Delimitation of cryptic species of the *Scytosiphon lomentaria* complex (Scytosiphonaceae, Phaeophyceae) in Japan, based on mitochondrial and nuclear molecular markers

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SUMMARY

Scytosiphon lomentaria (Scytosiphonaceae, Ectocarpales) is believed to include some cryptic species, particularly in the Pacific. We attempted to delimit these species in Japan using mitochondrial *cox*1 and *cox*3 and nuclear ITS2 and the second intron of the centrin gene (*cetn*-int2). Fifty-three *cox*1+*cox*3 mitotypes, 26 ITS2 ribotypes and 45 *cetn*-int2 haplotypes were found in 107 samples collected from 33 localities in Japan. Based on phylogenetic analyses, similar sequence types grouped into ten mitogroups, eight ribogroups and six *cetn*-int2 haplogroups (sequence-type groups). From the molecular trees and combinations of the mito-, ribo- and haplogroups, three cryptic species were apparent (Groups I-III). Group I, widely distributed on Pacific coasts, was highly supported by all molecular trees whereas Groups II (North Pacific) and III (Northwestern Pacific and Australasia) were more closely related to each other. However, sequence-type-group combinations that would be characteristic of hybrids between Groups II and III were not detected, suggesting no gene flow between the two Groups. Further investigations of additional 127 sympatrically growing plants supported the absence of gene flow between Groups II and III. Four samples did not belong to any of the Groups I-III and possibly represent additional species.

Key words: *cetn*-int2; *cox*1; *cox*3; gene flow; ITS2; reproductive isolation

INTRODUCTION

Studies of cryptic species are important for an accurate understanding of biodiversity and the process of speciation. Many cryptic algal lineages have been found by methods of molecular-based species delimitation (Leliaert et al. 2014 and included references).

The brown alga *Scytosiphon lomentaria* (Lyngbye) Link (Scytosiphonaceae, Ectocarpales) is a widespread species, occurring in cold and warm waters worldwide (Lüning 1990). The physiology, cell biology and molecular biology of the species have been intensively studied (e.g., Lüning & Dring 1975; Tom Dieck 1987; Nagasato *et al*. 2004; Fujita *et al*. 2005; Katsaros *et al*. 2006; Kimura *et al*. 2010). However, molecular phylogenetic studies have shown that morphospecies under this name show considerable genetic diversity (Camus *et al*. 2005; Cho *et al*. 2007). Sequence data from the *rbc*L gene suggested that the Atlantic and the Pacific entities belong to different species (Cho *et al*. 2007), although morphological distinction between the two entities is not possible. The Atlantic entity also occurs on the coast of Chile (Camus *et al*. 2005; Contreras *et al*. 2007). Additionally, the Pacific entities were separated into two major clades in the phylogenetic trees based on the internal transcribed spacer (ITS) region of the nuclear ribosomal cistron, suggesting the possibility that they consist of at least two species (Cho *et al*. 2007). By contrast, *rbc*L sequences did not clearly divide the two Pacific groups (Cho *et al*. 2007).

Species are the basic biodiversity unit, but species delimitation is actually often difficult. Many species concepts have been proposed, and, of course, a different species concept may result in a different species boundary (e.g., Hausdorf 2011). The

biological species concept is one of the most basic and influential species concepts but it is applicable only for sexually reproductive organisms (Templeton 1989). The Pacific entities of the *S. lomentaria* complex include Japanese material, which can reproduce sexually (Nakamura & Tatewaki 1975; Kogame 1998). Hence, it is possible to define a species from this complex using the biological species concept.

Multiple molecular markers are particularly effective in revealing the boundaries of biological species. Analyses with multiple unlinked genes can indicate the presence or absence of genetic exchange and the evolutionary independence of lineages (Sites & Marshall 2004). The phylogenetic concordance among genes indicates evolutionary independent lineages that have not exchanged genes for a long time, whilst the phylogenetic discordance suggests the occurrence of genetic exchange between the lineages (Shaw 2002; Le Gac *et al*. 2007; Tronholm *et al*. 2010).

In order to delimit putative biological species in Japanese *S. lomentaria* in the present study, we used ITS of nrDNA and the intron region of the centrin gene (*cetn*) as nuclear markers and the mitochondrion-encoded *cox*1 and *cox*3 genes as unlinked molecular markers to the nuclear markers. Nuclear molecular markers, including introns—other than ribosomal regions such as ITS and IGS—have recently been used for phylogenetic analyses at genus and species levels (Grob *et al*. 2004; Whittall *et al*. 2006; Duarte *et al*. 2010). However, it is difficult to use genes that belong to gene families since such genes may lead to a risk of comparison among paralogous loci due to difficulties in obtaining orthologous regions by PCR with degenerate primers (Whittall *et al*. 2006). In contrast, use of single-copy genes carries a low risk of paralogous comparisons (Duarte *et al*. 2010). Centrin is a protein associated with

flagellar basal bodies and centrioles (Salisbury 1995); in *S. lomentaria*, it is a single-copy gene consisted of five fragments split by introns (Nagasato *et al*. 2004). The second intron of the centrin gene (*cetn*-int2) was used as a nuclear molecular marker in addition to ITS in the present study.

Scytosiphon lomentaria has a heteromorphic alternation of generations between a macroscopic, erect gametophyte (15-50 cm in height) and a microscopic, discoid sporophyte (1-5 mm in diameter) (Tatewaki 1966; Nakamura & Tatewaki 1975; Kogame 1998). Thus, only haploid gametophytes are generally collected as samples for DNA extraction. The use of haploid samples is advantageous because nuclear haplotype can be directly determined by a PCR direct sequence method even in polymorphic nuclear genes in a population. In the analyses of diploid samples a hybrid is possibly a first filial generation that is sterile. By contrast, in the analyses of haploid samples, a sample in which genetic exchange was detected is an individual that was derived from a meiospore produced in a hybrid. Therefore, genetic exchange detected in the haploid analyses more strongly suggests gene flow.

Japanese *S. lomentaria* also shows almost the same ITS genetic diversity as collections from other parts of the Pacific (Kogame *et al*. 2005; Cho *et al*. 2007). Here, we recognize three putative cryptic species (Groups I-III) based on phylogenetic analyses and combinations of mitochondrial and nuclear markers.

MATERIALS AND METHODS

Samples of *Scytosiphon lomentaria* were collected at 33 localities in Japan

(Fig. 1, Table S1 in the Supporting Information). All plants, except for seven cases, were dried as voucher herbarium specimens and are deposited in SAP (Table S1). One unialgal isolate from each individual was established by isolating gametes or parthenogenetic germlings of gametes. Thallus sex was determined by observing gamete fusion or odor of sexual pheromone as described by Kogame *et al*. (2005). The isolates were maintained in plastic Petri dishes (90 x 20 mm) containing PESI medium (Tatewaki 1966) at 10˚C under a 16:8 h light:dark regime at low photon irradiances (ca. 1 µmol photons m^{-2} s^{-1}). Samples used in previous studies (Kogame *et al*. 2005; Cho *et al*. 2007) were also examined (Table S1). In total, 107 isolates were used for phylogenetic analyses. For the investigation of gene flow between the Groups II and III, samples were collected in 2003, 2009 and 2010 from the coasts of Ishikari Bay in Hokkaido (43.25N, 141.14E; Fig. 1), including a large number of samples from Otamoi (43.2294N, 140.9551E) where both Groups are found sympatrically (Tables S2 and S3 in the Supporting Information). In Otamoi, positions of collected plants were recorded using a quadrat (50 cm × 50 cm) during sampling. Silica gel-dried material for molecular analyses and pressed voucher specimens (Tables S2, S3) were prepared from the collected samples.

Total genomic DNA was extracted from the cultured thalli or silica gel-dried samples and was purified as described by Kogame *et al*. (1999). The purified DNA was used as template DNA in a PCR to amplify the *cox*1, *cox*3, ITS2 and *cetn*-int2 regions. The pairs of primers used for PCR and sequencing were : COIS1F (5'-TTTCTCTGGAGTATTAGGAA-3'; forward) and GazR2 (Lane *et al*. 2006) for *cox*1; CAF4A and CAR4A (Kogame *et al*. 2005) for *cox*3; 5.8SBF (Yoshida *et al*. 2000)

and 25BR2 (Kogame & Masuda 2001) for ITS2; Slcen1357F (5'-CGCGGAGGCGCAAAGTCGAA-3'; forward) and Slcen2237R (5'-GTCGCTGATCATCTTCTTGAT-3'; reverse) for the first round of *cetn*-int2 PCR, and Slcen1377F (5'-AAAAGTTCGAGCTGACGGAG-3'; forward) and Slcen2215R (5'-CTCCTTCTTGGGCTCGAAGC-3'; reverse) for nested PCR. The following primers were used for the investigation of gene flow between the Groups II and III in the coasts of Ishikari Bay in Hokkaido: 5.8SFscy (5'-CGTCTTGCGACTTGCAGAAT-3'; forward) and 25BR2 for ITS2; cox3F and cox3R (Kato *et al*. 2006) for *cox*3. PCR was performed using Amplitaq Gold DNA Polymerase (PE Applied Biosystems, Foster City, CA, USA) or TaKaRa Ex Taq DNA Polymerase (TAKARA Bio Inc., Otsu, Japan). Dimethylsulfoxide (5% in reaction volume) was added to the PCR mix for the ITS2 region. Amplification conditions consisted of 1 min (Ex Taq DNA Polymerase) or 10 min (Amplitaq Gold DNA Polymerase) at 96˚C for denaturation, followed by 40-50 cycles of 30 s at 94˚C, 30 s at 55-58˚C (for ITS2 and *cetn-int2*) or 50˚C (for *cox*1 and *cox*3) and 30 s at 72˚C, with a final extension of 5 min at 72˚C. The PCR was performed with a GeneAmp PCR System 9600 or 9700 (PE Applied Biosystems). PCR products were precipitated using PEG (polyethylene glycol #6000, Nakalai Tesque, Kyoto, Japan) to remove residual primers and dNTP and directly sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit v. 1.1 (PE Applied Biosystems) and an ABI Prism 310 or 3130 Genetic Analyzer (PE Applied Biosystems), following the manufacturer's protocols. The ITS2 sequencing results of the samples of 23-Mitsumatsu-11m, 29-Kannonzaki-3m and 30-Katsuura-3m and some of samples from Otamoi showed double peaks after a certain site in an electropherogram,

indicating a mix of two sequences that probably differ by an indel. The single-peak portion of the mixed sequence was identical to sequences determined in other samples. Then, the identical sequence was subtracted from the mix by eye, and the other remaining sequence was read.

The obtained sequences, together with previously published sequences, were aligned by eye (Table S1). The alignments used in the present study are available upon request from the corresponding author. Four data sets were analyzed: mitotypes (combined data of *cox*1 and *cox*3), ribotypes (ITS2), *cetn*-int2 haplotypes and mitotype+*cetn*-int2. Phylogenetic analyses were performed using maximum likelihood (ML), maximum parsimony (MP) and neighbor joining (NJ) methods in PAUP*4.0b (Swofford 2002). In the ITS2 analyses, gaps were treated as single events regardless of gap length (Peters *et al*. 1997), and a gap matrix was added to the end of the alignment. In the *cetn*-int2 and mitotypes+*cetn*-int2 analyses, gaps were treated as missing data. Nucleotide differences among sequences were calculated as 'uncorrected *p*-distance' with the 'pairwise distances' command in PAUP. ML trees were searched with the best-fit model selected and parameters assumed by Modeltest v. 3.7 (Posada and Crandall 1998). The hierarchical likelihood ratio test selected TrN+G for the *cox*1+*cox*3 data set and the *cox*1+*cox*3+*cetn*-int2 data set, and K80+G for the *cetn*-int2 data set as the best-fit model. MP trees were constructed by heuristic search with simple step-wise addition and TBR branch swapping. NJ trees were constructed using Tamura-Nei distance (Tamura & Nei 1993) except for ITS2 for which standard distances (total character distance) in PAUP was used. Mid-point rooting was adapted for all trees, and a bootstrap analysis (Felsenstein 1985) was performed with 100 replicates for ML and

500 for NJ and MP. Closely related sequences were grouped as a sequence-type group (mitogroup, ribogroup and *cetn*-int2 haplogroup) based on an alignment or inferred phylogenetic trees to facilitate easy reference during discussion, especially on genetic exchange. Scatter plots of pairwise genetic distances (Tamura-Nei distance) for *cetn*-int2 and *cox*1+*cox*3 were made using Microsoft Excel. An additional ITS2 NJ analysis using Tamura-Nei distance was conducted based on the alignment of Cho *et al*. (2007), to which data of Contreras *et al*. (2007) and ITS2 ribotypes of the present study were added.

RESULTS

Cox*1*+cox*3 analyses*. The combined alignment of *cox*1+*cox*3 was 1144 bp (*cox*1: 643 bp, *cox*3: 501 bp) in length and contained no gaps. Maximum sequence difference was 9.5% in *cox*1 and 11.2% in *cox*3. Fifty-three different sequences were grouped into ten mitogroups (Fig. 2), which were divided into two clades, mtK-S and mtT-U with high statistical supports in all analyses.

ITS2 analyses. The lengths of ITS2 sequences were variable, ranging from 240 to 255 bp, thus there were a lot of gaps in the alignment (Fig. S1 in the Supporting Information). The maximum pairwise sequence difference was 5.31% when gaps were treated as single events. Twenty-six ITS2 ribotypes were found and were grouped into eight ribogroups (itA-H, Fig. S1, Table S1 in the Supporting Information). Each ribogroup had unique indels whose lengths were 2 to 18 bp. In the NJ tree (Fig. S2 in the Supporting Information), the ribotypes were first divided into the clade of ribogroup

itH and the clade of other ribogroups. In the additional ITS2 analysis, which included previously published sequence data of Pacific *S. lomentaria* samples, ribogroup itH and the other ribogroups were included in the clade A and B of Cho *et al*. (2007), respectively. (Fig. S3 in the Supporting Information).

Cetn-*int2 analyses*. The *cetn*-int2 alignment was 774 bp (only intron region) in length with gaps in 32 positions. A microsatellite-like region consisting of a TCG repeat (2-17 or more times) was present in the intron. Due to the length of the repeat, DNA sequence reading collapsed after the repeat, and complete sequences were only obtained by joining the incomplete forward and reverse sequences. In this case, the exact length of the repeat could not be determined, so the simple repeat region was excluded in the analyses. The maximum pairwise difference was 20.7%. Forty-five haplotypes were grouped into six haplogroups, which were divided into two clades, cnA-E and cnF (Fig. 3). Two subclades, cnA-C and cnD-E, were recognized in the former clade.

Comparison of the cox*1*+cox*3, ITS2 and* cetn-*int2 trees*. In the *cox*1+*cox*3 tree, samples in the mtK, mtL and mtM clades had a single ribogroup itA. Samples in the mtQ clade had ribogroups itF or itG, and samples in the mtR clade had itG. Regarding the *cetn*-int2 tree (Fig. 3), the samples of cnA and cnC included only ribogroup itA. The sample of cnB (25-Tsuyazaki-4f) was isolated in all analyses and showed no similar sequence to those of any other samples. The samples of cnD included ribogroups itB, itG and itF and mitogroups mtQ, mtR and mtS. The mtN clade in the *cox*1+*cox*3 tree corresponded to the clade of cnE in *cetn*-int2 trees. The samples of cnF formed a

separate clade in all analyses. Meanwhile, samples of mtT in the *cox*1+*cox*3 tree did not form a clade in the *cetn*-int2 trees.

Thirteen combinations of mitogroups, ribogroups and *setn*-int2 haplogroups was observed (Fig. 3). The combinations KAA (mt, it, cn), KAC, LAA and MAA shared itA and partly shared mtK and cnA. No sequence-type group was shared between these and other combinations. These combinations were recognized as Group II. The combinations QFD, QGD, RGD and SBD shared cnD and partly shared mtQ and itG, and shared no sequence-type groups with other combinations. These combinations were recognized as Group III. Similarly, combinations THF and UHF were recognized as Group I. The combination PEB was distinct from others, sharing no sequence-type group with other combinations, and was tentatively recognized as Group IV(?) because only one sample showed this combination. The combinations NCE and NDE were also tentatively recognized as Group V(?) for the same reason. Moreover, Group I was widely distributed from Kyushu to Hokkaido while Groups II and III were found in Hokkaido and the Japan Sea coast of Honshu but were absent along the Pacific coast of Honshu (Fig. 1). The samples belonging to Groups IV(?) and V(?) came from southern (Kyushu) and northern (Hokkaido) localities, respectively, and were missing in Honshu.

Cox*1*+cox*3*+cetn-*int2 analyses*. Phylogenetic analyses were conducted for combined data of *cox*1+*cox*3+*cetn*-int2 in samples of Groups II, III, IV(?) and V(?) (Fig. 4). Two samples of Group I were added to the alignment as outgroup. ML, MP and NJ trees were the same in main branches. Groups II, III and V(?) were well supported (Fig. 4). Group IV(?) was the sister to Group II, but its supports were not high. Group V(?) was

the sister to the clade of Group III, but support for the clade of these two groups was poor.

Sequence differences for cox*1 and* cox*3*. Pairwise sequence differences (uncorrected *p*-distance) in *cox*1 were less than 2.6% within each of Groups I-III and 4.2-9.5% among the Groups (Table S4). In *cox*3, sequence differences were less than 5.8% within each Group and 2.0-11.0% among the Groups (Table S4).

Scatter plot analyses. A scatter plot of pairwise genetic distances was made for *cetn*-int2 and *cox*1+*cox*3 among samples of Groups II, III, IV(?) and V(?). In the scatter plot (Fig. 5), points in the circle 1 were values among the groups, and points in the circle 2 were values within each group. Points in the circle 3 show samples that are largely different in *cox* but are similar in *cetn*-int2. By contrast, points in the circle 4 show samples that are similar in *cox* but are largely different in *cetn*-int2. Points among groups (circle 1) and points within each group (circle 2) were separated. However, some points (circle 5) within Group II were positioned closely to points (circle 1) among groups. These points in the circle 5 were derived from five samples of Group II: 23-Mitsumatsu-7f, 13-Kakijima, 2-Oshoro 000510-11m, 16-Seseki and 20-Tappizaki-1f. These samples were similar to other samples of Group II in *cox* or *cetn*-int2. In the case of the former, the points were plotted in the circle 4, and in the case of the latter points were plotted in the circle 3.

In terms of *cox*1+*cox*3 pairwise distances, there were gaps around 0.015 (dotted line 6 in Fig. 5) and 0.024 (dotted line 7). Values larger than the line 6 were ones

among mitogroups (mtK-mtS), and values smaller than the line 6 were ones within each mitogroup. Three points between lines 6 and 7 were values between mtR and mtS. In terms of *cetn*-int2 pairwise distances, there was a gap around 0.032 (dotted line 8). Values larger than the line 8 were ones among *cetn*-int2 haplogroups (cnA-cnE), and values smaller than the line 8 were ones within each haplogroup.

Investigation of gene flow between the Groups II and III. One hundred seventy six (176) samples collected from 12 localities in the coasts of Ishikari Bay were sequenced for *cox*3 and ITS2, including 127 samples from Otamoi, which is a sympatric site of the Groups II and III. Six combinations of *cox*3 mitogroups (K, N, Q and T) and ribogroups (A, D, F, G and H) were found: KA (Group II, 94 samples), ND (V(?), 2), QF (III, 35), QF/G (III, 7), QG (III, 21) and TH (I, 17) (Fig. 6, Tables S2, S3). QF/G means that the sample (individual) may have two sequences belonging to itF and itG ribogroups. Combinations that could be derived from hybrids between the Groups II and III such as KF, KG and QA were not found.

The region of *cetn*-int2 was additionally sequenced for 119 samples collected from Otamoi in 2009 and 2010. Samples having KA (*cox*3, ITS2) showed the haplogroup cnA or cnC, and samples having QF, QF/G or QG showed the haplogroup cnD (Table 1). No combination that can be derived from hybrids between Groups II and III was detected. In Otamoi, plants of the Groups II and III grew beside each other, and there seemed no significant difference in habitat position between the Groups (Fig. S4 in the Supporting Information).

DISCUSSION

In our study, most isolates of *Scytosiphon lomentaria* were sexual—this mode of reproduction is common for *S. lomentaria* in Japan. The three molecular markers used herein, the mitochondrial *cox*1 and *cox*3 and the nuclear *cetn*-int2, showed substantial variation that is suitable for phylogenetic analyses. Although ITS2 sequences showed low resolution in phylogenetic analyses, they were used for analyses of sequence-type combinations. Based on the results of the phylogenetic analyses and combinations of sequence-type groups, we recognized three groups (Groups I-III) within this morphospecies in Japan.

Group I was strongly supported by all of the markers in the phylogenetic analyses The phylogenetic concordance among these genes also suggested no genetic exchange between this Group and the other Groups II-V(?). Thus, we considered Group I and the others as representatives of different species. Group I was divided into two mitogroups (mtT and mtU) in the *cox*1+*cox*3 analyses, suggesting that samples of mtT and mtU may be of different species. In the analyses of the nuclear markers, however, samples of mtT and mtU were mixed. For example, 30-Katsuura-2f that showed mtT had the same *cetn*-int2 sequence as that of 3-Shimamaki-2m that showed mtU. If samples of mtT and mtU were not conspecific, the identical *cetn*-int2 sequences that they shared resulted from incomplete lineage sorting or were remains of sequences from a common ancestor (see Avise 1989; Smouse 2000; Nichols 2001). However, it is unlikely that ancestral *cetn*-int2 sequences have been retained in both mtT and mtU samples because the *cetn*-int2 sequences are more diverse than the mitochondrial

markers in Group I. The identical nuclear sequences (*cetn*-int2) and the largely different mitochondrial sequences between mtT and mtU samples are more adequately explained by gene flow within Group I (McGuire *et al*. 2007). Therefore, we did not recognize significant subgroups in Group I and regarded this group as a single species.

Group II was supported in the *cox*1+*cox*3 and the *cox*1+*cox*3+*cetn*-int2 trees, forming well or moderately supported clades. Three mitogroups, mtK, mtL and mtM, in this Group were largely different from each other, but the samples (13-Kakijima and 23-Mitsumatsu-7f) that showed mtL and mtM had similar ITS2 and *cetn*-int2 sequences to those of mtK samples. Although samples of the Group II showed different mitogroups and *cetn*-int2 haplogroups in some cases, they can be connected by partly identical or similar sequences of other markers, suggesting that all these samples belong to the same species.

Group III formed a clade in all analyses except for ITS2. Ribogroups itF and itG differed by a large indel, but four samples (1-Asari7f, 1-Asari-8f, 1-Asari10f and 4-Setana) of itG showed the same or similar *cox*1+*cox*3 sequences (mtQ) to those of itF samples. The sample 22-Himi-1 showed unique sequences in *cox*1+*cox*3 (mtS) and ITS (itB) but had a similar *cetn*-int2 sequence (cnD) to the other samples of Group III. These results indicate that samples of the Group III are connected partly by having identical sequence-type groups and are thus recognized as a single species.

The sample 25-Tsuyazaki-4f of Group IV (?) did not have similar sequences to those of other samples in any analyses. This may support our hypothesis that this sample belongs to a different species. The three samples (2-Oshoro 881025-Cr5m, 2-Oshoro 980407-1f and 19-Kitami-Esashi) of Group V(?) formed a highly-supported

clade (mtN and cnE) except for ITS2 analyses. Further, these three samples did not share any sequence-type group with Groups I-III, showing no close relationships to them. However, the small number of Group V(?) samples may have limited the detection of relationships. Thus, we considered these three samples, as well as 25-Tsuyazaki-4f (Group IV(?)), as entities that are of unclear affiliation.

Gaps between intraspecific and interspecific pairwise distances have been reported for some molecular markers including *cox*1 even in closely related seaweed species although such gaps are not necessarily found (Sites & Marshall 2004; Mattio & Payri 2010; Yang *et al*. 2014). In the scatter plot analyses, distributions of pairwise genetic distances showed gaps (lines 6-8 in Fig. 5) in each of *cox*1+*cox*3 and *cetn*-int2, but the gaps did not correspond between the *cox* and *cetn*-int2. By contrast, points among Groups II, III, VI(?) and V(?) were separately plotted from points within each group. This result supports recognizing the four groups. Since random combinations of intraspecific variations are expected within a species, the cases of combinations of small *cox* distances and large *cetn*-int2 distances and the opposite, i.e., combinations of large *cox* and small *cetn*-int2 distances, may occur. Points in circles 3 and 4 in Figure 5 are applicable to these cases, suggesting intraspecific variations. Points in circle 5 are applicable to the case that intraspecific distances are large in both *cox* and *cetn*-int2. On the other hand, interspecific pairs are expected to have relatively large values in both *cox* and *cetn*-int2, and the circle 1 is comparable to this case.

In order to test the separation of the Groups II and III, a large number of samples collected from Ishikari Bay were examined, but no sequence-type-group combinations indicative of hybrids between the two were detected. Considering Groups

II and III did not share any sequence-type group in any analyses, gene flow is absent between them and they therefore represent different species. No significant differences in habitat position were observed between the two Groups in Otamoi, and the thalli of the two Groups were reproductively mature when collected. Considering that both Groups have similar habitat position and reproductive periodicity, it is unlikely that these two factors act as reproductive barriers. Similar situation has been reported in the red algal genus *Pyropia*, in which two cryptic species grew sympatrically, even on the same rocks (Niwa and Kobiyama 2014; Niwa *et al*. 2014).

The present study also showed that asexually-reproducing samples detected in a previous study (Kogame *et al*. 2005) in the localities of 1-Asari, 2-Oshoro and 9-Muroran belong to Group I. Some sequence types of the asexual samples were congruent with some of the sexual samples of Group I, suggesting that the asexual lineages differentiated recently in Group I. These asexual samples were only found in the cold waters of Hokkaido, suggesting the evolution of asexual populations in response to a colder environment. In the Rhodophyta, particularly in *Caloglossa* species, life history studies and analyses using spacer sequences of the RUBISCO operon revealed asexual lineages, although the actin sequences of sexual and asexual plants were identical in some populations (West *et al*. 2001; Kamiya 2004; Kamiya *et al*. 2011). Meanwhile, asexual isolates of *Mastocarpus* were not distinguished from sexual isolates in both RuBisCo spacer and cox2-3 spacer (Zuccarello *et al*. 2005).

The diversity of the ITS2 in Japanese *S. lomentaria* was consistent with that of the Pacific entities previously reported in Cho *et al*. (2007) where the Pacific clade consisted of the two large clades (A and B) in ITS trees. The ribogroup itH and the other

ribogroups were congruent with the clade A and B of Cho *et al*. (2007), respectively. According to Cho *et al*. (2007), ribogroup itH (Group I) is widely distributed in the Pacific coasts (North Pacific, Australia and New Zealand), ribogroup itA (Group II) in Korea, USA and Russia, and itB (Group III) in Korea, Australia and New Zealand as well as in Japan. Group I is also distributed in Chile (Camus *et al*. 2005; Contreras *et al*. 2007). Since in some instances the same sequences were found in localities far from each other, Cho *et al*. (2007) considered that artificial transfer (perhaps by shipping or by aquaculture) may have caused extended distributional range. By contrast, the ITS2 ribogroup itF is found only in Korea and Japan (Cho *et al*. 2007).

The maximum sequence differences (9.5%) in *cox*1 were similar to but slightly lower than that (11.2%) in *cox*3 in Japanese *S. lomentaria*. In *cox*1, sequence differences within each Group of I-III were smaller than those among the Groups, but in *cox*3 intra- and inter-Group sequence differences overlapped. In *cox*1 of species of the kelp *Saccharina*, intraspecific divergence was within 0-1.2% and interspecific divergence was generally greater than 4% (McDevit and Saunders 2010). In Japan, *Undaria pinnatifida* (Harvey) Suringar had 1.9% of *cox*3 divergence (Uwai *et al*. 2006a, b, 2007), and *Sargassum horneri* (Turner) C. Agardh showed 4.5% *cox*3 divergence (Uwai *et al*. 2009). The brown algal genus *Padina* showed up to 2.6% intraspecific divergence in *cox*3 in the western Pacific (Ni-Ni-Win *et al*. 2010). *Colpomenia claytoniae* S.M. Boo *et al*. (as '*claytonii*') from the Pacific and South Africa differed from one another by 0-4.6% and from closely related *Colpomenia* species by 5.6-8.0% in *cox*3 (Boo *et al*. 2011). These intraspecific and interspecific divergences are comparable to those of the Groups I-III of Japanese *S. lomentaria*.

This is probably the first time that the intron region of *cetn* has been used for phylogenetic analyses among closely related species. Here, the *cetn*-int2 was highly variable, yielding more variable and informative sites than ITS, *cox*1 and *cox*3. In addition, the *cetn*-int2 trees had a greater number of well-supported clades. These results suggest that the *cetn*-int2 can be a suitable phylogenetic marker within a species or among closely related species and that introns of nuclear single-copy genes can be a useful marker at a species level in brown algae as well as animals and embryophytes (e.g., Whittall *et al*. 2006; Rosell *et al*. 2010).

The Chilean kelps *Lessonia berteroana* Montagne and *L. spicata* (Suhr) Santelices are sibling species with no apparent gene flow between them and are distinctly distributed in the northern and central Chile (Tellier *et al*. 2009, 2011; González et al. 2012). These two species are strictly separated in space even in the contact zone of their distributions (Tellier *et al*. 2009, 2011). In a *Fucus* species complex from the NE Atlantic, the component entities maintain their own morphology sympatrically but are found in high (*F. spiralis* L. and *F. guiryi* Zardi *et al*.) and mid-intertidal habitats (*F. vesiculosus* L. complex), despite the occurrence of gene flow among them (Coyer *et al*. 2011; Zardi *et al*. 2011). It has been hypothesized that environmental gradients (temperatures, desiccation and competition) promote diversification and speciation in the species complexes of *Fucus* and *Lessonia* (Coyer *et al*. 2011; Tellier *et al*. 2011; Zardi *et al*. 2011). By contrast, the Groups II and III of *S. lomentaria* complex exist sympatrically and seem to have similar niches. Thus, such environmental gradients are unlikely factors for speciation of the two Groups. Another possible factor may be geographical separation. It has been suggested that repeated

separation and joining of populations during Pleistocene glaciations had a significant influence on diversification and speciation of marine organisms including brown algae (Lane *et al*. 2007; Coyer *et al*. 2011).

At least three cryptic species were found in Japanese *S. lomentaria* in this study, and they are different from *S. lomentaria* that are widely distributed in the NE Atlantic and Mediterranean (Cho *et al*. 2007). *Chorda lomentaria* Lyngbye, the basionym of *Scytosiphon lomentaria*, was described from syntype localities in the Faroe Islands and Bornholm, Denmark (Type in C). *Scytosiphon lomentaria* currently includes three varieties and nine heterotypic synonyms (Guiry & Guiry 2013). Further morphological and molecular studies are required, especially for European material, in order to assign appropriate taxonomic names to the candidate cryptic species of *S. lomentaria* in Japan and elsewhere.

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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:

Fig. S1. An alignment of ITS2 ribotypes found in Japanese *Scytosiphon lomentaria*.

Fig. S2. Neighbor joining (NJ) midpoint-rooted tree for ITS2 ribotypes in Japanese *S. lomentaria*.

Fig. S3. NJ tree of ITS2 sequences in *S. lomentaria* widely collected from Pacific coasts.

Fig. S4. Growing position of *S. lomentaria* in Otamoi, Otaru, Hokkaido, Japan.

Table S1. Collection data of samples used in this study.

Table S2. Samples of *Sc. lomentaria* collected from Ishikari Bay, Hokkaido, Japan in

2003.

Table S3. Samples of *S. lomentaria* collected from Otamoi, Otaru, Hokkaido, Japan in 2009 and 2010.

Table S4. Pairwise sequence differences (uncorrected *p*-distance) in *cox*1 and *cox*3 for Groups I-III, IV(?) and V(?) in *S. lomentaria* from Japan.

Figure legends

Fig. 1. Map of Japan showing collection locations of samples used in this study. Numerals (1-33) are locality codes, and locality names are listed in Table S1 in the Supporting Information. Sequence-type-group combinations of *cox*1+*cox*3 (K-U), ITS2 (A-H) and *cetn*-int2 (A-F), that were found in the locality, are shown after the locality codes. I, II, III, IV(?) and V(?) in superscript indicate the Group that the combination belongs to. Asterisk: Ishikari Bay.

Fig. 2. Maximum likelihood (ML) midpoint-rooted tree for *cox*1+*cox*3 sequences of *Scytosiphon lomentaria* in Japan. Sample names consist of the locality code (numerals) and the sample code listed in Table S1 in the Supporting Information. "f", "m", "ax" and no character after numerals of sample codes mean female, male, asexual and unknown for sexuality, respectively. Mitogroups (mtK-mtU) are also indicated. Bootstrap values (≥ 70%) for ML/maximum parsimony (MP)/neighbor joining (NJ) are shown near branches. Asterisk indicates 100% bootstrap value.

Fig. 3. ML midpoint-rooted tree for *cetn*-int2 sequences of *S. lomentaria* in Japan. Mitogroups (mt), ribogroups (it) and haplogroups of *cetn*-int2 (cn) are indicated. I-III, IV(?) and V(?) indicate the Groups (putative cryptic species) that are demonstrated in this study. See the caption in Fig. 2 for detail.

Fig. 4. ML midpoint-rooted tree for *cox*1+*cox*3+*cetn*-int2 sequences in samples of

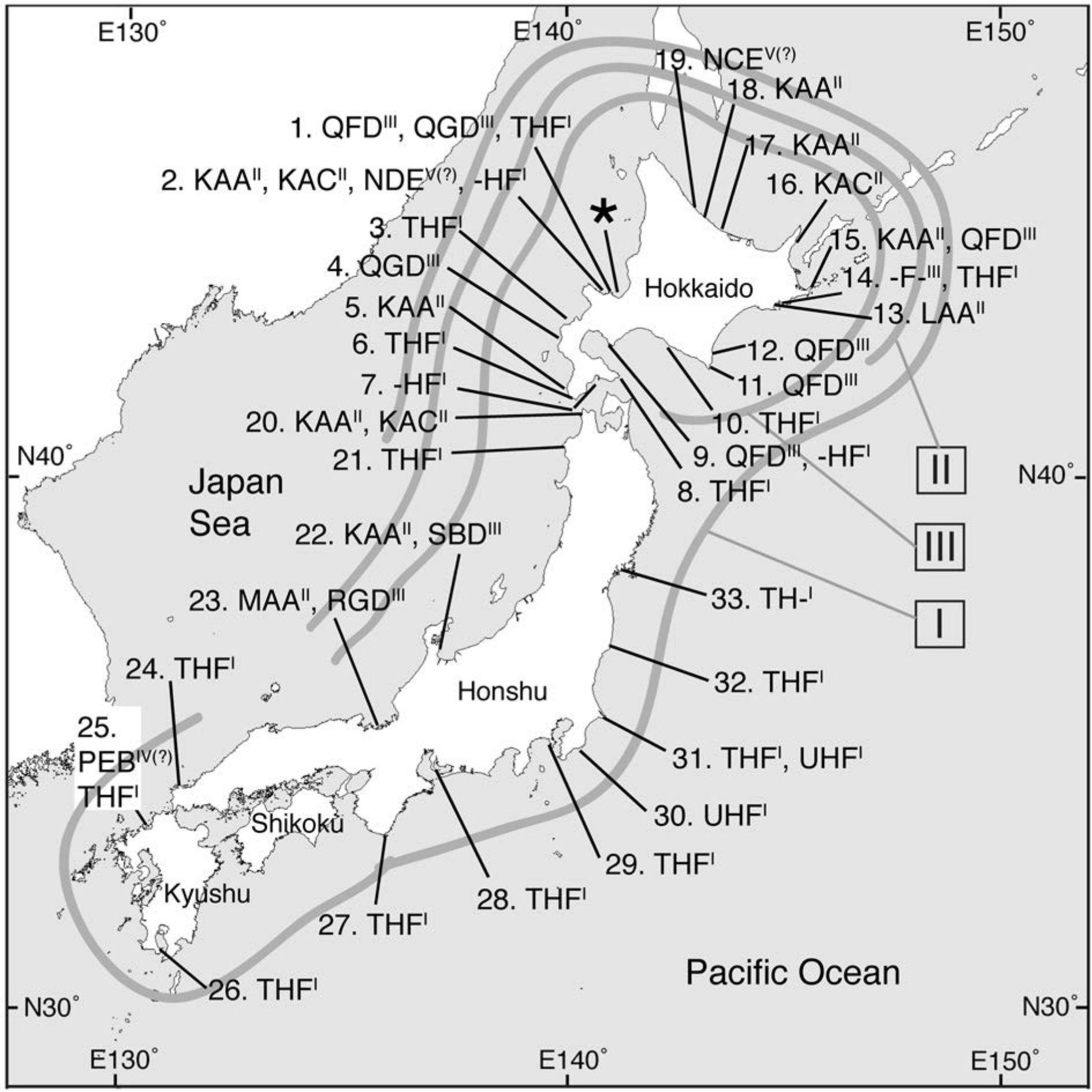
Groups II, III, IV(?), V(?) and two samples of Group I. See the captions in Figs 2 and 3 for detail.

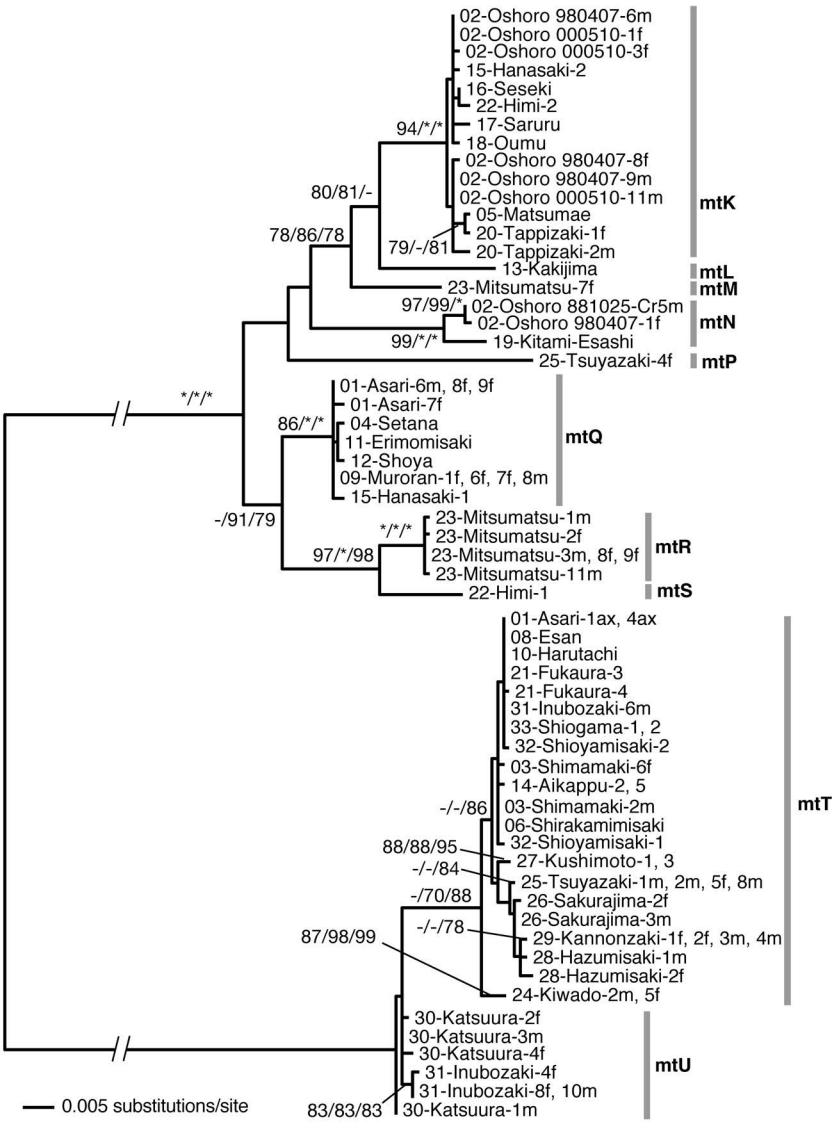
Fig. 5. Scatter plot of pairwise genetic distances for *cetn*-int2 and *cox*1+*cox*3 among samples of Groups II, III, IV(?) and V(?). 'II - II' in sample labels indicates 'within Group II'. 'II - III' indicates 'between Groups II and III'. For labels of 1-8, see text.

Fig. 6. Collection localities of *S. lomentaria* on the coasts of the Ishikari Bay, Hokkaido, Japan. Combinations of mitogroups (K, N, Q, T) and ribogroups (A, D, F, G, H) that were found from the localities are shown. The numeral after the combination indicates the number of samples that showed the combination.

Table 1. Combinations of sequence-type groups (*cox*3, ITS2 and *cetn*-int2) found in Otamoi, Otaru, Hokkaido, Japan, and the number of samples that showed the combinations.

Collection date	Combination of sequence-type groups (*cox*3, ITS2, *cetn*-int2)					Total
	Group II		Group III			
	KAA	KAC	QFD	QGD	QF/GD	
29 April 2009	21	15	3	4	2	45
17 April 2010	30	11	18	10	5	74
Total	51	26	21	14	7	119


E130°
E140°
E150°
N40°
N30°
19. NCE V(?)
18. KAA II
1. QFD III, QGD III, THF I
17. KAA II
2. KAA II, KAC II, NDE V(?), -HF I
16. KAC II
3. THF I
15. KAA II, QFD III
4. QGD III
14. -F- III, THF I
Hokkaido
5. KAA II
13. LAA II
6. THF I
12. QFD III
7. -HF I
11. QFD III
20. KAA II, KAC II
10. THF I
21. THF I
9. QFD III, -HF I
8. THF I
II
III
I
Japan Sea
22. KAA II, SBD III
33. TH- I
23. MAA II, RGD III
24. THF I
32. THF I
Honshu
25. PEB IV(?) THF I
31. THF I, UHF I
30. UHF I
Shikoku
29. THF I
Kyushu
28. THF I
27. THF I
26. THF I
Pacific Ocean


02-Oshoro 980407-6m
02-Oshoro 000510-1f
02-Oshoro 000510-3f
15-Hanasaki-2
16-Seseki
22-Himi-2
17-Saruru
18-Oumu
02-Oshoro 980407-8f
02-Oshoro 980407-9m
02-Oshoro 000510-11m
05-Matsumae
20-Tappizaki-1f
20-Tappizaki-2m
13-Kakijima
23-Mitsumatsu-7f
02-Oshoro 881025-Cr5m
02-Oshoro 980407-1f
19-Kitami-Esashi
25-Tsuyazaki-4f
01-Asari-6m, 8f, 9f
01-Asari-7f
04-Setana
11-Erimomisaki
12-Shoya
09-Muroran-1f, 6f, 7f, 8m
15-Hanasaki-1
23-Mitsumatsu-1m
23-Mitsumatsu-2f
23-Mitsumatsu-3m, 8f, 9f
23-Mitsumatsu-11m
22-Himi-1
01-Asari-1ax, 4ax
08-Esan
10-Harutachi
21-Fukaura-3
21-Fukaura-4
31-Inubozaki-6m
33-Shiogama-1, 2
32-Shioyamisaki-2
03-Shimamaki-6f
14-Aikappu-2, 5
03-Shimamaki-2m
06-Shirakamimisaki
32-Shioyamisaki-1
27-Kushimoto-1, 3
25-Tsuyazaki-1m, 2m, 5f, 8m
26-Sakurajima-2f
26-Sakurajima-3m
29-Kannonzaki-1f, 2f, 3m, 4m
28-Hazumisaki-1m
28-Hazumisaki-2f
24-Kiwado-2m, 5f
30-Katsuura-2f
30-Katsuura-3m
30-Katsuura-4f
31-Inubozaki-4f
31-Inubozaki-8f, 10m
30-Katsuura-1m
94/*/*
80/81/-
78/86/78
79/-/81
97/99/*
99/*/*
//*
86/*/*
-/91/79
//*
97/*/98
-/-/86
88/88/95
-/-/84
-/70/88
-/-/78
87/98/99
83/83/83
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mtN
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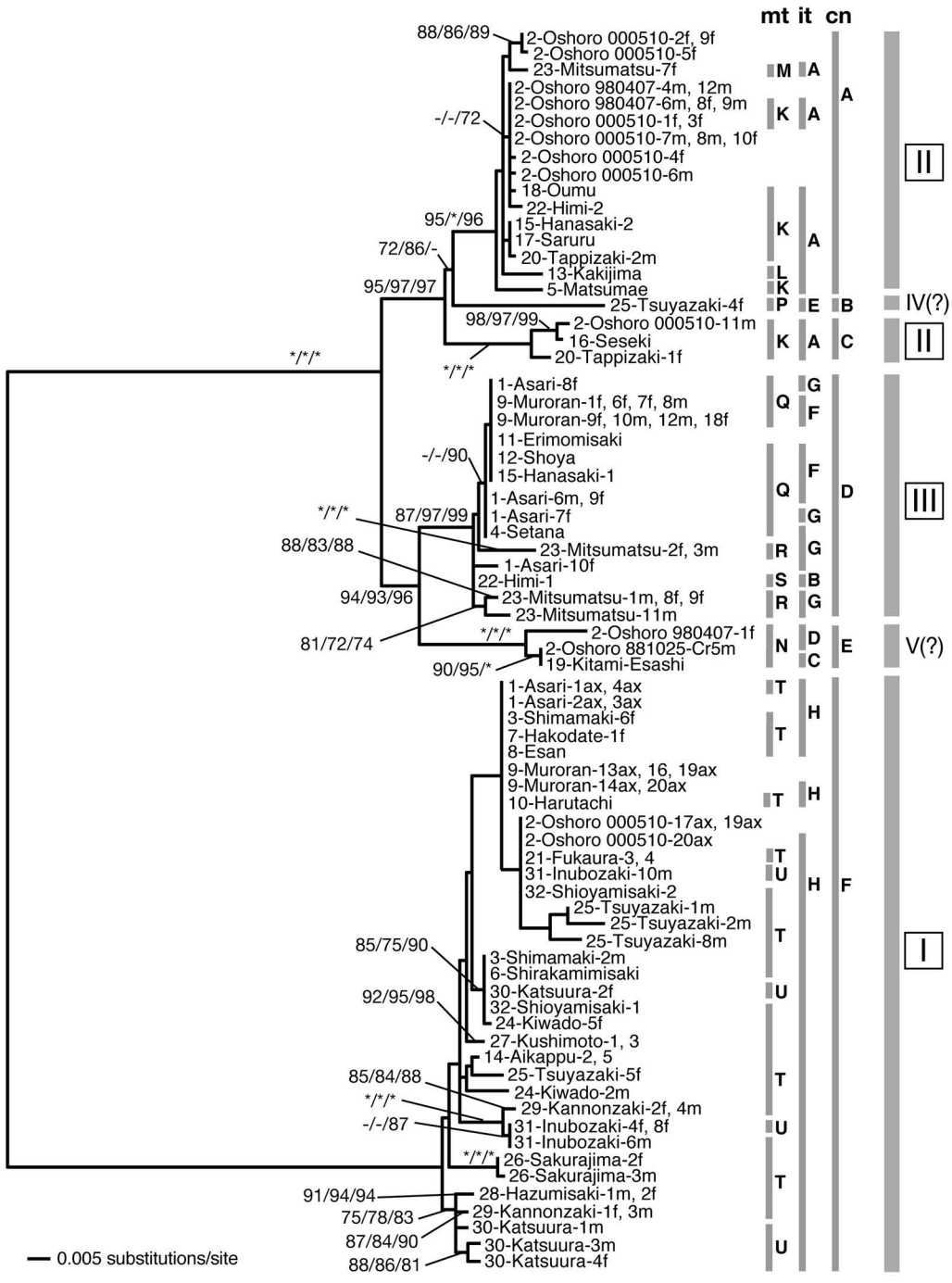
mt it cn
88/86/89
2-Oshoro 000510-2f, 9f
2-Oshoro 000510-5f
23-Mitsumatsu-7f
M A
2-Oshoro 980407-4m, 12m
2-Oshoro 980407-6m, 8f, 9m
-/-/72
2-Oshoro 000510-1f, 3f
K A
A
2-Oshoro 000510-7m, 8m, 10f
2-Oshoro 000510-4f
2-Oshoro 000510-6m
18-Oumu
22-Himi-2
II
95/*/96
15-Hanasaki-2
17-Saruru
72/86/-
20-Tappizaki-2m
13-Kakijima
5-Matsumae
95/97/97
K A
L
K
25-Tsuyazaki-4f
P E B
IV(?)
98/97/99
2-Oshoro 000510-11m
16-Seseki
20-Tappizaki-1f
K A C
II
//*
//*
1-Asari-8f
9-Muroran-1f, 6f, 7f, 8m
9-Muroran-9f, 10m, 12m, 18f
Q G F
11-Erimomisaki
-/-/90
12-Shoya
15-Hanasaki-1
Q F
D
III
1-Asari-6m, 9f
//*
87/97/99
1-Asari-7f
G
4-Setana
88/83/88
23-Mitsumatsu-2f, 3m
R G
1-Asari-10f
S B
22-Himi-1
94/93/96
23-Mitsumatsu-1m, 8f, 9f
R G
23-Mitsumatsu-11m
//*
2-Oshoro 980407-1f
81/72/74
2-Oshoro 881025-Cr5m
N D C E
V(?)
19-Kitami-Esashi
90/95/*
1-Asari-1ax, 4ax
T
1-Asari-2ax, 3ax
3-Shimamaki-6f
H
7-Hakodate-1f
T
8-Esan
9-Muroran-13ax, 16, 19ax
9-Muroran-14ax, 20ax
H
10-Harutachi
T
2-Oshoro 000510-17ax, 19ax
2-Oshoro 000510-20ax
21-Fukaura-3, 4
T
31-Inubozaki-10m
U
H F
32-Shioyamisaki-2
25-Tsuyazaki-1m
25-Tsuyazaki-2m
T
25-Tsuyazaki-8m
I
85/75/90
3-Shimamaki-2m
6-Shirakamimisaki
30-Katsuura-2f
U
92/95/98
32-Shioyamisaki-1
24-Kiwado-5f
27-Kushimoto-1, 3
14-Aikappu-2, 5
85/84/88
25-Tsuyazaki-5f
T
24-Kiwado-2m
//*
29-Kannonzaki-2f, 4m
-/-/87
31-Inubozaki-4f, 8f
U
31-Inubozaki-6m
//*
26-Sakurajima-2f
26-Sakurajima-3m
T
91/94/94
28-Hazumisaki-1m, 2f
75/78/83
29-Kannonzaki-1f, 3m
30-Katsuura-1m
87/84/90
U
30-Katsuura-3m
88/86/81
30-Katsuura-4f
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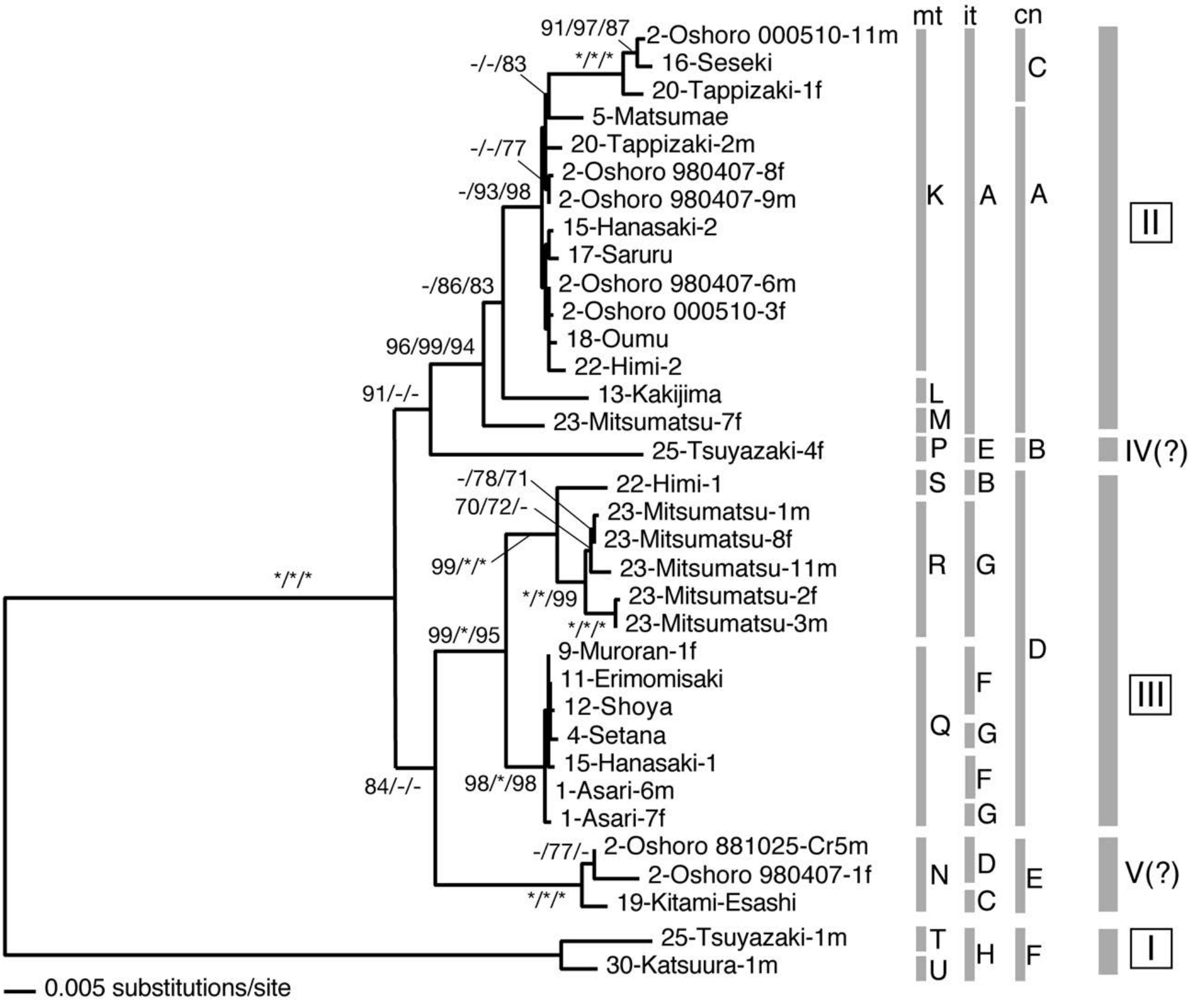
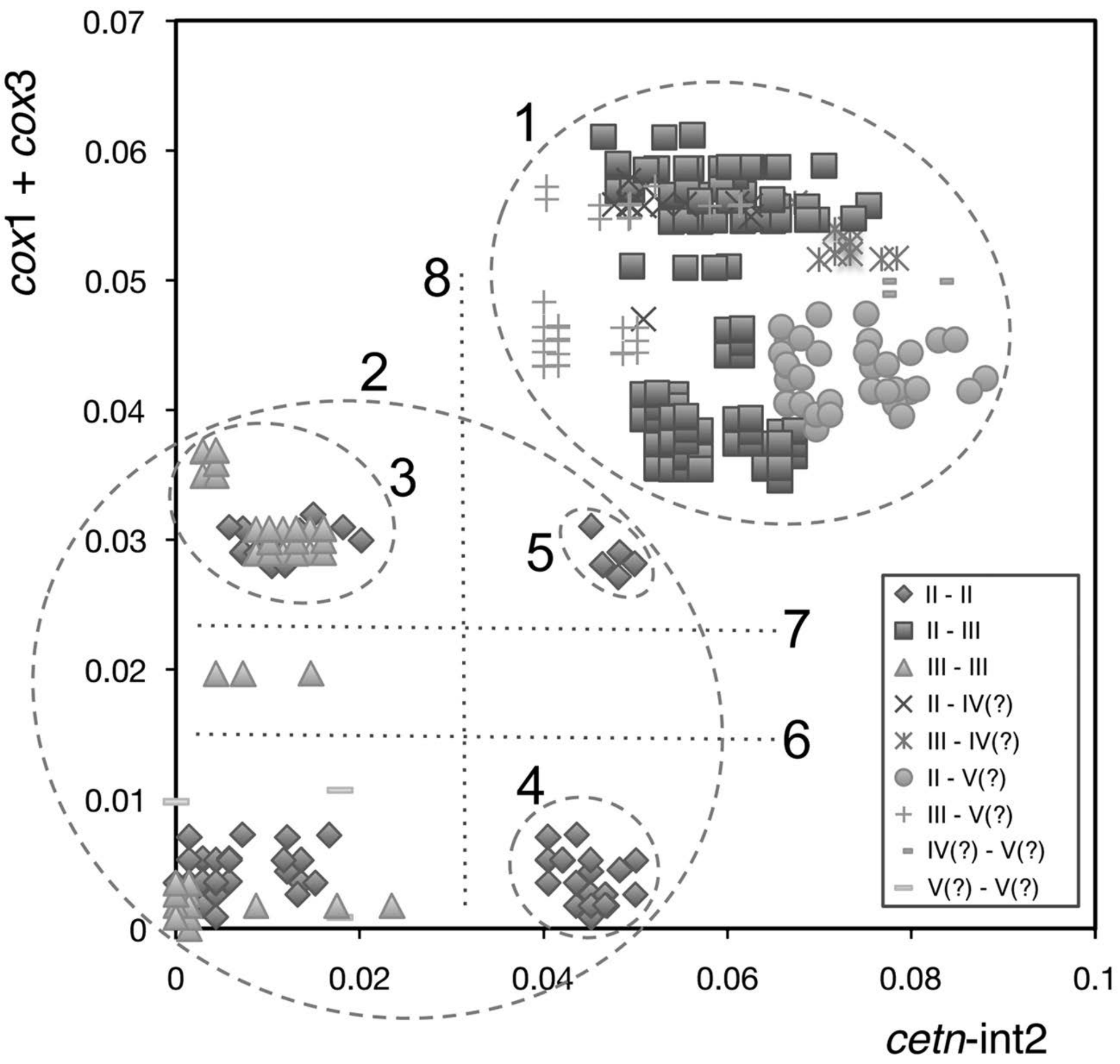

mt
it
cn
91/97/87
2-Oshoro 000510-11m
//*
16-Seseki
-/-/83
20-Tappizaki-1f
5-Matsumae
-/-/77
20-Tappizaki-2m
2-Oshoro 980407-8f
-/93/98
2-Oshoro 980407-9m
15-Hanasaki-2
17-Saruru
-/86/83
2-Oshoro 980407-6m
2-Oshoro 000510-3f
18-Oumu
96/99/94
22-Himi-2
91/-/-
13-Kakijima
23-Mitsumatsu-7f
25-Tsuyazaki-4f
-/78/71
22-Himi-1
70/72/-
23-Mitsumatsu-1m
23-Mitsumatsu-8f
99/*/*
23-Mitsumatsu-11m
//*
//99
23-Mitsumatsu-2f
//*
23-Mitsumatsu-3m
99/*/95
9-Muroran-1f
11-Erimomisaki
12-Shoya
4-Setana
15-Hanasaki-1
84/-/-
98/*/98
1-Asari-6m
1-Asari-7f
-/77/-
2-Oshoro 881025-Cr5m
2-Oshoro 980407-1f
//*
19-Kitami-Esashi
25-Tsuyazaki-1m
30-Katsuura-1m
K
L
M
P
S
R
Q
N
T
U
A
E
B
G
F
D
C
H
II
III
IV(?)
V(?)
I
0.005 substitutions/site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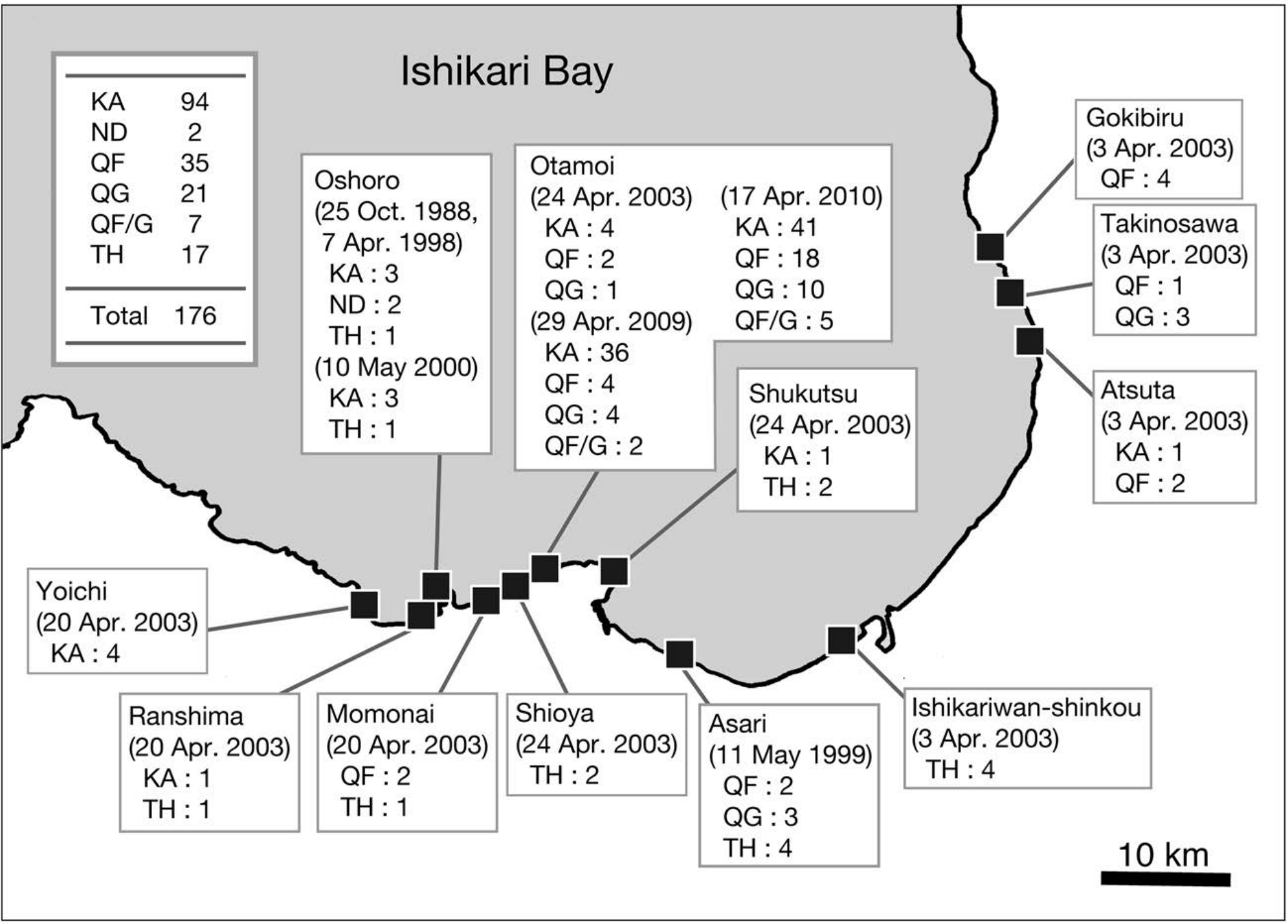
0.06
0.05
0.04
0.03
0.02
0.01
0
cox1 + cox3
0
0.02
0.04
0.06
0.08
0.1
cetn-int2
1
2
3
4
5
6
7
8
II - II
II - III
III - III
II - IV(?)
III - IV(?)
II - V(?)
III - V(?)
IV(?) - V(?)
V(?) - V(?)


Ishikari Bay
KA 94
ND 2
QF 35
QG 21
QF/G 7
TH 17
Total 176
Oshoro
(25 Oct. 1988,
7 Apr. 1998)
KA : 3
ND : 2
TH : 1
(10 May 2000)
KA : 3
TH : 1
Otamoi
(24 Apr. 2003)
KA : 4
QF : 2
QG : 1
(29 Apr. 2009)
KA : 36
QF : 4
QG : 4
QF/G : 2
(17 Apr. 2010)
KA : 41
QF : 18
QG : 10
QF/G : 5
Shukutsu
(24 Apr. 2003)
KA : 1
TH : 2
Gokibiru
(3 Apr. 2003)
QF : 4
Takinosawa
(3 Apr. 2003)
QF : 1
QG : 3
Atsuta
(3 Apr. 2003)
KA : 1
QF : 2
Yoichi
(20 Apr. 2003)
KA : 4
Ranshima
(20 Apr. 2003)
KA : 1
TH : 1
Momonai
(20 Apr. 2003)
QF : 2
TH : 1
Shioya
(24 Apr. 2003)
TH : 2
Asari
(11 May 1999)
QF : 2
QG : 3
TH : 4
Ishikariwan-shinkou
(3 Apr. 2003)
TH : 4
10 km