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Caloglossa beccarii (Delesseriaceae, Rhodophyta) from freshwater rivers in Kerala, India, a critical new record

John A. West^{1,*}, Mitsunobu Kamiya², E. K. Ganesan³, Susan Louiseaux-de Goër⁴ and L. Jose⁵

¹School of Botany, University of Melbourne, Parkville, Victoria 3010, Australia

²Faculty of Marine Bioscience Fukui Prefectural University, 1-1 Gakuencho, Obama, Fukui 917-0003, Japan

³Instituto Oceanográfico, Universidad de Oriente, Cumaná 6101, Venezuela

⁴11 Rue des Moguerou, 29680 Roscoff, France

⁵Department of Botany, St. Albert's College, Ernakulam, Kerala 682018, India

Caloglossa species occurs in freshwater streams around Southeast Asia. We report it from 2 different riverine sites in Kerala, India. Tetrasporangiate plants were observed in field collections from the Periyar River and Chalakkudy River. The Chalakkudy isolate did not reproduce in culture but the Periyar isolate developed abundant tetrasporangial sori in culture. Many spores were discharged and most were abortive, but some germinated normally, sporelings forming male gametophytes with numerous spermatangial sori and females with many procarps, viable carposporophytes and some nonfunctional (no carpospores) pseudocystocarps. Some carpospores germinated forming new tetrasporophytes. Molecular evidence (28S rDNA and *rbcl*) placed the Indian specimens close to *C. beccarii* and *C. fluviatilis*. Considering the freshwater habitat and morphology of vegetative thalli (blade shape, rhizoid arrangement, and number of rhizoid filament per cell), the Indian specimens should be assigned to *C. beccarii*.

Key Words: *Caloglossa beccarii*; *Caloglossa fluviatilis*; India; Kerala; LSU; *rbcl*

INTRODUCTION

The Ceramiales is the most varied order among red algae (ca. 420 genera, 2,660 species) (Guiry and Guiry 2015), but only a few genera have been found from freshwater habitats. The genus *Caloglossa* (Delesseriaceae, Ceramiales) is widespread in tropical to temperate mangrove, estuarine and freshwater habitats. It is comprised of 22 currently accepted species (Guiry and Guiry 2015), and the following six species have been reported from freshwater streams.

Caloglossa beccarii (Zanardini) DeToni was described as *Delesseria beccarii* Zanardini that occurred with *Tho-*

rea flagelliformis Zanardini in a freshwater stream near Gunung Pueh in Sarawak, Malaysia (Zanardini 1872, Bec-cari 1904). The species is recorded as “epilithic on stones in freshwater coastal streams and epiphytic on mangrove trunks, prop roots and pneumatophores in areas with moderate water flow and high turbidity and restricted to south-east Asia (India, Burma, Malaysia, Indonesia, Singapore), the western Pacific, and northern Australia as far south as 28° S” (King and Puttock 1994, Kamiya et al. 2003). On Guadalcanal, Solomon Islands, Womersley and Bailey (1970) recorded it growing in a swimming hole near

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*Corresponding Author

E-mail: jwest@unimelb.edu.au

Tel: +61-3-8344-8080, Fax: +61-3-9347-5460

Fig. 1. (A) Map of Kerala, India showing Periyar and Chalakkudy River collecting sites. (B) Ganesan and Jose collecting at Ezhattumugham, Chalakkudy River in 2013, showing solid rock substrate and topography. (C) Habit of field specimens from Ezhattumugham, Chalakkudy River.

the mouth of a freshwater stream where it was subject to seawater at high tide. The first reported Indian specimen was obtained by Biswas in 1936 from the Hooghly River at the Calcutta Botanical Garden (now Ancharya Jagadish Chandra Bose Indian Botanic Garden) (<http://www.nhn.leidenuniv.nl/index.php/institute>). Unfortunately no herbarium specimens are available in the National Herbarium in Leiden, The Netherlands or the National Botanical Garden in Calcutta so this record is unsubstantiated. *C. beccarii* also has been found in other SE Asian countries including Thailand (Sato and Akiyama 2001), Vietnam (Kamiya et al. 2003) and in freshwater rivers (24–29°C, pH 7–8, conductivity 40–50 $\mu\text{S cm}^{-2}$) in Selangor and Negeri Sembilan of peninsular Malaysia (Johnston et al. 2014).

C. beccarii, which had been recorded from a freshwater habitat in the Pédro Miguel Locks of the Panama Canal (US Alg. Type Coll. 217746) (Lin et al. 2001), was redesignated as a new species, *C. fluviatilis* Kratesky, Fredericq

et J. N. Norris (Kratesky et al. 2012), because, in addition to the distinct phylogenetic relationship, the rhizoidal arrangement and nodal constriction of blades are distinct from those of *C. beccarii* (Kratesky et al. 2012).

The closely related *C. ogasawaraensis* Okamura was first described from the Ogasawara Islands of southern Japan (Okamura 1897) and is now widely recorded in marine, estuarine and freshwater habitats around the world (eastern USA; King and Puttock 1994, West and Zuccarello 1995) (Central and South America; Sheath et al. 1993, Pedroche et al. 1995, Kratesky et al. 2012, Zuccarello et al. 2012) (Australia, Micronesia, southeast Asia and Madagascar; Kamiya and West 2014). *Caloglossa ogasawaraensis* (as *C. ogassuarensis* Skuja) was observed in freshwater streams of Costa Rica by Soto (1982) and was first recorded from freshwater in India by Jose and Patel (1990).

Caloglossa leprieurii (Montagne) G. Martens was col-

lected from a freshwater stream in Puerto Rico (Sheath et al. 1993). In Australia *C. vieillardii* (Kützinger) Setchell was recorded from freshwater at the Hopkins River Waterfall, Victoria and Jacksons Creek, Organ Pipes National Park, Victoria, Australia (as *C. lepriurii*) (West and Kraft 1996, West et al. 2001). In China *C. saigonensis* (as *C. lepriurii* var. *angusta*) was found in Szechwan Province near Pehpei on the Jialing River, over 1,500 km from the Yangtze River mouth near Shanghai (Jao 1941).

We have investigated the *Caloglossa* populations in Kerala for several years and compared them with various species using morphological, culture and molecular analyses and think it is now important to clarify general biological and taxonomic aspects of the Kerala populations.

MATERIALS AND METHODS

Collection details are shown in Fig. 1A map and Table 1. The Chalakkudy River general habitat and the collectors, L. Jose and E. K. Ganesan, are shown in Fig. 1B and the typical habit of a field collected plant is in Fig. 1C. Herbarium specimens of *C. beccarii* from Neriampalam, Kerala are deposited in University of Michigan Herbarium (MICH 684329) and University and Jepson Herbaria, University of California, Berkeley (UC/JEPS 2050401).

Culture methods

Isolation and culture methods are similar to those used in earlier work (West et al. 2001, West 2005). Irradiance was 10 : 14 h diurnal light-dark, cool white LEDs or cool white fluorescent lamps (3-12 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and temperature 18-22°C air-conditioning. Although the field specimens were collected in very low salinity (<0.5) we cultured them in salinities of 2 or 5 with standard modified Provasoli's medium (MPM) supplemented with peat extract to mimic tannin levels in tropical freshwater rivers. Sterile peat extract was prepared as 500-mL MilliQ water, 15 g peat moss (commercial garden package), steam sterilized for 30 min, cooled, vacuum filtered with two sheets of Whatman No. 1 (9 cm), re-sterilized and stored at 4°C. The extract fluid was clear and dark brown. One litre of culture medium was prepared as 975-mL MilliQ water, 5 mL peat extract, 10 mL seawater (salinity of 30), and 10 mL MPM nutrient enrichment. This has been useful for culturing many red algae from freshwater / low salinity habitats.

Molecular analyses

The DNA extraction method, polymerase chain reaction (PCR) conditions, and sequencing procedure

Table 1. *Caloglossa beccarii* collection locations, substrates, temperatures, salinities, reproduction, collectors, and dates, Kerala, India

Location	Collecting date	Collector	Note
4 km upstream from Ezhattumugham, Chalakkudy R. (10°17.746' N, 76°22.084' E)	Nov 24, 2012	Jose	India1 (sample name); tetrasporophyte; on solid rocks in fast running clear water
Ezhattumugham, Chalakkudy R.	Apr 16, 2013	Jose	Vegetative; on solid rocks, fast running clear water, elevation 140 m
	Oct 9, 2013	Jose and Ganesan	Vegetative
	Dec 7, 2013	Jose and Ganesan	Vegetative
Maniyanpara (between Chembankuzhi and Nereamangalam), Periyar R., Idukki District (10°3.384' N, 76°48.110' E)	Oct 6, 2013	Jose	India2 (sample name); vegetative; elevation 45 m
Ezhattumugham, Chalakkudy R. (10°17.746' N, 76°22.084' E)	Feb 13, 2014	Jose, Ganesan, and West	India3 (sample name); tetrasporophyte; on shaded solid rocks in fast running clear water, water temp. 32°C, salinity 0.1
Nereamangalam cacao plantation, Periyar R. (10°2.547' N, 76°47.777' E)	Feb 14, 2014	Jose, Ganesan, and West	India4 (sample name); vegetative; on submerged rocks and sandbags (30-50 cm deep) in wide, slow moving, murky water, water temp. 26°C, salinity 0.2

Fig. 2. Field specimens collected from Chalakkudy River, Kerala, India on November 24, 2012. (A) Whole thallus. Several rhizoidal filaments are produced from the ventral side of the nodes (arrowheads). (B) Slightly constricted node. (C) Part of thallus at second node from apex. Lateral axis (large arrow) is still diminutive. Nodal cell (diamond) produces one cell row (small arrow) toward main axis side. First axial cell of lateral axis (triangle) forms one cell row on adaxial side. First axial cell of the main axis (star) produces two cell rows (arrowheads) opposite lateral blade. (D) Rhizoidal filaments (arrowheads) produced from pericentral cells at node. (E) Adventitious blade initials (arrows) developed from lateral pericentral cells of first axial cell above node. (F) An adventitious blade initial (arrow) derived from internodal marginal cell. (G) Immature tetrasporangia (arrows). Scale bars represent: A, 500 µm; B, 200 µm; C, E & G, 50 µm; D & F, 100 µm.

Table 2. The information of primers for PCR and sequencing

Target	Primer name	Primer sequence (5'-3')	Direction
LSU rDNA (for PCR)	LSU-YF	GCAGGACGGTGGCCATGGAAGT	Forward
	LSU-ZR	TGATAGGAAGAGCCGACATCGA	Reverse
LSU rDNA (for sequencing)	LSU-ZF2	CCGTAACTTTGGGAAAAGGGTTGGC	Forward
	LSU-YR2	CGGATTCCTTTGCCCGTTGCRGTTTC	Reverse
<i>rbcL</i> (for PCR)	<i>rbc</i> -F3	ATGTCTAACTCTGTAGAAGAACGGAC	Forward
	<i>rbc</i> -R4c	GGRTGTGGATCWTCWGTATAYTC	Reverse
<i>rbcL</i> (for sequencing)	<i>rbc</i> -R6b	GCWACACGRITTWGCTGTTGCWCC	Reverse
	<i>rbc</i> -F7	GGTATYCAITGYGGWCARATGC	Forward
	<i>rbc</i> -R8	ATCTYTCTTTCCARCGCATRAAWGG	Reverse

PCR, polymerase chain reaction; LSU rDNA, large subunit of rDNA.

followed Hayakawa et al. (2012); specifically PCR and sequencing were carried out on the large subunit of rRNA (LSU) gene and the large subunit of RuBisCO (*rbcL*) gene. DNA was extracted using field-collected specimens from Chalakkudy River (India1) and from Periyar River (India2). The sequences of primers used for PCR and sequencing are presented in Table 2. The sequence data were deposited in the DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp>) (LC065028-LC065031).

The methods for alignment of sequence data and construction of phylogenetic trees were the same as those described by Kamiya and West (2014). Adjustments of the resulting alignments were performed manually, resulting in 1,329 bp for LSU and 1,417 bp for *rbcL*. Using the program Kakusan4 (Tanabe 2011), the best-fitted substitution model was estimated for maximum likelihood (ML) analysis on the basis of the corrected Akaike information criterion (TN + G and GTR + G for LSU and *rbcL*, respectively) and for Bayesian inference (BI) analysis based on the Bayesian information criterion (K80 + G and GTR + G for LSU and *rbcL*, respectively).

RESULTS

Morphological observations

Thalli collected in the Periyar River on February 14, 2014 were growing on shallow rocks, dark red to brown, forming densely branched tufts up to 2 cm high (Figs 1C & 2A). The blades branched subapically and were linear to narrowly lanceolate, slightly constricted at the nodes, up to 4.5 mm long (Fig. 2B). Blade width was 50–160 μm at the upper part, 90–320 μm at the middle part, and 40–120 μm at the lower part. Initials of lateral axes were frequently seen not only at the first but at the second or third nodes because of their slower elongation than the main axes (Fig. 2C). The numbers of cell rows around the node, which are the important characters for identifying *Caloglossa* species, were as follows: one or occasionally two adaxial cell rows form from the first axial cell at the lateral axis; the nodal axial cell producing one cell row (small arrow in Fig. 2C) and the first axial cells of the main axis (FMA) forming one or two cell rows opposite the lateral blade (arrowheads in Fig. 2C). A single rhizoid filament, 20–35 μm in diameter and up to 4 mm long (Fig. 2D), was derived from a pericentral cell at the node or immediately above and below the node. No wing cells produced rhizoid filaments. Adventitious secondary blades occasionally arose from the first axial cell above the node

at the same plane as that of the thallus (Fig. 2E), rarely from the marginal cells at the internodes (Fig. 2F); endogenous blades were not seen. We observed only a few plants with immature tetrasporangia (Fig. 2G). Gametophytes with sexual reproductive structures were never found in the field. All the above observations were made with dried field specimens.

Both strains (4840 from Chalakkudy River and 4841 from Periyar River) were cultured, initially in a salinity of 5 at low photon fluence rates (3–5 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) for 2–3 months and then shifted to >10 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 4 months. Isolate 4840 grew more slowly than 4841 and did not reproduce in over one year of culture. By contrast 4841 grew robustly, plants reaching >20 mm in 3 months (Fig. 3A). After four months robust tetrasporangial sori developed (Fig. 3A & B) and many spores were discharged but germination was poor. However surviving sporelings (Fig. 3C) grew well and after 7 months male gametophytes developed spermatangial sori (Fig. 3D & E) with normal spermatia released. As in field specimens, adventitious blades of cultured blades were derived from internodal marginal wing cells of vegetative blade (Fig. 3F). Female gametophytes developed carpogonial branches along the midrib (Fig. 4A & C). Numerous carposporophytes developed in about one month (Fig. 4B & D) and discharged some carpospores from which a few sporelings developed. Also some ‘pseudocystocarps’ developed that did not form carpospores (Fig. 4E). Although at a salinity of 5 the thalli grew well and reproduced, the overall viability of spores was low. We attempted culturing field collected plants initially at a salinity of 1–2 but survival and growth were not as good as in a salinity of 5. It is puzzling that tetraspore and carpospore survival and germination were low at a salinity of 5.

Phylogenetic analyses

The two Indian specimens (India1 from Chalakkudy River and India2 from Periyar River) showed only one bp substitution in the LSU sequences. The LSU analyses revealed that these Indian specimens made a clade with *C. fluviatilis* from Panama (57% ML support and 0.61 BI support) (Fig. 5). The relationship between this clade and *C. beccarii* from Singapore, Vietnam and Thailand was unresolved.

The *rbcL* sequences of the two Indian specimens (India1 and India2) were identical. In the *rbcL* phylogeny, they were also related to *C. fluviatilis* but it was weakly supported (56% ML support and 0.84 BI support) (Fig. 6). Their relationship with *C. beccarii* was unresolved.

Fig. 3. (A) Habit of culture 4841 showing branching, nodes and tetrasporangial sori. (B) Tetrasporangial sori (single row of sporangia on each side of midrib) showing developing, mature and discharged sporangia. (C) Young tetraspore germling with upper blade and rhizoids at base. (D) Mature male with spermatangial sori on many blades. (E) Blade tip with elongate spermatangial sori. (F) Adventitious blades from intermodal marginal wing cells of vegetative blade like that of field specimen, Fig. 2F. Scale bars represent: A & D, 1 mm; B, C, E & F, 100 µm.

Fig. 4. (A) Female trichogyne with numerous attached spermatia. (B) Female with numerous developing and mature carposporophytes. (C) Female with supporting cell (sc), carpgonial branch (prc) and trichogyne (arrowhead) on blade midrib. (D) Lateral view of young cystocarp with normal pericarp and gonimoblast with developing carposporangia visible underneath. Lightly stained with anilin blue. (E) Face view of pseudocystocarp with pericarp enclosing ostiole in center. Panels C, D, and E were treated with 10 s. 800 W microwave to shrink cells. Scale bars represent: A & D, 100 μ m; B, 500 μ m; C, 25 μ m; E, 50 μ m.

Fig. 5. Maximum-likelihood (ML) phylogeny of *Caloglossa* species inferred from the partial large subunit of rRNA gene sequences. *Taenioma perpusillum* (J. Agardh) J. Agardh was used as an outgroup. The bootstrap values for ML (>50%; left) and posterior probabilities for Bayesian inference (≥ 0.80 ; right) are presented for each branch. Accession number, strain number or sample name is shown in each parenthesis.

Fig. 6. Maximum-likelihood phylogeny of *Caloglossa* species inferred from the partial *rbcL* gene sequences. *Centroceras gasparrinii* (Meneghini) Kützinger was used as an outgroup. Other information is in the Fig. 5 legend.

DISCUSSION

Although the freshwater *Caloglossa* species are present in various temperate and tropical regions of the world neither the general biology nor molecular relationships have been investigated adequately. How did these transitions from the marine and estuarine habitats to freshwater occur? How have growth, reproduction and physiology changed in these radically different conditions? They are also subjected to a different set of cohabitants, plants, animals and pathogens. Grazing by different animals may require different defense mechanisms (Goodman and Hay 2013).

In both LSU and *rbcL* trees, the present alga made a clade with *C. fluviatilis*, which was not supported by high values of bootstrap and BI analyses. *C. fluviatilis* is separated from *C. beccarii* by forming rhizoids from nodal pericentral cells and adjacent wing cells (type F in Kamiya et al. 2003) and multiple rhizoid filaments per cell (Krayesky et al. 2012). It is occasionally difficult to delineate the arrangement pattern of rhizoids around the node, especially type D, E, and F, which are distinguished by the presence / absence of rhizoids derived from wing cells (Kamiya et al. 2003). This feature may be affected by blade width because the number of wing cells around the nodes tend to be fewer in slender thalli (Kamiya unpublished data). By contrast, the number of rhizoid derived from a cell is relatively stable and clear-cut in most *Caloglossa* species (Kamiya et al. 2003), and the Indian alga, which has single rhizoid filament per cell, is distinguishable from *C. fluviatilis*.

This alga is similar to *C. ogasawaraensis* based on the slender blades and the production of rhizoids only from pericentral cells around the nodes (type E in Kamiya et al. 2003). Actually Jose and Patel (1990) identified the specimen collected from Sholayar River, Kerala India as *C. ogasawaraensis*. However, *C. ogasawaraensis* produces only one cell row from FMA (Kamiya and West 2014), whereas the present alga sometimes forms two cell rows (Fig. 2C). This feature is used to distinguish *C. vieillardii* (mainly one cell row) from *C. leprieurii* (2-4 cell rows), which are phylogenetically distinct from each other (Kamiya et al. 2003). The disjunction between the Indian specimens and *C. ogasawaraensis* is also supported by our phylogenetic analyses.

A monophyletic relationship of *C. beccarii* was not supported in this study; the LSU tree shows that the specimens collected from brackish water habitats in Singapore and Vietnam were distinct from that from a freshwater habitat in Thailand. Kamiya et al. (2003) carried out the

detailed morphological observation of *C. beccarii* collected from both brackish and freshwater habitats but could not find any remarkable difference between them. Nevertheless, considering the common features, one or two cell rows numbers from FMA and single rhizoid filament per cell, the present alga should be assigned to *C. beccarii*. Because populations of *C. beccarii* examined is limited, more extensive samplings and culture experiments is necessary to delimitate this species.

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