Aquatic Invasions (2009) Volume 4, Issue 4: 567-574
DOI 10.3391/ai.2009.4.4.1

Research article

Recent introduction of a freshwater red alga *Chantransia macrospora* (Batrachospermales, Rhodophyta) to Okinawa, Japan

Aki Kato[1*], Naohiro Morita[1], Tomoko Hiratsuka[2] and Shoichiro Suda[1]
[1]*Department of Chemistry, Biology and Marine Science, Faculty of Science, University of the Ryukyus, Senbaru 1, Nishihara, Okinawa 903-0213, Japan*
[2]*Department of Natural Environmental Studies, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwanoha, Kashiwa-city, Chiba 277-8653, Japan*
E-mail: h081716@sci.u-ryukyu.ac.jp (AK), sudas@sci.u-ryukyu.ac.jp (SS)
**Corresponding author*

Received 2 June 2009; accepted in revised form 5 November 2009; published online 19 November 2009

Abstract

The freshwater red algal form taxon, *Chantransia macrospora* Wood, was recently collected at an artificial reservoir pond in Okinawa, Japan. This species is chiefly characterized by a bluish-green thallus and erect filaments irregularly branched with cylindrical to tapered cells and monosporangia on lateral branchlets. Our culture study confirmed that *C. macrospora* from Okinawa was the 'chantransia' sporophyte of *Batrachospermum* species. The *rbc*L sequence of *C. macrospora* from Okinawa was identical to isolate FG15 of this species from French Guiana in South America. The sporophyte *Chantransia macrospora* as well as the gametophyte *B. macrospora* Montagne are distributed in North and South America. However, there have been no records of either *C. macrospora* or *B. macrosporum* in Japan or in eastern Asia. Therefore, *C. macrospora* specimens originally from South America may have been introduced to Okinawa, Japan, through the vector such as ornamental macrophytes.

Key words: *Audouinella*; *Batrachospermum*; *Chantransia macrospora*; freshwater algae; introduced species; macroalgae

Introduction

Many introduced species of marine macroalgae have been reported worldwide, for example, the green algae *Caulerpa taxifolia* (Vahl) C. Agardh and *Codium fragile* ssp. *tomentosoides* (van Goor) P. C. Silva, the brown algae *Undaria pinnatifida* (Harvey) Suringar and *Sargassum muticum* (Yendo) Fensholt, the red algae *Grateloupia turuturu* Yamada and *Hypnea musciformis* (Wulfen) J.V. Lamouroux (Inderjit et al. 2006; Defeo et al. 2009). In addition, several introduced species of freshwater macroalgae, *Bangia* Lyngbye, *Compsopogon* Montagne, *Hildenbrandia* Nardo, have also been found, and the possibility of their introduction was usually reinforced by molecular phylogenetic analyses (Müller et al. 1998; Rintoul et al. 1999; Sherwood and Sheath 1999).

Boudouresque and Verlaque (2002) distinguished between introduced or invasive species: an introduced species shows no discernible negative impact, whereas an invasive species has an identified negative impact. Inderjit et al. (2006) suggest that it is better to use the term "introduced" or "non-indigenous" and state whether or not the introduced species has demonstrated a harmful effect. However, they pointed out the difficulties with the exact definition of an invasive species in the absence of a clearly demonstrated negative effect. We followed the perspective of Boudouresque and Verlaque (2002) and regarded a species without discernible negative impact as an introduced species.

Chantransia macrospora Wood, a freshwater filamentous red alga, usually grows in streams or rivers in North and South America [Necchi et al. 1993; Necchi and Zucchi 1995; Kumano 2002, as *Audouinella macrospora* (Wood) Sheath & Burkholder]. This species is mainly characterized by a bluish-green thallus and erect filaments irregularly branched with cylindrical to tapered cells. Pit plug ultrastructure of *C. macro-*

spora indicated that this species belongs to the Batrachospermales Pueschel & Cole (Pueschel et al. 2000), not the Acrochaetiales Feldmann including *Audouinella* Bory which once contained this species. Recent culture and molecular studies indicate that *C. macrospora* is the sporophyte, 'chantransia stage', of *Batrachospermum macrospora* Montagne (Pueschel et al. 2000; Necchi and Zuccchi 1997; Chiasson et al. 2005). Especially, the *rbc*L has been utilized in the past to link *Chantransia* sporophytes with *Batrachospermum* species identified based on gametophyte characteristics (Chiasson et al. 2005; 2007; Vis et al. 2005; 2008). Both the sporophyte *C. macrospora* and gametophyte *B. macrosporum* are found in North and South America (Kumano 2002). However, there have been no records of either species from other continents and regions, including Japan.

In this study, we identified algal specimens growing on dead grasses and floating plastic trash in an artificial reservoir pond, Senbaru-ike, in Okinawajima Island, Japan, based on morphology as *C. macrospora*. To further investigate this occurrence, the specimens were cultured to determine if gametophytes would be produced and *rbc*L gene sequences were compared with the literature to confirm the taxonomic identification and find a potential origin.

Material and Methods

Specimens and culturing

Chantransia macrospora specimens were collected in 2006 from an artificial reservoir pond, Senbaru-ike, on the premises of the University of the Ryukyus, Okinawa, Japan (26°14'66"N, 127°45'55"E). Voucher herbarium specimens were deposited in the Herbarium of the Faculty of Science, University of the Ryukyus, Okinawa (RYU-A). The phenology of *C. macrospora* was surveyed every one or two weeks from November in 2007 to October in 2008. Unialgal cultures were established by removing attached zooplankton and diatoms in agar plates. Culture was maintained in freshwater enriched PES medium (Provasoli 1968), C medium (Ichimura 1971) and IMK medium (Nippon Pharm. Co., Tokyo). The temperatures and photoperiods were regulated at 20 or 25°C, 14:10 h L:D and lighting was 25-35 µmol photons $m^{-2}s^{-1}$. The established culture was deposited in National Institute for Environmental Studies, Japan (NIES). For light microscopy, living field and culture materials were used.

Electron microscopy

Material for thin sections was prepared as follows: equal volumes of fixative [5% glutaraldehyde, 0.5 M sucrose, in 0.1 M cacodylate buffer (pH 7.2)] with or without a drop of osmium tetroxide and culture medium containing growing cells were mixed together at room temperature for 1 h. A fixative based on the culture medium instead of cacodylate buffer was also used. After rinsing twice with 0.05 M cacodylate buffer, cells were postfixed in 2% osmium tetroxide for 1 h, and rinsed once with the same buffer. Thalli were embedded in Spurr's resin (Spurr 1969) after dehydration in a graded ethanol series. Sections were cut with a diamond knife using a Leica EM UC6 Ultramicrotome (Leica Microsystems K.K., Tokyo, Japan) and collected on slot grids coated by Formvar. Sections were double stained with 2% uranyl acetate and lead citrate (Reynolds 1963). Observations were carried out with a JEOL JEM 1011 (JEOL, Tokyo, Japan) transmission electron microscope.

Genetic analyses

Total DNA was extracted from a fresh culture thallus using Nucleon PhytoPure Genomic DNA Extraction Kits (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's protocol. The following gene fragments were amplified and sequenced with the oligonucleotide primers stated below: 1) the nuclear SSU rDNA – three primer pairs SR1 and SR5, SR4 and SR9, SR8 and SR12 (Nakayama et al. 1996); and 2) the chloroplast *rbc*L – a primer pair F160 and *rbc*L R and two internal primers R472.2 and R 897 (Vis and Sheath 1999). The PCR products directly sequenced using a CEQ8800 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA) with the dye-terminator method. Sequences of SSU rDNA and *rbc*L were deposited in DDBJ (AB503215 and AB503216). A BLAST (Basic Local Alignment Search Tool) search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed to determine closely related sequences.

The SSU rDNA alignment, including a sequence determined here and previously published sequences of 27 species of the Batrachospermales and related orders (Vis et al. 1998; Pueschel et al. 2000), was aligned using

Clustal W 1.83 (Thompson et al. 1997). *Rhodogorgon carriebowensis* J. Norris et Bucher (AF006089) served as an outgroup. The *rbc*L alignment, including a sequence determined here and 10 previously published sequences of *Chantransia macrospora* and 19 sequences of *Batrachospermum macrosporum* (Chiasson et al. 2005; Vis et al. 2008), was aligned manually as there were no gaps among *rbc*L sequences. Identical sequences were represented by a single sequence in the alignment. Thirteen closely related *Batrachospermum* species were used as outgroups following Vis et al. (2008).

The phylogenetic relationships among the sequences of the SSU rDNA and *rbc*L were inferred using maximum parsimony (MP), minimum evolution (ME) and maximum likelihood (ML), which was performed using PAUP*4.0b10 (Swofford 2002). MP and ME analyses were performed using the heuristic search option (with 100 random sequence additions for MP) and the tree-bisection-reconnection (TBR) branch swapping algorithm. All sites were treated as equally weighted, and gaps were treated as missing. To evaluate bootstrap support for individual clades (Felsenstein 1985), the MP and ME bootstrap analyses consisted of 1000 replications of the full heuristic search option with 10 random additions and TBR branch swapping (for MP). The maximum likelihood (ML) analysis was carried out using PhyML 2.4.4 (Guindon and Gascuel 2003; Guindon et al. 2005). The evolutionary models for ME and ML that best fit the data set were determined using a hierarchical likelihood ratio test in Modeltest 3.7 (Posada and Crandall 1998). The selected model for the *rbc*L data set was the GTR model (Rodriguez et al. 1990) with invariable sites (0.5249) and a gamma distribution parameter of 0.9279. The ML bootstrap analyses were conducted with 500 replications with subtree pruning-regrafting branch swapping.

Results

Morphology

Chantransia macrospora from the artificial reservoir pond Senbaru-ike was bluish green in colour (Figure 1) and growing on dead grasses, driftwood or floating plastic with water transparency that was approximately 50 cm throughout the year. There was a seasonal pattern and *C. macrospora* was not observed in late July to September. During this period, the water temperature was almost always more than 30°C.

This species formed dense tufts and was 2.0-2.5 mm in height. The thallus was composed of a uniseriate erect filament branching with narrow angles (Figure 1A) and well-developed rhizoids. The erect filaments were generally cylindrical to tapered along their height. Axial cells of the erect filaments were 14-22 µm in diameter and 41-72 µm in height. Chloroplasts were discoid to elongate and with three to five pyrenoids (Figure 1B). Lateral branches irregularly arose from the axial cell and formed monosporangia (19-23 µm in diameter and 31-39 µm in height) on apices of the ultimate branches. Germination of monospores occurred by unipolar elongation to form a rhizoidal filament leaving the spore empty of protoplast (Figure 1C). The dimensions of vegetative and reproductive structures of a culture isolate overlapped with the ranges of field specimens. After 6-10 months in culture from small segments (< 5mm) of *C. macrospora*, *Batrachospermum* gametophytes developed (Figure 1D-F). The filaments of juvenile gametophytes were clearly distinct from those of *C. macrospora* in shape and size, as gametophyte cells were disk shaped and significantly shorter and wider than the cylindrical vegetative cells of *C. macrospora* (Figure 1D). The gametophytes in later stages formed young whorled filaments and were branched (Figure 1E, F). The *Batrachospermum* gametophytes did not form reproductive structures and it was not possible to identify the species. We started culturing small segments (< 5mm) of *C. macrospora* and observed the formation of *Batrachospermum* gametophytes twice. However, the particular conditions of the temperature, light intensity and culture media that induced formation of gametophytes were not identified.

The ultrastructure of the pit plugs from the Senbaru-ike culture isolate are shown in Figure 2. Pit plug cores were 0.6-1.0 µm in diameter at the narrow waist, 0.9-1.2 µm in diameter at the ends, and 1.2-1.5 µm in height. The plug core often had projections near the waist (Figure 2A). The outer layer closer to the cytoplasm was unevenly thick and plate-like (Figure 2A) and sometimes appeared to be assembled from spheroidal units (Figure 2B), 0.1-0.2 µm thick. Some outer caps were discontinuous (Figure 2C) and therefore, it was possible to confirm that no membrane was present.

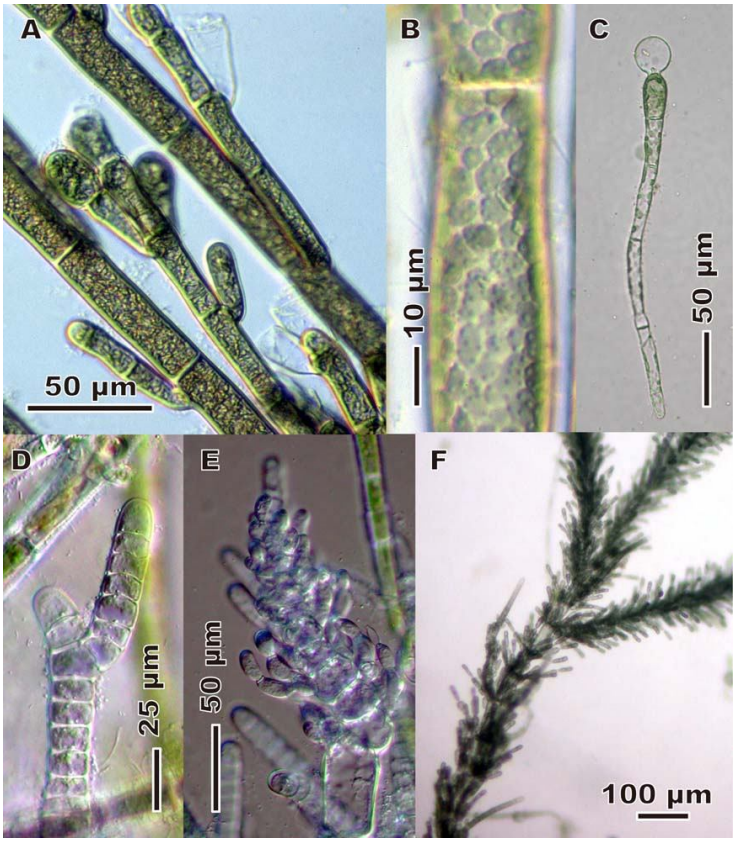

Figure 1. Morphology and reproduction of *Chantransia macrospora* in culture: (A) Erect filaments with lateral branches bearing monosporangia; (B) Discoid to elongate chloroplasts possessing pyrenoids; (C) Monospore germination with rhizoidal filament and empty spore; (D) Juvenile gametophyte in early stage; (E) Juvenile gametophyte with whorled filaments in later stage; (F) Branched *Batrachospermum* gametophyte

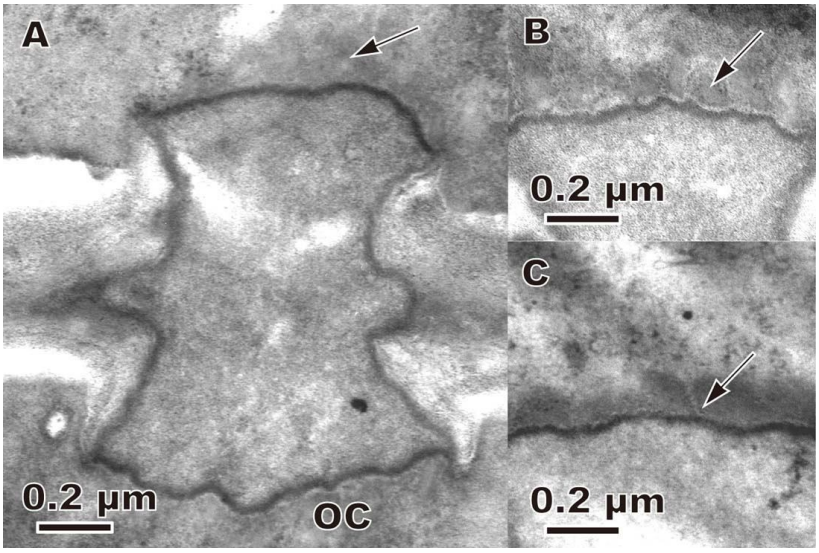

Figure 2. Ultrastructure of pit plugs of *Chantransia macrospora* in culture: (A) Pit plug with thick plate-like outer cap (arrow); (B) Outer cap appeared to be assembling from spheroidal units (arrow); (C) Discontinuous portion of the outer cap showing the absence of a cap membrane (arrow)

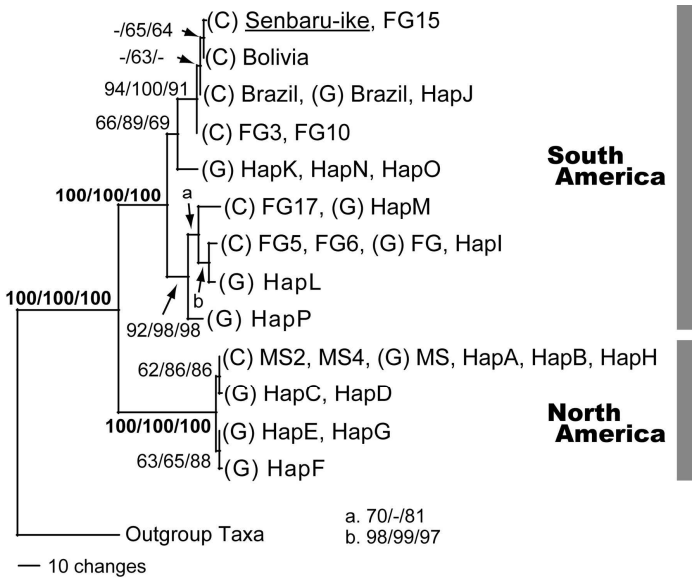

Figure 3. Phylogenetic tree from maximum parsimony (MP) analysis of *rbc*L sequences of *Chantransia macrospora* (C) and *Batrachospermum macrosporum* (G) (Length = 1156 steps, CI = 0.528, RI = 0.806). Sequence determined in this study is underlined. Numbers shown on each branch represent bootstrap values >60% for MP (left), ME (center) and ML (right) analyses. Outgroup taxa represent 13 *Batrachospermum* species. Abbreviations of samples followed Chiasson et al (2005) and Vis et al. (2008)

Molecular analyses

The BLAST search indicated that our determined sequence of SSU rDNA was closely related to two sequences (AF199505 and AF199506) of three isolates of *Chantransia macrospora* (as *Audouinella macrospora*, Pueschel et al. 2000), which were the only available in Genbank (searched 15 May 2009). The phylogenetic analysis using SSU rDNA sequences of the Batrachospermales and related orders also supported this.

The *rbc*L sequence of the isolate from Senbaru-ike was identical to isolate FG15 of *C. macrospora* from French Guiana (AY423414) in South America. The analyzed *rbc*L data set consisted of 38 sequences of *Chantransia macrospora* including the Senbaru-ike isolate and *Batrachospermum macrosporum*. Of the 1227-bp portion analyzed, 797 bp were variable, and 52 bp were phylogenetically informative. The MP analyses produced four MP trees, one of which is shown in Figure 3 (Length = 1156 steps, CI = 0.528, RI = 0.806). The ML (-ln L = 7270.695734) and ME trees are not shown. The topologies of the MP, ML and ME trees were identical to each other except for two arrangements that were not supported by any analytical methods. The Senbaru-ike isolates and FG15 formed a fully-supported clade with other isolates of *C. macrospora* and *B. macrosporum* gametophytes from South America. This South American clade was related as a sister clade to a fully-supported clade comprising of isolates of *C. macrospora* and *B. macrosporum* from North America (Figure 3).

Discussion

The form genus *Chantransia* including three species was proposed to distinguish these specimens from taxa of the genera *Audouinella* and *Ptilothamnion* Thuret (Vis et al. 2006). *Chantransia* species represent an alternate life history phase (sporophyte stage) of species of the Batrachospermales and Thoreales Müller, Sherwood, Pueschel, Gutell & Sheath. The three *Chantransia* species are distinguished from each other by the thallus color in field, the development of erect filaments and cell dimensions. Our *Chantransia* specimens from Senbaru-ike was identical to *C. macrospora* in having a bluish-green thallus and erect filaments irregularly branched with cylindrical to tapered cells and the size of vegetative cells and monosporangia (Glazer et al. 1997; Kumano 2002; Chiasson et al. 2005; 2007; Vis et al. 2006). Pit plugs of the Senbaru-ike isolate were characterized by a thick plate-like outer cap layer and the absence of a cap membrane, which are consistent with the

C. macrospora (as *A. macrospora*) in Pueschel et al. (2000). Moreover, the Senbaru-ike isolate produced juvenile gametophytes of *Batrachospermum* species, indicating that the Senbaru-ike isolate was morphologically identical to other specimens of *C. macrospora* in previous studies (Glazer et al. 1997; Necchi and Zucchi 1997). The *rbc*L sequence of *C. macrospora* from the Senbaru-ike was identical to an isolate from French Guiana which was closely related to other isolates of this species (Chiasson et al. 2005). Therefore, we identified our *Chantransia* specimens as *C. macrospora*. This is the first report of *C. macrospora* from the northwest Pacific Ocean region.

Chantransia macrospora is distributed in North and South America (Kumano 2002, as *A. macrospora*). This species has been found to be the chantransia stage of *B. macrosporum* by culture and molecular studies (Necchi and Zucchi 1997; Pueschel et al. 2000; Chiasson et al. 2005). The geographic distribution of *B. macrosporum* is also North and South America (Kumano 2002). In molecular phylogenetic analyses, *C. macrospora* and *B. macrosporum* were distantly related to the members of the Batrachospermales known from eastern Asia, for example, *B. arcuatum* Kylin and *B. atrum* (Hudson) Harvey (Vis et al. 2006; Chiasson et al. 2007).

Kumano (2002) reported eight species of bluish-green *Audouinella* including *C. macrospora* and *C. pygmaea* (as *A. macrospora* and *A. pygmaea*), and these have not been reported from Japan to date. Geographical distribution of five species includes China, and one of these species, *Audouinella chalybaea* (Roth) Bory has been showed to produce *Batrachospermum* gametophytes (Israelson 1942). However, *A. chalybaea* is clearly distinguished from *C. macrospora* by much smaller monosporangia and from *C. pygmaea* by narrower erect filaments (Kumano 2002).

The *rbc*L sequence differences of *C. macrospora* and *B. macrosporum* ranged between 1-2.5% even in South America (Chiasson et al. 2005), and 5.5-6.5% between North and South America (Chiasson et al. 2005, Vis et al. 2008). These results indicate that *C. macrospora* has relatively high genetic intraspecific divergence. Therefore, considering the geographical distribution and the low genetic variations between the Japan specimens and French Guiana, the most likely explanation of the presence of *C. macrospora* in Okinawa is via introduction. *Chantransia macrospora* from this study may have been secondarily transported from South America, directly or indirectly, to Okinawa, Japan. The freshwater red alga *Compsopogon* has been suspected to be introduced in the same way that the freshwater green alga *Hydrodictyon* Roth was introduced by the aquarium or ornamental fish and macrophyte trade (Rintoul et al. 1999). *Chantransia macrospora* has also been reported to grow on the aquatic fern *Ceratopteris* in fish tanks (Glazer et al. 1997). Moreover, introduced fishes and water plants have been reported in Okinawa, Japan (Tachihara et al. 2002; Kadono 2002; Yokota 2003). For example, an introduced freshwater fish, *Liposarcus disjunctivus* Weber, which is native to Amazon, South America, was recently found spreading in Okinawajima Island, including at our collection site, Senbaru-ike, the artificial reservoir pond of University of the Ryukyus (Takeshima and Yoshino 1996). According to Takeshima and Yoshino (1996), after this pond was built in 1977, it was drained completely for over 1 week to construct irrigation facilities in 1983. At that time, *L. disjunctivus* was not found there. However, *L. disjunctivus* was found for the first time in the Makiminato River system, which originates from Senbaru-ike, in 1989; subsequently more than 40 specimens have been collected at Senbaru-ike. These results suggest that *L. disjunctivus* was introduced into Senbaru-ike after 1984. Considering this evidence, it is possible that *C. macrospora* was also introduced in recent decades by a vector, such as ornamental macrophytes.

Chantransia macrospora can grow in the very eutrophic pond Senbaru-ike, although *Chantransia* and *Batrachospermum* species are usually reported in less polluted streams (Necchi et al. 1993; Necchi and Zucchi 1995). This result may indicate that *C. macrospora* has a high tolerance for polluted conditions. Moreover, the fact that tiny freshwater algae, such as *C. macrospora*, have been found may indicate that unknown introduced creatures on which *C. macrospora* grows potentially exist. Rivers on Okinawajima Island, Japan, are small-scale and their environments are unstable (Nishijima 1984). Some reports have previously dealt with freshwater fishes introduced to Okinawajima Island, and have demonstrated ecosystem disturbances by these introductions (Senou 1985; Kouchi 1995). A survey of introduced aquatic plants and algae is urgently needed in Okinawajima Island and also on the other Ryukyu Islands in Japan.

Acknowledgements

We thank Dr. James Davis Reimer for his critical reading and valuable comments for the manuscript. This study was supported by a grant for the 21st Century COE Program "The Comprehensive Analyses on Biodiversity in Coral Reef and Island Ecosystems in Asian and Pacific Regions" at University of the Ryukyus financed by the Ministry of Education, Culture, Sports, Science and Technology, Japan (Monbukagakusho).

References

Boudouresque CF, Verlaque M (2002) Biological pollution in the Mediterranean Sea: invasive versus introduced macrophytes. Marine Pollution Bulletin 44: 32-38, doi:10.1016/S0025-326X(01)00150-3

Chiasson WB, Sabo NJ, Vis ML (2005) Affinities of freshwater putative chantransia stages (Rhodophyta) from molecular and morphological data. Phycologia 44: 163-168, doi:10.2216/0031-8884(2005)44[163:AOFPCS]2.0.CO;2

Chiasson WB, Johanson KG, Sherwood AR, Vis ML (2007) Phylogenetic affinities of the form taxon *Chantransia pygmaea* (Rhodophyta) specimens from the Hawaiian Islands. Phycologia 46: 257-262, doi:10.2216/06-79.1

Defeo O, McLachlan A, Schoeman DS, Schlacher TA, Dugan J, Jones A, Lastra M, Scapini F (2009) Threats to sandy beach ecosystems: A review. Estuarine Coastal and Shelf Science 81: 1-12, doi:10.1016/j.ecss.2008.09.022

Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783-791

Glazer AN, Chan CF, West JA (1997) An unusual phycocyanobilin-containing phycoerythrin of several bluish-colored, Acrochaetioid, freshwater red algal species. Journal of Phycology 33: 617-624, doi:10.1111/j.0022-3646.1997.00617.x

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology 52: 696-704

Guindon S, Lethiec F, Duroux P, Gascuel O (2005) PHYML Online - a web server for fast maximum likelihood-based phylogenetic inference. Nucleic Acids Research 33: W557-W559

Ichimura T (1971) Sexual cell division and conjugation-papilla formation in sexual reproduction of *Cloasterium strigosum*. Proceedings of the Seventh International Seaweed Symposium, University of Tokyo Press, Tokyo: 208-214

Inderjit, Chapman D, Ranelletti M, Kaushik S (2006) Invasive marine algae: An ecological perspective. Botanical Review 72: 153-178, doi:10.1663/0006-8101(2006)72[153:IMAAEP]2.0.CO;2

Israelson G (1942) The freshwater Florideae of Sweden. Studies on their taxonomy, ecology, and distribution. Symbolae Botanica Upsaliensis 6: 1-134

Kadono Y (2002) *Pistia stratiotes*. 1. Alien species-Plants. Chapter 3. Instances of alien and ecosystems affected by them in Japan. In: The Ecological Society of Japan (ed), Handbook of Alien species in Japan. Chijinshokan, Tokyo, pp 202 (In Japanese)

Kouchi R (1995) Present status and problems of the riverine environment on Okinawajima Island. The Biological Magazine Okinawa 33: 69-77 (in Japanese)

Kumano S (2002) Freshwater Red Algae of the World. Biopress, Bristol, England. 375 pp

Müller KM, Sheath RG, Vis ML, Crease TJ, Cole KM (1998) Biogeography and systematics of *Bangia* (Bangiales, Rhodophyta) based on the Rubisco spacer, *rbc*L gene and 18S rRNA gene sequences and morphometric analyses. 1. North America. Phycologia 37: 195-207

Nakayama T, Watanabe S, Mitsui K, Uchida H, Inouye I (1996) The phylogenetic relationship between the Chlamydomonadales and Chlorococcales inferred from 18S rDNA sequence data. Phycological Research 44: 47-55, doi:10.1111/j.1440-1835.1996.tb00037.x

Necchi O, Zucchi MR (1995) Systematics and distribution of freshwater *Audouinella* (Acrochaetiaceae, Rhodophyta) in Brazil. European Journal of Phycology 30: 209-218, doi:10.1080/09670269500650991

Necchi O, Zucchi MR (1997) *Audouinella macrospora* (Acrochaetiaceae, Rhodophyta) is the *Chantransia* stage of *Batrachospermum* (Batrachospermaceae). Phycologia 36: 220-224

Necchi OJ, Sheath RG, Cole KM (1993) Systematics of freshwater *Audouinella* (Acrochaetiales, Rhodophyta) in North America. 2. The bluish species. Algological Studies 71: 13-21

Nishijima S (1984) Uniqueness of inland waters environments. In: Okinawa Association of Biology Education (ed), Creatures of Okinawa (Okinawa no Seibutsu). Okinawa Association of Biology Education, Okinawa, pp 103-114 (in Japanese)

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 14: 817-818

Provasoli L (1968) Media and prospects for the cultivation of marine algae. In: Watanabe A, Hattori A (eds) (1968) Cultures and Collections of Algae, Proceedings of the U.S.-Japan Conference, Hakone, Sept. 1966. Japanese Society of Plant Physiology, Hakone, Japan, pp 63-75

Pueschel CM, Saunders GW, West JA (2000) Affinities of the freshwater red alga *Audouinella macrospora* (Florideophyceae, Rhodophyta) and related forms based on SSU rRNA gene sequence analysis and pit plug ultrastructure. Journal of Phycology 36: 433-440, doi:10.1046/j.1529-8817.2000.99173.x

Reynolds EC (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. Journal of Cell Biology 17: 208-212, doi:10.1083/jcb.17.1.208

Rodriguez F, Oliver JF, Marin A, Medina JR (1990) The general stochastic model of nucleotide substitution. Journal of Theoretical Biology 142: 485–501

Rintoul TL, Sheath RG, Vis ML (1999) Systematics and biogeography of the Compsopogonales (Rhodophyta) with emphasis on the freshwater families in North America. Phycologia 38: 517-527

Senou H (1985) The crisis of extinctions of freshwater fish in Okinawa. Annual Bulletin of the Freshwater Fish Protection Association 11: 73-78 (in Japanese)

Sherwood A, Sheath R (1999) Biogeography and systematics of *Hildenbrandia* (Rhodophyta, Hildenbrandiales) in North America: inferences from morphometrics and *rbc*L and 18S rRNA gene sequence analyses. European Journal of Phycology 34: 523-532, doi:10.1080/09541449910001718881

Spurr AR (1969) A low viscosity epoxy resin embedding medium for electron microscopy. Journal of Ultrastructure Research 26: 31-42, doi:10.1016/S0022-5320(69)90033-1

Swofford DL (2002) PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, MA

Tachihara K, Tokunaga K, Chimura Y (2002) Alien fishes in Okinawa Island. 2. Landscape or ecosystems affected by invasive alien species-Inlands. Chapter 3. Instances of alien and ecosystems affected by them in Japan. In: The Ecological Society of Japan (ed), Handbook of Alien species in Japan. Chijinshokan, Tokyo, pp 248-249

Takeshima M, Yoshino T (1996) *Liposarcus disjunctives* (Loricariidae: Siluriformes): a New Exotic Fish Colonized in Okinawa Island. The Biological Magazine Okinawa 34: 35-41 (in Japanese and English abstract)

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25: 4876-4882, doi:10.1093/nar/25.24.4876

Vis ML, Sheath RG (1999) A molecular investigation of the systematic relationships of *Sirodotia* species (Batrachospermales, Rhodophyta) in North America. Phycologia 38: 261-266

Vis ML, Saunders GW, Sheath RG, Dunse K, Entwisle TJ (1998) Phylogeny of the Batrachospermales (Rhodophyta) inferred from *rbc*L and 18S ribosomal DNA gene sequences. Journal of Phycology 34: 341-350, doi:10.1046/j.1529-8817.1998.340341.x

Vis ML, Chiasson WB, Sheath RG (2005) Phylogenetic relationship of *Batrachospermum* species (Batrachospermales, Rhodophyta) from coastal streams in French Guiana. Phycologia 44: 441-446, doi:10.2216/0031-8884(2005)1[441:PROBSB]2.0.CO;2

Vis ML, Entwisle TJ, West JA, Ott FD (2006) *Ptilothamnion richardsii* (Rhodophyta) is a chantransia stage of *Batrachospermum*. European Journal of Phycology 41: 125-130, doi:10.1080/09670260500418504

Vis ML, Hodge JC, Necchi O, Jr. (2008) Phylogeography of *Batrachospermum macrosporum* (Batrachospermales, Rhodophyta) from North and South America. Journal of Phycology 44: 882-888, doi:10.1111/j.1529-8817.2008.00540.x

Yokota M (2003) 4. Aquatic Vascular Plants. Chapter 6. The Plants of Inland Waters. In: Nishida M, Shikatani N and Shokita S (eds), The Flora and Fauna of Inland Waters in the Ryukyu Islands. Tokai University Press, Tokyo, pp 99-114 (In Japanese)