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Reproductive and genetic distinction between broad and narrow entities of *Caloglossa continua* (Delesseriaceae, Rhodophyta)

MITSUNOBU KAMIYA[1]*, JIRO TANAKA[2], ROBERT J. KING[3], JOHN A. WEST[4], GIUSEPPE C. ZUCCARELLO[3] AND HIROSHI KAWAI[1]

[1]*Kobe University Research Center for Inland Seas, Iwaya, Awaji-cho 656-2401, Japan*
[2]*Laboratory of Phycology, Tokyo University of Fisheries, Konan-4, Minato-ku, Tokyo 108-0075, Japan*
[3]*School of Biological Science, The University of New South Wales, Sydney, NSW 2052, Australia*
[4]*School of Botany, University of Melbourne, Parkville, VIC 3052, Australia*

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The relationship of *Caloglossa continua* ssp. *postiae* to closely related taxa was assessed through an analysis of morphological characters, life history and culture studies, and molecular sequencing. Reproductive morphology provided no features by which subspecies *postiae* might be characterized. However, the distinctive narrow blades on which the taxon was originally described proved to be stable in a wide range of culture conditions. Crosses between isolates of subspecies *postiae* from Australia and Japan resulted in pseudocystocarps, but neither group of isolates was interfertile with *C. continua* ssp. *continua* or with *C. monosticha*. DNA sequence data from the RUBISCO spacer and the flanking regions of the *rbc*L and *rbc*S genes showed that subspecies *postiae* does not form a sister group with subspecies *continua* but is basal to the clade of taxa that exhibit endogenous branching. On the basis of these observations, subspecies *postiae* is described as a distinct species, *Caloglossa postiae* Kamiya et R.J. King.

INTRODUCTION

The relatively few characters and character states exhibited in many macroalgae, together with variation in their expression due to habitat, age, season, and broad geographical distribution, create considerable difficulties in the circumscription of algal taxa. This is especially true of the euryhaline red alga *Caloglossa*. The members of this genus are widely distributed and occur in habitats ranging from marine to brackish water. Post (1936) recorded wide morphological variation within species and, as a result, recognized several infraspecific taxa. In their monographic treatment of the genus *Caloglossa,* King & Puttock (1994) also recognized several subspecies, in particular *C. continua* (Okamura) King et Puttock, including *C. continua* ssp. *postiae,* which was differentiated only by narrow blades and short internodes. Although that subspecies could be clearly separated from other subspecies based on this character alone, its circumscription rested on field collections from only one population. Given the variability in blade width due to growth conditions exhibited in the genus (Kamiya *et al.* 1995), the taxonomic significance of the character in the recognition of *C. continua* ssp. *postiae* warrants attention. This is especially critical given the study of Wynne and De Clerck (1999), in which they conclude that differences in blade width are insufficient to differentiate two other *Caloglossa* species, *C. saigonensis* Tanaka et P.-H. Hô and *C. monosticha* Kamiya.

The principal characteristics for classifying *Caloglossa* species have been morphological, particularly branching pattern (Post 1936). Lateral axes in *Caloglossa* are formed exogenously, and additional secondary blades are borne either endogenously or adventitiously. Only one species exhibited endogenous branching of its secondary blades (Post 1936), and subsequently several species and subspecies have been distinguished based on rhizoidal position and the number of cell rows around nodes (King & Puttock 1994; Kamiya *et al.* 1997). Although some studies have demonstrated the taxonomic significance of these phenotypic traits (Kamiya *et al.* 1995, 1997, 1998), it is still unclear whether each character can be used for clarifying evolutionary relationships between species or subspecies. DNA sequence analysis, a recent trend for inferring phylogenetic lineage, appears to be an appropriate method for resolving this question. The spacer and its flanking regions of ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO) genes were selected for the present molecular phylogenetic analysis. These regions have been used successfully for phylogenetic and biogeographic studies at the levels of genera, species, and populations of red algae (Maggs *et al.* 1992; Goff *et al.* 1994; Brodie *et al.* 1996, 1998; Zuccarello & West 1997; Müller *et al.* 1998; Woolcott & King 1998).

In the present study, sequence data are combined with more traditional information on morphology and reproductive crossability to address the following questions: (1) is this slender form of *Caloglossa continua* ssp. *postiae* stable, even under various growth conditions; (2) if so, does reproductive incompatibility exist between the isolates showing different blade width; and (3) is each morphological feature, including blade width, that is used for distinguishing species or subspecies valid for establishing phylogenetic relationships?

MATERIALS AND METHODS

Collections were made at the localities indicated in Table 1 and Fig. 1. Methods of collection, culturing, and crossing testing are described in Kamiya *et al.* (1997).

DNA analyses were completed for two isolates of *Caloglossa continua* ssp. *continua,* two of *C. continua* ssp. *postiae,*

* E-mail: mkamiya@kobe-u.ac.jp

Table 1. Abbreviation of species and locality, species name, locality, date of collection, culture number of plants used in this study, and these isolator. Accession numbers are recorded in the DDBJ, EMBL, and GenBank sequence databases.

Abbreviation	Species	Locality	Date	Culture no.	Accession no.[1]	Isolator
AP-MEX	*C. apomeiotica*	Espiritu Santo Is., Mexico	19 May 92	910	D89948	Kamiya
CO-JPN1	*C. continua* ssp. *continua*	Kuriyama River, Chiba, Japan	4 Dec 90			Kamiya
	C. continua ssp. *continua*	Kuriyama River, Chiba, Japan	14 Jul 91	639, 640	D89950	Kamiya
CO-JPN2	*C. continua* ssp. *continua*	Kido River, Chiba, Japan	4 Dec 90			Kamiya
	C. continua ssp. *continua*	Kido River, Chiba, Japan	14 Jul 91			Kamiya
CO-JPN3	*C. continua* ssp. *continua*	Hidaka River, Wakayama, Japan	10 Mar 92			Kamiya
CO-JPN4	*C. continua* ssp. *continua*	Sumiyou River, Amami Is., Kagoshima, Japan	7 Apr 91	447, 500	AB023379	Kamiya
CO-JPN5	*C. continua* ssp. *continua*	Nasada River, Okinawa, Japan	8 Dec 91	728, 729	AB023380	Kamiya
LE-AUS1	*C. leprieurii*	Mangrove Bay, Exmouth, Western Australia	8 Oct 91	922	D89954	Kamiya
LE-AUS2	*C. leprieurii*	Oak Beach, Queensland, Australia	20 Sep 91	9	D89955	Kamiya
LE-AUS3	*C. leprieurii*	Nielsen Park, Sydney, NSW, Australia	17 Oct 91	880	D89956	Kamiya
LE-JPN2	*C. leprieurii*	Ôura River, Okinawa Is., Japan	8 Dec 91	902	D89949	Kamiya
LE-JPN3	*C. leprieurii*	Shimajiri, Miyako Is., Japan	2 Apr 91	736	D87813	Kamiya
LE-JPN4	*C. leprieurii*	Shimajiri, Miyako Is., Japan	2 Apr 91	490	D89951	Kamiya
LE-PER	*C. leprieurii*	Puerto Pizarro, Tumbes, Peru	10 Feb 90	1048	D89959	West
LE-SAF	*C. leprieurii*	Umlalazi, Natal, South Africa	4 Oct 91	1053	D89957	West
LE-SGP1	*C. leprieurii*	Changi, Singapore	13 Jan 93	937	D89953	Kamiya
LE-SGP2	*C. leprieurii*	Ubin Is., Singapore	13 Jan 93	932	D89952	Kamiya
LE-VZL	*C. leprieurii*	Laguna des Restinges, Isla Margarita, Venezuela	13 Apr 91	1052	D89958	West
MO-AUS1	*C. monosticha*	Derby, Western Australia, Australia	2 Oct 91	890, 892	D89960	Kamiya
MO-AUS2	*C. monosticha*	Small Lagoon, Denham, Western Australia, Australia	11 Oct 91			Kamiya
MO-SGP1	*C. monosticha*	Kg. Melayu, Pulau Ubin, Singapore	13 Jan 93			Kamiya
MO-SGP2	*C. monosticha*	Batu Puteh, Changi, Singapore	13 Jan 93			Kamiya
	C. monosticha	Batu Puteh, Changi, Singapore	13 Mar 4	986, 987	AB023381	Kamiya
OG-JPN	*C. ogasawaraensis*	Kido River, Chiba, Japan	22 Nov 91	596	D89961	Kamiya
PO-AUS	*C. continua* ssp. *postiae*	Georges River, Sydney, N.S.W., Australia	1 Jul 92	1122		Karsten
	C. continua ssp. *postiae*	Georges River, Sydney, N.S.W., Australia	4 Mar 96			King
	C. continua ssp. *postiae*	Georges River, Sydney, N.S.W., Australia	24 May 97	post98	AB023383	Zuccarello
PO-JPN1	*C. continua* ssp. *postiae*	Miyara River, Ishigaki Is., Okinawa, Japan	26 Feb 94	961, 962	AB023382	Kamiya
	C. continua ssp. *postiae*	Miyara River, Ishigaki Is., Okinawa, Japan	1 Mar 95			Kamiya
PO-JPN2	*C. continua* ssp. *postiae*	Isobe River, Ishigaki Is., Okinawa, Japan	23 Jan 98			Kamiya
PO-JPN3	*C. continua* ssp. *postiae*	Shiira River, Iriomote Is., Okinawa, Japan	2 Mar 95	1061		Kamiya
PO-JPN4	*C. continua* ssp. *postiae*	Tahara River, Yonaguni Is., Okinawa, Japan	28 Feb 95	1065		Kamiya
ST-SGP	*C. stipitata*	Kranji, Singapore	12 Jan 93	972	AB023384	Kamiya

[1] Sequence data of specimens with accession numbers beginning with "D" are published in Kamiya *et al.* (1998).

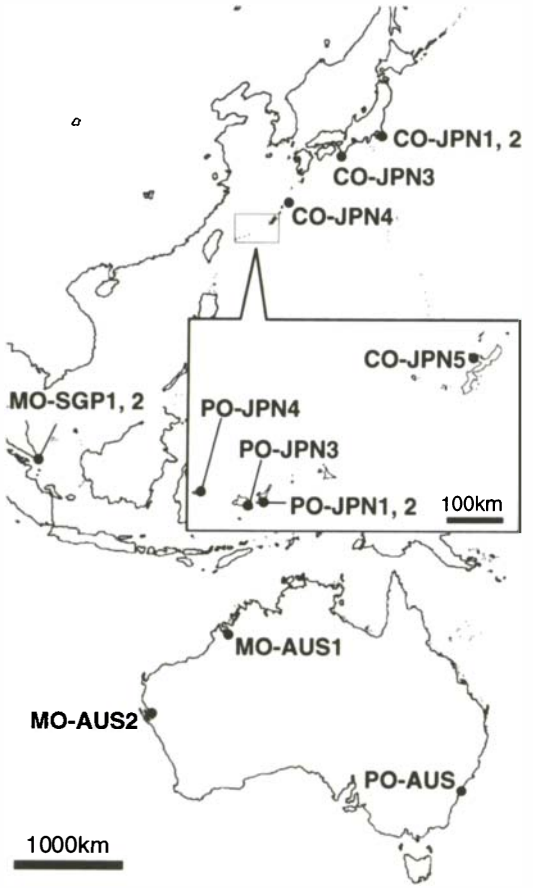


Fig. 1. A map showing collection sites in Australia, Japan, and Singapore. The abbreviations correspond to those in Table 1.

one of *C. monosticha,* and one of *C. stipitata* Post, with sequence data for 15 additional isolates from Kamiya *et al.* (1998). The DNA extraction and sequencing were performed as described in Kamiya *et al.* (1998) or by the following method. Approximately 20 mg wet weight of algal tissue was ground in liquid nitrogen, and DNA was extracted by using DNeasy Plant Mini Kit (Qiagen Inc., California). Total DNA served as a template for polymerase chain reaction (PCR). The RUBISCO spacers were amplified using primers containing sequences from the region 54 bp upstream from the 3′-terminus of the *rbc*L gene and complementary to the region 151 bp downstream from the 5′-terminus of the *rbc*S gene, 5′-TATACTTCTACAGACACAGCTGA-3′ (primer rbcL-M1), and 5′-ATGTCAAATAATGGTAGTCCCCA-3′ (primer rbcR2-M2), respectively. For dye-primer sequencing, M13-40 (25 bp) and M13 reverse sequence (25 bp) were added at the 5′-terminus of primer rbcL-M1 and rbcR2-M2, respectively. Each 50 μl of PCR reaction mixture contained 20.5 μl sterile water, 5 μl 10× LA PCR buffer (Takara Biomedicals, Tokyo), 200 μM each dNTP, 0.2 μM each primer, 2.5 units Ex Taq polymerase (Takara Biomedicals, Tokyo), and 1 μl (0.01–1 μg) genomic DNA. PCR amplifications were performed in GeneAmp® PCR System 2400 (Perkin-Elmer Applied Biosystems, California) with 30 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Excess primers and dNTP were removed from the PCR products by incubating at 0°C for 1 h with 0.6 volumes of precipitation solution [20% polyethylene glycol (MW 7500) and 2.5 M NaCl]. After centrifugation at 14,000 rpm for 10 min, the pellet was washed with cold 70% ethanol, air-dried, then dissolved in 15 μl sterilized distilled water.

Double-stranded PCR products were sequenced directly using an ALF express™ DNA sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden) and the dye-primer method according to the manufacturer's instructions. Complete sequences were determined for both strands of the regions. The nucleotide sequence data reported herein have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases.

The sequences obtained were aligned for phylogenetic analyses using the Clustal W computer program (Thompson *et al.* 1994). Two independent types of data analyses were used to assess the stability of evolutionary relationships resolved in the molecular phylogenies: the distance-based neighbor-joining (NJ) method and the character-based maximum parsimony (MP) analysis. Pairwise nucleotide distance estimates of the sequences were calculated using Kimura's two-parameter method (Kimura 1980) in the program DNADIST in PHYLIP 3.5c (Felsenstein 1993). Phylogenetic trees were inferred from these distance estimates using the NJ method (Saitou & Nei 1987, program NEIGHBOR in PHYLIP 3.5c). The MP analysis was performed with the program PAUP 3.1.1 (Swofford 1993) using branch-and-bound search procedures. Stability of the resulting groups was tested by bootstrap analyses (1000 replications, Felsenstein 1985) in both the distance matrix and MP method. In the MP trees, the decay index of each branch was also calculated based on 20,000 trees less than or equal to 10 steps longer than the most parsimonious tree.

RESULTS AND OBSERVATIONS

Vegetative morphology

Terms used in this study representing thallus orientation and cell rows are used in Fig. 2. The thalli of *Caloglossa continua* ssp. *postiae* are commonly leaf-like, flat, light brown, subdichotomously branched, and up to 10 mm high (Fig. 3). Each blade (0.3–2.2 mm long, 0.1–0.5 mm wide) is only slightly constricted at the nodes and consists of a conspicuous midrib flanked on either side by wings. Each axial cell (33–143 μm long, 5–18 μm diam.) is surrounded by four pericentral cells (40–145 μm long, 8–30 μm diam.). Each axial cell produces a second-order cell row toward both sides, and the second-order cell row forms two to five third-order cell rows abaxially (Fig. 4). A fourth-order cell row sometimes occurs from the third-order cell row at the edge of the blade. Each second-order cell row is composed of three to nine cells (8–38 μm diam.) at the internodes.

The dome-shaped apical cell (5–10 μm high, 5–18 μm diam.) divides transversely and cuts off two to eight disk-shaped segments (2 μm high, 10–20 μm diam.) posteriorly. Monosiphonous rhizoids (5–10 μm diam.) are produced between the main and lateral axes (Fig. 5). A few new blades arise from the first axial cell above the node and out of the plane of the thallus (termed secondary endogenous branches), usually on the dorsal side (Fig. 6).

The nodal and internodal anatomy was analyzed (Table 2). *Caloglossa continua* ssp. *postiae* from Australia and Japan

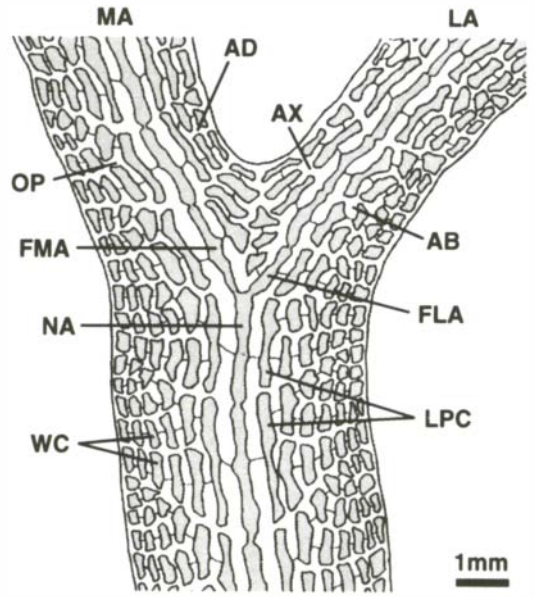


Fig. 2. Drawing of a part of thallus at the node of *Caloglossa continua* ssp. *postiae*. Terminology follows King & Puttock (1994). Transverse pericentral cells and secondary pit connections are omitted. AB, abaxial side; AD, adjacent side; AX, adaxial side; FLA, first axial cell of the lateral axis; FMA, first axial cell of the main axis; LA, lateral axis; LPC, lateral pericentral cell; MA, main axis; NA, nodal axial cell; OP, opposite side; WC, wing cell.

possesses one adaxial cell row from the first axial cell of the lateral axis (Fig. 7), which *C. leprieurii* (Montagne) J. Agardh does not form at all. Subspecies *postiae* produces multiple cell rows from the nodal axial cell opposite the formation of an exogenous branch (Figs 8, 9) as in *C. continua* ssp. *continua* (Fig. 10), whereas *C. monosticha* and *C. saigonensis* possess a single cell row (Fig. 11). *Caloglossa continua* ssp. *postiae* forms significantly fewer cells in a second-order row at both the nodes and internodes than either *C. continua* ssp. *continua* or *C. monosticha* (Table 2).

In the field-collected specimens, the blade width of *C. continua* ssp. *postiae* is consistently less than in the other three taxa and hardly more than 0.3 mm (Table 3). Blade widths of *C. continua* ssp. *continua* and *C. monosticha* are 0.4 to 1.5 mm and 0.4 to 1.9 mm, respectively. *Caloglossa saigonensis* is separable from *C. continua* ssp. *postiae* and *C. monosticha* by its narrow and long blade (Fig. 12).

Culture studies

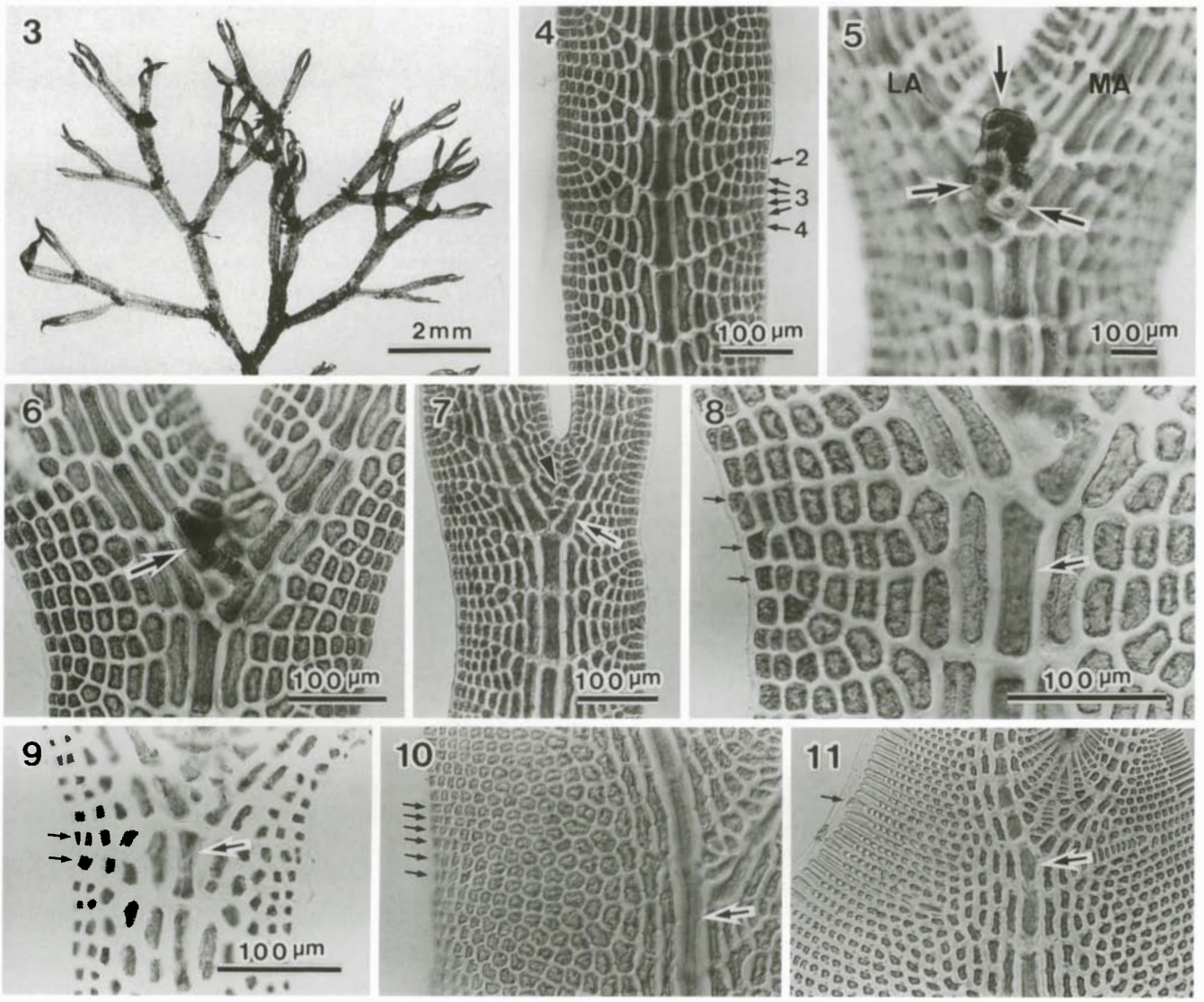
The stability of the blade width was examined at a range of temperatures (10°, 15°, 20°, 25°, and 30°C) and salinities (0, 8, 16, 24, and 32 psu). Twenty carpospores obtained from each of the isolates of *Caloglossa continua* ssp. *continua* (Japan), *C. monosticha* (Australia), and *C. continua* ssp. *postiae* (Japan) were cultured for 40 d (Figs 13, 14). In the temperature experiment, only *C. continua* ssp. *continua* germinated at 10°C. In the salinity experiment, no germlings survived at 0 psu. In both experiments, a relatively wide range of blade widths was shown in *C. continua* ssp. *continua* (0.5–1.1 mm) and *C. monosticha* (0.2–1.3 mm), whereas *C. continua* ssp. *postiae* maintained its narrow blades (0.1–0.3 mm) under all conditions.

A *Polysiphonia*-type life history was observed in the culture isolates of *C. continua* ssp. *postiae*. In the tetrasporophyte, formation of tetrasporangia takes place in acropetal succession and from the lateral pericentral cells toward the margins of the blade. Sporangial sori (300–800 μm long, 130–150 μm wide) are produced on both sides of the midrib in the upper part of the branches (Fig. 15). Initially the 10th to 22nd lateral pericentral cells and wing cells divide into two, forming the tetrasporangial initial toward the apex and stalk cell. The stalk cell forms cover cells on each surface, and the cover cells enlarge and divide laterally and posteriorly over the sporangia (Fig. 16). Mature tetrasporangia (30–50 μm diam.) divide cruciate-decussately or tetrahedrally (Fig. 17).

On the male gametophyte, spermatangial sori are found on both sides of the midrib at the upper and middle parts of a thallus, 0.6 to 1.3 mm long and 180 to 300 μm wide (Fig. 18). In the first step of spermatangial formation, wing cells cut off one to three cortical cells toward each surface. Each cortical cell functions as a spermatangial mother cell and is irregularly oval or subquadrate, 5.0 to 8.8 μm long, and 3.8 to 5.0 μm wide (Fig. 19). A spermatangial mother cell cuts off three to five spherical spermatangia (2.0–3.8 μm diam.) toward the outer surface by anticlinal divisions (Fig. 20).

On the female gametophyte, the reproductive structures are usually formed on the ventral side at the upper parts of a thallus, sometimes on the dorsal side. The tip of a thallus bearing several procarps along the central axis is typically incurved. Each procarp is composed of a four-celled carpogonial branch borne on the supporting cell (Fig. 21), which cuts off a sterile cell toward the surface (Fig. 22). The carpogonium of the carpogonial branch forms an elongated trichogyne 2.5 to 10 μm diam. and up to 140 μm long. One or two procarps per blade become fertile, forming cystocarps. Cystocarps are ovate, 160 to 250 μm in height, and 180 to 270 μm diam., covered by eight to 10 longitudinal pericarp filaments, with a narrow ostiole at the tip (Fig. 23). Each mature cystocarp includes 23 to 68 ovoidal or ellipsoidal carposporangia, 28 to 63 μm long and 20 to 30 μm diam. (Fig. 24), and normal cystocarps release carpospores in 2 wk. Both carpospores and tetraspores show typical bipolar germination. Conical or cylindrical pseudocystocarps resulted from the interpopulational crossings between Australian and Japanese strains (Figs 25, 26). Although such pseudocystocarps appear to possess normal pericarp filaments and an ostiole, they do not produce any carposporangia even after a few months in culture.

Reproductive compatibility was examined among three strains of *Caloglossa continua* ssp. *postiae,* one of *C. continua* ssp. *continua,* and two of *C. monosticha* (Fig. 27). Some of the results between *C. continua* ssp. *continua* and *C. monosticha* are based on published data in Kamiya *et al.* (1997). No cystocarps were formed on the separated female gametophytes used as negative controls. In almost all crosses, spermatia were observed attached to trichogynes on female gametophytes. Successful crossings between male and female strains that had originated from the same tetrasporophyte were regarded as positive controls. In all the positive controls, carpospores from the cross developed into fertile F_1 tetrasporophytes, which discharged tetraspores that developed into dioecious F_1 gametophytes, the female gametophytes of which formed normal cystocarps.



Figs 3–11. Vegetative morphology of field-collected *Caloglossa* taxa.

Figs 3–8. Details of field-collected specimens of *Caloglossa continua* ssp. *postiae* from Isobe River, Ishigaki Island, Japan.

Fig. 3. Whole thallus.

Fig. 4. Internodal blade. The arrows numbered 2, 3, and 4 indicate the second-, third-, and fourth-order cell rows, respectively.

Fig. 5. Monosiphonous rhizoids (arrows) produced at the node on the ventral side. Note rhizoidal filaments arising between the main (MA) and the lateral axes (LA).

Fig. 6. A secondary endogenous branch (arrow). Initial branch arises from the first axial cell of the lateral axis toward the dorsal side.

Fig. 7. An adaxial cell row (arrowhead) derived from the first axial cell (arrow) of the lateral axis.

Fig. 8. Three cell rows (small arrows) produced from the nodal axial cell (large arrow) opposite the formation of the lateral branch.

Figs 9–11. Part of thalli showing the order of cell rows around nodes of field specimens. Note the number of cell rows on the opposite side (small arrows) derived from the nodal axial cell (large arrows).

Fig. 9. *Caloglossa continua* ssp. *postiae* from Georges River, Sydney, Australia.

Fig. 10. *Caloglossa continua* ssp. *continua* from Kido River, Chiba, Japan.

Fig. 11. *Caloglossa monosticha* from Derby, Australia.

The strains of *C. continua* ssp. *postiae* from two Japanese islands, Iriomote and Yonaguni, were interfertile, whereas they gave rise to only pseudocystocarps when crossed with the strain from Australia (Figs 25, 26). These slender strains were not compatible with either *C. continua* ssp. *continua* or *C. monosticha.*

DNA analyses

The sequence data obtained were aligned with already published sequence data on 11 isolates of *C. leprieurii* and one isolate each of *C. apomeiotica* West et Zuccarello, *C. continua* ssp. *continua, C. monosticha,* and *C. ogasawaraensis* Okamura (Table 1). The aligned data set was 271 nucleotides long (*rbc*L 31 bp, RUBISCO spacer 90 bp, *rbc*S 150 bp). There were 84 variable sites (excluding gaps) identified among all the isolates; 55 of these were phylogenetically informative. Nine site changes and one length mutation were found between *C. continua* ssp. *postiae* from Australia and Japan, whereas there were 18 to 22 site differences from *C. continua* ssp. *continua.*

Table 2. Comparison of cell number and cell row number derived from the axial cells at the node. Forty nodes were examined from each of the 10 populations, and 40 nodes of 20 carposporelings after 40 d culture were examined in each of the four isolates. Numbers in parentheses are rare observations.[1]

Species-locality	n	Cell number in a 2nd-order row at internode	Cell number in a 2nd-order row at node	Cell row number from an intermodal axial cell	Cell row number from NA		Cell row number from FMA		Cell row number from FLA	
					Opposite	Abaxial	Opposite	Adjacent	Adaxial	Abaxial
Field specimens										
PO-AUS	10	4–8	4–8	3–5	2–3	1–2	(1)–(4)	1–(3)	1	1–3
PO-JPN1	20	3–9	3–9	3–5	2–4	1–3	1–4	1 (2)	1	1–3
PO-JPN3	20	3–7	3–6	3–4	2–4	1–3	2–3	1 (2)	1	1–3
PO-JPN4	20	3–8	4–8	3–7	2–5	1–4	1–5	1–3	1	1–3
CO-JPN2	20	7–19	9–18	4–9	4–6	1	4–6	2–3	1	1–4
CO-JPN3	20	7–20	9–20	5–10	(4)–6	1–4	2–6	1–(4)	1	1–4
CO-JPN5	20	7–18	12–19	3–5	(2)–6	1–3	3–(6)	(1)–(4)	1	1–3
MO-AUS1	20	14–28	14–26	4–5	1	1	1–(4)	1–2	1	1
MO-AUS2	20	10–20	10–20	3–6	1	1	1–2	1	1	1
MO-SGP1	20	8–20	11–25	5–7	1–4	1	1–7	1–(4)	1	1–3
SA-VTN	18	6–11	5–10	3–6	1	1	2–4	1–2	1	1–2
Cultured specimens										
PO-JPN1	20	2–7	2–6	2–6	(1)–4	1–3	1–5	1–3	1	1–3
CO-JPN1	20	7–18	7–19	4–5	(1)–5	1	2–5	(1)–3	1	1–3
MO-AUS1	20	4–25	7–26	3–5	1	1	1–3	1–2	1	1
MO-SGP2	20	3–18	7–18	3–5	1–2	1	1–4	1–2	1	1

[1] n, number of examined specimens; NA, nodal axial cell; FMA, first axial cell at the main axis; FLA, first axial cell at the lateral axis.

Kimura's two-parameter distances were estimated between taxa and the phylogeny was inferred with the NJ method including gaps (Fig. 28). The MP analysis using the branch and bound search procedure resulted in five most parsimonious trees (length = 175 steps, consistency index = 0.766). Each gap site was treated as a new state. A thousand bootstrap data sets were generated from the original data. Each new data set was used to construct the most parsimonious tree, and a 50% majority rule consensus tree was generated from these trees (Fig. 29).

Both trees clearly separated *C. ogasawaraensis* and *C. stipitata* from the other species (100% bootstrap value). Geographically distant strains of *C. continua* ssp. *postiae* were included in a same clade. Both trees suggested that this subspecies diverged first from the group of endogenously branched species. Data from three populations of *Caloglossa continua* ssp. *continua* demonstrated low sequence divergence with each other (0%–0.4%). The evolutionary relationship of *C. monosticha* from Australia and Singapore was not resolved. Excluding gaps, the topology was unchanged in the MP analysis, but in the distance analysis, *C. monosticha* from Singapore was a sister group to *C. continua* ssp. *postiae* but was supported by a low bootstrap value (29%, data not shown). *Caloglossa leprieurii,* including the morphologically similar but asexual species *C. apomeiotica,* was shown to be monophyletic, confirming the conclusion reached by Kamiya *et al.* (1998).

DISCUSSION

In many algal studies, morphological and anatomical traits considered to be definitive for taxa are later shown to be too variable when applied to large collections or to material grown under a range of environmental conditions. In this study, blade width was the only essential difference found between the two subspecies of *Caloglossa continua.* This difference, however, appears to be constant under the range of conditions tested, and because the ranges do not overlap, subspecies *postiae* can be reliably identified by this feature alone. Crossing experiments demonstrate complete reproductive isolation of subspecies *postiae* from subspecies *continua,* and DNA sequence data also indicate that these two subspecies are distinct at the level of species. The sexual and asexual reproductive structures of subspecies *postiae* are described for the first time here. They are fundamentally identical to those described for subspecies *continua* (as *C. leprieurii* f. *continua*; Tanaka 1992) and *C. leprieurii* (Papenfuss 1961).

The taxonomic significance of blade width has been discussed in relation to another species in the genus, *Caloglossa leprieurii* (Kamiya *et al.* 1995). The occurrence of phenotypically intermediate thalli in both field and laboratory cultured materials made it difficult to discriminate clearly between the typical and narrow types. This was consistent with the much lower degree of divergence in the DNA sequences (2.7%–3.9%) compared to that shown between the two subspecies of *C. continua* (7.4%–9.2%).

In a more recent study, the value of blade width has again been brought into question. Wynne and De Clerck (1999) suggested that *Caloglossa monosticha* is conspecific with *C. saigonensis,* a species that was separated from *C. continua* ssp.

Table 3. Comparison of blade length and width of field-collected specimens. The length was measured between nodes and internode width at the second, third, and fourth blades from the apex. Numbers represent ranges; mean values are given in parentheses.

Species-locality	n	2nd blade (mm)		3rd blade (mm)		4th blade (mm)	
		Length	Width	Length	Width	Length	Width
PO-AUS	10	0.3–2.2 (0.9)	0.1–0.3 (0.2)	0.6–1.4 (0.9)	0.2–0.3 (0.2)	0.9–1.4 (1.1)	0.2–0.3 (0.2)
PO-JPN1	20	0.5–1.5 (0.8)	0.1–0.2 (0.2)	0.3–1.6 (0.8)	0.1–0.3 (0.2)	0.6–1.6 (1.0)	0.2–0.3 (0.2)
PO-JPN3	20	0.3–1.0 (0.6)	0.1–0.2 (0.1)	0.5–1.4 (0.8)	0.1–0.2 (0.1)	0.5–1.8 (1.1)	0.1–0.3 (0.2)
PO-JPN4	20	0.4–1.1 (0.6)	0.1–0.2 (0.2)	0.3–1.1 (0.7)	0.1–0.3 (0.2)	0.5–1.6 (0.8)	0.2–0.3 (0.2)
CO-JPN2	20	0.9–2.5 (1.3)	0.4–0.8 (0.6)	0.9–2.4 (1.6)	0.5–1.4 (0.7)	0.7–3.7 (1.9)	0.4–1.0 (0.8)
CO-JPN3	20	0.8–1.5 (1.1)	0.4–1.0 (0.6)	0.9–2.3 (1.4)	0.5–1.4 (0.8)	0.9–2.4 (1.5)	0.5–1.5 (0.9)
CO-JPN5	20	0.7–1.7 (1.0)	0.4–1.0 (0.7)	0.8–1.6 (1.2)	0.4–1.0 (0.8)	0.8–2.1 (1.2)	0.4–1.0 (0.8)
MO-AUS1	20	1.1–3.1 (1.9)	0.7–1.4 (1.0)	1.6–3.3 (2.3)	1.0–1.7 (1.3)	1.7–3.3 (2.6)	1.0–1.9 (1.4)
MO-AUS2	20	1.1–2.8 (1.9)	0.5–1.4 (0.9)	1.0–2.9 (2.0)	0.7–1.8 (1.1)	1.9–2.8 (2.3)	1.1–1.7 (1.3)
MO-SGP1	20	0.8–2.0 (1.2)	0.5–1.0 (0.8)	0.7–1.9 (1.3)	0.6–1.2 (0.9)	0.7–1.9 (1.3)	0.4–1.1 (0.9)
SA-VTN	16	1.0–3.1 (1.8)	0.2–0.5 (0.3)	1.4–3.2 (2.3)	0.3–0.6 (0.5)	1.6–3.8 (2.3)	0.4–0.6 (0.5)

[1] n, number of examined specimens.

continua by its long and narrow blades. Reexamination of the type specimen of *C. saigonensis* confirms the formation of a single cell row from a nodal cell, which is the unique trait of *C. monosticha* (Table 2). On the basis of the limited observations made here on *C. saigonensis,* it appears to be distinguishable by blade shape from *C. monosticha* (Fig. 12). Because blade width appears to be indicative of underlying differences in *C. postiae,* the relationship between *C. saigonensis* and *C. monosticha* should be considered uncertain until appropriate culture experiments and molecular analyses have been performed.

The results presented here allow further speculation on relationships within the genus *Caloglossa.* Post (1936) was the first to give a synoptic overview of the genus and subsequently King & Puttock (1994) rearranged her classification by introducing some new morphological criteria. The two branching patterns, endogenous and adventitious, had been recognized early. The endogenous branching type, to which *C. continua, C. monosticha,* and *C. leprieurii* (including *C. apomeiotica*) belong, produces secondary branches from the first axial cell above the node and out of the plane of the thallus. The adventitious type expresses branching from pericentral cells in the same plane as the thallus and includes five species, *C. adherens* King et Puttock, *C. beccarii* (Zanardini) De Toni, *C. bengalensis* (Martens) King et Puttock, *C. ogasawaraensis,* and *C. stipitata.* The members of the endogenously branched group show similar gross morphology with each other, and all of them have been recognized as a single species for a long time (King & Puttock 1994). The endogenously branched group was shown to be monophyletic by the molecular data, although only two species were examined in the adventitiously branched group. More conservative gene regions should be analyzed in future molecular phylogenetic studies because the alignment of the RUBISCO spacer region

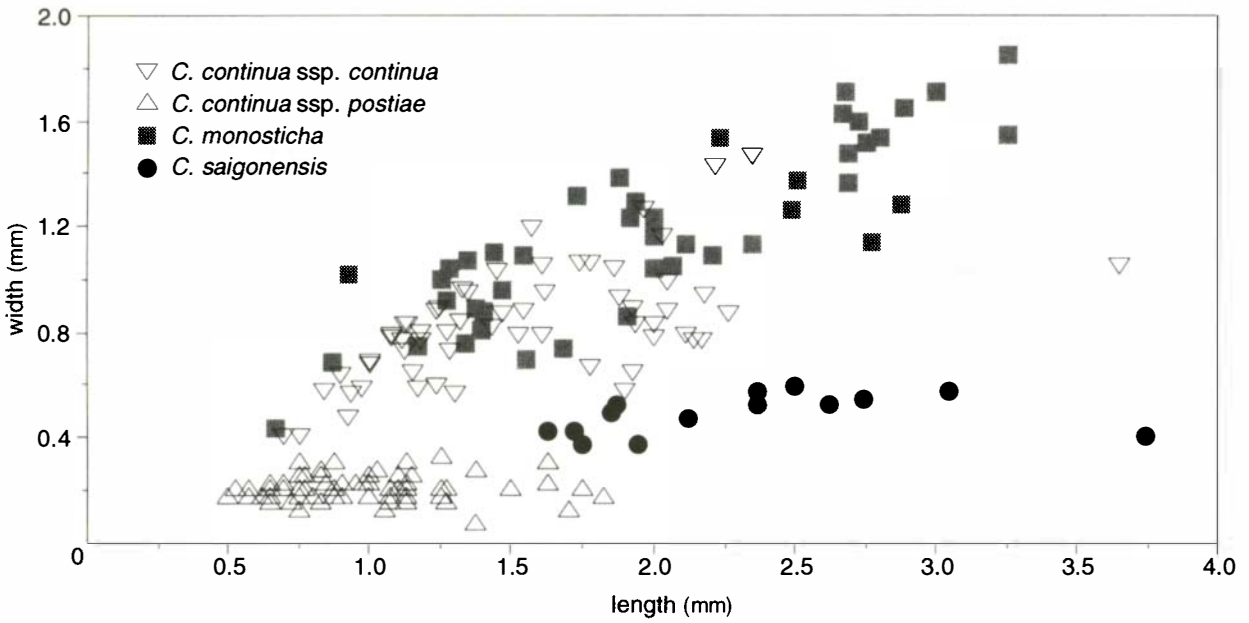
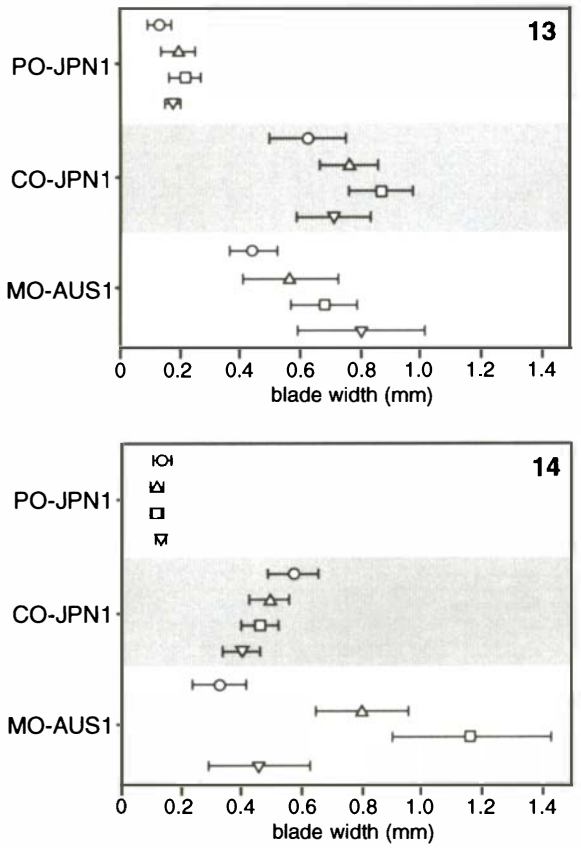


Fig. 12. Morphological variation in *Caloglossa continua* ssp. *postiae* (PO-AUS and PO-JPN1, 3, 4), *C. continua* ssp. *continua* (CO-JPN2, 3, 5), *C. monosticha* (MO-AUS1, 2 and MO-SGP1), and *C. saigonensis* (SA-VTN). Twenty field-collected specimens (10 specimens in PO-AUS and 16 in SA-VTN) were examined in each population. The vertical axis represents internodal blade width between the third and fourth nodes; the horizontal axis represents blade length from the third to fourth nodes.



Figs 13, 14. Internodal blade width between the first- and second-order nodes from the apex of the three isolates, *Caloglossa continua* ssp. *postiae, C. continua* ssp. *continua,* and *C. monosticha,* after 40 d of culturing carpospores under various conditions. The symbols indicate the mean (n = 10), and the error bars represent ± 1 SD about the mean.

Fig. 13. Temperature experiment at the salinity of 14 psu, 21 photons m^{-2} s^{-1}; (–○–) 15°C; (–△–) 20°C; (–□–) 25°C; (–▽–) 30°C.

Fig. 14. Salinity experiment at 25°C, 17 photons m^{-2} s^{-1}; (–○–) 8 psu; (–△–) 16 psu; (–□–) 24 psu; (–▽–) 32 psu.

within the other adventitious branching group members can be expected to be difficult given that their gross morphology is more diverse.

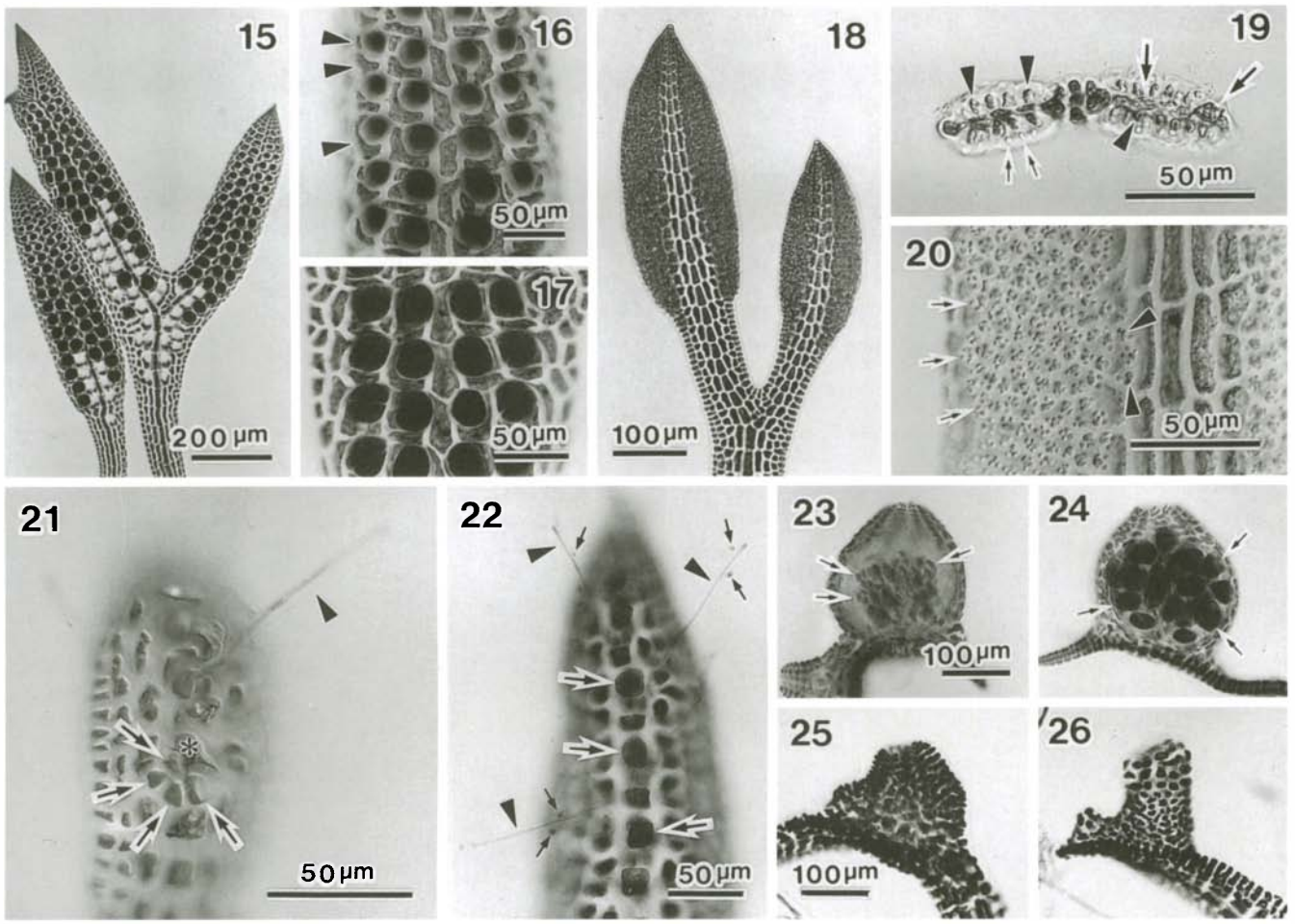
The position of rhizoids is also an important character to classify *Caloglossa* species. *Caloglossa continua* and *C. monosticha* produce rhizoids in an axillary cluster developing between the main and lateral axes at the node (axil rhizoid type). Rhizoids arising from the nodal pericentral cell and the two pericentral cells above the node are seen in *C. beccarii, C. leprieurii, C. ogasawaraensis, C. stipitata,* and *C. triclada* (Post) King et Puttock (nodal rhizoid type). The molecular analyses suggest a paraphyletic group of the axil rhizoid type and it can be assumed that the rhizoidal position changed at least twice within the endogenous branching lineage (Fig. 29). One can suppose the acquisition of axillary rhizoids happened more than once if nodal rhizoids are plesiomorphic. Alternatively, nodal rhizoids in *C. leprieurii* can be considered to be a result of parallelism. The latter hypothesis seems more likely because *C. leprieurii* often forms rhizoids clustered at the node, generally coalescing to form a conspicuous stipe, which is unique to that species.

Recent systematic studies have considered the number of cell rows around nodes as a new taxonomic criterion for *Caloglossa* species. *Caloglossa leprieurii* was characterized by the absence of the adaxial cell row from the first axial cell on the lateral axis (King & Puttock 1994). This feature was already suggested as a synapomorphy in the endogenous branching lineage in the most recent molecular study (Kamiya *et al.* 1998), and this was confirmed in the present analysis with the additional species. *Caloglossa monosticha* was separated from *C. continua* by possession of a single cell row from the nodal axial cell opposite the formation of an exogenous branch (Kamiya *et al.* 1997). Only a single cell row is formed at this position in *C. monosticha* from Australia, and specimens from Singapore sometimes produce both single and multiple cell rows in the same thallus (Kamiya *et al.* 1997). This difference may be related to the present molecular phylogeny in which *C. monosticha* has not been shown to be monophyletic. Furthermore, it is unclear whether the single or multiple cell row type is the more primitive because other species show an intermediate feature (e.g. *C. leprieurii* = one to three, *C. ogasawaraensis* = one or two, *C. stipitata* = one or two, [Kamiya, unpublished data]). Although this character may be useful to distinguish *C. monosticha* from *C. continua,* it may be phylogenetically uninformative in this genus.

The formation of pseudocystocarps, having no carposporangia with normal pericarp filaments, was observed in the cross between geographically distant isolates of *C. continua* ssp. *postiae* or *C. monosticha* (Fig. 27). The development of pseudocystocarps has been reported by several authors in crossing experiments between morphologically similar or identical algae (see Kamiya *et al.* 1998). In recent studies, pseudocystocarps formed in crosses of algae showing a low degree of sequence divergence [*Bostrychia radicans* (Montagne) Montagne = 0.6%–1.7% (Zuccarello & West 1997); *Caloglossa leprieurii* = 0.4%–2.6% (Kamiya *et al.* 1998)]. Although this tendency is recognized in *C. continua* ssp. *postiae* in this study (3.7% sequence divergence), the Australian and Singaporean isolates of *C. monosticha* exhibit a much higher distance value (7.0%). The crossability, including production of pseudocystocarps, can be considered to be a maintained ancestral feature—a symplesiomorphy; therefore, the entities that demonstrate a positive mating reaction may not necessarily be close genealogically (Donoghue 1985). Furthermore, it is still unknown which genes and how many are involved in the establishment of intersterility in algal speciation. Some authors have reported inconsistency in results based on crossability and genetic distance (see for examples Pakker *et al.* 1996; Zuccarello & West 1997), and this urges caution in the use of reproductive compatibility as a phylogenetic indicator.

In conclusion, we have demonstrated that the narrowness of the blades, the noninterfertility with other subspecies, and the molecular sequence data show that the entity discussed as a subspecies by King & Puttock (1994) actually represents a distinct species—one that is ancestral to the *Caloglossa* clade in which endogenously branched species occur.

According to Article 39.1 of the International Code of Botanical Nomenclature (Greuter *et al.* 1994), the name of ssp. *postiae* is invalid because no figure or illustration accompanied its description (see King & Puttock 1994). We therefore provide the specific diagnosis of *C. postiae* herein.



Figs 15–26. Reproductive features of cultured *Caloglossa* thalli.
Figs 15–17. Cultured tetrasporophyte of *C. continua* ssp. *postiae* from Georges River, Sydney, Australia.
Fig. 15. Tetrasporangial sori.
Fig. 16. Cover cells (arrowheads) over the sporangia.
Fig. 17. Cruciate-decussately divided tetrasporangia.
Figs 18–20. Cultured male gametophyte of *C. continua* ssp. *postiae* from Georges River, Sydney, Australia.
Fig. 18. Spermatangial sori.
Fig. 19. Transverse section of spermatangial sorus. Spermatangial mother cells (arrowheads) cut off from wing cells (large arrows) ventrally and dorsally. These mother cells form spermatangia (small arrows) toward the outer surface.
Fig. 20. Three to five spherical spermatangia (arrows) are anticlinally cut off from each spermatangial mother cell (arrowheads).
Figs 21, 22. Cultured female gametophyte of *C. continua* ssp. *postiae* from Georges River, Sydney, Australia.
Fig. 21. Procarps produced on the axial cells of the apical blade. Each procarp is composed of four carpogonial branch cells (arrows) on a supporting cell (asterisk). A trichogyne (arrowhead) elongates from the terminal cell of the carpogonial branch.
Fig. 22. Sterile cells (large arrows) cut off from supporting cells toward the surface. Several spermatia (small arrows) attach on the trichogynes (arrowheads).
Figs 23, 24. Cystocarps resulted from an artificial crossing between male and female gametophytes of *C. continua* ssp. *postiae* from Georges River, Sydney, Australia.
Fig. 23. Several chains of gonimoblasts (arrows) formed in the immature cystocarp.
Fig. 24. Many carposporangia (arrows) produced in the matured cystocarp.
Figs 25, 26. Pseudocystocarps induced by an artificial crossing of *C. continua* ssp. *postiae.*
Fig. 25. Conical pseudocystocarp produced on female gametophyte from Georges River, Sydney, Australia, with male from Tahara River, Yonaguni Is., Japan.
Fig. 26. Cylindrical pseudocystocarp produced on female gametophyte from Shiira River, Iriomote Is., Japan, with male from Georges River, Sydney, Australia.

Caloglossa postiae Kamiya et R.J. King sp. nov.

Figs 3–8

DIAGNOSIS: Rami endogeni nodi a cellulis axalibus oriundi; laminae leviter sive vix nodis constrictae, 0.3–2.2 mm longae, 0.1–0.5 mm latae; rhizoidea divergentia ventralia intra axes efficientia; cellula prima axialis axis lateralis cellulas adaxiales habet. Cellula nodi adversum axem lateralem cellulae multifariarum habet. Affinis *C. continuae,* sed lamina valde angusta differt.

Endogenous branches at the nodes produced from the axial cells, blades slightly or scarcely constricted at the nodes, 0.3 to 2.2 mm long and 0.1 to 0.5 mm wide, divergent rhizoids produced within the axes on the ventral side. The first axial

♂ \ ♀		CO-JPN1	MO-SGP2	MO-AUS1	PO-JPN3	PO-JPN4	PO-AUS
		639	987	892	1061	1065	1122
CO-JPN1	640	+	−	−	−	−	−
MO-SGP2	986	−	+	−	−	−	−
MO-AUS1	890	−	−*	+	−	−	−
PO-JPN3	1061	−	−	−	+	ND	ND
PO-JPN4	1065	−	−	−	+	+	−*
PO-AUS	1122	−	−	−	−*	−*	+

Fig. 27. Results of the hybridization experiments. Each number indicates a strain number listed in Table 1. +, F_1 sporophytes and subsequent F_1 gametophytes were fertile (shaded); −*, pseudocystocarps were produced; −, no reproduction occurred; ND, no data.

cell of the lateral axis forms adaxial cells. Multiple cell rows derived from the nodal cell opposite the lateral axis. This species is similar to *C. continua* in the preceding characters but different in its much narrower blades.

HOLOTYPE: TNS-AL-45189 (M. Kamiya, 23-I-1998; Figs 3–8).

TYPE LOCALITY: Isobe River, Ishigaki Island, Okinawa, Japan (24°21′N, 124°12′E).

ADDITIONAL SPECIMENS EXAMINED: Miyara River, Ishigaki Is. Okinawa, Japan (24°21′N, 124°12′E), TNS-AL-45190 (*M. Kamiya,* 26 February 1994); Shiira River, Iriomote Is., Okinawa, Japan (24°20′N, 123°55′E), TNS-AL-45191 (*M. Kamiya,* 2 March 1995); Tahara River, Yonaguni Is., Okinawa, Japan (24°29′N, 123°00′E), TNS-AL-45192 (*M. Kamiya,* 28 February 1995); Georges River at Georges Hall, Sydney, New South Wales, Australia (33°52′S, 151°13′E), UNSW 15205 (*R.J. King & C. F. Puttock,* 21 September 1983).

HABITAT: Distributed in estuaries and sheltered coasts in subtropical to temperate regions, often epiphytic on mangroves. In Japan, grown abundantly on trunks and pneumatophores of *Kandelia candel* (Linnaeus) Druce and *Rhizophora stylosa* Griffith or rocks, intermingling with *Caloglossa ogasawaraensis, Bostrychia radicans, Catenella caespitosa* (Withering) Irvine, or *Catenella impudica* (Montagne) J. Agardh. In Australia, found on trunks of *Avicennia marina* (Forsskal) Vierhapper with *C. leprieurii* and *B. moritziana* (Sonder) J. Agardh.

ETYMOLOGY: The specific epithet honors the late Dr. Erika Post, Kiel, Germany, who contributed significantly to the study of the algae associated with mangroves.

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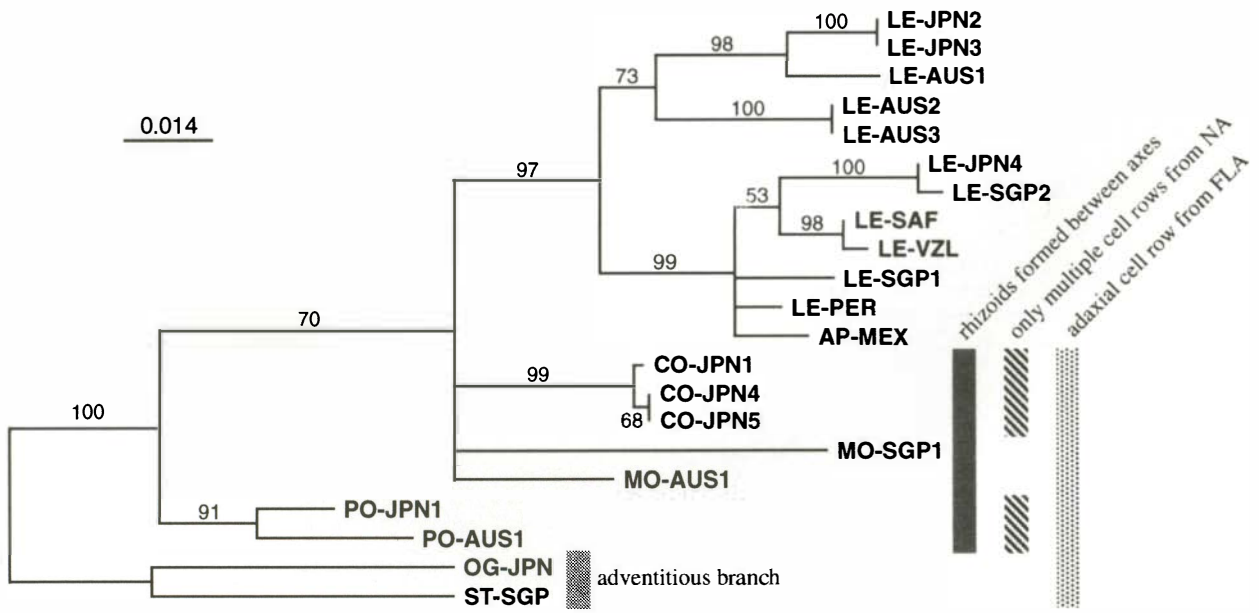

Fig. 28. Phylogenetic tree inferred by the neighbor-joining method from RUBISCO spacer and the flanking regions of the *rbc*L and *rbc*S genes. Branch lengths are proportional to evolutionary distances. The numbers indicate bootstrap values computed for 1000 resamplings.

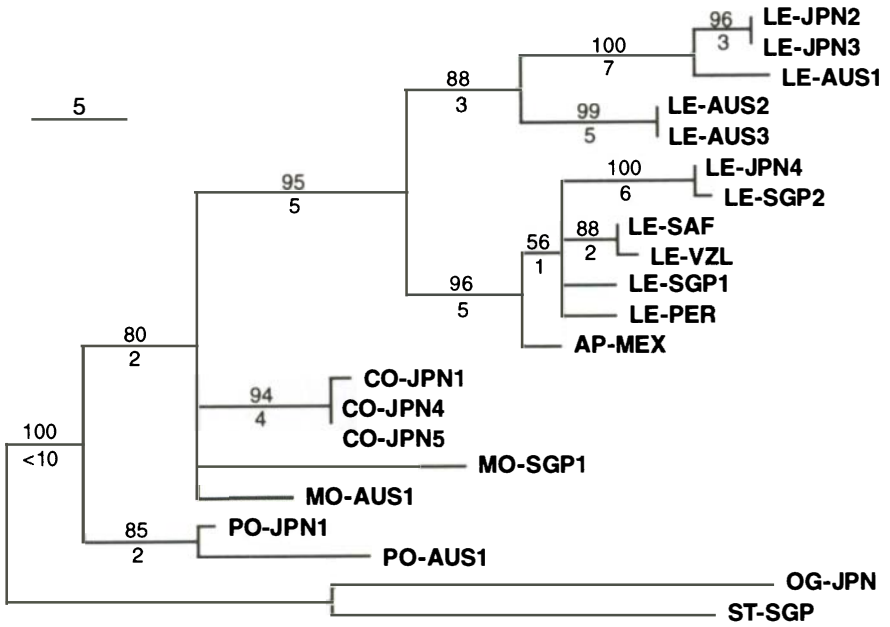

Fig. 29. Bootstrap 50% majority rule consensus tree inferred by parsimony analysis from RUBISCO spacer and the flanking regions of the *rbc*L and *rbc*S genes. Branch lengths are proportional to nucleotide changes. Bootstrap values computed for 1000 resamplings are indicated above branches and decay indices are below branches.

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