Phycological Research

Phycological Research 2016; **64:** 95–101 doi: 10.1111/pre.12126

Phylogeography of *Asparagopsis taxiformis* revisited: Combined mtDNA data provide novel insights into population structure in Japan

Akira Kurihara,[1,2]* Hanna Horiguchi,[1] Takeaki Hanyuda[1] and Hiroshi Kawai[1]

[1]Kobe University Research Center for Inland Seas, Kobe, Hyogo, and [2]Faculty of Agriculture, Kyushu University, Fukuoka, Japan

SUMMARY

Six intraspecific lineages (Lineages 1–6) of *Asparagopsis taxiformis* have been previously established based on mitochondrial *cox*2-*cox*3 intergenic spacer and a partial *cox*1 sequences. 'Lineage 2' (L2) was suggested to be a recent introduction to the Mediterranean Sea, but its source population has not yet been identified. In order to clarify the nature of northwestern Pacific populations, we performed extensive sampling in Japan (60 individuals from 16 locations) and molecular phylogenetic analyses based on mitochondrial sequences. Sixteen additional individuals, collected from eight locations in the Indo-Pacific, Caribbean, and Mediterranean regions, were also analyzed. Combined sequence analyses revealed that the Japanese populations only consisted of L2. Out of 19 combined haplotypes identified within L2, two are shared between Japan and the Mediterranean Sea and the Hawaiian Islands, and 12 were identified as endemic to Japan. Genetic analyses of population differentiation suggested that Japanese populations are genetically isolated from the Mediterranean and the Hawaiian populations. A genetic disjunction appears to separate two subpopulations within Japan: one between Toi and Kagoshima and the other between Ojikajima Island and Kagoshima in the Kyushu area.

Key words: Bonnemaisoniales, *cox*1, *cox*2-*cox*3 spacer, genetic disjunction, introductions, native, nonindigenous, Rhodophyta.

INTRODUCTION

The establishment of nonindigenous marine species may have negative ecological, economic and social impacts (Schaffelke & Hewitt 2007; Andreakis & Schaffelke 2012). For example, the introduced species of *Caulerpa taxifolia* (Vahl) *C. Agardh*, *C. racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman et Boudouresque, *Sargassum muticum* (Yendo) Fensholt and *Undaria pinnatifida* (Harvey) Suringer have altered the species composition of local biota in a wide range of temperate regions (Schaffelke & Hewitt 2007). In the tropics, *Kappaphycus alvarezii* (Doty) Silva was introduced to Moku 'O Loe in Kāneohe Bay, O'ahu, but now overgrows and smothers corals in the Bay (Glenn & Doty 1981; Smith *et al.* 2002). Currently, approximately 100 macroalgal species are recognized as nonindigenous worldwide (Boudouresque & Verlaque 2010).

Molecular phylogenetics represent a powerful approach for delineating species within taxonomically difficult groups and resolving genealogical relationships (e.g., Olsen *et al.* 1998; Conklin *et al.* 2009; Sun *et al.* 2012). Furthermore, the origins and introductory pathways of nonindigenous species are often investigated by comparing the haplotype diversities among native and introduced populations (e.g., Verlaque *et al.* 2003; Voisin *et al.* 2005; Miller *et al.* 2007; Provan *et al.* 2008; Kogishi *et al.* 2010).

Asparagopsis taxiformis (Delile) Trevisan de Saint-Léon is a red alga that is widely distributed in warm temperate and tropical waters. Molecular phylogenetic analyses based mainly on mitochondrial *cox*2-*cox*3 spacer sequences have uncovered four intraspecific lineages (Andreakis *et al.* 2007; Sherwood 2008) and a fifth and a sixth lineages were recently identified from the southern Pacific (Dijoux *et al.* 2014; Andreakis *et al.* 2016). Of the six lineages, 'Lineage 2' (L2) has the widest distribution in the Indo-Pacific region and the Mediterranean Sea.

A common *cox*2-*cox*3 spacer haplotype in L2 has been observed in the Indo-Pacific (Hawaii, Japan, Lord Howe Islands, Réunion, South Korea, Taiwan, and Vietnam), the Mediterranean Sea, the NE Atlantic (Azores), and South Africa (Andreakis *et al.* 2007, 2016; Bolton *et al.* 2011; Dijoux *et al.* 2014). Along with the sharing of this common haplotype in the Mediterranean Sea, *A. taxiformis* has been reported from the western and central regions since the early 1990s (Ballesteros & Rodríguez-Prieto 1996; Barone *et al.* 2003; Altamirano *et al.* 2008; Tsiamis *et al.* 2010). It was proposed that this was evidence for a recent introduction from the Indo-Pacific (i.e., nonindigenous origin) and then westward range expansion (Andreakis *et al.* 2007). On the other hand, high levels of *cox*2-*cox*3 spacer sequence variation were recorded in the central Mediterranean, particularly Italy, and it was hypothesized that this indicated multiple introductions in this area (Andreakis *et al.* 2007, 2009). As yet the sources of this 'putative' introduction have not been determined. The South African population of L2 is also considered to be nonindigenous (Bolton *et al.* 2011).

Asparagopsis taxiformis is distributed from subtropical to warm temperate waters in southern Japan (Yoshizaki 1993; Yoshida 1998) and is generally recognized as one of the most common red algal species of the subtropical algal flora (e.g., Terada & Suzuki 2011). Earlier studies have showed that:

*To whom correspondence should be addressed.
Email: akuriha@agr.kyushu-u.ac.jp
Communicating editor: Giuseppe C. Zuccarello
Received 21 November 2014; accepted 17 March 2016.

© 2016 Japanese Society of Phycology

(i) the Japanese population belonged to L2 but only three samples (from Hiroshima, Okinawa and Shimane Prefectures) were analyzed, and (ii) the three samples listed above shared a single haplotype (Andreakis *et al.* 2007; Dijoux *et al.* 2014). This lack of variation suggests that *A. taxiformis* is nonindigenous to Japan, although this alga has not ever been recognized as an invasive taxon to Japan. Due to the limited number of samples in earlier studies, this question cannot be answered. Good coverage of distributional ranges in sampling is important for reliable inference of source populations of nonindigenous species (Muirhead *et al.* 2008).

In earlier phylogenetic studies of *Asparagopsis*, a partial mitochondria-encoded *cox*1 gene and the *cox*2-*cox*3 spacer region, the plastid-encoded RUBISCO spacer region, and a partial nuclear LSU rDNA region have been used, but the latter two markers have lower intraspecific sequence variations (Andreakis *et al.* 2004; Robba *et al.* 2006; Sherwood *et al.* 2010). The low resolution of the genetic marker used for the analyses might have resulted in spurious apparent sharing of the common *cox*2-*cox*3 spacer haplotype among various locations. In this study, we examined Japanese *A. taxiformis* populations and performed molecular phylogenetic and phylogeographic analyses to reconstruct genetic structures among populations of L2, based on a combined dataset of the *cox*2-*cox*3 spacer and *cox*1 sequences.

MATERIALS AND METHODS

Sample collection, DNA extraction, PCR and sequencing

We collected 76 individuals of *A. taxiformis* from the Indo-Pacific, Caribbean Sea and Mediterranean Sea, 60 of which were collected from 16 localities that cover a wide area of

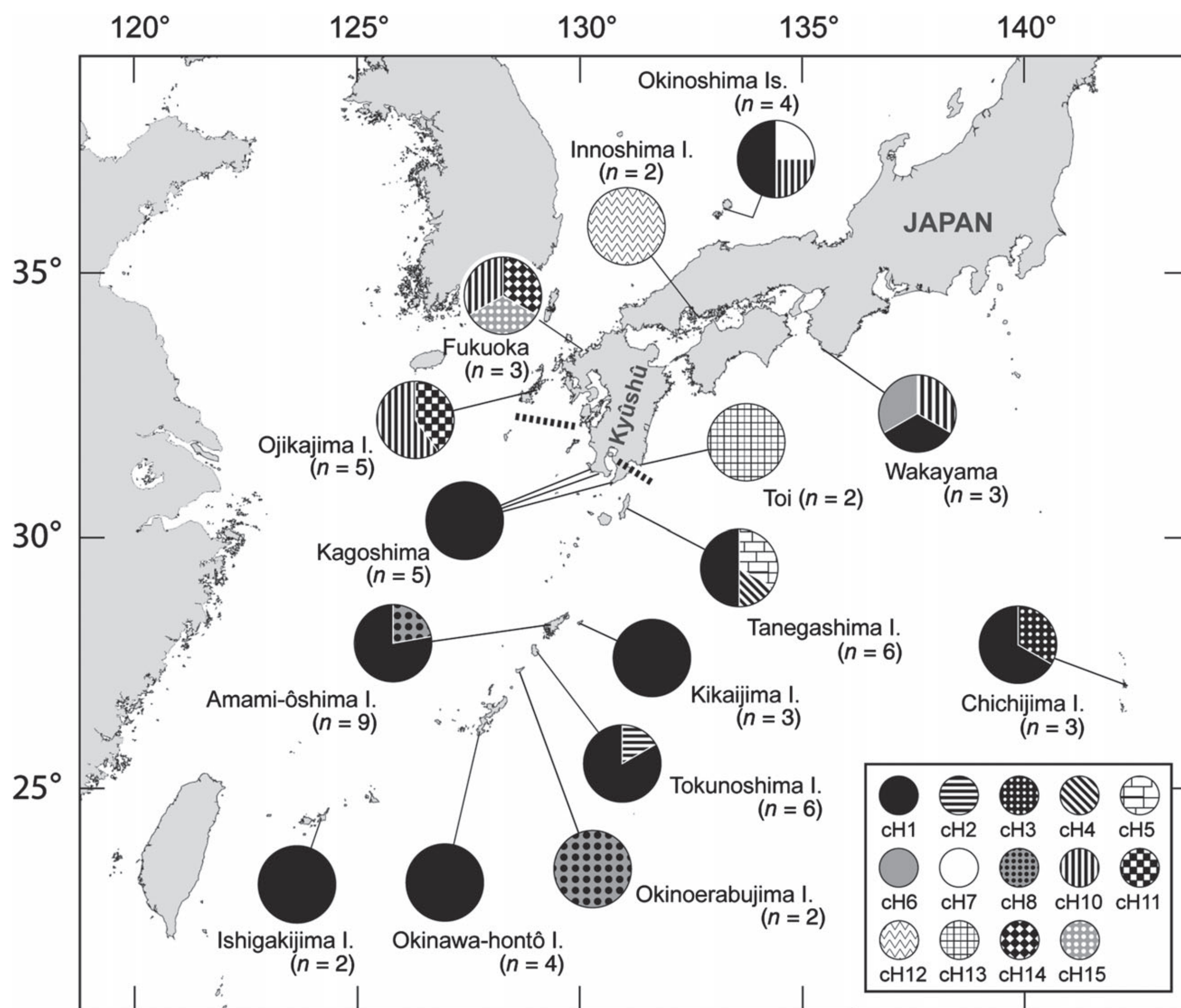

Fig. 1. Geographic distribution of combined haplotypes (*cox*2-*cox*3 spacer + *cox*1) of Japanese *Asparagopsis taxiformis*. Different combined haplotypes are depicted by different patterns. n = Number of samples examined. Haplotype keys in bottom-right corner. Three locations in Kagoshima Prefecture (Marukihama, Banshobana, and Tajiri) are merged with Kagoshima. Dashed lines represent a hypothetical boundary between the northern and southern subpopulations within the Japanese population.

© 2016 Japanese Society of Phycology

southern Japan (Fig. 1, Appendix S1 in the Supporting Information). Fresh collections were desiccated in silica gel and were deposited in the Kobe University Research Center for Inland Seas (Kobe, Japan). Unialgal culture strains were established from vegetative tissues of the *Falkenbergia*-stage (i.e., tetrasporophytic) thalli collected from Ishigakijima Island (Japan) and Chuuk (Federated States of Micronesia). These culture strains were deposited in the Kobe University Macroalgal Culture Collection (KU-MACC, Kobe, Japan). Genomic DNA was extracted from silica-dried materials, culture strains or herbarium specimens using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. The DNA extraction protocol for herbarium materials were modified as such: ground material was incubated in the lysis buffer overnight at 40°C, then re-incubated for another 15 min at 60°C the next day before continuing kit protocol. The targeted regions, the *cox*2-*cox*3 spacer and partial *cox*1 region, were amplified using primer sets shown in Zuccarello *et al.* (1999) and Saunders (2005). When we failed to amplify the regions with the primer sets, we tried a nested PCR method with the following primer sets: cox2-for3 (5′-CTAGARGTRGATAATCGSATG-3′) and cox3-rev3 (5′-GATGACCTTCAAAAGTWGATT-3′) (first round) – and cox2-for3 and cox3-rev4 (5′-GARGCTACAAAAGGTCAAGG-3′) (second round) for *cox*2-*cox*3 spacer; GazF1 (Saunders 2005) and cox1-R1 (5′-TAAHCCTARAAAATGCATDGG-3′) (first round) – and GazF1 and GazR1 (Saunders 2005; second round) or GazF1 and GazR1 (first round) – GazF2 (Saunders 2005) and GazR1 for *cox*1. The first round of PCR was performed with an initial denaturation of 95°C for 2 min, followed by 25 cycles of 95°C for 30 s/50°C for 1 min/72°C for 30 s, with a final 5-min extension at 72°C. A 1:100 dilution of the first round PCR products were used for the second round. The second round PCR was performed with an initial denaturation of 95°C for 2 min, followed by 35 cycles of 95°C for 30 s/50°C for 30 s/72°C for 30 s with a final 5-min extension at 72°C. PCR reaction mixture (total 10 μL) contained 1X Ex Taq Buffer, 0.25U of TaKaRa Ex Taq (Takara Bio Inc., Shiga, Japan), 0.2 mM each of dNTP, 0.4 μM of each primer, and genomic DNA. The PCR amplification was performed in a TaKaRa PCR Thermal Cycler Dice (Takara Bio Inc., Shiga, Japan). Amplified PCR products were checked by 1.5% agarose gel electrophoresis and were precipitated with a 13% polyethylene glycol (#6000, Nakalai Tesque, Kyoto, Japan)/ 1.6 M NaCl solution to remove unincorporated primers and dNTPs (John 1980). Direct sequencing was performed on a Beckman Coulter CEQ 8000 Series Genetic Analysis System (Beckman Coulter Inc., Fullerton, CA, USA). Sequence fragments were edited and assembled using MEGA5 (Tamura *et al.* 2011) and Geneious v.5.2.2 (Kearse *et al.* 2012). Newly generated *cox*2-*cox*3 spacer and *cox*1 sequences in this study were deposited under DDBJ accession numbers AB774444-AB774454, AB774209-AB774223, and LC075724-LC075726 (Appendix S1 in the Supporting Information).

Data analysis

When choosing data from GenBank, we chose only samples of which both *cox*2-*cox*3 spacer and *cox*1 sequences had been determined. Consequently, 12 sets of the sequences (Appendix S2 in the Supporting Information) were added into our newly generated sequences. The dataset was aligned with ClustalW (Thompson *et al.* 1994), implemented in MEGA5. All identical sequences were removed from the datasets prior to phylogenetic analyses. Homogeneity of base frequencies across taxa for each dataset was tested by a χ^2 test, using Phylogears2 ver. 2013.10.22 (Tanabe 2013).

Because the *cox*2-*cox*3 spacer region contained 143 bp of the 3′ end of the *cox*2 gene and 196 bp of the intergenic spacer, the best-fit substitution model for the combined dataset was selected using Kakusan4 ver. 2015.01.23 (Tanabe 2011) for each codon position (*cox*2 and *cox*1) and the intergenic spacer, based on the corrected Akaike's information criterion (AICc) (Sugiura 1978) and the Bayesian information criterion (BIC) (Schwarz 1978). The nucleotide substitution models selected by Kakusan4 were summarized in Appendix S3 in the Supporting Information.

Molecular phylogenetic trees were constructed using the maximum likelihood (ML) and the Bayesian inference (BI) methods. The ML analysis was performed with 1000 iterations of the likelihood-ratchet method (Vos 2003), implemented in Phylogears2 and Treefinder (Jobb *et al.* 2004). Nodal support values were calculated using the bootstrap method in ML, with 1000 replicates for each dataset. The BI analysis was performed with the selected model, using MrBayes5d ver. 3.1.2.2012.12.13 (Tanabe 2012), a modified version of MrBayes 3.1 (Ronquist & Huelsenbeck 2003). The analysis was carried out by two simultaneous runs, each of which consisted of running four simultaneous chains for 1 million generations and sampling trees every 1000 generations. Bayesian posterior probabilities were calculated after discarding the first 10% of the trees saved during MCMC as 'burn-in,' based on results of the stationary states of log-likelihood analyzed using Tracer 1.6 (Rambaut *et al.* 2014). Additionally, *cox*2-*cox*3 spacer and *cox*1 datasets were separately analyzed in the similar way (Appendix S4 in the Supporting Information).

A preliminary analysis, which included *cox*2-*cox*3 spacer and *cox*1 sequences of *Asparagopsis armata* Harvey as an outgroup (Salcombe, England, KU-d11009), clearly showed monophyly of the entire collection of *A. taxiformis* and high genetic divergence between the two species (not shown). Thus, we analyzed *A. taxiformis* sequences only, applying the midpoint rooting function implemented in MEGA5.

Statistical parsimony analyses were performed using TCS ver. 1.21 (Clement *et al.* 2000), with a 95% confidence level for the *cox*2-*cox*3 spacer, *cox*1, and combined datasets. A gap (insertion/deletion) in the *cox*2-*cox*3 spacer dataset was deleted.

The combined dataset was tested for population differentiation using two different approaches in Arlequin ver. 3.5.1.2 (Excoffier & Lischer 2010). We calculated population pairwise fixation indices, F_{st}, inferring population structure using only haplotype frequencies, and Φ_{st} estimates, incorporating both haplotype frequencies and the number of variable nucleotides observed between haplotypes (Baker *et al.* 2005), and significance was tested with a nonparametric permutation approach with 10,000 permutations. Kimura-2-parameter distances (Kimura 1980) were used to calculate the degree of differentiation between haplotypes in conducting Φ_{st} analysis. An exact

© 2016 Japanese Society of Phycology

test of population differentiation was performed to test the null hypothesis of random distribution of haplotypes, and significance tested using Markov chain with 100,000 steps, 10,000 of which were dememorization steps, at the significance level of 0.05.

RESULTS

A total of 74 sets of a partial *cox*1 (643 bp) and *cox*2-*cox*3 spacer (339 bp) sequences were newly generated in this study, 59 sets of which were derived from *A. taxiformis* collected from Japan. After eliminating identical sequences, the combined dataset consisted of 982 bp and 31 sequences, in which no gaps were included. Homogeneity of base frequencies among taxa was not rejected ($\chi^2_{84} = 2.2530$, $P = 1.00$).

The BI- and ML-inferred trees were mostly congruent, so only the ML tree is shown (Fig. 2a). Five of the six major lineages (L1 to L5) that were proposed in earlier studies (Andreakis *et al.* 2007; Dijoux *et al.* 2014) were recovered. A sample from the Bahamas clustered in L3 while samples from Chuuk, Guam and Phuket were placed into L4.

Based on the combined haplotypes of the *cox*2-*cox*3 spacer and *cox*1, a total of 19 combined haplotypes (hereafter called combined haplotypes: cH1–19) were identified in L2 (Table 1, Appendix S1 in the Supporting Information): 14 combined haplotypes were found from Japan, 12 of which were endemic (cH1–8, cH11–14), while cH10 was found in both Greece and Japan (Wakayama and Ojikajima Island) and cH15 was found in Hawaii and Japan (Fukuoka). Additional endemic combined haplotypes also were detected from the Mediterranean Spain (cH16) and the Hawaiian Islands (cH9, cH17–19) (Fig. 2b). While samples from Greek (cH10) and Spain (cH16) had different combined haplotypes, they shared a *cox*2-*cox*3 spacer haplotype, which we called Haplotype B1 (Appendix S5 in the Supporting Information). The Haplotype B1 has been found from various locations in the Mediterranean Sea, northern Atlantic, and the Indo-Pacific regions.

Within Japanese samples, cH1 was located in the center of the haplotype network and was most frequent. cH1 was

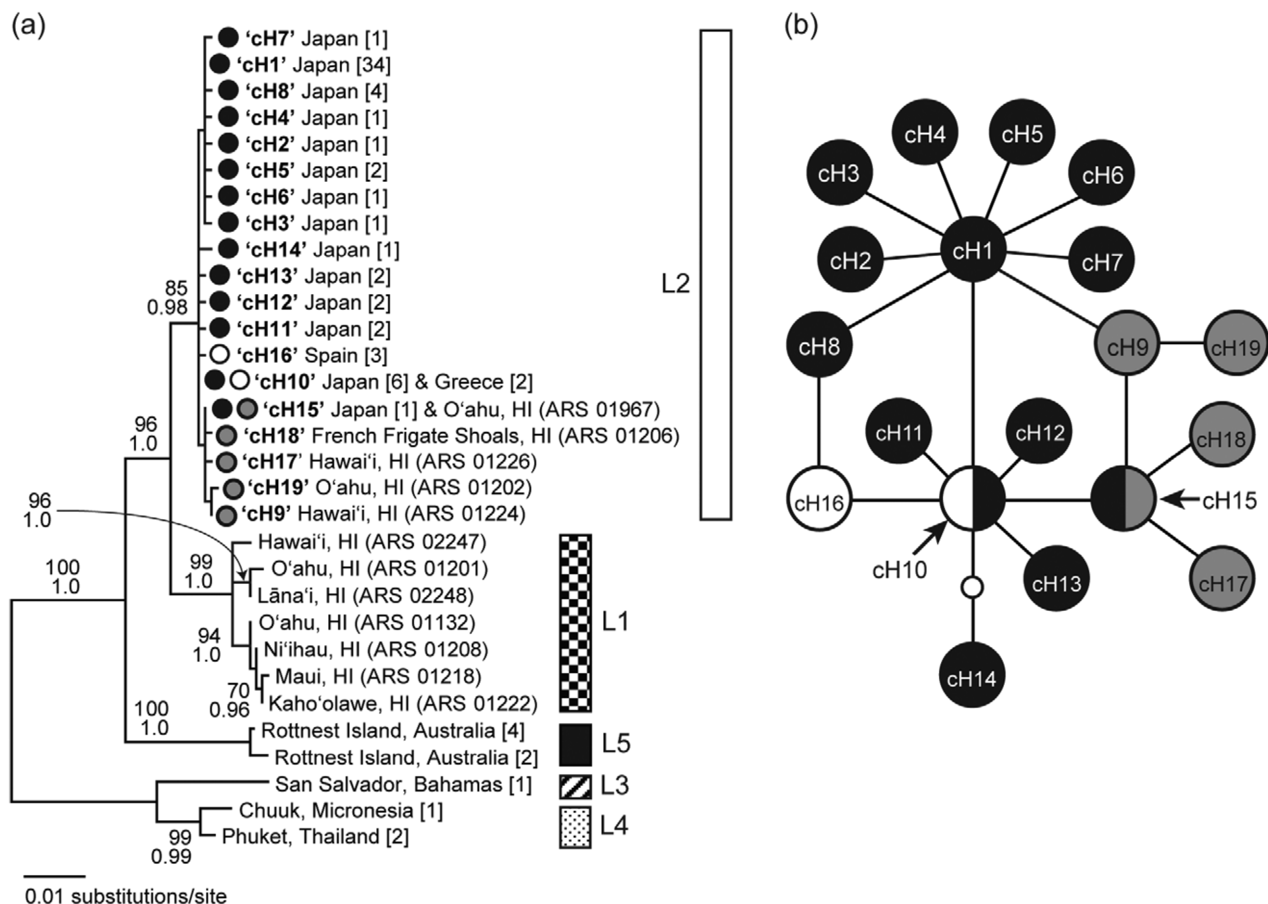

Fig. 2. Phylogeny of *A. taxiformis* inferred from the combined dataset (*cox*2-*cox*3 spacer + *cox*1). (**a**) Mid-point rooted maximum-likelihood (ML) tree. Upper and lower values at nodes represent ML bootstrap percentages (MLBs) and Bayesian posterior probabilities (BPPs). Only values of MLBs >70% and BPP >0.90 are shown. Refer to Appendix S1 in the Supporting Information for the specific codes of combined haplotypes (cH1–19). Numbers in brackets indicate number of individuals identified with specific sequences. GenBank accessions of *cox*2-*cox*3 spacer and *cox*1 sequences for the Hawaiian samples are summarized in Appendix S2 in the Supporting Information. (**b**) A statistical parsimony network for L2. Small open circles represent undiscovered haplotypes. Lines between haplotypes represent one mutational step. Black = Japan samples; Grey = Hawaii samples; White = Mediterranean samples.

© 2016 Japanese Society of Phycology

Table 1. Codes of combined haplotypes based on the *cox*2-*cox*3 spacer and the partial *cox*1 regions, by geographic regions examined

Combined haplotype	Geographic regions	*cox*2-*cox*3 spacer	*cox*1
cH1	Japan	B1	H1
cH2	—	B1	H5
cH3	—	B4	H1
cH4	—	B1	H8
cH5	—	B1	H4
cH6	—	B1	H3
cH7	—	B1	H2
cH8	—	B1	H7
cH11	—	B2	H9
cH12	—	B3	H9
cH13	—	B1	H10
cH14	—	B1	H11
cH10	Japan, Greece	B1	H9
cH15	Japan, Hawaiian Islands	B5	H9
cH16	Mediterranean Spain	B1	H14
cH9	Hawaiian Islands	B5	H1
cH17	—	B5	H13
cH18	—	B5	H15
cH19	—	B5	H6

observed at 9 of the 13 locations and was predominant from Kagoshima south to Ishigakijima Island (Fig. 1). cH10 was also located in the center of the star-shaped network where six combined haplotypes were connected. Geographically, cH1 and its derived combined haplotypes were recorded from Toi north to Okinoshima Islands, while cH10 and its derived combined haplotypes were found from Kagoshima south to Ishigakijima Island.

Population differentiation was found between samples from Japan and the Mediterranean Sea ($F_{st} = 0.3322$, $P < 0.001$; $\Phi_{st} = 0.4312$, $P < 0.001$) and from Japan and the Hawaiian Islands ($F_{st} = 0.2271$, $P < 0.01$; $\Phi_{st} = 0.5049$, $P < 0.001$). Exact tests also supported the above results. On the other hand, no differentiation was found between the Mediterranean Sea and the Hawaiian Islands in the pairwise F_{st} analysis and the exact test based on haplotype frequencies. Assumed two subpopulations (northern *vs.* southern) within Japanese *A. taxiformis* population, its hypothesis was received support at both the nucleotide and the haplotype levels ($F_{st} = 0.3122$, $P < 0.001$; $\Phi_{st} = 0.4244$, $P < 0.001$). The northern subpopulation included Ojikajima Island Fukuoka, Toi, Wakayama, Hiroshima, and Okinoshima Islands, and only cH10–15 were recorded from these subpopulations; whereas only cH1–8 were found in the southern subpopulation (Fig. 1).

DISCUSSION

Molecular phylogenetic analyses using mtDNA markers have refined the relationships among intraspecific lineages of *Asparagopsis taxiformis* worldwide (Andreakis *et al.* 2007; Sherwood 2008; Bolton *et al.* 2011; Dijoux *et al.* 2014). In this study, we report additional localities in the Pacific (Guam, Chuuk, and Phuket: L4) and the Atlantic (Bahamas: L3) (Fig. 2a, Appendices S5 and S6 in the Supporting Information), and that the Japanese population consists only of L2.

This study is the first to analyze intraspecific relationships using a combined dataset of *cox*2-*cox*3 spacer and *cox*1, and clearly shows that the use of combined data increase levels of measured diversity and lead to more resolved phylogenetic relationships (Fig. 2 *vs.* Appendix S5 in the Supporting Information). In addition, when comparing the number of haplotypes or combined haplotypes recovered with the three different datasets, the number is highest for the combined data and is the lowest for the *cox*2-*cox*3 spacer (Appendix S7 in the Supporting Information). Sequence variation in the *cox*1 region is likely contributed to this. For example, samples with *cox*2-*cox*3 spacer haplotypes B1 and B5 are subdivided into 11 and 5 *cox*1 haplotypes, respectively (Table 1). This also indicates that sharing Haplotype B1 is not strong evidence to support recent introductions, although this haplotype is widely distributed throughout the range of L2.

Future research characterizing combined sequence analysis and the fine-scale phylogeographic diversity of *A. taxiformis* over the extent of its range (e.g., Dijoux *et al.* 2014) would help to specify candidates of populations that were introduced into new habitats. The founder of the Mediterranean L2 populations was reported to come from the Indo-Pacific region (Andreakis *et al.* 2007), but its specific source has not yet been identified. Better sampling in the Indo-Pacific and in the Mediterranean Sea, especially around Italy, with combined data may answer the source of the introduction.

The first records of *A. taxiformis* distribution in Japan date back to the early 1900s. The oldest literature record (as *A.* '*sanfordiana*') is in Okamura (1902), in which the locality 'Ogasawarajima' (= Islands of Ogasawara-shotô) is given, and herbarium specimens collected from the Kyushu area (such as Nagasaki, Ôita, Kagoshima, and Okinawa Prefectures) in 1906–1909 are deposited in the Herbarium of the Faculty of Science, Hokkaido University (reference specimens: SAP 076652, 076655, 076657, 076660, 076669–076671). Unfortunately, we were unable to determine the mtDNA haplotypes of these specimens. On the other hand, our analyses have revealed not only genetic differentiation between the samples from Japan and the Mediterranean Sea/Hawaiian Islands, but also the fact that the Japanese populations only consist of L2. The number of samples from the Mediterranean Sea and the Hawaiian Islands is limited, and additional analyses would be required to draw more precise conclusion; nevertheless our discovery would indicate that Japanese L2 is the native lineage in Japan, not likely the origin of a recently established founder population.

Our data also suggested differentiation within Japan (southern (subtropical type) *vs.* northern (temperate type)). The potential genetic disjunction appears to separate two populations: one between Toi and Kagoshima and the other between Ojikajima Island and Kagoshima in the Kyushu area (Fig. 1). A major phylogeographic break is well known in this area (e.g., Kojima *et al.* 1997; Akihito *et al.* 2008; Hirase *et al.* 2012a,b; Hirase & Ikeda 2014), including a brown alga *Sargassum horneri* (Turner) C. Agardh (Uwai *et al.* 2009).

A northward range expansion of subtropical coral species has been reported (Nojima 2008; Nojima & Okamoto 2008; Sugihara *et al.* 2009), and this rapid expansion is correlated

© 2016 Japanese Society of Phycology

with rising sea surface temperatures (Yamano *et al.* 2011). Hence, in addition to fine-scale phylogeographic studies of coastal benthic organisms in the southern Sea of Japan through western to southern Kyushu area, physiological studies for the effects of rising sea temperatures could help predicting their future distributions in response to global climate change.

ACKNOWLEDGEMENTS

We are grateful to Dr. Eric Henry and Mrs. Kimberly Y. Conklin for their valuable comments on the manuscript, Dr. Noritaka Hirohashi and Mr. Masanori Nishizaki (Shimane University, Japan), Dr. Maria Altamirano and Dr. Antonio Flores-Moya (University of Malaga, Spain), Dr. John Huisman (Murdock University, Australia), and Dr. Heung-Sik Park (Korean South Pacific Ocean Research Center, Korea) for their help in collecting specimens; and Dr. Mitsunobu Kamiya, Dr. Kazuhiro Kogame, and Dr. Taiju Kitayama for loans of specimens. A part of this study was supported by the Strategic International Cooperative Program (2009–2012) by JST to H. K, and a TOTAL Foundation research grant for ‘Brown algal diversity and ecology in the Eastern Mediterranean Sea’ led by Christos Katsaros (University of Athens, Greece).

REFERENCES

Akihito, Fumihito, A., Ikeda, Y. *et al.* 2008. Evolution of Pacific Ocean and the Sea of Japan populations of the gobiid species, *Pterogobius elapoides* and *Pterogobius zonoleucus*, based on molecular and morphological analyses. *Gene* **427**: 7–18.

Altamirano, M., Munoz, A. R., de la Rosa, J. *et al.* 2008. The invasive species *Asparagopsis taxiformis* (Bonnemaisoniales, Rhodophyta) on Andalusian coasts (Southern Spain): reproductive stages, new records and invaded communities. *Acta Bot. Malacit.* **33**: 5–15.

Andreakis, N., Costello, P., Zonolla, M., Saunders, G. W. and Mata, L. 2016. Endemic or introduced? Phylogeography of *Asparagopsis* (Florideophyceae) in Australia reveals multiple introductions and a new mitochondrial lineage. *J. Phycol.* **52**: 141–7.

Andreakis, N., Kooistra, W. H. C. F. and Procaccini, G. 2009. High genetic diversity and connectivity in the polyploid invasive seaweed *Asparagopsis taxiformis* (Bonnemaisoniales) in the Mediterranean, explored with microsatellite alleles and multilocus genotypes. *Mol. Ecol.* **18**: 216–26.

Andreakis, N., Procaccini, G., Maggs, C. and Kooistra, W. H. C. F. 2007. Phylogeography of the invasive seaweed *Asparagopsis* (Bonnemaisoniales, Rhodophyta) reveals cryptic diversity. *Mol. Ecol.* **16**: 2285–99.

Andreakis, N., Procaccini, G., and Kooistra, W. H. C. F. 2004. *Asparagopsis taxiformis* and *Asparagopsis armata* (Bonnemaisoniales, Rhodophyta): genetic and morphological identification of Mediterranean populations. *Eur. J. Phycol.* **39**: 273–83.

Andreakis, N. and Schaffelke, B. 2012. Invasive marine seaweeds: pest or prize? *In* Wiencke, C. and Bischof, K. (Eds.) *Seaweed Biology: Novel Insights into Ecophysiology, Ecology and Utilization*. Ecological Studies, *Vol. 219*. Springer-Verlag, Berlin, pp. 235–62.

Baker, A. R., Loughlin, T. R., Burkanov, V. *et al.* 2005. Variation of mitochondrial control region sequences of Steller sea lions: the three-stock hypothesis. *J. Mammal.* **86**: 1075–84.

Ballesteros, E. and Rodríguez-Prieto, C. 1996. Presència d'*Asparagopsis taxiformis* (Delile) Trevisan a Balears. *Boll. Soc. Hist. Nat. Balears* **39**: 135–8.

Barone, R., Mannino, A. M. and Marino, M. 2003. *Asparagopsis taxiformis* (Bonnemaisoniales, Rhodophyta): first record of gametophytes on the Italian coast. *Bocconea* **16**: 1021–5.

Bolton, J. J., Andreakis, N. and Anderson, R. J. 2011. Molecular evidence for three separate cryptic introductions of the red seaweed *Asparagopsis* (Bonnnemaisoniales, Rhodophyta) in South Africa. *Afr. J. Mar. Sci.* **33**: 263–71.

Boudouresque, C.-F. and Verlaque, M. 2010. Is global warming involved in the success of seaweed introduction in the Mediterranean Sea? *In* Israel, A., Einav, R. and Seckbach, J. (Eds.) *Seaweeds and their Role in Globally Changing Environments*. Springer, London, pp. 33–50.

Clement, M., Posada, D. and Crandall, K. A. 2000. TCS: a computer program to estimate gene genealogies. *Mol. Ecol.* **9**: 1657–60.

Conklin, K. Y., Kurihara, A. and Sherwood, A. R. 2009. A molecular method for identification of the morphologically plastic invasive algal genera *Eucheuma* and *Kappaphycus* (Rhodophyta, Gigartinles) in Hawaii. *J. Appl. Phycol.* **21**: 691–9.

Dijoux, L., Viard, F. and Payri, C. 2014. The more we search, the more we find: discovery of a new lineage and a new species complex in the genus *Asparagopsis*. *PLoS One* **9**: e103826.

Excoffier, L. and Lischer, H. E. L. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Mol. Ecol. Resour.* **10**: 564–7.

Glenn, E. P. and Doty, M. S. 1981. Phytosynthesis and respiration of the tropical red seaweeds, *Eucheuma striatum* (Tambalang and Elkhorn varieties) and *E. denticulatum*. *Aquat. Bot.* **10**: 353–64.

Hirase, S. and Ikeda, M. 2014. Divergence of mitochondrial DNA lineage of the rocky intertidal goby *Chaenogobius gulosus* around the Japanese Archipelago: reference to multiple Pleistocene isolation events in the Sea of Japan. *Mar. Biol.* **161**: 565–74.

Hirase, S., Ikeda, M., Kanno, M. and Kijima, A. 2012a. Detection of regional allozyme divergence in the rocky intertidal goby *Chaenogobius annularis*. *Ichtyol. Res.* **59**: 264–7.

Hirase, S., Ikeda, M., Kanno, M. and Kijima, A. 2012b. Phylogeography of the intertidal goby *Chaenogobius annularis* associated with paleoenvironmental changes around the Japanese Archipelago. *Mar. Ecol. Prog. Ser.* **450**: 167–79.

Jobb, G., von Haeseler, A. and Strimmer, K. 2004. TREEFINDER: a powerful graphical analysis environment for molecular phylogenetics. *BMC Evol. Biol.* **4**: 18.

John, T. L. 1980. Fractionation of DNA fragments by polyethyleneglycol induced precipitation. *Methods Enzymol.* **65**: 347–53.

Kearse, M., Moir, R., Wilson, A. *et al.* 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **28**: 1647–9.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**: 111–20.

Kogishi, K., Kitayama, T., Miller, K. A., Hanyuda, T. and Kawai, H. 2010. Phylogeography of *Cutleria cylindrica* (Cutleriales, Phaeophyceae) in northeastern Asia, and the identity of an introduced population in California. *J. Phycol.* **46**: 553–8.

Kojima, S., Segawa, R. and Hayashi, I. 1997. Genetic differentiation among populations of the Japanese shell *Turbo* (*Batillus*) *cornutus* corresponding to warm currents. *Mar. Ecol. Prog. Ser.* **150**: 149–55.

Miller, K. A., Engle, J. M., Uwai, S. and Kawai, H. 2007. First report of the Asian seaweed *Sargassum filicinum* Harvey (Fucales) in California, USA. *Biol. Invasions* **9**: 609–13.

Muirhead, J. R., Gray, D. K., Kelly, D. W., Ellis, S. M., Heath, D. D. and Macisaac, H. J. 2008. Identifying the source of species invasions: sampling intensity vs. genetic diversity. *Mol. Ecol.* **17**: 1020–35.

Nojima, S. 2008. Coral communities in Amakusa, western Kyushu. *Midoriishi* **15**: 5–11 (in Japanese).

Nojima, S. and Okamoto, M. 2008. Enlargement of habitats of scleractinian corals to north and coral bleaching events. *Nippon Suisan Gakkaishi* **74**: 884–8 (in Japanese with English abstract).

© 2016 Japanese Society of Phycology

Okamura, K. 1902. *Nippon Sôrui-meii*. Keigyosha, Tokyo (in Japanese).

Olsen, J. N., Valero, M., Meusnier, I., Boele-Bos, S. and Stam, W. T. 1998. Mediterranean *Caulerpa taxifolia* and *C. mexicana* (Chlorophyta) are not conspecific. *J. Phycol.* **34**: 850–6.

Provan, J., Booth, D., Todd, N. P., Beatty, G. E. and Maggs, C. A. 2008. Tracking biological invasions in space and time: elucidating the invasive history of the green alga *Codium fragile* using old DNA. *Divers. Distrib.* **14**: 343–54.

Rambaut, A., Suchard, M. A., Xie, D. and Drummond, A. J. 2014. Tracer v1.6. Available from URL: http://beast.bio.ed.ac.uk/Tracer/ (accessed: 7 Feb. 2015)

Robba, L., Russell, S. J., Barker, G. L. and Brodie, J. 2006. Assessing the use of the mitochondrial *cox*1 marker for use in DNA barcoding of red algae (Rhodophyta). *Am. J. Bot.* **93**: 1101–8.

Ronquist, F. and Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–4.

Saunders, G. W. 2005. Applying DNA barcoding to red macroalgae: a preliminary appraisal holds promise for future applications. *Philos. Trans. R. Soc. B* **360**: 1879–88.

Schaffelke, B. and Hewitt, C. L. 2007. Impacts of introduced seaweeds. *Bot. Mar.* **50**: 397–417.

Schwarz, G. 1978. Estimating the dimension of a model. *Ann. Stat.* **6**: 461–4.

Sherwood, A. R. 2008. Phylogeography of *Asparagopsis taxiformis* (Bonnemaisoniales, Rhodophyta) in the Hawaiian Islands: two mtDNA markers support three separate introductions. *Phycologia* **47**: 79–88.

Sherwood, A. R., Sauvage, T., Kurihara, A., Conklin, K. Y. and Presting, G. G. 2010. A comparative analysis of COI, LSU and UPA marker data for the Hawaiian florideophyte Rhodophyta: implications for DNA barcoding of red algae. *Cryptogamie Algol.* **31**: 451–65.

Smith, J. E., Hunter, C. L. and Smith, C. M. 2002. Distribution and reproduction characteristics of nonindigenous and invasive marine algae in the Hawaiian Islands. *Pac. Sci.* **56**: 299–315.

Sugihara, K., Sonoda, N., Imafuku, T., Nagata, S., Ibusuki, T. and Yamano, H. 2009. Latitudinal changes in hermatypic coral communities from west Kyushu and Oki Islands in Japan. *J. Jpn. Coral Reef Soc.* **11**: 51–67 (in Japanese with English abstract).

Sugiura, N. 1978. Further analysis of the data by Akaike's information criterion and the finite corrections. *Commun. Stat. Theory* **A7**: 13–26.

Sun, Z., Hanyuda, T., Lim, P.-E., Tanaka, J., Gurgel, C. F. D. and Kawai, H. 2012. Taxonomic revision of the genus *Lobophora* (Dictyotales, Phaeophyceae) based on morphological evidence and analyses *rbc*L and *cox*3 gene sequences. *Phycologia* **51**: 500–12.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**: 2731–9.

Tanabe, A. S. 2011. Kakusan4 and Aminosan: two programs for comparing nonpartitioned, proportional, and separate models for combined molecular phylogenetic analyses of multilocus sequence data. *Mol. Ecol. Resour.* **11**: 914–21.

Tanabe, A. S. 2012. MrBayes5d Version 3.1.2.2012.12.13. Available from: http://www.fifthdimension.jp/ (accessed: 7 Feb. 2015)

Tanabe, A. S. 2013. Phylogears Version 2.0.2013.1022, Available from: http://www.fifthdimension.jp/ (accessed: 7 Feb. 2015)

Terada, R. and Suzuki, T. 2011. Marine algal flora and its community structure of Kuroshima Island, Mishima Village, Kagoshima, Japan. *Kagoshima Univ. Res. Center Pacific Island, Occas. Pap.* **51**: 6–15 (in Japanese with English abstract).

Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids. Res.* **22**: 4673–80.

Tsiamis, K., Montesanto, B., Panayotidis, P., Katsaros, C. and Verlaque, M. 2010. Updated records and range expansion of alien marine macrophytes in Greece (2009). *Mediterr. Mar. Sci.* **11**: 61–79.

Uwai, S., Kogame, K., Yoshida, G., Kawai, H. and Ajisaka, T. 2009. Geographical genetic structure and phylogeography of the *Sargassum horneri*/*filicinum* complex in Japan, based on the mitochondrial *cox*3 haplotype. *Mar. Biol.* **156**: 901–11.

Verlaque, M., Durand, C., Huisman, J. M., Boudouresque, C.-F. and Le Parco, Y. 2003. On the identity and origin of the Mediterranean invasive *Caulerpa racemosa* (Caulerpales, Chlorophyta). *Eur. J. Phycol.* **38**: 325–9.

Voisin, M., Engle, C. R. and Viard, F. 2005. Differential shuffling of native genetic diversity across introduced regions in a brown alga: Aquaculture vs. maritime traffic effects. *Proc. Natl. Acad. Sci. U.S.A.* **102**: 5432–7.

Vos, R. A. 2003. Accelerated likelihood surface exploration: the likelihood ratchet. *Syst. Biol.* **52**: 368–73.

Yamano, H., Sugihara, K. and Nomura, K. 2011. Rapid poleward range expansion of tropical reef corals in response to rising sea temperatures. *Geophys. Res. Lett.* **38**: L04601.

Yoshida, T. 1998. *Marine Algae of Japan*. Uchida-Rokakuho, Tokyo (in Japanese).

Yoshizaki, M. 1993. *Asparagopsis taxiformis* (Delile) Trevisan. *In* Hori, T. (Ed.) *An Illustrated Atlas of the Life History of Algae*. Brown and Red Algae, *Vol. 3*. Uchida Rokakuho Publishing, Tokyo, pp. 236–7 (in Japanese).

Zuccarello, G. C., Burger, G., West, J. A. and King, R. J. 1999. A mitochondrial marker for red algal intraspecific relationships. *Mol. Ecol.* **8**: 1443–7.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Collection information of *Asparagopsis taxiformis* specimens analyzed in this study.
Appendix S2. List of specimen codes used in the combined sequence analysis. GenBank accession numbers of *cox*2-*cox*3 spacer (left) and *cox*1 (right) for each code are in parentheses (see Fig. 1).
Appendix S3. Nucleotide substitution models selected by Kakusan4.
Appendix S4. List of GenBank accession numbers that included in the *cox*2-*cox*3 spacer and cox1 datasets.
Appendix S5. Phylogeny of the *Asparagopsis taxiformis* samples inferred from the *cox*2-*cox*3 spacer dataset.
Appendix S6. Phylogenetic relationships among/within lineages inferred from the *cox*1 dataset of *Asparagopsis taxiformis*.
Appendix S7. Number of haplotypes (or combined haplotypes) found from the samples where both *cox*2-*cox*3 spacer and cox1 sequences have been identified. n = Number of individuals examined.

© 2016 Japanese Society of Phycology