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# Phylogeographic analysis of the brown alga *Cutleria multifida* (Tilopteridales, Phaeophyceae) suggests a complicated introduction history

**Hiroshi Kawai,[1]* Keita Kogishi,[1] Takeaki Hanyuda,[1] Shogo Arai,[2] C. Frederico Gurgel,[3] Wendy Nelson,[4] Alexandre Meinesz,[5] Kostas Tsiamis[7] and Akira F. Peters[6]**

[1]Kobe University Research Center for Inland Seas, Kobe, Hyogo, [2]Marine Algal Research, Minatozaka, Kasuya, Fukuoka, Japan, [3]Departamento de Botânica, CCB, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil, [4]National Center for Aquatic Biodiversity and Biosecurity, NIWA, Wellington, New Zealand, [5]Faculté des Sciences, Université de Nice, Nice, [6]Bezhin Rosko, Santec, France, and [7]Hellenic Centre for Marine Research, University of Athens, Anavissos, Greece

## SUMMARY

In depth genetic comparisons of populations of *Cutleria multifida* (Tilopteridales, Phaeophyceae) collected from Europe, the northwestern Pacific Ocean, Australia and New Zealand using the DNA sequences of four gene regions (the mitochondrial *cox*2 and *cox*3 genes, the intergeneric spacer region adjacent to *cox*3, and the open reading frame) suggested that the northwestern European and Japanese populations were considerably greater in terms of their genetic divergence than Mediterranean, Australian or New Zealand populations. The haplotypes of the populations in northwestern European (distribution range including the type locality, seven haplotypes) and Japanese populations (seven haplotypes) were unique except for one shared haplotype. There were weak but positive correlations between the geographical distance and the genetic divergence among northwestern European and Japanese populations. Moreover, both female and male gametophytes occurred in eight of the nine Japanese localities, suggesting Japanese populations showed normal sexual heteromorphic life history of the species. In light of these results, it appears that Japanese populations were native to the area despite earlier hypothesis. In contrast, Australian and New Zealand populations were composed of only one haplotype that is very close to those found in northwestern Europe and Japan, suggesting a recent introduction history from Europe (or from northeastern Asia via Europe) by ship transport to Australia and New Zealand. The Mediterranean populations included two haplotypes identical to those found in northwestern Europe and Japan, and it is suggestive of transoceanic introductions of some populations between Mediterranean and Japanese coasts.

Key words: *cox*2, *cox*3, introduction, marine macroalgae, molecular phylogeny.

## INTRODUCTION

Due to the globalization of shipping, fishery and aquaculture activities, increasing transoceanic introductions of marine organisms have become universal threats to coastal ecosystems. Introductions of marine organisms represent a serious problem, with about 10 000 species being associated with ship transport (ballast water and ship-hull communities) around the globe (Carlton 1999; Bax *et al.* 2001). Some macroalgae [e.g. *Codium fragile* subsp. *tomentosoides* (van Goor) P.C.Silva, *Undaria pinnatifida* (Harvey) Sugingar, *Caulerpa taxifolia* (M.Vahl) C.Agardh] are believed to be introduced or secondarily spread by means of ship transport, and have caused serious impacts to coastal ecosystems (Jousson *et al.* 2000; Uwai *et al.* 2006; Miller *et al.* 2007; Provan *et al.* 2008).

*Cutleria multifida* (Turner) Greville (Tilopteridales, Phaeophyceae) is an annual brown alga, generally growing in subtidal habitat of sheltered areas such as bays and inlets, and is frequently found in harbors. *Cutleria multifida* was first described from the British Isles (Turner 1801; Greville 1830). Since then, the species has been recorded from a wide range of temperate and subtropical regions in Europe (Newton 1931; Ribera *et al.* 1992; Bárbara & Cremades 1996; Bartsch & Kuhlenkamp 2000; Bárbara *et al.* 2005; Serio *et al.* 2006), Atlantic Islands (Price *et al.* 1978; Audiffred & Weisscher 1984; Neto *et al.* 2001; Haroun *et al.* 2002; John *et al.* 2004), Chile (Santelices *et al.* 1989), Argentina (Piriz *et al.* 2003), Africa (Price *et al.* 1978; Ben Maïz *et al.* 1987; Ribera *et al.* 1992), Australia (Womersley 1987; Sanderson 1997; Hewitt *et al.* 2004; Kraft 2009), New Zealand (Adams 1983, 1994; Nelson 1999), Pacific Islands (Samoa: Skelton & South 1999) and Japan (Migita & Ichiki 1962; Yoshida 1998; Yoshida *et al.* 2005).

Because of the distribution patterns of the species in the vicinity of international ports in the Southern Hemisphere like Wellington Port, New Zealand and Coquimbo, Chile, as well as the dominance of female gametophytes in those populations, they were considered to be the parthenogenetic descendants of individuals transported from their native range by shipping (Adams 1983, 1994; Santelices *et al.* 1989; for details see below). Similarly, the occurrence of *C. multifida* in Japan was noticed for the first time in 1957 near the port area of

*To whom correspondence should be addressed.
E-mail: kawai@kobe-u.ac.jp
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Nagasaki, an international trading port, and therefore Migita and Ichiki (1962) considered that the species was non-indigenous in Japan, and speculated that those populations originated from introductions by shipping. Since then, the species has been reported from diverse localities in Japan, and were regarded as spread from the primary introduction sites (Kitayama 1993; Tanaka 2002). In contrast, in Australia the species has not been particularly recognized as a non-indigenous species (Womersley 1987), perhaps because the distributional range of the species within Australia is rather broad (from Western Australia to South Australia) and its early collections date back at least to the late 19$^{th}$ Century (e.g. ADU A01577, Torrens Island, Gulf St. Vincent, South Australia dated September 1887).

Nevertheless, because of the scanty nature of the floristic data of macroalgae, especially for subtidal communities, it is often difficult to prove that a species was not present in a locality prior to its first discovery, and to conclude that a particular population originated from recent anthropogenic introductions. In the present study we address just this issue with in-depth phylogeographic analyses with the goal of clarifying native ranges of the species, and examining whether some disjunct populations such as Japanese, Australian and New Zealand populations are introduced populations.

We compared the genetic divergence of worldwide populations using mitochondrial gene sequence data, and estimated the native ranges and the origin and invasion pathway for the putative introduced populations. For elucidating the genetic divergence of *C. multifida* populations, the DNA sequences of four gene regions [the mitochondrial *cox*2 and *cox*3 genes, the intergenic spacer region adjacent to *cox*3, and the open reading frame (ORF)] were used after Kogishi *et al.* (2010) where the genetic divergence of a related species *Cutleria cylindrica* Okamura [ = *Mutimo cylindricus* (Okamura) H.Kawai et T.Kitayama; Kawai *et al.* (2012)] was successfully demonstrated. Furthermore, in order to examine the occurrence of the dominance of parthenogenetic female gametophytes, sex of gametophytes newly collected in Japan were examined.

## MATERIALS AND METHODS

For the genetic comparisons within and among populations, the following specimens of *Cutleria multifida* were collected from Japan (44 individuals/10 localities), Ireland (6/1), UK (9/1), France (27/5), Italy (3/1), Greece (5/1), Spain (1/1), Australia (17/2), New Zealand (3/1), and used for DNA extractions (Table 1). Small fragments of each specimen were quickly dried in silica gel and used for DNA extractions. Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. PCR (polymerase chain reaction) amplifications and sequencing of the mitochondrial *cox*2 and *cox*3 genes, ORF region [homolog of ORF379 (*Fucus vesiculosus* L., AY494079)], and the intergenic region between the *cox*3 gene and ORF region were performed. Primers for PCR amplification and sequencing are listed in Table 2. PCR was carried out with a TaKaRa PCR Thermal Cycler Dice (Takara Bio, Shiga, Japan) using the TaKaRa ExTaq Reaction Kit (Takara Bio) or the KOD FX (ToYoBo, Osaka, Japan). PCR condition followed Kojima *et al.* (2015). Amplicons were PEG purified (Lis 1980), and were sequenced using a CE DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) and a CEQ8000 DNA Analysis System (Beckman Coulter) following the manufacturer's instructions.

**Table 1.** Populations of *Cutleria multifida* examined in the present study. KU-####, culture strain housed in Kobe University Macroalgal Culture Collection (KU-MACC)

| Locality code | Collection information (Locality, collection date, collector) | Haplotyes (No. samples) |
|---|---|---|
| 1 | Carna, Galway, Ireland (13 June 2011, H. Kawai) | H4 (5), H6 (1) |
| 2 | Restronguet Point, Cornwell, Great Britain (17 June 2011, H. Kawai) | H1 (1), H2 (1), H7 (4) |
| 3 | Roscoff, Brittany, France (A. F. Peters, KU-1459/23 July 2010, H. Kawai) | H2 (2), H3 (2) |
| 4 | Le Caro, Radede Bresr, Finistere, France (25 May 2013, A. F. Peters) | H7 (2), H9 (1) |
| 5 | Ría de Arousa, Pontevedra, Spain (5 July 2012, M. Altamirano) | H3 (1) |
| 6 | Cannes, Côte d'Azur, France (16 April 2008, H. Kawai) | H15 (8) |
| 7 | Antibes, Côte d'Azur, France (16 April 2008, H. Kawai) | H7 (1), H15 (7) |
| 8 | Villefranche, Côte d'Azur, France (D. G. Müller, KU-1189/17 April 2008, H. Kawai) | H15 (4) |
| 9 | Naples, Campania, Italy (14 March 2012, 17 March 2012, A. F. Peters) | H7 (2), H15 (1) |
| 10 | Lesbos Island, Greece (5 March 2009, H. Kawai) | H15 (5) |
| 11 | Tsukumo Bay, Ishikawa, Japan (22 January 2006, S. Arai) | H7 (4) |
| 12 | Sekumi, Wakasa, Fukui, Japan (9 June 2005, S. Arai) | H7 (2), H13 (1) |
| 13 | Saigo Port, Oki Island, Shimane, Japan (21 April 2006, S. Arai) | H7 (6), H10 (1) |
| 14 | Ie Islands, Himeji, Hyogo, Japan (1 June 2013, M. Watanabe) | H15 (2) |
| 15 | Omi Island, Nagato, Yamaguchi, Japan (24 May 2006, S. Arai) | H7 (3), H15 (1) |
| 16 | Yamaguchi, Yamaguchi Japan (9 May 2005, S. Arai) | H15 (2) |
| 17 | Yura, Awaji Island, Hyogo, Japan (9 April 2006, S. Arai/12 March 2013, H. Kawai) | H15 (4) |
| 18 | Kitanada, Naruto, Tokushima, Japan (8 April 2006, S. Arai) | H7 (1), H15 (7) |
| 19 | Yanoshima, Fukuyama, Hiroshima, Japan (10 May 2006, S. Arai) | H15 (4) |
| 20 | Onyu Island, Saeki, Oita Prf., Japan (6 June 2005, 15 March 2006, S. Arai) | H7 (3), H11 (1), H12 (1), H14 (1) |
| 21 | Coobowie, SA, Australia (10 October 2009, H. Kawai) | H8 (10) |
| 22 | Aldinga, SA, Australia (8 October 2009, H. Kawai) | H8 (7) |
| 23 | Golden Bay, Stewart Island, New Zealand (November 2002, W. Nelson) | H5 (3) |

**Table 2.** List of primers used for polymerase chain reaction (PCR) and sequencing

| Code | PCR / Sequencing | Forward / Reverse | Sequence (5'-3') | Annealing position |
|---|---|---|---|---|
| cox2-P5† | + / + | F | GAKGAGATAAAAGAAATKTTATC | *cox2* (2359–2381) |
| cox2-Cu1† | – / + | F | GATGACGATTTAGCTATTCC | *cox2* (2812–2831) |
| cox2-Cu2† | – / + | R | TTMGTAGGMACAARAAGACG | *cox2* (2909–2890) |
| cox2-Cu3† | – / + | R | GCCCAWGAATGYARAACATC | *cox2* (2960–2941) |
| cox2-P2† | + / + | R | GAGCATAAYCTTTTWCCAACCC | *cox2* (3152–3131) |
| trnY-P2 | + / – | F | GKCAGATTGTAAATCTGTTGG | *trn*Y (27–47) |
| trnY-P1‡ | + / – | F | TCYATCRTAGGTTCGAATCC | *trn*Y (52–71) |
| cox3-P1‡ | + / – | F | GAYCCWAGTCCMTGGCCWTTAG | *cox3* (49–70) |
| cox3-P5.2† | + / – | F | KCHCCHGTYTTTAATATTGG | *cox3* (340–359) |
| cox3-P6† | – / + | R | CDACAATHGCATGATGAGCCC | *cox3* (478–457) |
| cox3-Cm1 | – / + | F | TGGGCTTTTTTTACGTCTTC | *cox3* (316–335) |
| cox3-Cm2 | – / + | R | TGCCTAAACCCTGCAGAGCC | *cox3* (514–495) |
| cox3-Cm3 | – / + | F | TCGAATATATGAACGCACCC | cox3 (557–576) |
| cox3-P2‡ | + / – | R | ACAAARTGCCAATACCAAGC | *cox3* (755–736) |
| ORF379-P1† | + / – | R | CACAATATTTAACTTTATCG | ORF379 (133–114) |

Annealing positions correspond to the sequences of *Fucus vesiculosus* (*cox2*, *trn*Y, *cox*3, and ORF379, AY494079, Oudot-Le Secq *et al.* 2006).
†Kogishi *et al.* (2010).
‡Ni-Ni-Win *et al.* (2008).

To examine genetic relationships among the haplotypes of *C. multifida*, a statistical parsimony network was created using TCS v.1.21 (Clement *et al.* 2000) based on the DNA sequences of mitochondrial haplotypes with a 95% connection limit. Alignments were prepared using the Clustal X program (Thompson *et al.* 1997) and then manually adjusted by eye. The correlations between geographical distance and the genetic distance between populations (measured based on Phist) were performed with 10 000 randomizations, for the whole populations in Europe and Japan using IBDWS v.3.14 (Jensen *et al.* 2005). The geographic distances used in the analysis were estimated based on both straight line and rough distance along coastline. The calculated genetic distances less than zero were removed, and the remaining values were used to obtain approximation straight line. For the statistical analyses, correlation coefficient *(R)*, coefficient of determination *($R^2$)*, *F* values, and *P*-values were calculated using Microsoft Excel 2011 (Microsoft Corp., Redmond, WA, USA.). Haplotype (Hd) and nucleotide ($\pi$) diversities were measured for each population using DNAsp v.5 (Librado & Rozas 2009). Pairwise $\varphi_{st}$ was estimated using Arlequin v.3.5 (Excoffier & Lischer 2010). Mismatch distribution analyses to explore demographic patterns of populations were performed by DNAsp v.5.

The sex of the newly collected fertile gametophytes in the nine Japanese localities (population code 11–13, 15–20 in Table 1) were determined by examining the morphology of gametangia using a BX50 compound microscope (Olympus, Tokyo, Japan) with Nomarski optics.

## RESULTS

In the DNA sequences of the mitochondrial *cox2* (513 bp) and *cox*3 (646 bp) genes, the intergenic region adjacent to *cox*3 and the ORF region (56 bp), and the intergenic spacer region (12 bp) adjacent to *cox*3 and the ORF region, a total of 15 haplotypes (halploid genotypes) were recognized in the individuals collected from Japan, Galway (Ireland), Cornwall (Great Britain, UK), Roscoff and Le Caro (Brittany, France), Mediterranean France, Italy, Greece, Australia and New Zealand (Tables 1,3, Table S1). Genetic relationships among the haplotypes from a spanning network tree (Fig. 1a) with the distribution of the haplotypes and their ratios in each local population (Fig. 1b–d) are depicted. The Japanese (Fig. 1c) and the northwestern European (Ireland, Cornwall and Brittany, Fig. 1b) populations showed relatively high genetic divergence.

**Table 3.** List of haplotypes revealed from DNA sequences of mitochondrial *cox2*, *cox*3 genes, and the open reading frame (ORF) region adjacent to *cox*3 gene

| Haplotypes | Accession No. (*cox2*/*cox*3 + ORF) |
|---|---|
| H1 | AB683455 / AB683459 |
| H2 | AB683454 / AB683458 |
| H3 | AB540610 / AB540620 |
| H4 | AB683456 / AB683460 |
| H5 | AB540611 / AB540621 |
| H6 | AB683457 / AB683461 |
| H7 | AB540609 / AB540619 |
| H8 | AB540608 / AB540618 |
| H9 | LC074883 / LC074884 |
| H10 | AB540612 / AB540622 |
| H11 | AB540614 / AB540624 |
| H12 | AB540615 / AB540625 |
| H13 | AB540613 / AB540623 |
| H14 | AB540616 / AB540626 |
| H15 | AB540617 / AB540627 |

The populations in northwestern Europe (Galway, Cornwall, Le Caro, and Roscoff) included seven of the 15 haplotypes identified in the present study, and the combinations of each of the two haplotypes within the populations were different from each other (Fig. 1a). However, Mediterranean populations (Fig. 1b) only included two haplotypes (H7 and H15), which were genetically rather distant. In the *cox*3 region, the genetic divergence between two haplotypes (H14 and H15)

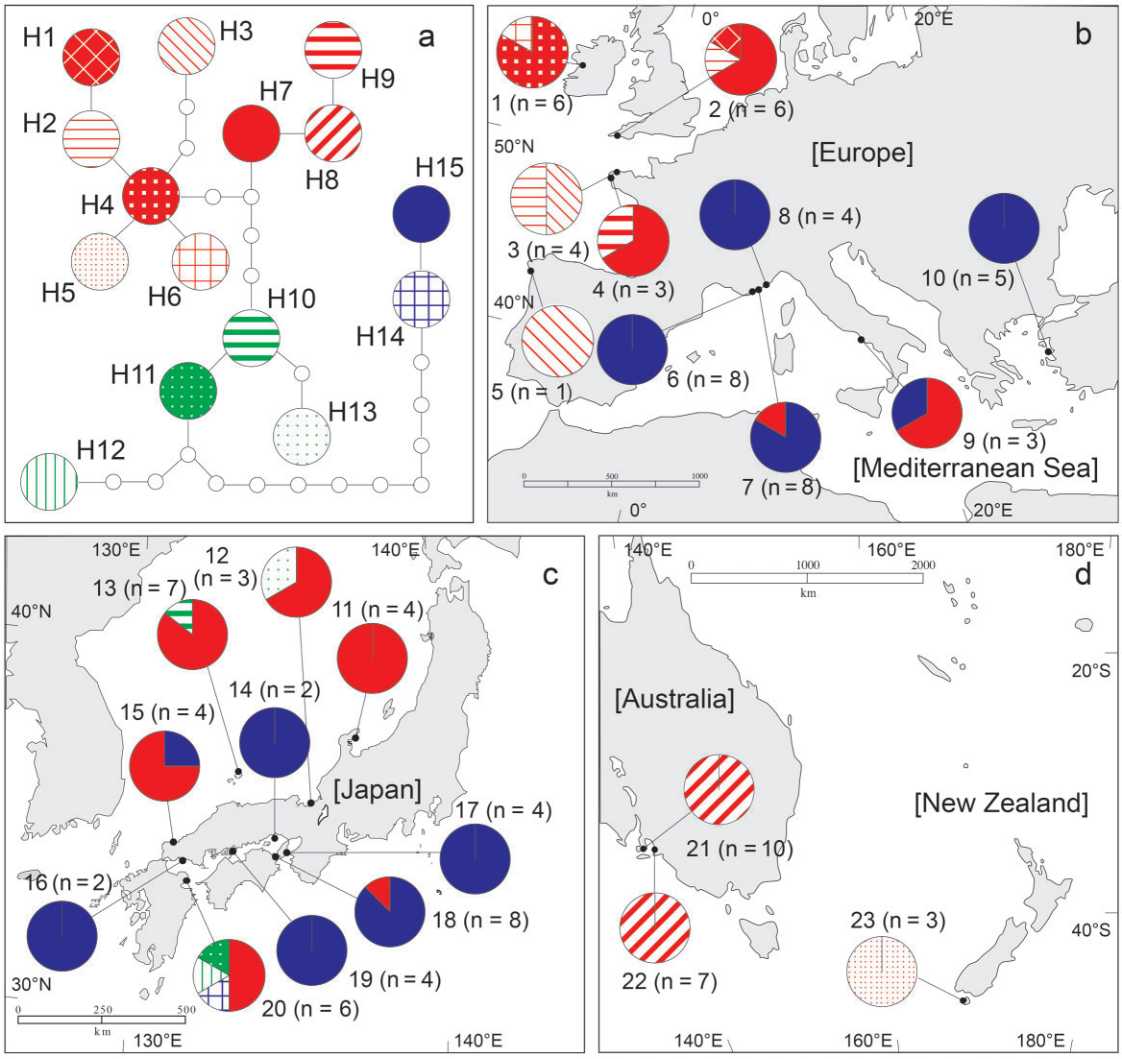


**Fig. 1.** Relationships and geographical distributions of mitochondrial DNA haplotypes (H1–H15) of *Cutleria multifida* based on the sequences of *cox2*, *cox3* genes, the intergeneric spacer region adjacent to cox3, and the open reading frame region. (a) Statistical parsimony network tree of mitochondrial DNA haplotypes. Each line connecting haplotypes corresponds to a one-base mutation; (b–d). Distribution and ratio of the haplotypes in the local populations in Europe (b), Japan (c) and Australia and New Zealand (d). Combinations of color and pattern represent different haplotypes. Each number in Figure 1b–d indicates the locality code in Table 1, and 'n' represents the number of samples sequenced in each population.

and the other haplotypes (H1–H13) was 0.31–1.24%. There was no clear correlation ($R^2 = 0.23$) between the geographical distance and genetic divergence within European populations. The correlation value became larger ($R^2 = 0.37$) when Mediterranean populations (localities no. 6–10) were removed from the dataset; however, the linear regression was not statistically significant ($P > 0.05$).

In Japan, 7 of the 15 haplotypes were identified, with half of the Japanese populations including multiple (up to four) haplotypes (Fig. 1c). There was a weak but positive correlation ($R^2 = 0.40$) between the geographical distance and the genetic divergence within Japanese individuals (Fig. 2b). Among the haplotypes found in Japan, H7 was also found in Cornwall, Le Caro, Antibes and Naples, and H15 was also found widely on the Mediterranean coasts (i.e. Cannes, Antibes, Villefranche, Naples and Lesbos Island). In contrast, the haplotypes H10–H14 were not found in Europe, Australia and New Zealand. Oonyujima in Kyushu (locality no. 20 in Fig. 1c) included four haplotypes and showed the highest genetic divergence in the analyses.

The haplotype found in the two localities in Australia (H8) were unique (Fig. 1a,d), but genetically very close (1 bp substitution) to H7 found in Cornwall, Le Caro (northwestern Europe), and Japan. Similarly, the haplotype from New Zealand (H5) was unique but genetically very close to H4 (1 bp substitution) found in Galway in northwestern Europe (Fig. 1a,d). Genetic diversities of four geographic regions were summarized in Table S2. Haplotype diversities for northwestern Europe and Japan were relatively high (0.805 and 0.618, respectively), and those of Mediterranean and Australia/New

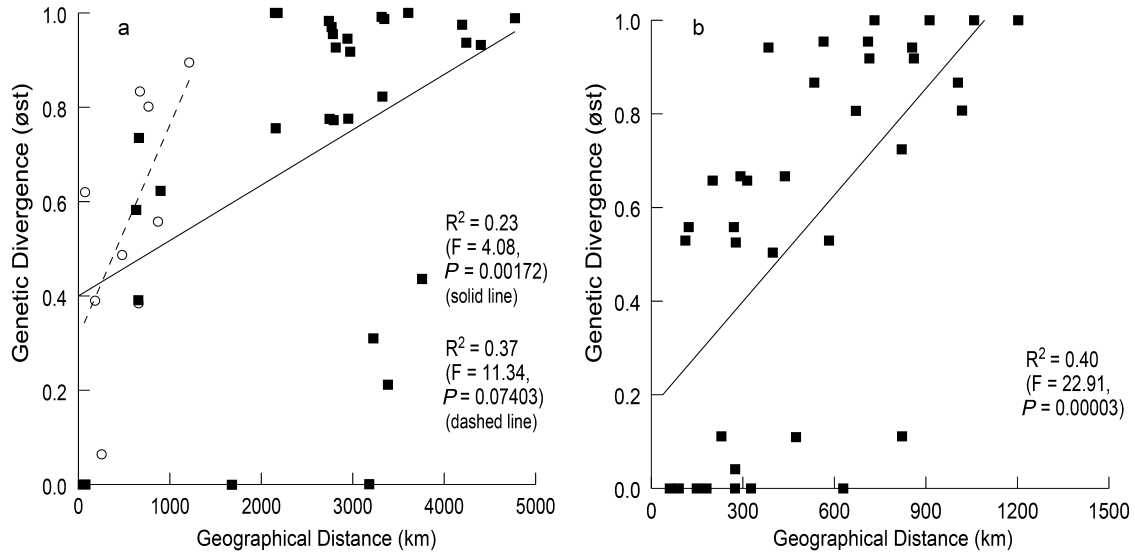


**Fig. 2.** Relationship between the geographical distance and genetic divergence between populations of *Cutleria multifida* in Europe (a) and Japan (b). The broken line in Figure 2a indicates the regression line based on the populations from northeastern Europe (solid square) excluding the Mediterranean Sea (open circle, locality code: 1–5). The solid line in Figure 2a indicates the regression line based on all European populations.

Zealand were low (0.198 and 0.268, respectively). Pairwise $\Phi_{st}$ values among four geographical regions were summarized in Table S3. All pairwise $\Phi_{st}$ values were statistically significant ($P < 0.001$).

As to the demographic patterns of regional populations, in the mismatch distribution analyses based on the concatenated sequences, the northwestern European populations showed multiple peaks, whereas those in the Mediterranean, Australia and New Zealand showed single peaks (Fig. 3). In contrast, the Japanese populations showed an intermediate pattern between the two patterns, with a single major peak and smaller multiple peaks.

Of the fertile gametophytes collected in various localities in Japan, the number of female and male gametophytes were as follows: female:male = 3:1 in Tsukumo Bay; 2:2 in Sekumi; 11:1 in Oki; 3:1 in Oomijima; 1:5 in Oonyujima; 1:1 in Yamaguchi; 3:2 in Kitanada; 19:7 in Yanoshima; and 2:0 in Yura. Therefore, it was shown that both female and male gametophytes occur in eight of the nine localities examined in Japan.

## DISCUSSION

The haplotype comparisons of populations of *Cutleria multifida* using mitochondrial gene sequences revealed that the populations in northwestern Europe and Japanese coasts showed relatively high genetic divergences, whereas those in the Mediterranean Sea, Australia and New Zealand were rather low. All of the three northwestern European populations and about half of the eight Japanese populations included multiple (up to four) haplotypes, and there was a weak but positive correlation ($R^2 = 0.40$) between the geographical distance and the genetic divergence among the individuals examined within Japan. The $R^2$ value of the northwestern European populations ($R^2 = 0.37$) was comparable to Japan, although the value reduced to 0.23 when the Mediterranean populations were included. This may suggest the presence of geographical barriers for gene flow depending on distance in northwestern Europe and Japan. Furthermore, Japanese and the northwestern European populations appeared genetically independent (H7, H10–H15 in Japan and H1–H4, H6–H7, H9 in northwestern Europe), except for haplotype H7, between the two regional groups. The haplotypes H10–H14 were only found in Japan and the genetic divergence among them (higher than 10 bp substitutions) was comparable to that in Europe. Therefore, the hypothesis that the Japanese populations are non-indigenous, e.g. introduced by shipping, is not supported.

In contrast, Australian and New Zealand populations were composed of only one haplotype. Therefore, in spite of the great geographical distances separating them, the haplotypes found in Australia and New Zealand were genetically close to those found in Europe and Japan. Considering these features, Australian and New Zealand populations are considered to be the result of anthropogenic transport from Europe and/or Asia. As to the Mediterranean populations, the genetic divergence was remarkably low, and the two haplotypes found in the area (i.e. H7 and H15) were rather distant. The haplotype H7 was both found in northwestern Europe and Japan, but H15 was found in Japan and over a wide range of the Mediterranean Sea. The haplotype H14, genetically closest to H15, is only found in Japan, and rather distant from other Japanese haplotypes H10–H13. Therefore, it is difficult to estimate the original distributional ranges of H7 and H15, but it is likely that there have been some transoceanic introductions of these haplotypes.

In the *cox*3 region, the genetic divergence between two haplotypes (H14 and H15) and the other haplotypes (H1–H13) was 0.31–1.24%. In contrast, it was 3.87–4.95% between *C. multifida* and *C. chilosa* (Falkenberg) P.C.Silva

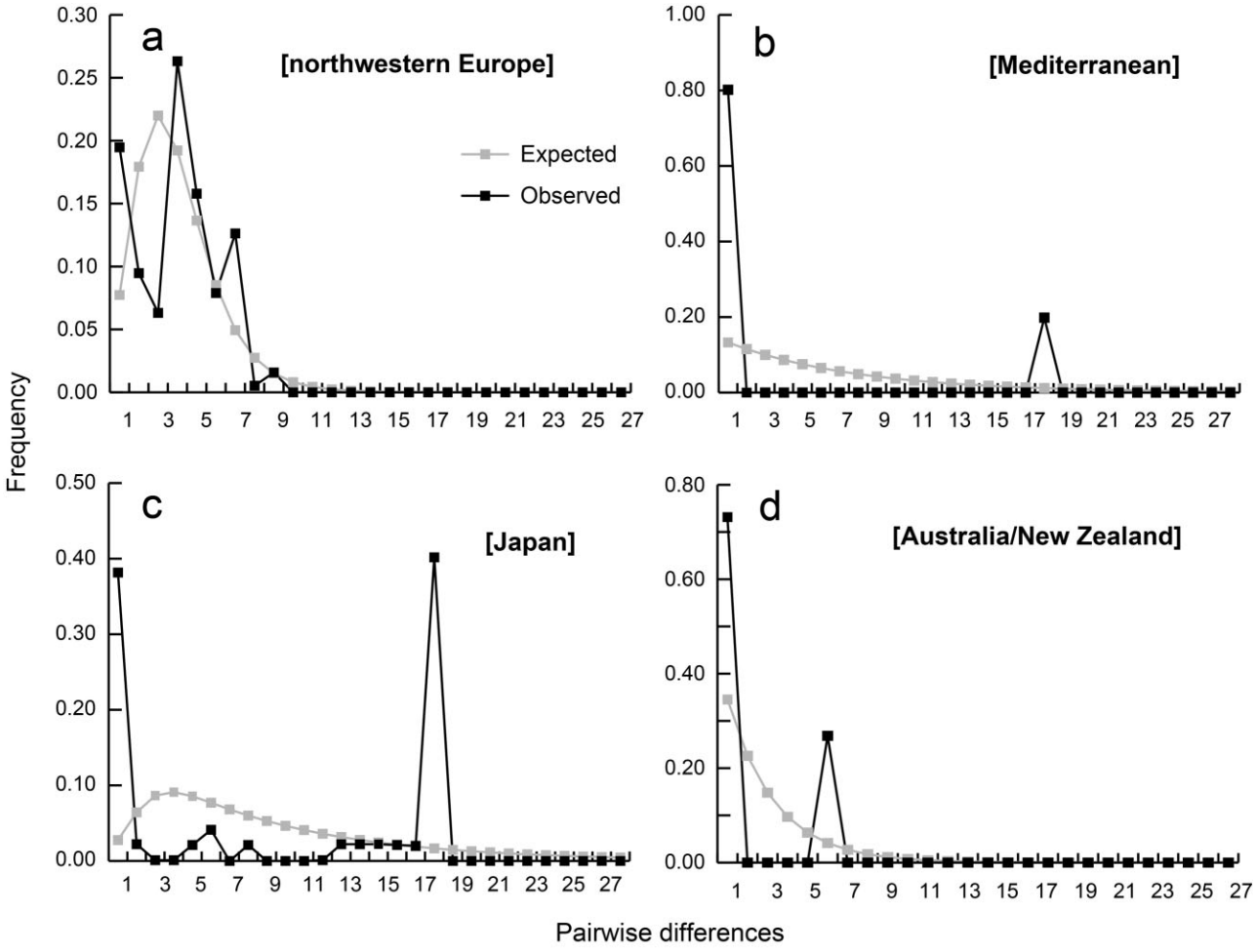


**Fig. 3.** Mismatch distributions for each regions based on the concatenated sequences. For the mismatch distributions, the solid lines show observed frequency distribution while the dashes lines show the distribution expected under the sudden-expansion model. (a) northwestern Europe (locality code: 1–5), (b) Mediterranean (6–10), (c) Japan (11–20), (d) Australia/New Zealand (21–23).

[(Kawai *et al.* 2012) and present study]. Furthermore, in the *psa*A region (Kawai *et al.* 2012), the genetic differences within *C. multifida* (0.11–0.63%), including the specimens correspond to H4, H8, H13, and H15 in the present study, were smaller than that of within *C. adspersa* (Mertens ex Roth) De Notaris (0.95%). Therefore, in spite of the wide distributional range, and although there is relatively large genetic gap between the haplotypes H14 and H15, and other haplotypes, they are considered to belong to a single species with potential gene flow.

In the mismatch distribution analyses, it was suggested that the northwestern European populations have a long history after the establishment (multiple peaks), whereas those in the Mediterranean, Australia and New Zealand were new after the establishment by the anthropogenic introductions (single peaks). In contrast, the Japanese populations showed an intermediate pattern between the two patterns. This may suggest that Japanese populations are a mix of both native and introduced populations.

*Cutleria multifida* typically shows a heteromorphic life history alternating between relatively large dioecious, erect branched gametophytes and small (*Aglaozonia*-stage) prostrate sporophytes (Kuckuck 1899; Yamanouchi 1912). Although the ratio of male and female gametophytes is theoretically expected to be 1:1, dominance of female gametophytes in field populations has been repeatedly reported within the presumptive native range (i.e. Europe) as well as in isolated localities in the Southern Hemisphere (i.e. New Zealand, Chile) (Thuret 1850; Church 1898; Coppejans 1981; Adams 1983, 1994; Fletcher 1987; Santelices *et al.* 1989). This phenomenon was considered to be the result of frequent occurrence of parthenogenetic female gametophytes. In Europe, Thuret (1850) observed parthenogenetic germination of female gametes into young filamentous gametophytes. Church (1898) reported that female gametes of *C. multifida* germinated parthenogenetically and developed into crustose thalli, which were later shown to be haploid (Yamanouchi 1912). Since only female gametophytes of *C. multifida* were found in Wellington Port, New Zealand and Coquimbo, Chile, and since these populations were limited to the vicinity of international ports, they were considered to be the parthenogenetic descendants of individuals transported from their native range by shipping (Adams 1983, 1994; Santelices *et al.* 1989). However, both female and male gametophytes

were found in most of the populations in Japan, and differed from the populations found in New Zealand and Chile.

The prostrate sporophytes of *C. multifida* (*Aglaozonia*-stage) are considered to be more tolerant to poor environmental conditions (e.g. low light intensity, high temperature, desiccation, etc.) than the erect gametophytes (Kitayama *et al.* 1992), and the sporophytes attached on ship-hulls or ballast rocks are more likely to survive the long cruise across the tropical regions traversed between Europe, northwestern Asia, Australia and New Zealand. The introduction to the Mediterranean Sea is also likely to have been associated with young oysters introduced for shellfish farming, similar to many other macroalgae introduced to the area from Japan (Verlaque 2001; Verlaque *et al.* 2005).

We have not been able to obtain specimens suitable for molecular studies from the western coast of Africa, the Indian Ocean, or South America. Considering the natural distributional patterns of the species it is possible that the Indian Ocean populations connecting the Western Pacific Asia are native populations, but it is also highly likely that they are traces of long-distance introductions from Europe to southern Pacific regions (i.e. Australia/New Zealand and Pacific South America) since the Age of Discovery. Provan *et al.* (2008) showed that the worldwide introduction of *Codium fragile* ssp. *tomentosoides*, originally distributed in Japan and its vicinity, had occurred at least 100 years ago. International ports might have played the role of oases in the journey across vast marine "deserts" for the introduction of marine organisms by maritime activities. Therefore, worldwide genetic analyses of species will provide an interesting model for elucidating the spread patterns of marine organisms by maritime activities.

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## SUPPORTING INFORMATION

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

**Table S1.** List of haplotypes and sample code for each population.

**Table S2.** Genetic diversity based on the mitochondrial region for each geographic region.

**Table S3.** Pairwise $\varphi_{st}$ estimates among four region of *Cutleria multifida* on the basis of mitochondrial DNA sequences.