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Phylogenetics of lichens in the genus *Cladonia* (Cladoniaceae) in northern and northeastern Thailand

Die Phylogenese von Flechten der Gattung *Cladonia* (Cladoniaceae) im Norden und Nordosten Thailands

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Key words: *Cladonia*, ITS, parsimony, phylogenetics.

Schlagwörter: *Cladonia*, ITS, parsimony, Phylogenese.

Summary: Phylogenetic relationships of lichens in the genus *Cladonia* were cladistically examined with morphological, chemical and internal transcribed spacer (ITS) region of the nuclear ribosomal DNA sequence data. Data matrix of the ITS region included 39 specimens of 32 taxa representing most of the currently recognised sections of *Cladonia* and the outgroup in the genus *Cladia*. Cladistic analyses were carried out using parsimony method. In the combined analysis of morphological and chemical data the strict consensus tree revealed a well supported clade in the *Cocciferae* section. Other taxa in the sections *Cladonia*, *Ascyphiferae* and *Perviae* showed unresolved relationships. Results of the molecular analysis yielded a better resolution of the phylogenetic tree with four important clades. Clades I and IV were placed in the Subdivision III while clades II and III represented the Subdivision II. The analyses of both the combined morphological and chemical data as well as molecular data confirmed the monophyly of the section *Cocciferae*.

Zusammenfassung: Die phylogenetischen Beziehungen von Flechten der Gattung *Cladonia* wurden mit morphologischen, chemischen und molekularen Methoden (ITS-Region der nrDNA) untersucht Die Analyse umfaßt 39 Exemplare von 32 Taxa der meisten der gegenwärtig bekannten Sektionen der Gattungen *Cladonia* und *Cladia* als Außengruppe. Kladistische Analysen wurden mit der "Maximum parsimony"-Methode durchgeführt. Mit der kombinierten Analyse von morphologischen und chemischen

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Daten zeigte sich der "Strict consensus tree" in der Sektion *Cocciferae* als am besten geeignet. Die übrigen Taxa der Sektionen *Cladonia, Ascyphiferae* und *Perviae* zeigten unbestätigte Beziehungen. Die Ergebnisse der molekularen Analyse zeigten ein besseres Ergebnis des phylogenetischen Stammbaums mit vier wichtigen Verzweigungen. Die Zweige I und IV wurden der Unterabteilung III zugeteilt, wogegen die Zeige II und III von der Unterabteilung II repräsentiert werden. Die kombinierten Analysen von morphologischen und chemischen, wie auch molekularer Daten bestätigten die monophyletische Abstammung der Gattung *Cocciferae*.

Introduction

The genus *Cladonia* HILL ex BROWNE, consisting of lichen-forming fungi (Ascomycotina: Lecanorales: suborder Cladoniinae), includes more than 400 species which are distributed throughout the world (AHTI 2000). This genus is characterised by a dimorphic thallus, consisting of horizontal, foliose or crustose primary thallus and vertical secondary thallus called podetia. *Cladonia* species contain a wide variety of secondary compounds, especially β-orcinol depsides and depsidones such as atranorin, barbatic, squamatic, thamnolic, sekikaic, fumarprotocetraric and psoromic acids. One challenge with *Cladonia* is that the morphology of most of the species is highly variable. Therefore, characters of secondary chemistry are often useful, but even then many species are difficult to identify and the taxonomy of several groups is still problematic. Both morphological structures and chemistry compositions are affected by habitats and factors related to the genetic component, age and the environmental conditions (CULBERSON et al. 1983). All previous evolutionary studies of lichens in the genus *Cladonia* used only morphological and chemical data. CULBERSON (1986) illustrated that biogenetic relationships of secondary products in lichens could be used for cladistic analyses among taxa of the *Cladonia chlorophaea* group. HYVÖNEN et al. (1995) presented a cladistic analysis based on morphological and chemical characters of the genus *Cladina* by using the section *Unciales* of the genus *Cladonia* as outgroup. The results indicated the paraphyly of the genus *Cladina* and polyphyly of the section *Unciales*. Furthermore, when *Pycnothelia papillaria, Cladia aggregata* and *Cladia retipora* were employed as outgroup taxa the results did not support some sectional divisions of the genus *Cladonia*. The sections *Cocciferae* and *Helopodium* have some members more closely related to species of the other sections. This maybe because some characters which overlap among species were homoplastic, making species determination difficult (STENROOS et al. 1997). Most recent studies were based on sequence data. Internal transcribed spacer (ITS) region has proved a valuable source of characters for delimiting lichen genera and determining phylogeny at the infrageneric level. This region consists of two spacers, including ITS1 and ITS2. The ITS1 region is located between the 18S and the 5.8S genes, and the ITS2 region is located between the 5.8S and the 28S genes. These spacer regions are routinely used for

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studies on phylogenetic reconstruction, genetic variability and divergence of closely related species for a wide range of Cladoniaceae (STENROOS et al. 2002, MYLLYS et al. 2003). The first molecular studies of Cladoniaceae were carried out by KASHEVAROV (1992). These studies supported the generic status of *Cladina* using nucleotide sequence homologies of the DNA among four species of *Cladina* and six of *Cladonia*. STENROOS et al. (2002) compared phylogenies of the genus *Cladonia* including *Cladina* based on analyses of the ITS region, β-tubulin gene, morphological and chemical data. The results of both morphological and chemical characters showed homoplasy, especially in the section *Cladonia*. On the other hand, the molecular data revealed a better resolution of the current sections of *Cladonia*, including *Ascyphiferae*, *Helopodium*, *Strepsiles*, *Unciales* and one section of *Cladina*.

The main purpose of this study was to investigate the phylogenetic relationships of *Cladonia* in northern and northeastern Thailand using simultaneous analyses of the morphological and chemical data and the ITS sequences. The information obtained will be employed to conserve the diversity and bring about sustainable uses of the lichens in this genus.

Materials and methods

Study sites

The study was primarily based on fresh material from the northern and northeastern parts of Thailand covering Doi Inthanon National Park 18° 48′ 26.6″N 98° 53′ 16.6″E, and Doi Suthep Pui National Park 12°55′57.5″N 100° 45′29.5″E in Chiang Mai Province, Phu Hin Rong Kla National Park 16°59′ 14″N 100°59′50″E in Phitsanulok Province and Phu Luang Wildlife Sanctuary 17°16′ 48.5″N 101°31′29.3″E in Loei Province. The areas span from 1,000 – 2,565 meters above sea level (Fig. 1).

Taxon sampling

Thirty-nine representative samples of 32 taxa were selected for analyses Fourteen taxa were collected from Thailand (Tab. 1). These samples included *Cladonia* sections *Cladonia* (10 samples/7 taxa), *Ascyphiferae* (4/3), *Cocciferae* (4/2) and *Perviae* (1/1). One outgroup species was selected from the genus *Cladia* (2 samples/1 taxon). Three undescribed species were discovered, and they are described in a separate paper (AHTI et al. 2008). Eighteen ITS sequences belonging to core taxa of different sections of *Cladonia* were downloaded from GenBank (Tab. 2). These sequences were selected based on current classification (AHTI 2000) and tentative phylogeny of the genus *Cladonia* (STENROOS et al. 2002).

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Morphological and chemical data

Most data used to construct a matrix of morphology, anatomy and secondary chemistry characters were obtained from the literature e.g. HYVÖNEN et al. 1995, STENROOS et al. 1997 & 2002. Standard methods for thin layer chromatography (CULBERSON & KRISTINSSON 1969, CULBERSON 1972, WHITE & JAMES 1985) were used to analyse secondary chemistry of the genus *Cladonia* and *Cladia*. The matrix comprised 41 characters of morphology, podetia anatomy, reproductive structures and lichen substances. Twenty-one of the characters were morphological and 20 chemical. Thirty-three characters were binary and 8 multistate.

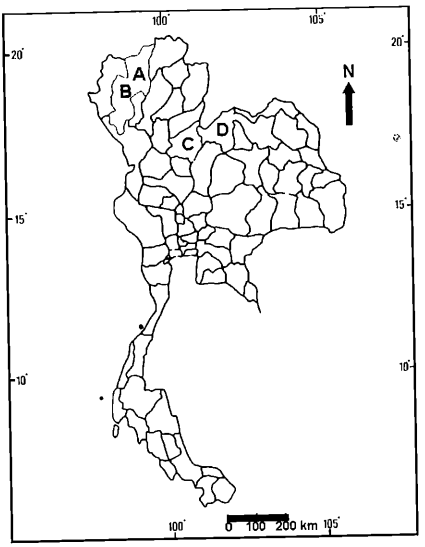

Fig. 1: Collecting localities of the genera *Cladia* and *Cladonia* in northern and northeastern Thailand covering **A**) Doi Inthanon national park, **B**) Doi Suthep Pui National Park, **C**) Phu Hin Rong Kla National Park and **D**) Phu Luang Wildlife Sanctuary.

Phylogenetic analysis of the morphological and chemical characters

Morphological and chemical data contained 21 and 20 characters, respectively. These characters were coded either as binary or multistate. All character states were coded with 0, 1 and 2. Valid character state symbols were 0, 1, 2 and P Missing data were identified by '?' With *Cladia aggregata* as the outgroup, phylogenetic trees were produced using PAUP* version 4.0b (SWOFFORD 1998). The branch-and-bound search setting optimality criterion with parsimony was employed. All characters were of type unordered and had equal weight. MulTrees option was in effect. Starting tree (s) was obtained via stepwise addition. Branch-swapping algorithm used the tree-bisection-reconnection (TBR) method.

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Bootstrap analysis was conducted with 1,000 replicates of random addition sequences in order to test the support for branches of phylogenetic trees.

Tab. 1: Twenty-one specimens of the genera *Cladia* and *Cladonia*, with GenBank accession numbers were used in this study. All specimens are deposited in RAMK herbarium. Doin = Doi Inthanon National Park, PH = Phu Hin Rong Kla National Park, PL = Phu Luang Wildlife Sanctuary and SU = Doi Suthep Pui National Park.

Species	Sectional classification	Collection	Collecting site	GenBank No.
Cladia aggregata (SW.) NYL.		SP268RAMK	PH	EU091338
Cladia aggregata (SW.) NYL		SP286RAMK	PL	EU113276
Cladonia cf. *awasthiana* AHTI & UPRETI	*Cladonia*	SP271RAMK	PH	EU113290
Cladonia cf. *awasthiana* AHTI & UPRETI	*Cladonia*	SP283RAMK	SU	EU113289
Cladonia corymbescens NYL. ex LEIGHT.	*Ascyphiferae*	SP285RAMK	PL	EU113288
Cladonia furcata (HUDS.) SCHRAD.	*Ascyphiferae*	SP279RAMK	Doin	EU113287
Cladonia furcata (HUDS.) SCHRAD.	*Ascyphiferae*	SP284RAMK	PL	EU113286
Cladonia fruticulosa KREMP.	*Cladonia*	SP276RAMK	PH	EU113294
Cladonia homchantarae AHTI & PARNMEN	*Cocciferae*	SP270RAMK	PH	EU113279
Cladonia homchantarae AHTI & PARNMEN	*Cocciferae*	SP265RAMK	PH	EU113280
Cladonia homchantarae AHTI & PARNMEN	*Cocciferae*	SP277RAMK	Doin	EU113281
Cladonia macilenta HOFFM.	*Cocciferae*	SP267RAMK	PH	EU113295
Cladonia mauritiana AHTI & J.C.DAVID	*Cladonia*	SP275RAMK	PH	EU113291
Cladonia mauritiana AHTI & J.C.DAVID	*Cladonia*	SP282RAMK	SU	EU113292
Cladonia ochrochlora FLÖRKE	*Cladonia*	SP264RAMK	PH	EU113284
Cladonia ochrochlora FLÖRKE	*Cladonia*	SP280RAMK	SU	EU113285

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Cladonia rappii A.EVANS	*Cladonia*	SP269RAMK	PH	EU113293
Cladonia recticaulis AHTI & PARNMEN	*Perviae*	SP266RAMK	PH	EU113278
Cladonia rudis AHTI & PARNMEN	*Ascyphiferae*	SP272RAMK	PH	EU113277
Cladonia scabriuscula (DELISE) NYL.	*Ascyphiferae*	SP278RAMK	Doin	EU113282
Cladonia singhii AHTI & DIXIT	*Cladonia*	SP274RAMK	PH	EU113283

Tab. 2: Eighteen ITS sequences belonging to core taxa of different sections of Cladonia used in this study.

Taxon name	Current classification	Collection	GenBank No.
Cladonia caespiticia (PERS.) FLÖRKE	*Helopodium*	Canada, Nova Scotia, 1999 AHTI 57084 (H)	AF455205
Cladonia cariosa (ACH.) SPRENG.	*Helopodium*	Finland, 1999 PUOLASMAA s.n. (TUR)	AF455230
Cladonia carneola (FR.) FR.	*Cocciferae*	Finland, 1995 STENROOS 5583 (TUR)	AF454452
Cladonia ceratophylla (SW.) SPRENG.	*Helopodium*	Brazil, Minas Gerais, 1997 STENROOS 5081 (TUR)	AF455171
Cladonia cervicornis ssp. *verticillata* (HOFFM.) AHTI	*Cladonia*	Canada, Newfoundland, 1999 AHTI 56951 (H)	AF453845
Cladonia crispata (ACH.) FLOT.	*Perviae*	Finland, 1999 STENROOS 5214 (TUR)	AF457888
Cladonia déformis (L.) HOFFM.	*Cocciferae*	Finland, 1995 STENROOS 5584 (TUR)	AF454448
Cladonia didyma (FE´E) VAIN.	*Cocciferae*	USA, North Carolina, 1998 AHTI 56216 (H)	AF453703
Cladonia digitata (L.) HOFFM.	*Cocciferae*	Finland, 1999 STENROOS 5164 (TUR)	AF453701
Cladonia fimbriata (L.) FR.	*Cladonia*	Chile, Prov. Magallanes, 1999 FEUERER 60132 (TUR)	AF455224
Cladonia gracilis (L.) WILLD. subsp. *gracilis*	*Cladonia*	Sweden, 1999 THELL 9931 (TUR)	AF455194
Cladonia merochlorophaea ASAHINA	*Cladonia*	Finland, 1999 STENROOS 5168 (TUR)	AF455227

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Cladonia multiformis G. MERR.	*Ascyphifera e*	USA, Nova Scotia, 1999 AHTI 57065 (H)	AF4552 13
Cladonia rangiferina (L.) F. H. WIGG. subsp. *rangiferina*	*Cladina: Cladina*	Finland, 1999 STENROOS 5173 (TUR)	AF4583 06
Cladonia signata (ESCHW.) VAIN.	*Ascyphifera e*	Guyana, 1997 STENROOS 4876 (TUR)	AF4579 01
Cladonia subconistea ASAHINA	*Cladonia*	China, Hunan, 1998 KOPONEN et al. 55878 (H)	AF4552 10
Cladonia subulata (L.) F. H. WIGG.	*Cladonia*	Finland, 1997 STENROOS 5106 (TUR)	AF4551 80
Cladonia uncialis (L.) F. H. WIGG. subsp. *uncialis*	*Unciales*	Sweden, 1997 STENROOS 5116 (TUR)	AF4552 50

DNA extraction

Either fresh or herbarium material was used for extracting total genomic DNA. Each podetium was scraped free of squamules, most of the soredia and the algal layer to reduce contamination with symbiotic algae and epiphytes. Thallus fragments of 2-15 mg were ground in liquid nitrogen. DNA was extracted using either the CTAB method (DOYLE & DOYLE 1987) and the DNeasy™ Plant Mini Kit (QIAGEN).

PCR amplification and DNA sequencing

The entire ITS region of the nuclear ribosomal DNA were amplified with the primers ITS1F 5´CTTGGTCATTTAGAGGAAGTAA3´ (GARDES & BRUNS 1993) and ITS4 5´TCCTCCGCTTATTGATATGC3´ (WHITE et al. 1990). PCR reactions were performed using the thermal cycler (Eppendorf). Each solution contained 8.75 µl of nuclease-free water, 2.5 µl of 10x buffer with 15 mM $MgCl_2$, 2.5 µl of 25 mM $MgCl_2$, 5 µl of 5x Q-solution, 0.5 µl of 10 mM dNTP's mix, 5 µl each of the primers at 10 mM concentration, 1.0 µl of DNA sample and 0.25 µl of *Taq* DNA polymerase. A 30 cycle reaction was carried out with PCR profile of 1 min at 95°C (denaturation), 1 min at 52-57°C (annealing) and 1 min at 72°C (extension) with final extension of 72°C for 2 min. Amplification products were cleaned using QIAquick PCR Purification Kit. DNA sequencing analyses were carried out by Macrogen Inc., Seoul, Korea.

Sequence characteristics and alignment

The sequence alignment of the complete ITS region for 39 taxa was accomplished by ClustalX (1.64b) (THOMPSON et al. 1997). Gaps were assumed to represent insertions or deletions (indels) that occurred as the sequences di-

verged from a common ancestor. The aligned sequences were further examined by eye using GeneDoc (2.5.000) (NICHOLAS & NICHOLAS 1997) and sequence characteristics were obtained.

Phylogenetic analysis of the ITS region

Aligned sequences of the entire ITS region were analysed with PAUP* version 4.0b after the outgroup was defined *(Cladia aggregata)*, using the method of parsimony and heuristic algorithm. All characters were of type unordered and had equal weight. MulTrees option was in effect. Starting tree (s) was obtained via stepwise addition. Branch swapping algorithm used the TBR method. Bootstrap analysis was performed with 1,000 replicates of random addition sequences.

Results

Morphological and chemical data used in the analysis

The data matrix used in the analysis included 41 characters. Twenty-one of the characters were morphological and 20 chemical. Thirty-three characters were binary and 8 multistate.

1. Primary thallus persistent (0), intermediate (1), evanescent (2)
2. Primary squamules sorediate (0), esorediate (1)
3. Pseudopodetia (0), podetia (1)
4. Pseudopodetia dichotomously branched (0), irregular (1)
5. Pseudopodetia solid (0), hollow (1)
6. Podetia laminal on primary squamules (0), phyllopodial (1)
7 Podetia repeatedly branched (0), unbranched or with occasional branches (1)
8. Branching equal (0), equal or unequal (1), unequal (2)
9. Branching dichotomic (0), dichotomic or dichotomic + trichotomic (1), dichotomic + trichotomic (2)
10. Branching trichotomic (0), dichotomic or trichotomic + tretachotomic (1), trichotomic + tretachotomic (2)
11. Axils of podetia closed (0), open partially (1)
12. Scyphi absent (0), facultative (1), obligatory (2)
13. Scyphi proliferating from margins (0), from the center (1)
14. Centrally proliferating scyphal plates divided (0), not divided (1)
15. Cylindrical stereome absent or very rudimentary (0), present (1)
16. Cortex present on podetia (0), discontinuously (1), absent (2)
17 Soredia absent (0), present (1)
18. Soredia diffuse granulose (0), diffuse farinose (1)
19. Podetia squamules absent (0), occasional (1), abundant (2)
20. Pycnidial jelly hyaline (0), jelly red (1)

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21. Hymenial discs brown (0), ochre-yellow (1), red (2)
22. Beta-orcinol p-depsides absent (0), present (1)
23. Atranorin absent (0), present (1)
24. Barbatic acid absent (0), present (1)
25. Squamatic acid absent (0), present (1)
26. Beta-orcinol m-depsides (thamnolic acid) absent (0), present (1)
27. Beta-orcinol depsidones absent (0), present (1).
28. Fumarprotocetraric acid absent (0), present (1)
29. Psoromic acid absent (0), present (1)
30. Norstictic acid absent (0), present (1)
31. Stictic acid absent (0), present (1)
32. Orcinol p-depsides (perlatolic acid, divaricatic acid) absent (0), present (1)
33. Orcinol m-depsides absent (0), present (1)
34. Homosekikaic acid absent (0), present (1)
35. Sekikaic acid absent (0), present (1)
36. Dibenzofurans absent (0), present (1)
37 Didymic acid absent (0), present (1)
38. Usnic acid absent (0), present (1)
39. Antraquinones absent (0), present (1)
40. Rhodocladonic acid absent (0), present (1)
41. Zeorin absent (0), present (1)

Phylogenetic analysis of the morphological and chemical characters

The specimens were selected from members of *Cladonia* in the sections *Ascyphiferae*, *Cladonia*, *Cocciferae* and *Perviae*. The data matrix used in the analysis included 41 characters (Tab. 3). Only 28 characters were parsimony informative. The analysis based on branch-and-bound algorithm yielded 64 equally parsimonious trees of length 69 steps with the consistency index (CI) of 0.5652, homoplasy index (HI) of 0.4348, retention index (RI) of 0.7619 and rescaled consistency index (RC) of 0.4306. Strict consensus tree obtained from the morphological and chemical data was not informative enough to fully resolve relationships among taxa. The result revealed two clades (Fig. 2). **Clade I,** consisting of *Cladonia homchantarae* and *C. macilenta*, shows a strong bootstrap support of 90%. **Clade II** (BS = 86%), contains two specimens of *C. ochrochlora*.

Tab. 3: A data matrix containing morphological and chemical characters of *Cladia* and *Cladonia* under study.

Taxon name	Character							
	5	10	15	20	25	30	35	41
Cladia aggregata (PH)	2?011	??10?	????0	00?0?	?1010	00000	00000	000000
C. aggregata (PL)	2?011	??10?	????0	00?0?	01010	00000	00000	000000
Cladonia homchantarae (PH)	011??	01110	10??1	00?21	21001	10000	00000	110110
C. homchantarae (Doin)	001??	01110	10??1	0111?	?1001	10000	00000	110110
C. homchantarae (PH)	011??	01110	10??1	00?21	21001	10000	00000	110110
C. macilenta (PH)	101??	0110?	00??1	2110?	10000	10000	00000	110110
Cladonia recticaulis (PH)	111??	00122	00??1	10?10	01011	10000	00000	000000
C. fruticulosa (PH)	101??	0111?	01??1	11110	00000	01010	00000	000000
C. mauritiana (PH)	001??	01???	01??1	1101?	00000	01100	00000	000000
C. mauritiana (SU)	001??	01???	0???1	1101?	?0000	01100	00000	000000
C. rappii (PH)	111??	?1???	02101	00?1?	00000	01100	00000	000000
C. ochrochlora (PH)	001??	01???	10??1	1101?	?0000	01100	00111	000000
C. ochrochlora(SU)	001??	01???	10??1	1101?	?0000	01100	00111	000000
C. furcata (PL)	2?1??	?0111	10??1	00?00	?0000	01100	00000	000000
C. furcata (Doin)	111??	01111	10??1	00?2?	?0000	01100	00000	000000
C. scabriuscula (Doin)	111??	?1111	10??1	00?2?	?0000	01100	00000	000000
C. corymbescens (PL)	2?1??	?0111	00??1	00?0?	?1100	01100	00000	000000
Cladonia cf. *awasthiana* (SU)	001??	01100	01??1	21P1?	00000	01100	00000	000000
Cladonia cf. *awasthiana* (PH)	111??	01100	01??1	21P1?	00000	01100	00111	000000
C. singhii (PH)	111??	01100	01??1	21P1?	00000	01100	00111	000000
C. rudis (PH)	111??	01111	10??1	1102?	?0000	01100	00000	000000

Note: ? = unknown and inapplicable character, P = polymorphism.

Phylogenetic analysis of the ITS region

The aligned length of the entire ITS region was 682 characters. All characters were of type unordered and had equal weight. 408 characters were constant. Within 274 variable characters, 81 were parsimony uninformative while 193 were parsimony informative. Based on heuristic algorithm, 14 equally parsimonious trees with the length of 667 steps and CI = 0.5817, HI = 0.4183, RI = 0.7475 and RC = 0.4348 were obtained. Strict consensus tree reveals six groups of four important clades (Fig. 3). **Clade I** (BS = 99%) comprises two species of the section *Perviae*, namely *Cladonia crispata* (AF457888, Finland) and *C. recticaulis*. **Clade II** (BS = 68%) contains two groups belonging to the section *Cladonia*. **Group I** (BS = 95%) includes *Cladonia* cf. *awasthiana* and *C. singhii* with *C. fimbriata* (AF455224, Chile) as a sister group. **Group II** (BS = 65%) contains *C. gracilis* subsp. *gracilis* (AF455194, Sweden) and *C. ochrochlora*. **Clade III** (BS = 63%) corresponds to the sections *Ascyphiferae, Cladonia* and *Helopodium*.

Tab. 4: Sequence characteristics of the ITS region for 39 taxa in the genera *Cladia* and *Cladonia* under study.

Sequence characteristics	ITS1	ITS2	ITS1 5.8S ITS2
Length range (total) (bp)	217-285	164-176	534-607
Length mean (total) (bp)	243.5	169.3	562.8
Length range (ingroup) (bp)	237-285	164-176	558-607
Length mean (ingroup) (bp)	244.9	169.4	564.4
Aligned length (total)	335	196	682
GC content range (%)	51-57	50-57	50-55
GC content mean (%)	54.4	53.9	52.6
Number of indels (total)	188	55	245
Number of constant characters	169	94	408
Number of variable characters	166	102	274
Number of parsimony uninformative characters	40	38	81
Number of parsimony informative characters	126	64	193
Parsimony informative percentage (%)	37.6	32.6	28.3
Transition/transversion ratio	3.11	3.74	2.97

This clade reveals three groups including **Group III** (BS = 64%) shows a relationship between *C. cariosa* (AF455230, Finland) and *C. signata* (AF457901, Guyana) belonging to the sections *Helopodium* and *Ascyphiferae*, respectively; **Group IV** (BS = 85%) contains five species of the section *Ascyphiferae* and one species, *C. subconistea* (AF455210, China) from the section *Cladonia* with *C. ceratophylla* (AF455171, Brazil) from the section *Helopodium* as a basal taxon; and **Group V** (BS = 77%) contains taxa belonging to the section *Cladonia* and *C. caespiticia* (AF455205, Canada) which is usually included in the section *Helopodium*. **Clade IV** (BS = 85%) composes of *Cladonia* in the sections *Cocciferae*, *Unciales* and *Cladina*. **Group VI** (BS = 61%) contains six taxa belonging to the section *Cocciferae*, *C. uncialis* subsp. *uncialis* (AF455250, Sweden) and *C. rangiferina* subsp. *rangiferina* (AF458306, Finland) which are classified in the sections *Unciales* and *Cladina*, respectively.

Discussion

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Sequence characteristics of the ITS region

The ITS1 region of the lichens under study was longer than the ITS2 region (Tab. 4). This type of length variation has been reported from other groups of lichens such as the genera *Arthonia* (ITS1 = 304/ ITS2 = 191), *Dendrographa* (220/167), *Dirina* (220/188), *Hubbsia* (224/191), *Lecanactis* (230/187) and *Roccella* (224/180) (MYLLYS et al. 1999). The length of ITS1 for the ingroup taxa ranged from 237 base pairs (bp) in one specimen of the section *Cladonia* (*C. fruticulosa*) to 285 bp in the section *Cocciferae* (*C. macilenta*). *Cladonia macilenta* possessed a large insertion of 36 bp. High levels of length variation among the ITS1 sequences were observed in the *Peltigera canina* species complex (MIADLIKOWSKA et al. 2003). In contrast, the conservative 5.8S region was almost uniform in length in all taxa, varying from 156 to 157 nucleotides. The length of the ITS2 sequences ranged from 164 bp in the section *Cladonia* (*C. fruticulosa*) to 176 bp in the same section (*C. rappii*). The outgroup taxon, *Cladia aggregata*, had the length of the ITS1 and ITS2 regions of 217 and 167 bp, respectively.

Phylogenetic relationships based on morphological, chemical and molecular data

The lichens in the genus *Cladonia* were divided into seven sections, namely *Ascyphiferae*, *Cladonia*, *Cocciferae*, *Helopodium*, *Perviae*, *Unciales* and *Strepsiles* by AHTI (2000). Earlier STENROOS et al. (1997) presented a cladistic analysis based on morphological and chemical characters of the genera *Cladonia* and *Cladina*. The results showed that the current sectional division of *Cladonia* was not well supported. In this study, the specimens were selected from members of *Cladonia* in the sections *Ascyphiferae*, *Cladonia*, *Cocciferae* and *Perviae*. A phylogenetic tree based on parsimony analysis of the morphological and chemical data revealed that the section *Cocciferae* was best supported. Red pigment in hymenia and conidiomata was confirmed to be a good character to define the section *Cocciferae*. At early stage, some specimens lack the red hymenial discs. However, the section *Cocciferae* could still be identified by some diagnostic chemical characteristics such as dibenzofurans (e.g. didymic, usnic acids), β-orcinol depsides (e.g. thamnolic acid), and rhodocladonic acid (STENROOS 1986, HYVÖNEN et al. 1989, GUO & KASHIWADANI 2004). Some of these compounds were also found in *Perviae*, but in somewhat different combinations. Compound combinations such as thamnolic and barbatic acids were typical in *Perviae*, whereas these compounds commonly occur together with usnic or didymic acids in *Cocciferae* (STENROOS et al. 2002). Relationships of other taxa under study were unresolved due to the presence of homoplastic characters such as soredia, scyphi, stereome and cortex which were used to define only small groups of species or even a single species (STENROOS et al. 2002, HYVÖNEN 1997).

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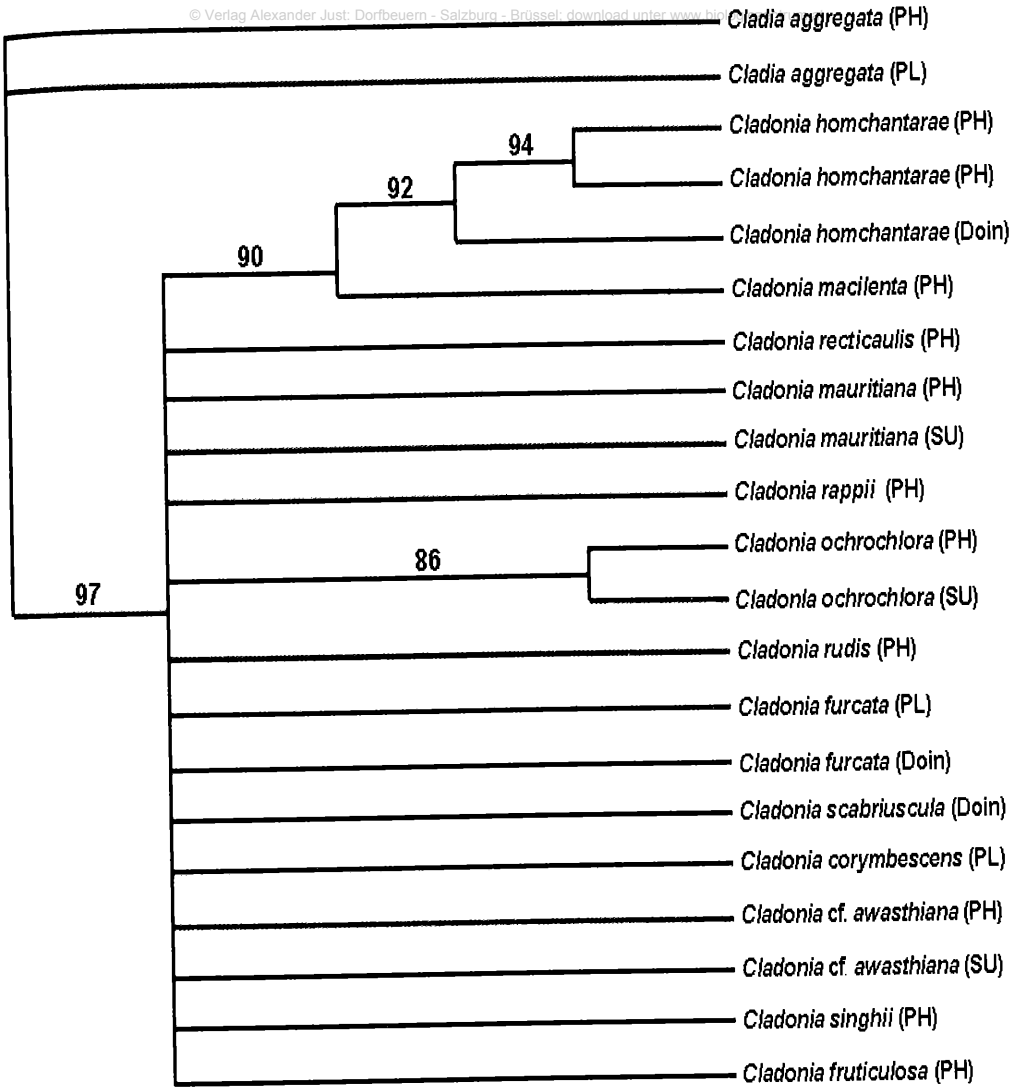

Fig. 2: Strict consensus tree obtained from 64 equally parsimonious trees based on the analysis of morphological and chemical data using branch-and-bound algorithm. Numbers above branches are bootstrap values (%) of 1,000 replicates. Fit measures of the tree: treelength = 69 steps, CI = 0.5652, RI= 0.7619 and RC = 0.4306. Doin = Doi Inthanon National Park, PH = Phu Hin Rong Kla National Park, PL = Phu Luang Wildlife Sanctuary and SU = Doi Suthep Pui National Park.

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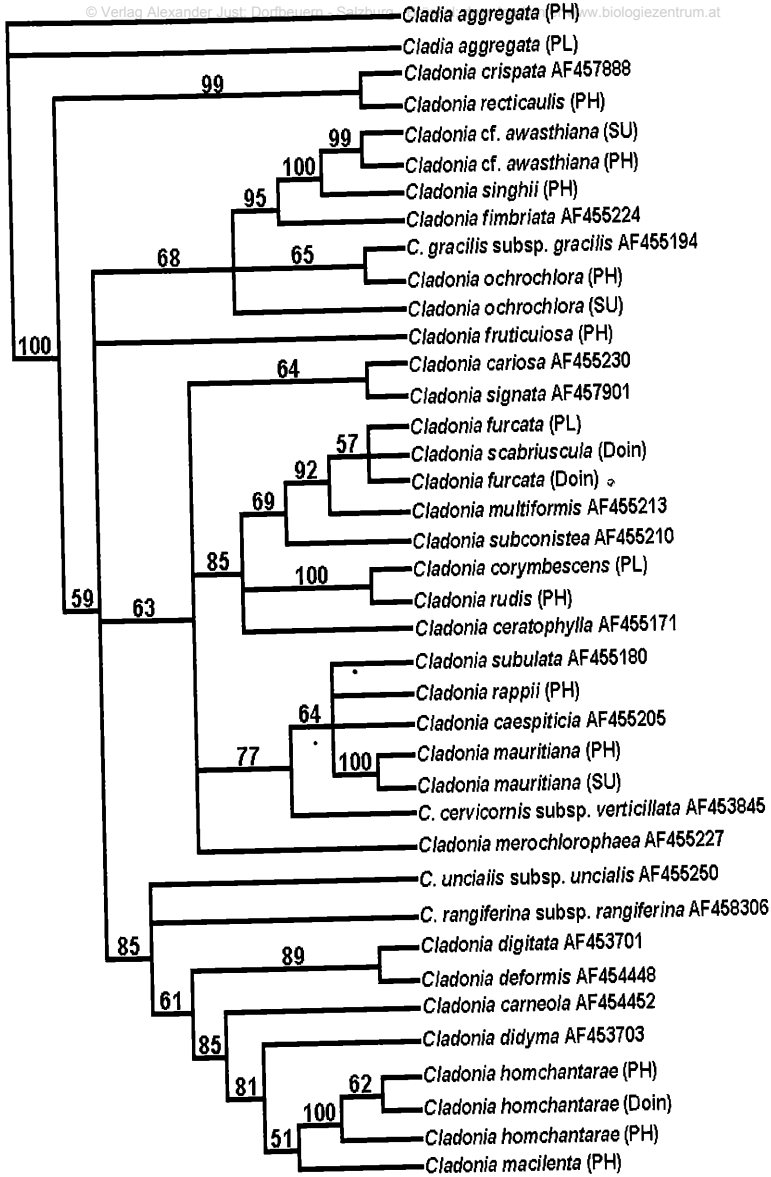

Fig. 3: Strict consensus tree obtained from 14 equally parsimonious trees based on the analysis of the entire ITS region using heuristic algorithm. Numbers above branches are bootstrap values (%) of 1,000 replicates. Fit measures of the tree: treelength = 667 steps, CI = 0.5817, RI = 0.7475 and RC = 0.4348. Doin = Doi Inthanon National Park, PH = Phu Hin Rong Kla National Park, PL = Phu Luang Wildlife Sanctuary and SU = Doi Suthep Pui National Park.

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The molecular data, on the other hand, yielded a better resolution of the phylogenetic tree with six groups of four important clades. All six groups obtained basically corresponded to the current classification illustrated by AHTI (2000) with slight differences. **Clade I** (BS = 99%) forms a distinct clade, including *C. crispata* and *C. recticaulis*. Both taxa were placed in the section *Perviae* (AHTI 2000). The result agreed with the informally classified sections illustrated by STENROOS et al. (2002). They tentatively referred to this clade as the Subdivision III, which includes groups corresponding to the *Cladonia* sections *Cocciferae, Perviae* and *Unciales* and the *Cladina* sections *Cladina, Impexae*, and *Tenues* (sensu AHTI). **Clade II** (BS = 68%) includes two groups (I & II) belonging to the section *Cladonia*. It is interesting to note that *C. ochrochlora* from Phu Hin Rong Kla National Park is more closely related to *C. gracilis* subsp. *gracilis* than *C. ochrochlora* from Doi Suthep Pui National Park. This suggested that *C. ochrochlora* is a variable species. Based on STENROOS et al. (2002) *C. gracilis* formed a monophyletic group with *C. ochrochlora* as well as with other taxa in the *C. gracilis* group (AHTI 1980) and referred to it as the Subgroup *Graciles*. **Clade III** (BS = 63%) comprises members of the sections *Ascyphiferae, Cladonia* and *Helopodium* which forms three groups (III, IV & V). **Clade III** could be placed in the Subdivision II which includes species representing the *Cladonia* sections *Ascyphiferae, Helopodium*, and *Cladonia* (sensu AHTI) (STENROOS et al. 2002). This clade showed unresolved relationships among *C. furcata* from Phu Luang Wildlife Sanctuary, *C. scabriuscula* from Doi Inthanon National Park and *C. furcata* from Doi Inthanon National Park. In terms of morphological variation, our results revealed that *C. furcata* possessed some characters which were different between two populations. The specimens from Phu Luang Wildlife Sanctuary showed fairy smooth and unbroken cortex while the specimens from Doi Inthanon National Park showed patchy and almost granular cortex with the presence of podetia squamules. BRODO et al. (2001) also observed similar morphological variations in North America. According to STENROOS et al. (2002) the *C. furcata*-complex was established and it was often difficult to distinguish *C. furcata* from its apparently very close relative, *C. scabriuscula*, due to the presence of overlapping morphological characters and many intermediate types. **Clade IV** (BS = 85%) encompasses species from the section *Cocciferae*, forming a monophyletic group with *C. uncialis* subsp. *uncialis* and *C. rangiferina* subsp. *rangiferina* as basal taxa. Based on a tentative phylogeny depicted by STENROOS et al. (2002) this clade was also referred to as the Subdivision III. In this clade, however, it is interesting to note that *C. rangiferina* which was once considered to be in a separate genus, *Cladina*, is found nested within the genus *Cladonia*. This result confirmed the analyses by HYVÖNEN et al. (1995) as well as by STENROOS et al. (2002) which gave no support for *Cladina* at the genus level.

Conclusions

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The results of this study can be concluded as follows: 1) strict consensus tree based on parsimony analysis of the morphological and chemical data revealed a strongly supported clade in the *Cocciferae* section. Other taxa in the sections *Cladonia*, *Ascyphiferae* and *Perviae* showed unresolved relationships, and 2) based on the entire ITS region clades I and IV were recognised in the Subdivision III while clades II and III represented the Subdivision II. The analyses of both the combined morphological and chemical data as well as molecular data confirmed the monophyly of the section *Cocciferae*.

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