

Phylogeography of *Caloglossa leprieurii* and related species (Delesseriaceae, Rhodophyta) based on the *rbcL* gene sequences

Mitsunobu Kamiya,^{1*} Giuseppe C. Zuccarello² and John A. West³

¹ Kobe University Research Center for Inland Seas, 2746, Iwaya, Awaji-cho 656-2401, Japan, ² National Herbarium of the Netherlands, Leiden University Branch, P.O. 9514, 2300 RA Leiden, The Netherlands and ³ School of Botany, University of Melbourne, Parkville, Victoria 3010, Australia

SUMMARY

The phylogeography of *Caloglossa leprieurii* and related species were examined by reconstructing evolutionary relationships based on the *rbcL* sequence data. Both *C. leprieurii* (nine strains) and *C. vieillardii* (four strains) are resolved as monophyletic, but their relationship to *C. intermedia* is unresolved. In *C. vieillardii*, which is restricted to the south-western and central Pacific regions, the genetic distance of the four Australian strains is correlated to their geographic distance, while such a correlation is not always seen in *C. leprieurii* showing more widespread distribution. The molecular phylogenetic data suggest that the dispersal of *C. leprieurii* between the Atlantic and Pacific American coasts may have happened more than once. Molecular clock calibration of divergence times within the *Caloglossa* species, based on the vicariant event of the last closure of the Isthmus of Panama, was performed to infer dispersal routes of the *Caloglossa* species. The estimated divergence time of *C. leprieurii* and *C. vieillardii* is in the early-middle Eocene, so the dispersal of these species may have occurred with the establishment of mangrove plants along the Tethys Sea which was a tropical connection route between the Atlantic and Pacific Oceans.

Key words: *Caloglossa*, *C. leprieurii*, *C. vieillardii*, divergence rate, mangrove algae, molecular clock, molecular phylogeny, phylogeography

INTRODUCTION

Studies on the morphology, reproductive compatibility, life cycle, physiological characteristics and molecular phylogeny of the euryhaline red alga *Caloglossa* have provided insights into the speciation events and biogeographical patterns (Kamiya *et al.* 1998; West *et al.* 2001). *Caloglossa leprieurii* (Montagne) G. Martens is especially abundant in mangrove environments and distributed worldwide. Previous studies have revealed that this species can be divided into two morphological types which are characterized by either single or multiple cell rows from the first axial cell at the main axis (Kamiya *et al.* 1995, 1998). Because the two morphological types are reproductively isolated as well as phylogenetically distinct, the single cell row type has recently assigned into *C. vieillardii* (Kützinger) Setchell (Kamiya

et al. 2003).

Previous molecular phylogenetic analyses on worldwide populations of *C. leprieurii* using the RuBisCo spacer and large subunit ribosomal RNA gene (LSU rDNA) sequences did not fully resolve the relationships of some populations (Kamiya *et al.* 1998, 2003; West *et al.* 2001). The low intraspecific resolution is possibly related to an insufficient number of informative sites in these sequences (13 sites in RuBisCo spacer and 33 sites in LSU rDNA). It has been shown that the RuBisCo large subunit (*rbcL*) gene shows a significantly faster evolutionary rate than LSU rDNA (Freshwater *et al.* 1999) and includes more informative sites than the RuBisCo spacer (Müller *et al.* 1998).

Estimates of divergence dates of lineages have generally provided useful information about biogeography and speciation, and fossil records have been often used as a basis for estimating rates of molecular evolution (Wendel and Albert 1992; Savard *et al.* 1994; Renner *et al.* 2000). In the case of marine organisms with no fossil record, the last closing of the Isthmus of Panama, which subdivided the tropical marine biota into Atlantic and Pacific populations, has been used as a calibration point (Knowlton and Weigt 1998; Zuccarello and West 2002). It is assumed that genetic divergence of marine organisms commenced after the final closure of the Isthmus, 3.5-3.0 Ma (Coates and Obando 1996).

Our goal is to reveal the evolutionary relationship between *C. leprieurii* and related species and to understand the phylogeography of these organisms based on the *rbcL* gene sequences. We also tried to estimate the divergence time of *Caloglossa* species in order to discuss whether past vicariant events are associated with these biogeographic patterns.

* To whom correspondence should be addressed.

Email: mkamiya@kobe-u.ac.jp

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Table 1. Source of *Caloglossa* species examined in this study. Genbank Accession numbers are given.

Species	Location	Date	Strain no.	Accession no.	Collector
<i>C. continua</i>	Tamsui R., Mangrove Station, Taipei. Pref., Taiwan	Mar. 12, 2000	MK1271	AY150313	Yokoyama
<i>C. intermedia</i>	Sapelo Island, Georgia, USA	Oct. 24, 1990	3092	AY150314	Darley
<i>C. leprieurii</i> *	I. Espiritu Santo, Baja California Sur, Mexico	Mar. 19, 1992	3276	AY150315	West
<i>C. leprieurii</i> *	Intercoastal Waterway, Ft. Pierce, Florida, USA	June 19, 1994	3421	AY150320	West
<i>C. leprieurii</i>	Mayagueyes I., La Parguera, Puerto Rico	Mar. 6, 1994	3402	AY150317	Ballantine
<i>C. leprieurii</i>	Boca R., San Juan, Chiapas, Mexico	Mar. 25, 1993	3378	AY150318	West
<i>C. leprieurii</i>	James I., South Carolina, USA	June 17, 1994	3407	AY150319	West
<i>C. leprieurii</i>	Cobhams Wharf, Virginia, USA	-	3156	AY150321	Ott
<i>C. leprieurii</i>	Isla Morada, Florida Keys, USA	June 15, 1994	3431	AY150316	West
<i>C. leprieurii</i>	Bali, Indonesia	Apr. 13, 1999	MK1208	AY150322	Kawachi
<i>C. leprieurii</i>	Shimajiri, Miyako Is., Japan	May 31, 1991	MK465	AY150323	Kamiya
<i>C. monosticha</i>	Batu Puteh, Changi, Singapore	Mar. 13, 1994	MK987	AY150324	Kamiya
<i>C. ogasawaraensis</i>	Georges Hall, Georges. R., New SouthWales, AU	Apr. 5, 1997	GZ61	AY150325	Zuccarello
<i>C. vieillardii</i>	Monkey Mia, Western Australia, AU	Aug. 31, 1998	D570	AY150326	Karsten
<i>C. vieillardii</i>	Tomkinson R., Northern Territory, AU	Aug. 22 1999	F442	AY150327	Zuccarello
<i>C. vieillardii</i>	Tewantin, Queensland, AU	Jul. 29, 1998	D494	AY150328	Zuccarello
<i>C. vieillardii</i> *	Garden I. Adelaide, South Australia, AU	Jan. 22, 1993	3328	AY150329	Karsten
<i>C. postiae</i>	Georges River, New SouthWales, AU	May 29, 1997	GZ98	AY150330	Zuccarello

* isolates lacking sexual reproduction

MATERIALS AND METHODS

Methods of collection and culture are described in Kamiya *et al.* (1998). Collections of *Caloglossa* species were made at the localities indicated in Table 1. The DNA extraction and sequencing were performed as described in Zuccarello *et al.* (2002). The sequences obtained were aligned for phylogenetic analyses using the Clustal X computer program (Thompson *et al.* 1997) and refined by eye. The maximum likelihood (ML), maximum parsimony (MP) and distance matrix methods were implemented with PAUP* (version 4.0b10, Swofford 1998). In MP analysis, characters were unordered, and all codon positions were weighted equally. Cladograms were produced using the branch and bound option. For distance matrix method, pairwise nucleotide distance estimates of the sequences were calculated using HKY85 model (Hasegawa *et al.* 1985) and phylogenetic trees were inferred from these distance estimates using neighbor-joining (NJ) method (Saitou and Nei 1987). For ML analysis, likelihood scores for the optimal trees obtained in the MP branch and bound search were calculated using a series of likelihood models incorporated into PAUP*. The program Modeltest version 3.0 (Posada and Crandall 1998) was also used to find the model of sequence evolution that best fits each data set by a hierarchical likelihood ratio test (hLRT). When the best sequence evolution model was determined, ML tree searches were performed using heuristic search option (5 random sequence additions) using the estimated parameters (base frequencies, among-site rate variation and proportion of invariable site). Bootstrap analyses (Felsenstein 1985) were performed to assess the robustness of clades (1,000 replicates in MP and NJ and 500 in ML). The statistical tests of the substitution rate were performed as described in Kamiya *et al.* (2003).

RESULTS AND DISCUSSION

The analyzed sequences, 1,362 bp in length, had a mean 37.1% of G + C content and included 409 variable sites identified among all the isolates; 268 of these were potentially phylogenetically informative. The number of variable sites was quite different among first, second and third codon positions; 64, 18 and 327, respectively. *Caloglossa ogasawaraensis* was used as an outgroup. Besides the full data set, data sets excluding third codon positions, or using amino acid sequences were used to reconstruct phylogenetic trees, but these trees were poorly resolved (lots of polytomies - data not shown). The best ML score (-ln = 5,925.90) was obtained using the general-time-reversible model (Rodríguez *et al.* 1990; base frequencies of A = 0.3217, C = 0.1430, G = 0.2050, T = 0.3302; rate matrix: A→C = 1.1190, A→G = 6.6810, A→T = 1.7017, C→G = 1.2353, C→T = 18.9485, G→T = 1.00; gamma distribution = 1.666; proportion of invariable site = 0.5956) and the topology shown in Fig. 1. MP analysis produced one most parsimonious tree (length = 865 steps, CI = 0.594, RI = 0.596).

The six ingroup species have been morphologically classified into two subgroups based on the position of rhizoids; *C. continua* (Okamura) King et Puttock, *C. monosticha* Kamiya and *C. postiae* Kamiya et King are in the axil rhizoid group, and *C. intermedia* Kamiya et West, *C. leprieurii* and *C. vieillardii* are the nodal rhizoid group (Kamiya *et al.* 2000). Both groups were monophyletic in the ML tree (Fig. 1) and MP one (data not shown), whereas the axil rhizoid group formed a clade with *C. vieillardii* in the NJ tree with low support (data not shown). The monophyletic relationship of either group was also resolved using LSU rDNA data and a larger data set (Kamiya *et al.* 2002). The low resolution of the axil group could be due to the low number of samples used in this study.

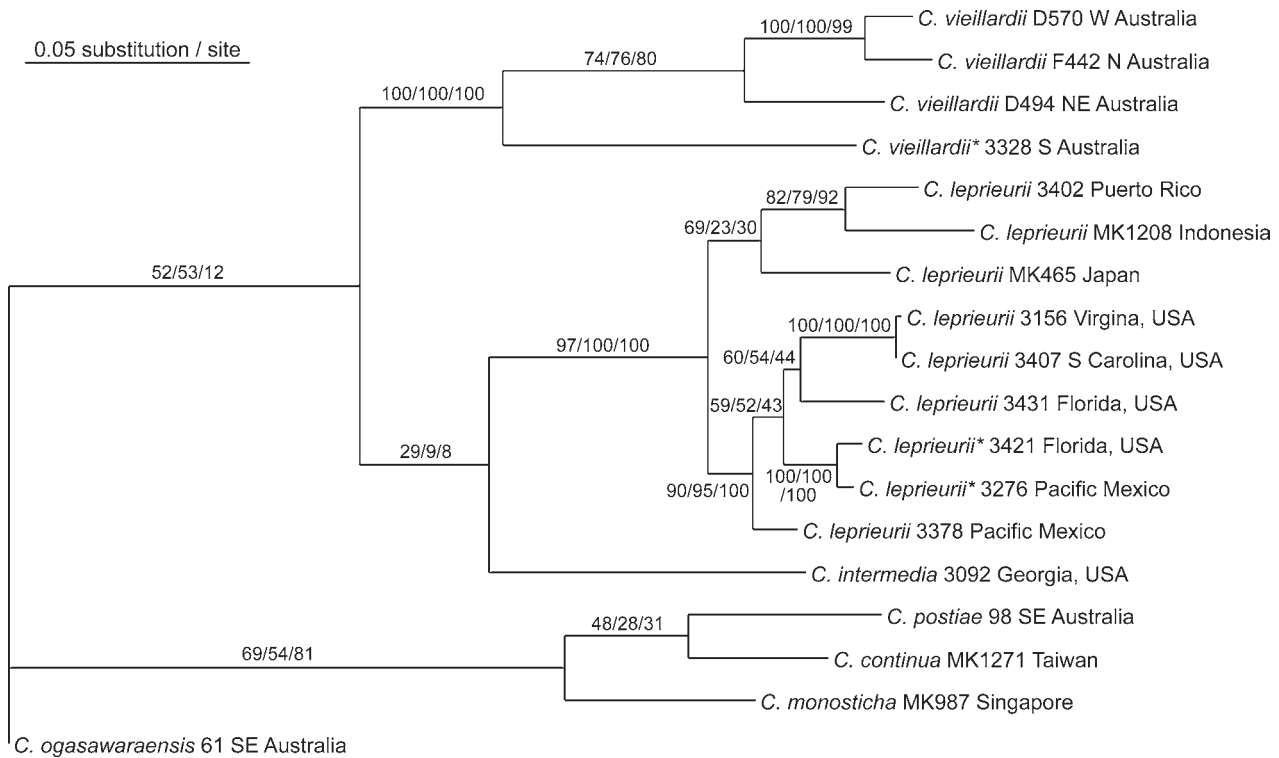


Fig. 1. Maximum-likelihood tree of *Caloglossa* species inferred from *rbcL* sequence data. Numerals at each node are bootstrap values (%) for ML, MP and NJ analyses. Asterisk indicates the asexual isolates.

Both *C. leprieurii* and *C. vieillardii* are monophyletic. In *C. vieillardii*, the evolutionary relationship of the four Australian isolates was correlated with the geographic distance of these samples. In contrast, such a correlation was not always seen in *C. leprieurii*; the strain from Puerto Rico made a clade with the Southeast Asian strains rather than the other Atlantic ones. Furthermore, the RuBisCo spacer analyses with additional Asian strains suggested that the south-western Pacific strains are not monophyletic and that the migration between Atlantic and Pacific Oceans occurred several times in the *C. leprieurii* lineage (West *et al.* 2001).

Two asexual strains of *C. leprieurii* and one of *C. vieillardii* were investigated in this study. Asexual populations of *C. leprieurii* were reported from Pacific Mexico at first and later found from the Atlantic and south Australian coasts (West *et al.* 1994, 2001). Asexual populations are found 1,200 km north of the northernmost sexual populations in Pacific Mexico, and West *et al.* (1994) considered that the asexual cycle occurred along the Pacific coasts. In contrast, the present study suggested the scenario that the asexualization happened along the western Atlantic coast and then the asexual plants colonized the eastern Pacific coast, where the sexual plants were already distributed. However, this evolutionary relationship was not supported by high bootstrap values in either trees, and it is necessary to analyze additional populations along the American coasts for better understanding the phylogeographic pattern of these entities.

The evolutionary position of *C. intermedia* has been prob-

lematic. This species is morphologically similar to *C. leprieurii* and *C. vieillardii* based on the rhizoidal position at the nodes and the number of rhizoids derived from each blade cell, but distinguishable by the presence of the adaxial cell row from the first axial cell at the lateral axis (Kamiya *et al.* 2000). This adaxial cell row, which is present in most other *Caloglossa* species, is considered as an ancestral character in the *Caloglossa* lineage. Thus *C. intermedia* was expected to be ancestral to *C. leprieurii* and *C. vieillardii* (Kamiya *et al.* 2000). This relationship is also suggested by the rhizoidal development of *C. intermedia*, which shows an intermediate characteristic between *C. leprieurii*/*C. vieillardii* and *C. monosticha* (Kamiya *et al.* 2000). The phylogenetic position of *C. intermedia* was, however, unresolved in the *rbcL* analyses (Fig. 1) as well as in RuBisCo spacer and LSU rDNA analyses (Kamiya *et al.* 2000, 2003).

In analysis of LSU rDNA sequences the substitution rate of *C. intermedia* was significantly greater than that of all the other species ($P < 0.01$) (Kamiya *et al.* 2003). This species showed LSU rDNA sequence divergence from other ingroup species of 10–17%, nearly equal to the intergeneric value (9–17% between *Caloglossa* and *Taenioma*) (Kamiya *et al.* 2003). This long-branch made the position of *C. intermedia* suspect. However, the evolutionary rate of the *rbcL* of *C. intermedia* is within the interspecific range (see below), and it is not known whether this difference is due to the difference between nuclear and chloroplast genes, or between multigene family and single copy genes.

Caloglossa vieillardii has been found from Australia,

Southeast Asia and central Pacific archipelagoes, while *C. leprieurii* is more widely distributed, in Atlantic and Pacific America, Southeast Asia, and Indian and Arabian coasts. Although *C. leprieurii* had not been found from Australia or central Pacific regions, recently an asexual population of this species was reported from Daintree River near Cooktown, northeastern Australia (West *et al.* 2001). Dispersal routes of *C. leprieurii* may have followed three possible paths, and in either direction. A first route across the Pacific Ocean would require long-distance dispersal by island hopping (Duke 1995), but this proposal seems improbable because *C. leprieurii* has not been found on central Pacific islands. A second route is through the Indian Ocean via the Cape of Good Hope. Quite recent genetic exchange of *C. leprieurii* between South Africa and Venezuela, which are 11,000 km distant, was suggested by their reproductive fertility and genetic distance (Kamiya *et al.* 1998). However, fossils of mangroves as well as corals were not found from the southern part of the African continent (McCoy and Heck 1976; Ellison *et al.* 1999), and this route was probably too cold for tropical organisms under paleoenvironmental conditions (Chapman 1984). Since contemporary *Caloglossa* species are found in temperate regions, without mangroves, this route may not be rejected.

A third alternative is via the ancient Tethys Sea, which has been considered for the biogeography of mangrove plants (McCoy and Heck 1976; Mephram 1983; Chapman 1984; Duke 1995; Ellison *et al.* 1999). There is general agreement that mangrove ecosystems first appeared in the late Cretaceous - early Tertiary on the shores of the Tethys Sea and then dispersed via the available coastal waterways (Saenger 1998; Ellison *et al.* 1999). The mild environment around the Tethys coasts may have developed not only mangrove plants but also mangrove algae. If so, we need to know if *C. leprieurii* existed during this Tethys time period.

The substitution rate of *rbcL* was statistically tested using the 1D and 2D methods (Tajima 1983; Miyashita *et al.* 1998). When *C. ogasawaraensis* Okamura was used as an outgroup, the molecular clock hypothesis was not rejected at the 1% level in any strains, including *C. intermedia*. Next, the divergence rate of this gene was calculated based on the vicariant event, the last closure of the Panama Strait. The present data indicated a well-supported clade of the two asexual isolates (3421 and 3276) from opposite sides of the Isthmus of Panama. If the sequence divergence between these two strains, which is 0.76% in HKY85 pairwise distance, was established after the Panama Strait was closed (3.5-3.0Ma), the divergence rate can be estimated to 0.109-0.127% per million years ($10.9-12.7 \times 10^{-10}$ substitutions per site per year). Because the mean sequence divergence between *C. leprieurii* and *C. vieillardii* is 11.2%, the divergence date of the two species can be estimated at 51-44 Ma. Many kinds of mangrove fossils were found around the Tethys Sea in this early-middle Eocene (Ellison *et al.* 1999), and these *Caloglossa* species were possibly growing under similar environmental conditions. In *C. leprieurii*, the pairwise distance is 5.9% between the strains from Indonesia and Puerto Rico, a well supported clade, so an estimated divergence date is calculated to 27-23 Ma. Considering a

period before closure of the Tethys Sea, 15-12 Ma (Rögl and Steininger 1984), dispersal of *C. leprieurii* between the future Atlantic and Pacific Oceans is conceivable via this Tethys route.

The substitution rate of *rbcL* has been estimated for land plants based on their fossil records; $1.4 - 2.4 \times 10^{-10}$ substitution rate per site per year (SSY) in the Atherospermataceae (Laurales) (Renner *et al.* 2000), 1.0×10^{-10} SSY in 38 woody seed plants (Albert *et al.* 1994), $2.5 - 3.5 \times 10^{-10}$ SSY in the cotton genus *Gossypium* (Wendel and Albert 1992), and 0.5×10^{-10} SSY in land plants including seed plant, fern and liverwort (Savard *et al.* 1994). These substitution rates are 10 to 20 times slower than for *Caloglossa*, and it is unknown whether the different evolutionary rate of the *rbcL* gene is related to the different evolutionary history between land plants and red algae, their different environments, or an error in rate calibration. Other algal data of the substitution rate of *rbcL* are necessary to resolve this problem.

In conclusion, the *rbcL* sequence analyses revealed the relationships within *C. leprieurii* and *C. vieillardii*, though the evolutionary position of *C. intermedia* was still ambiguous. Because of many informative sites, the *rbcL* gene is more useful for phylogenetic reconstruction than the RuBisCo spacer region. In addition, unlike LSU rDNA, the evolutionary rate of *rbcL* gene was almost equal in *Caloglossa* species examined in this study, and was useful in estimating the inter- and intraspecific divergence times.

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