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Biogeography of *Lychnothamnus barbatus* (Charophyta): molecular and morphological comparisons with emphasis on a newly discovered population from Taiwan

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Abstract — The genus *Lychnothamnus* (Rupr.) Leonhardi is a charophyte (Charophyta) found since the Late Eocene to the present. This is the first record of the extant species *L. barbatus* (Meyen) Leonhardi from Taiwan, and morphological analyses, molecular analyses and scanning electron microscopy have been used to explore the phylogeny and relationships between disjunct populations from Taiwan, Australia and Europe. The oospore/gyrogonites isopolarity index (ISI = 135-166) of the population in Taiwan is similar to the ISI of oospore/gyrogonites from Australia (ISI = 140-171), but quite different from the population in Europe (ISI = 115-140). The similarity of morphological features, and nearly identical *rbc*L sequences in the three populations from Taiwan, Australia and Europe, suggest the isolated populations originated from one population. We suggest that the Taiwanese population *L. barbatus* has been dispersed from/to Australia by aquatic migratory birds.

***Lychnothamnus barbatus* / biogeography / isopolarity index / *rbc*L gene / Taiwan**

Résumé — Biogéographie de *Lychnothamnus barbatus* (Charophyta) : comparaisons moléculaire et morphologique et découverte d'une nouvelle population à Taiwan. Le genre *Lychnothamnus* (Rupr.) Leonhardi est une charophyte (Charophyta) connue depuis l'Eocène supérieur jusqu'à maintenant. Les auteurs présentent la découverte d'une nouvelle population actuelle de *L. barbatus* à Taiwan. Des analyses morphologique, moléculaire et la microscopie électronique à balayage ont été utilisées pour étudier la phylogénie et les relations entre des populations disjointes de Taiwan, d'Australie et d'Europe. L'index d'isopolarité des oospores/gyrogonites (ISI = 135-166) de la population de Taiwan est similaire de l'ISI des oospores/gyrogonites d'Australie (140-171), mais assez différent de la population d'Europe (ISI = 115-140). La ressemblance morphologique ainsi que les séquences *rbc*L entre les trois populations de Taiwan, Australie et Europe suggèrent que ces populations isolées dérivent d'une seule population. Les auteurs suggèrent que la population de *L. barbatus* de Taiwan a été dispersée depuis ou vers l'Australie par des oiseaux migrateurs aquatiques.

***Lychnothamnus barbatus* / biogéographie / index d'isopolarité / gène *rbc*L / Taiwan**

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INTRODUCTION

The charophytes (Charales) are the closest relative to the land plants and are important both ecologically and evolutionarily (Karol *et al.*, 2001). They are one of the major carbonate producers in freshwater lakes (Petit-Maire *et al.*, 1981) and fossilize mainly by the means of gyrogonites, which are the typically spiraled and calcified female fructifications (Tappan, 1980).

The genus *Lychnothamnus* was proposed by Leonhardi (1863) based on the species described as *Chara barbata* Meyen (1827). Fossil representatives of *Lychnothamnus* occurred widely from the Late Eocene to the Holocene. It was particularly widespread in the Pliocene, and then declined (Casanova *et al.*, 2003). In the last few decades, *Lychnothamnus barbatus* (Meyen) Leonhardi, the extant species, has not been found in European or Asian localities, except for a population in Warrill Creek, Queensland, Australia (McCourt *et al.*, 1999), and also in Wallace Creek, Queensland (Casanova *et al.*, 2003; García, 2003). Recently, several new localities have been discovered from northern Australia, around the Gulf of Carpentaria (García & Chivas, this volume).

Very few studies have focused on the charalean algae of Taiwan (Imahori, 1951, 1953, 1954, 1957; Yang & Chiang, 1978) and this is the first time *L. barbatus* has been recorded from there. We analysed the morphological characteristics of *L. barbatus* under LM and SEM, and also used the *rbc*L gene for phylogenetic analysis. The *rbc*L gene was chosen because the gene's rate of change might suggest intraspecific differences (McCourt *et al.*, 1999). We also compared the phylogenetic and biogeographic relationships of *L. barbatus* from Taiwan, Europe and Australia.

MATERIALS AND METHODS

Study site

Lanyu Island is one of a series of volcanic islands between Taiwan and the Philippines in the western Pacific Ocean. The climate is tropical humid, with heavy rainfall (annual average 3081 mm) and high humidity (annual average over 90%). Hence, Lanyu Island is located at the northern limit of the tropical rain forest and characterized by a tropical rain forest plant community (Lin & Yen, 1982; Su & Ho, 1982).

Observations on the thalli

Lychnothamnus barbatus was collected from a freshwater water-body on Lanyu Island, Taiwan (Fig. 1). Specimens were preserved in 95% alcohol for molecular analysis, preserved in 4-5% formalin for morphological observations, and pressed in order to create a reference collection. Some specimens were kept in culture. Vegetative and reproductive structures were examined using a light microscope (LM) (Zeiss Axioskop 2) and a dissecting microscope (Zeiss Stemi SV11). Drawings were made with the aid of camera lucida. For scanning electron microscope (SEM) observations, mature oospores of *L. barbatus* were cleaned

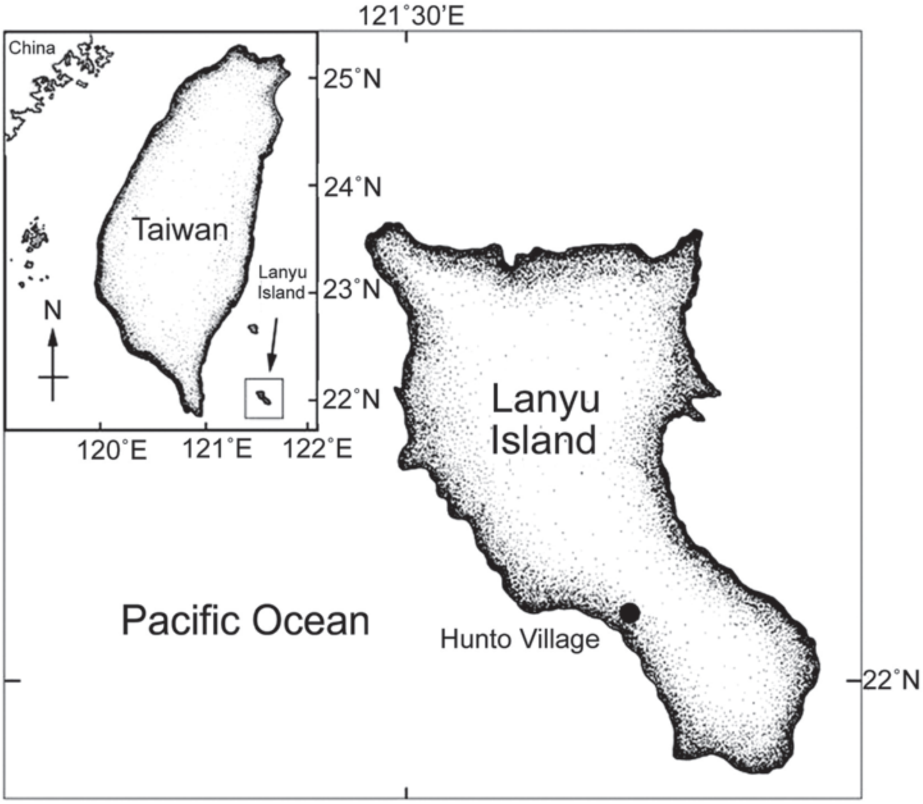


Fig. 1. Map showing Lauyu Island, Taiwan.

before examination, and then the remains of the spiral cells were removed with the aid of a fine needle and forceps under a dissecting microscope. Those selected oospores were placed for 12h in a 10% solution of Tween-20 detergent at 60°C (Casanova, 1991). The oospores were washed several times with distilled water, dehydrated by Freeze Dryer (Eyela FDU-506), then coated with Gold-Paladium by Sputter coater (Hitachi E - 1010), and finally observed under SEM (Hitachi 2460N) at an accelerated voltage of 20 kV. The terminology for charalean oospores used to describe the ornamentation patterns follows Wood (1965) and John & Moore (1987). Voucher specimens have been deposited at Department of Biology, National Changhua University of Education, Taiwan.

DNA extraction and PCR

Genomic DNA extraction from ethanol-preserved specimens was based on the protocol of the DNeasy Plant Mini Kit (Qiagen, Valencia, CA), and the selected gene was the chloroplast-encoded *rbc*L. The primers and protocols for gene amplification and automatic sequencing followed Sakayama *et al.* (2002). The generated *rbc*L sequences were compiled and aligned by using the software BioEdit v 5.0.9 (Hall, 2001) and then exported for phylogenetic analysis. In

Table 1. Species used in the *rbc*L sequence analysis.

Species	*Strain and locality*	*GenBank number*
Ingroup		
Chara rusbyana	X066, Argentina	AF097168[A]
C. vulgaris	X152, Denmark	AF097166[A]
C. globularis	F124C, Germany	AF097163[A]
C. connivens	X774, Spain	AF097160[A]
Lamprothamnim macropogon	X695, Australia	U27534[B]
L. papulosum	F137, France	AF097170[A]
Lychnothamnus barbatus	LY, Taiwan	AY707914[C]
Ly. barbatus	Ger, Germany	AF097172[A]
Ly. barbatus	Aus, Australia	AF097171[A]
Ly. barbatus	Croa, Croatia	U27533[B]
Nitellopsis obtusa	F131B, Western Germany	U27530[B]
Nitella gracilens	KINU, Japan	AB076063[D]
N. translucens	F108, Western France	AF097745[A]
N. pulchella	S011, Japan	AB076057[E]
Tolypella glomerata	F131A, Germany	AF097176[A]
T. nidifica	F138, France	U27531[B]
Outgroup		
Coleochaete nitellarum		AB013662[F]
C. orbicularis		L13477[G]
Zygnema peliosporum		U38701[H]

[A]McCourt, 1999; [B]McCourt *et al.*, 1996; [C]This study; [D]Morioka, 1941; [E]Sakayama, 2002; [F]Nishiyama & Kato, 1999; [G]Manhart, 1994; [H]McCourt *et al.*, 1995.

additions, 16 sequences of the charalean algae were directly obtained from the GenBank as ingroup, and three sequences of *Coleochaete nitellarum* Jost, *C. orbicularis* Pringsheim and *Zygnema peliosporum* Wittrock, were also downloaded as outgroup (Tab. 1).

Phylogenetic analysis

Phylogenetic analyses were performed using the neighbor joining (NJ) algorithms, the maximum parsimony (MP) available in the computer program PAUP (v. 4.0b10; Swofford, 2003) on a data matrix consisting of 1169 nucleotides of *rbc*L gene (Saitou & Nei, 1987). For NJ analysis, a distance matrix was calculated by applying the Kimura 2-parameter method (Kimura, 1980). Support for the nodes of NJ tree was determined by calculating bootstrap proportion values based on 1000 resamplings of neighbor-joining searches. The MP trees were constructed using a heuristic search with the stepwise addition of 1000 random replications, MULTREES (holding five trees at each step), and a tree bisection-reconnection (TBR) branch swapping algorithm. Support for nodes of the MP tree was assessed by calculating 1000 bootstrap resamplings of the heuristic searches based on random stepwise additions, MULTREES and TBR (Felsenstein, 1985).

RESULTS

Morphological observations

Lychnothamnus barbatus **(Meyen) Leonhardi 1863: 51.**

Type locality. Germany

Specimens examined. Hunto Village, Lanyu Island, Taiwan, *L.-C. Wang, S.-L. Liu & W.-L. Wang, NCUE-JYChou-920711-01*, 11.vii.2003.

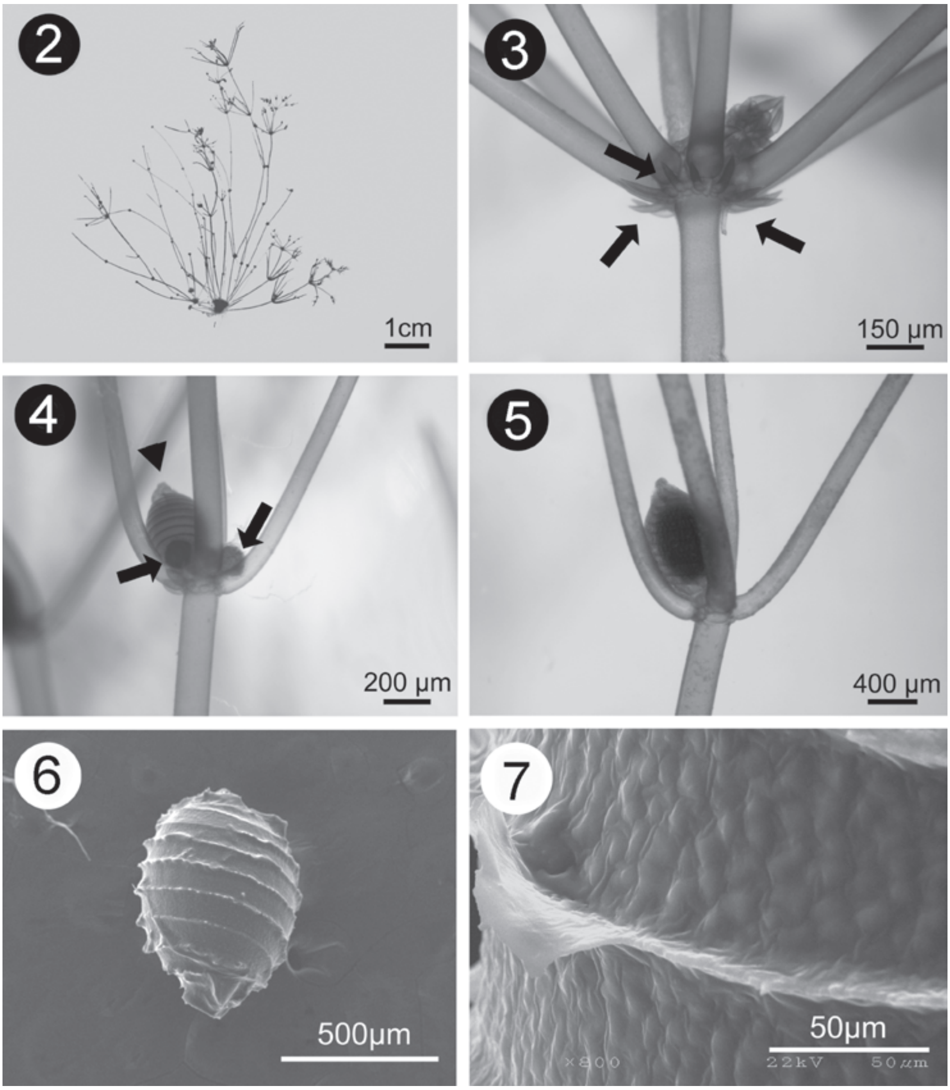
Vegetative morphology. Plants are up to 30 cm in height and light green in color (Fig. 2). Stem axes are ca 550 µm in diameter. The internodes of the lower part of the plant are longer than upper ones, and always longer than the branchlets. Spine cells are absent on axes. Stipulodes are in a single whorl, twice the number of the branchlets (Fig. 3). There are 6-7 branchlets in a whorl, each branchlet is up to 1-3 cm high and has 2-3 articulations. In culture, there were several shoots arising from one starchy bulbil at the base of the axis.

Reproduction. Plants are monoecious. Gametangia arise from all branchlet nodes, but not at the base of the whorls. At each node, one oogonium is produced between two antheridia (Fig. 4). Antheridia are 200-230 µm in diameter. The largest polar axis (LPA) of gyrogonites is 950-1075 µm and the largest equatorial diameter (LED) is 600-725 µm. Spiral cells show 8-10 convolutions and the coronula has 5 cells (Fig. 5). Oospores are brown in colour, 710-850 µm long and 500-580 µm broad with 8-9 striae (Fig. 6). In SEM, mature oospore walls show verrucate ornamentation (Fig. 7).

Molecular phylogenetic analysis

Partial *rbc*L sequences of *L. barbatus* from Taiwan have been generated for the first time in this study. No insertions or deletions were observed within the aligned sequences. For the phylogenetic analysis, 16 taxa of charalean algae were selected as an ingroup and three taxa as an outgroup (Tab. 1). The *rbc*L alignment consisted of 1164 sites, but the missing characters were excluded. Four equally parsimonious trees were found in maximum parsimony (MP) analysis based on a heuristic search using the stepwise addition of 1000 random replications. The neighbor joining (NJ) tree is shown in Fig. 8, and the minimum evolution score was 0.491. All the branches are supported by ≥ 50% bootstrap values in NJ tree, and the boostrap values are shown above the nodes. One of the four equally parsimonious MP trees is shown in Fig. 9, and branch lengths are shown above and boostrap values below each branch. The MP tree is 730 steps long, of these, 281 characters are parsimony informative, and the consistency index (CI) and retention index (RI) are 0.669 and 0.783 respectively. Monophyly of the family Characeae is strongly supported (100%).

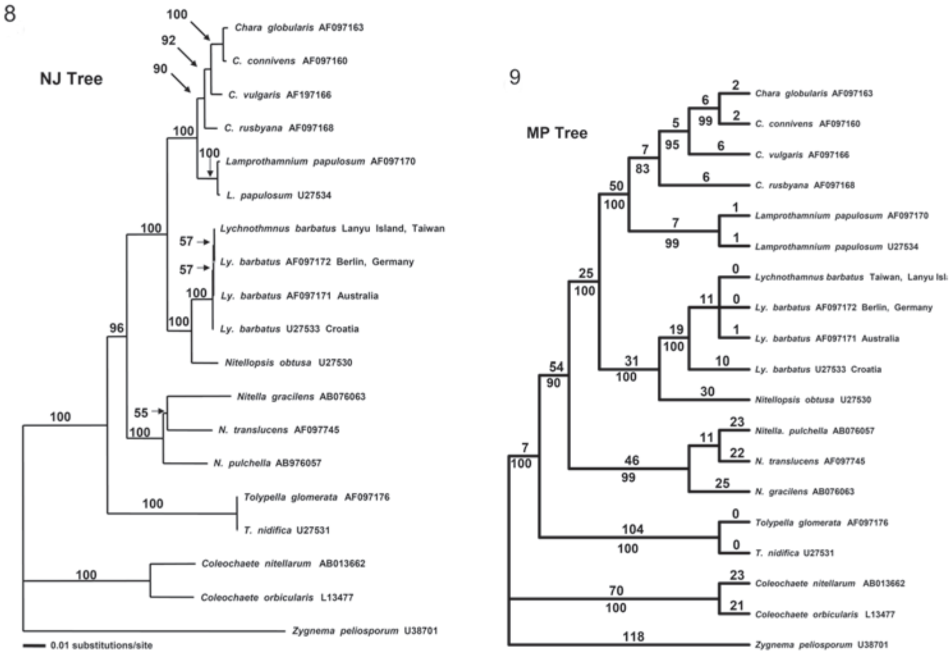
The pairwise distance measurements for the taxa shows that the differences among isolated localities within species of *Chara, Lamprothamnium* and *Lychnothamnus* (tribe Chareae) are not over 1% (data not shown). We only show the pairwise distance among different localities of *L. barbatus* (Tab. 2). There is not more than 0.3% variation in the pairwise distance between Taiwan, Germany, Australia and Croatia.



Figs 2-7. Morphological and reproductive structures of *Lychnothmnus barbatus*. **2.** Habit. **3.** Part of branchlets showing the single whorl of stipulodes (arrow). **4.** One oogonium (arrowhead) between two antheridia (arrow) arising at the node of a branchlet. **5.** Gyrogonite arising from the node. **7.** Oospores under SEM. **8.** Oospore wall showing verrucate ornamentation.

Table 2. Pairwise distance in *rbc*L (bp) of *Lychnothmnus barbatus* from Taiwan, Germany, Australia, and Croatia. Each number indicates absolute distances and sequence divergence.

	Taiwan	*Germany*	*Australia*	*Croatia*
Taiwan	-	0 %	0.085%	0.091%
Germany	0	-	0.074%	0.148%
Australia	1	1	-	0.222%
Croatia	1	2	3	-



Figs 8, 9. Phylogenetic trees based on 1169 base pairs in the encoding regions of the *rbc*L genes of 16 species of the charalean genera, *Chara*, *Lamprothamnium*, *Lychnothamnus*, *Nitellopsis*, *Nitella*, and *Tolypella* as well as three outgroup (Tab. 1). **8.** Neighbour-joining tree. Minimum evolution score = 0.497. Boostrap values (1000 replications, ≥ 50%) is shown above the nodes. Branch lengths are proportional to the amount of sequence change. **9.** One of the four equally maximum parsimony (MP) Tree length = 597 steps; CI = 0.737, RI = 0.795 (97 variable characters are parsimony-uninformative, 262 characters are parsimony-informative). Branch lengths shown above and bootstrap support (1000 replications, ≥ 50%) below each branch.

DISCUSSION

The gyrogonites of *Lychnothamnus* are distinctive among the Characeae due to the following structural features: (1) They show a characteristic elliptical shape; (2) The apical pole is flattened and the basal pole typically shows a star-shaped funnel formed by the protruding sutural crests around the basal opening; (3) The basal opening is pentagonal and located at the bottom of the funnel. Because of these features, *Lychnothamnus* gyrognites had long been classified as genus “*Rhabdochara*” (Mädler) Grambast (1957). Soulié-Märsche & Martín-Closas (2003) indicated that the apex and basal characteristics of fossil “*Rhabdochara*” are also present in the living *Lychnothamnus* and thus *Rhabdochara* was transferred to genus *Lychnothamnus*.

A comparison of gyrogonites of species of fossil *Lychnothamnus* and the extant species, *L. barbatus*, show similarities in the morphological features as well as biogeographical distribution. Thus, *L. barbatus* is considered as the only survivor of the genus *Lychnothamnus*. The ecological requirements for *L. barbatus* are permanent freshwater lakes or temporary as in some localities from Australia, with an optimal depth of one to five meters. Kargzmarz (1967) also pointed that *L. barbatus* can not fructify in a saline environment (*cf.* Petit-Maire & Riser, 1981).

Lychnothamnus barbatus has been recorded from Africa, Europe, China, India, and Australasia (Allen, 1888; Allen, 1925, 1928; Zaneveld, 1940; Jao, 1947; Corillion, 1957; Pal *et al.*, 1962; Wood, 1965; Bharati & Chennaveeraiah, 1983; Blazencic & Blazencic, 1983; Leach & Osborne, 1985; Krause, 1986; Casanova *et al.*, 2003), and its distribution is shown in Fig. 10. In this study, we found *L. barbatus* in Lanyu Island, Taiwan, which is formed by volcanism of Miocene-Pliocene age (Pelletier & Stephan, 1986). Later, García & Chivas (this volume) recorded *L. barbatus* from permanent freshwater rivers/creeks from north Queensland in areas affected by the summer monsoon. From the palaeobiogeographical point of view, *L. barbatus* seems to be an Eurasian taxon. The species was widely distributed from France to the southern part of Asia, with the fossil populations showing quite similar distribution. In Africa, the finding of fossil *Lychnothmnus* in the deposits of Erg Ine Sakane Lake is remarkable. This modern hyper-arid area had humid conditions around 7500 years ago (Petit-Maire & Riser, 1981).

The similarity of morphologic features, and nearly identical *rbc*L sequences in the populations from Taiwan, Europe, and Australia, indicate they originated from the same population. As we know, migratory birds usually disperse oospores of charophytes, which are viable after digestion (Proctor, 1962). Except the population of *L. barbatus* in Taiwan, that in Australia is the only stable population in the world at present (García, 2003).

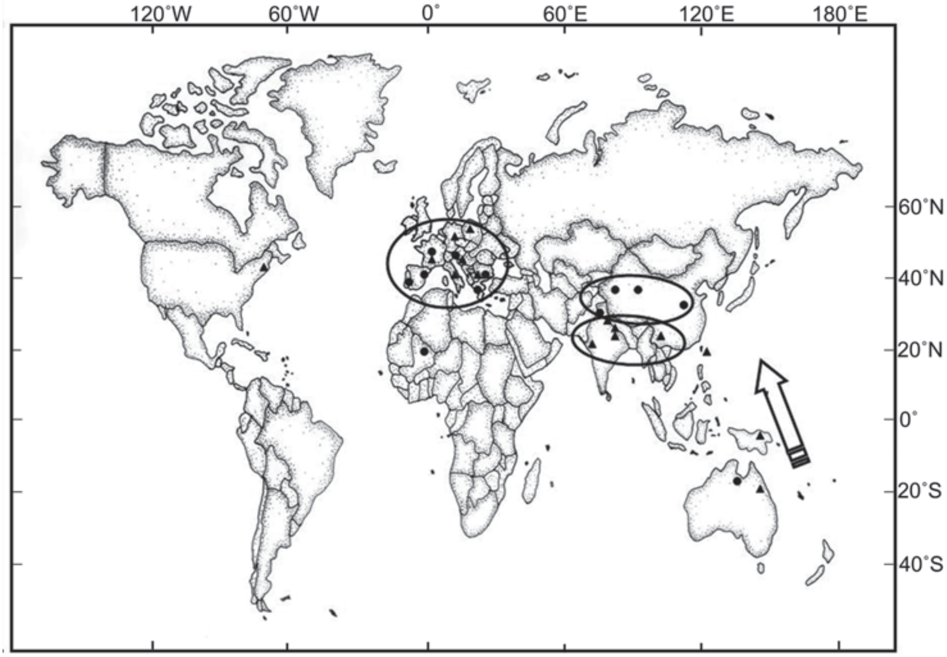


Fig. 10. Map showing the world-wide distribution of fossil *Lychnothmnus* (circle), and extant *Lychnothmnus barbatus* (triangle) and the possible path of dispersal of Taiwanese *L. barbatus*.

Table 3. Comparison of *Lychnothmnus barbatus* gyrogonites from Taiwan, Europe, and Australia.

	Lanyu Island Taiwan [A]	*Branderberg Germany* [B]	*Parsteiner-See Germany* [C]	*Wallace Creek Australia* [D]
Largest polar axis (LPA)	950-1075 µm	770-860 µm	700-975 µm	800-975 µm
Largest equatorial diameter (LED)	600-725 µm	590-680 µm	550-775 µm	(520)-560-640 µm
Number of circumvolutions	8-10	8-9	-	8-11
Isopolarity Index (ISI=(LPA/LED) *100)	135-166	117-139	115-140	(134)-140-171

[A]This paper; [B]Horn af Rantzien 1959; [C]Soulié-Märsche 1989; [D]García 2003.

In this study, gyrogonites of the population from Taiwan are compared with the populations from Europe and Australia by a comprehensive statistical analysis (Tab. 3). The gyrogonite width of the Taiwan material was within the mean of the Europe populations. The mean gyrogonite length is greater in Taiwan, and its isopolarity index (ISI) in the range of 135-166, quite different from the ISI of the Europe gyrogonites (ISI = 115-140), and is closest to the materials from Australia (ISI = 140-171). The results revealed similarities and differences among the gyrogonites of *L. barbatus* from Taiwan, Europe and Australia. The differences in length and the isopolarity index are interpreted as intraspecific variability (García, 2003), therefore in agreement with the molecular analysis of the different populations that indicates their high similarity.

Dispersal by aquatic birds is useful for algae and other organisms with resting eggs, extending the populations and accelerating the gene flow. The migration of some waterfowls between Australia and Taiwan (Driscoll & Ueta, 2002; Wei & Mundkur, 2003), provides the possible means of dispersion of oospores of algae to/from the shallow taro fields in Taiwan. Therefore, based on the similarity of oospore/gyrogonites of *L. barbatus* from Australia and Taiwan, we postulate that *L. barbatus* was dispersed by waterfowls from/to Australia to/from Taiwan. The similarity of morphological features, and nearly identical *rbc*L sequences in the three populations from Taiwan, Australia and Europe, suggest the isolated populations originated from one population. Further sampling of *L. barbatus* from SE Asia would test the hypotheses about its route of dispersal from/to Taiwan.

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