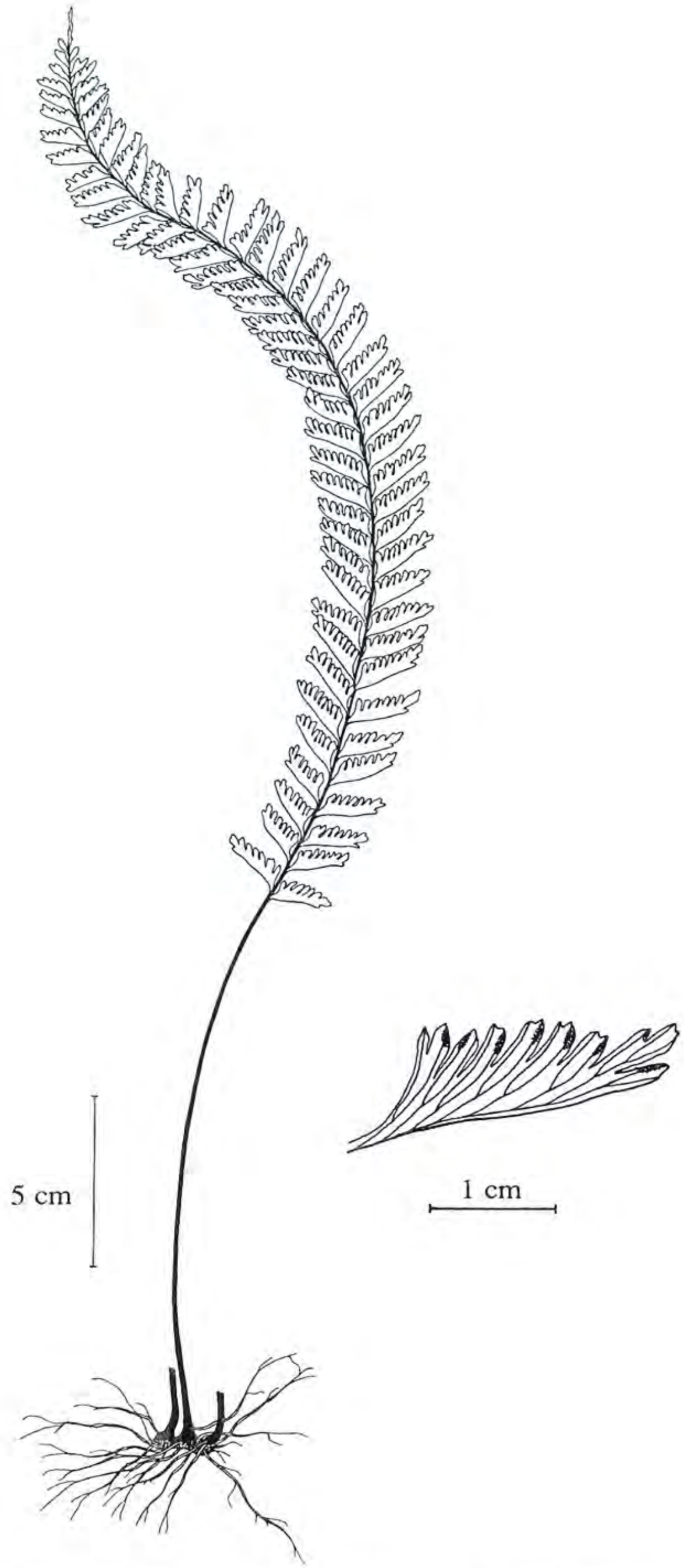


Figure 1b. *Hymenasplenium cheilosorum* (Kunze) Tagawa

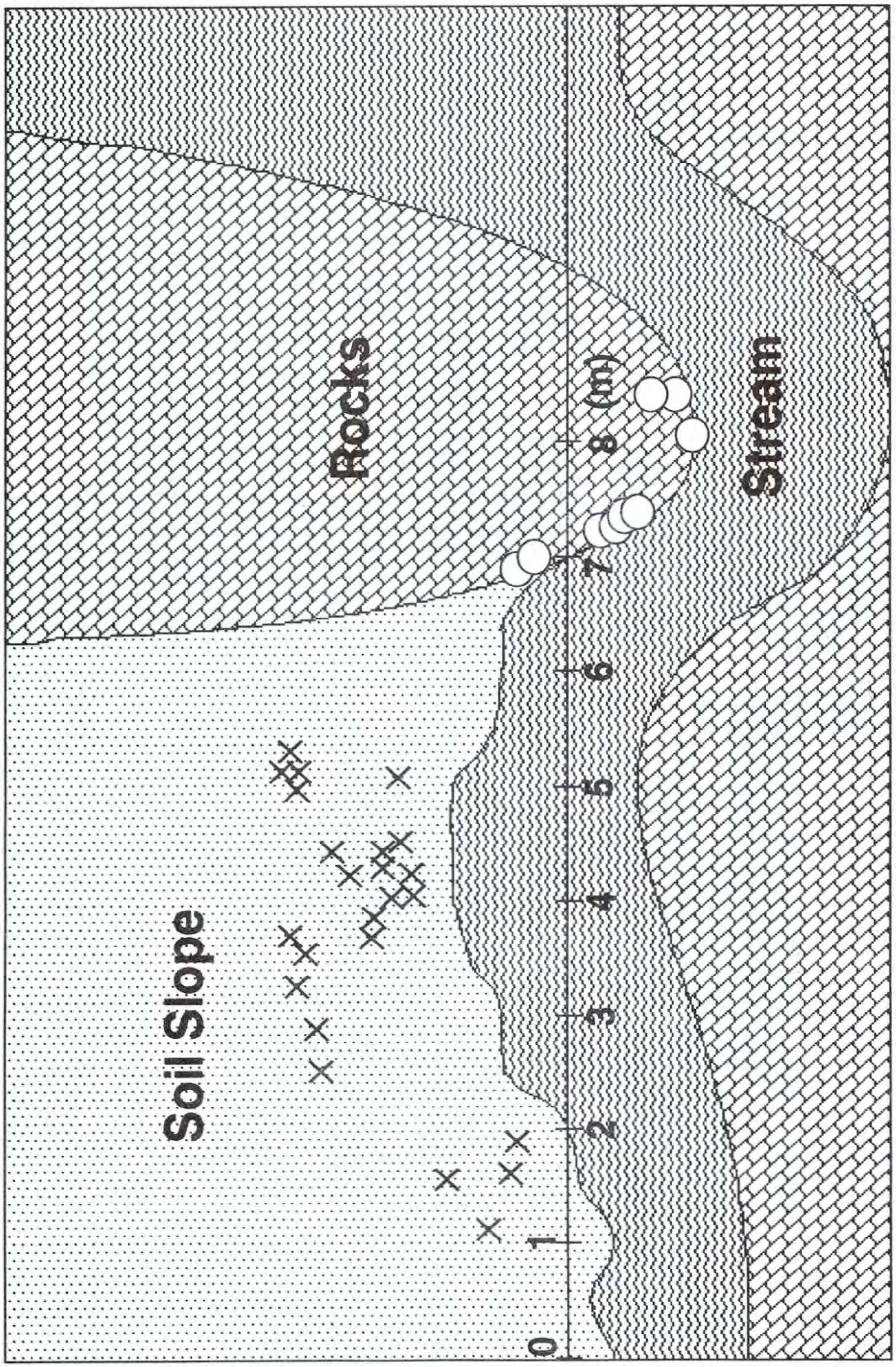


Figure 2a. Distribution map of *Hymenasplenium inthanonense* (X) and *H. cheilosorum* (O) in two areas near the Check Point, 1735 m alt., Doi Inthanon.

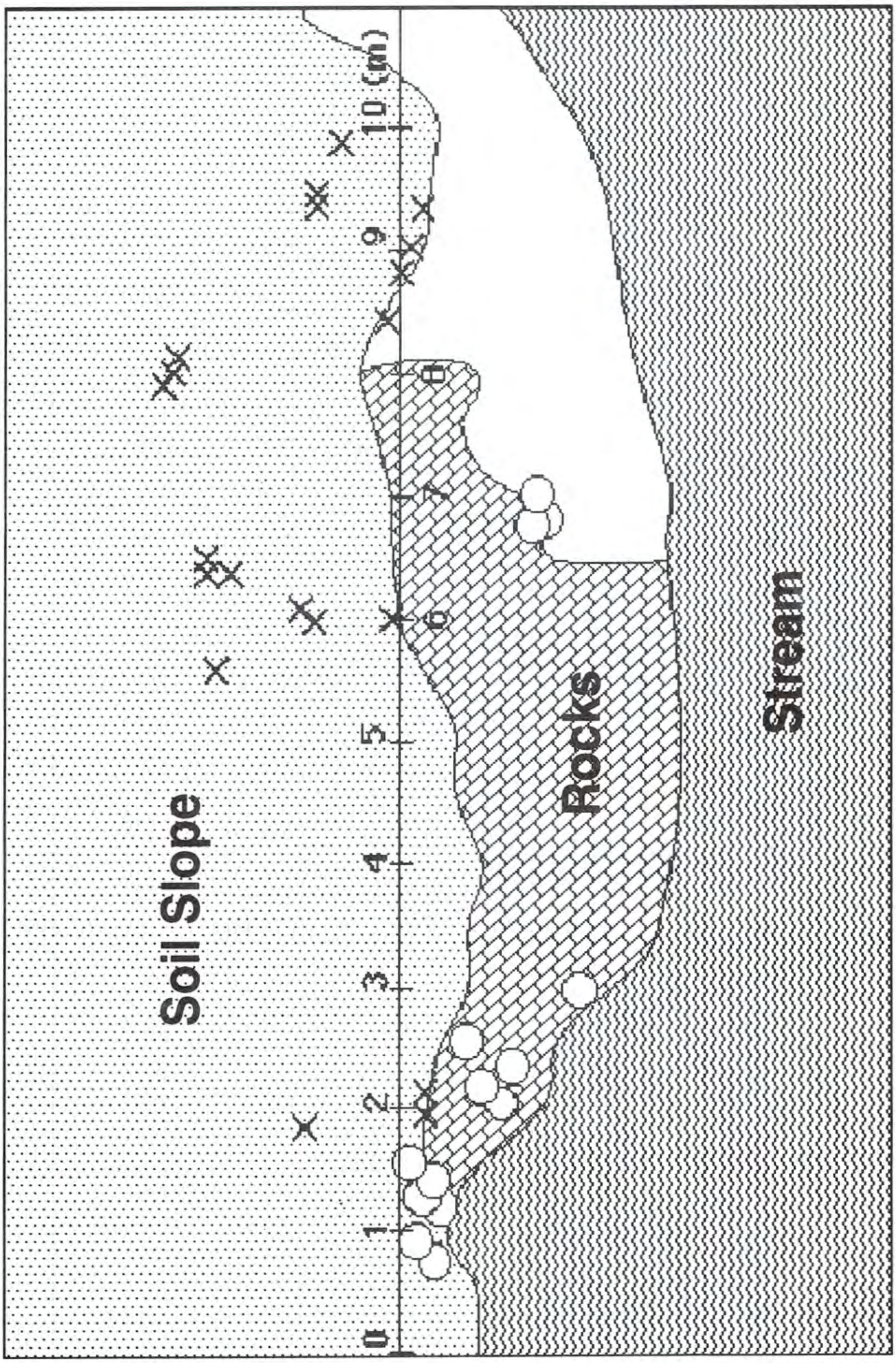


Figure 2a. Distribution map of *Hymenasplenium inthanonense* (x) and *H. cheilosorum* (O) in two areas near the Check Point, 1735 m alt., Doi Inthanon.

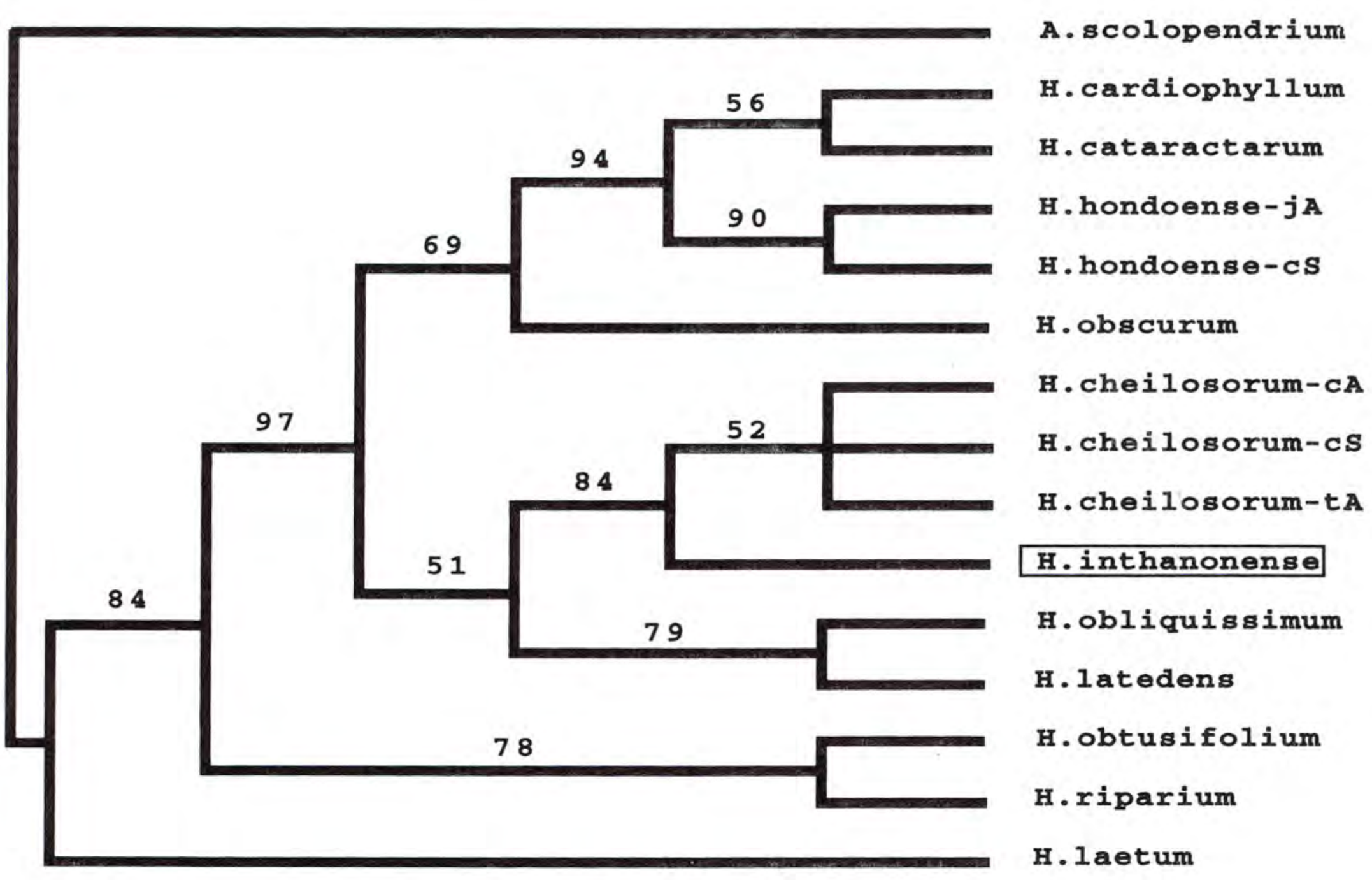


Figure 3. The single most parsimonious tree from the *rbc*L sequence data analysis of *Hymenasplenium inthanonense* and its possible related species. Numbers on branches are the bootstrap percentages.

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H. inthanonense       GACATCTTAGCAGCTTTCCGGATGACCCCACAACCCGGAGTACCAGCTGAAGAAGCCGGA   [60]
H. cheilosorum-thiA   ..T.....................................................
H. cheilosorum-chiA   ..T.....................................................
H. cheilosorum-chiS   ..T.....................................................

H. inthanonense       GCTGCGGTAGCTGCGGAATCCTCCACAGGTACGTGGACCACCGTATGGACAGATGGGTTG   [120]
H. cheilosorum-thiA   .....................................................
H. cheilosorum-chiA   .....................................................
H. cheilosorum-chiS   .....................................................

H. inthanonense       ACCAGCCTTGACCGTTATAAGGGCCGATGCTACGACATCGAGCCCGTCGCTGGGGAGGAA   [180]
H. cheilosorum-thiA   ................C....................................
H. cheilosorum-chiA   ................C....................................
H. cheilosorum-chiS   ................C....................................

H. inthanonense       AACCAGTATATCGCGTATGTAGCTTATCCCTTGGACCTCTTCGAAGAAGGTTCCGTCACC   [240]
H. cheilosorum-thiA   .....................................................
H. cheilosorum-chiA   .....................................................
H. cheilosorum-chiS   .....................................................

H. inthanonense       AACTTATTTACTTCCATTGTAGGTAACGTTTTCGGATTTAAGGCTCTACGTGCCATACGC   [300]
H. cheilosorum-thiA   .....................................................C.....
H. cheilosorum-chiA   .....................................................C.....
H. cheilosorum-chiS   .....................................................C.....

H. inthanonense       TTGGAAGACCTTCGAATCCCCCCCGCTTACTCCAAAACTTTCCTTGGACCGCCTCATGGT   [360]
H. cheilosorum-thiA   .....................................................
H. cheilosorum-chiA   .....................................................
H. cheilosorum-chiS   .....................................................

H. inthanonense       ATTCAGGTCGAAAGGGATAAATTGAACAAATATGGACGTCCCCTACTGGGATGTACGATC   [420]
H. cheilosorum-thiA   .....................................................
H. cheilosorum-chiA   .....................................................
H. cheilosorum-chiS   .....................................................

H. inthanonense       AAGCCAAAATTAGGCCTATCTGCTAAAAATTACGGTAGAGCTGTCTATGAATGCCTTCGT   [480]
H. cheilosorum-thiA   .....................................................
H. cheilosorum-chiA   .....................................................
H. cheilosorum-chiS   .....................................................

H. inthanonense       GGTGGACTTGATTTTACAAAAGATGATGAAAACGTAAACTCGCAACCATTCATGCGTTGG   [540]
H. cheilosorum-thiA   .....................................................
H. cheilosorum-chiA   ..........................................A..........
H. cheilosorum-chiS   .....................................................

H. inthanonense       AGAGATCGTTTCTTATTTGTTGCAGAAGCTCTCTTCAAATCCCAAGCTGAAACAGGCGAA   [600]
H. cheilosorum-thiA   ..........T..........................................
H. cheilosorum-chiA   ..........T..........................................
H. cheilosorum-chiS   ..........T..........................................

H. inthanonense       ATCAAGGGGCATTACTTAAACGCCACCGCAGGTACATGTGAAGAAATGTTAAAGAGAGCT   [660]
H. cheilosorum-thiA   .....................................................
H. cheilosorum-chiA   .....................................................
H. cheilosorum-chiS   .....................................................
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Figure 4. Alignment of *rbc*L sequences from *Hymenasplenium inthanonense* and apogamous forms of *H. cheilosorum* from Doi Inthanon (ta), and apogamous forms (ca) and sexual forms (cs) from Xishuangbanna, Yunnan, China.

Fig. 4. (continued)

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H. inthanonense       GTTTTTGCTAGAGAATTGGGTGCACCGATTGTCATGCATGACTACCTGACCGGGGGGTTT   [720]
H. cheilosorum-thiA   ...........................................................
H. cheilosorum-chiA   ...........................................................
H. cheilosorum-chiS   ...........................................................

H. inthanonense       ACCGCAAATACCAGTTTAGCTTTTTATTGCAGAGACAATGGACTGCTTCTCCATATTCAC   [780]
H. cheilosorum-thiA   ..........................................................T
H. cheilosorum-chiA   ..........................................................T
H. cheilosorum-chiS   ..........................................................T

H. inthanonense       CGCGCGATGCATGCTGTGATTGATAGACAACGAAATCACGGTATGCATTTCCGTGTACTG   [840]
H. cheilosorum-thiA   ..........................................T................
H. cheilosorum-chiA   ..........................................T................
H. cheilosorum-chiS   ..........................................T................

H. inthanonense       GCCAAAGCATTGCGCATGTCCGGTGGGGATCACGTACACGCCGGAACTGTAGTAGGTAAA   [900]
H. cheilosorum-thiA   ...........................................................
H. cheilosorum-chiA   ...........................................................
H. cheilosorum-chiS   ...........................................................

H. inthanonense       CTGGAAGGGGAAAGAGAAGTCACTTTGGGTTTCGTTGATTTACTCCGCGACGATTATATC   [960]
H. cheilosorum-thiA   ...........................................................
H. cheilosorum-chiA   ...........................................................
H. cheilosorum-chiS   ...........................................................

H. inthanonense       GAGAAAGATCGTGCACGCGGTATTTATTTCACCCAAGATTGGGTCTCCATGCCGGGTGTA   [1020]
H. cheilosorum-thiA   ......................G....................................
H. cheilosorum-chiA   ......................G....................................
H. cheilosorum-chiS   ......................G....................................

H. inthanonense       TTCCCCGTTGCTTCGGGGGGTATCCACGTATGGCACATGCCAGCTCTAACCGAAATATTT   [1080]
H. cheilosorum-thiA   ...........................................................C...
H. cheilosorum-chiA   ...........................................................C...
H. cheilosorum-chiS   ...........................................................C...

H. inthanonense       GGGGACGACTCTGTCCTACAGTTCGGTGGAGGAACTTTGGGACATCCCTGGGGAAATGCA   [1140]
H. cheilosorum-thiA   ...........................................................
H. cheilosorum-chiA   ...........................................................
H. cheilosorum-chiS   ...........................................................

H. inthanonense       CCCGGTGCGGTAGCCAACCGAG   [1162]
H. cheilosorum-thiA   .......................
H. cheilosorum-chiA   .......................
H. cheilosorum-chiS   .......................
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Figure 4. (continued) Alignment of *rbc*L sequences from *Hymenasplenium inthanonense* and apogamous forms of *H. cheilosorum* from Doi Inthanon (ta), and apogamous forms (ca) and sexual forms (cs) from Xishuangbanna, Yunnan, China.

Distribution: Endemic to Doi Inthanon.

Habitat: Terrestrial on shaded or half-shaded steep banks of soil along stream in the dense montane forest. *Additional specimen examined*: Thailand, Chiang Mai Prov., Doi Inthanon, valley along the driving road between the Check Point and the summit, at 1895 m alt., on soil slope near stream, in half-deep shade, 14 January, 1994, N. Murakami, J. Yokoyama and N. fukuoka 93-T623 (KYO, BKF).

CONSERVATION

Hymenasplenium inthanonense is an endemic species to Doi Inthanon. It has been recorded locally from only one valley along the road from the "Check Point" to the summit. This species has several large populations of 30–100 individuals on a steep slope by stream. Though it is not rare locally, the growing area is quite restricted.

We found several large populations of this species in January, 1994, and returned in December of the same year to find some of the populations seriously damaged by severe flooding and soil erosion along the valley. This flood was partly due to unusual high precipitation during the summer. However, it is no doubt that human activities had much more impacts on the natural montane forest habitat. We found forests scorched black by forest fires which we did not see during our January visit. As a consequence, soil erosion took place drastically on these burnt slopes.

It is highly probable that more endemic species are waiting to be discovered in this area. The Doi Inthanon mountain contains many rare plant species. Careful attention should be employed minimize human impacts on the threatened montane forests of Doi Inthanon and effectively conserve rare endemic plants like *H. inthanonense*.

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