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Host genetics and *Symbiodinium* D diversity in a stress-tolerant scleractinian coral, *Oulastrea crispata*, in the West Pacific

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ABSTRACT: Determination of the genetic diversity and structure of coral populations across their biogeographic range must include the investigation of the coral host and its associated *Symbiodinium*. We examined the genetic similarity of the stress-tolerant coral *Oulastrea crispata* and the diversity of *Symbiodinium* D across part of their geographic distribution, which ranges across 5800 km in the West Pacific from tropical Thailand (~7° N) to the outlying regions of temperate Japan (36°N). F_{ST}-statistics and AMOVA of directly sequenced coral ribosomal internal transcribed spacer (ITS) DNA sequences showed a high genetic homogeneity between temperate and subtropical populations, but showed a significant difference between temperate and subtropical populations and their tropical counterparts. Denaturing gradient gel electrophoresis (DGGE) of ITS DNA sequences identified 4 major *O. crispata*-associated *Symbiodinium* D types: D8, D8–12, D12–13, and D15; these were found in the regions extending from tropical Thailand towards the high latitude regions of Japan. F_{ST}-statistics and AMOVA of *Symbiodinium* ITS showed significant differences between tropical, subtropical, and temperate regions, with the D8 and D8–12 dominant in the tropical and subtropical regions, the D12–13 endemic in the subtropical northern South China Sea, and D15 restricted to the high-latitudinal outlying coral communities. Consistent variation in environmental factors, such as temperature and light, may have driven the regional-specific divergence of the *Symbiodinium* D types, suggesting that habitat-specific *Symbiodinium* types can assist *O. crispata* in acclimating to the environmental fluctuations found in the marginal range of coral distribution.

KEY WORDS: Coral–*Symbiodinium* · Clade D · Stress tolerance · Genetic subdivision · DGGE · ITS

INTRODUCTION

Corals, together with their endosymbiont dinoflagellates of the genus *Symbiodinium*, constitute part of the coral holobiont (Brown 1997). Coral holobiont plays an important role in building the coral reef ecosystem throughout tropical and subtropical waters between the equator and both the Tropic of

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Cancer and the Tropic of Capricorn (Veron 1995, Veron & Stafford-Smith 2000). In some exceptional cases, non-reefal coral communities can be established beyond this boundary and extend to high latitudinal regions, such as Japan, due to the influence of strong, warm oceanic current (e.g. Kuroshio) transportation (Veron 1995; also reviewed in Chen & Keshavmurthy 2009). The diversity of coral species gradually declines between coral reefs at the equator and non-reefal coral communities and outlying coral patches in high latitude regions (Veron 1995). This decline is likely due to either the dispersal limit of corals and their symbionts or to the fluctuation of environmental factors, such as temperature, light, turbidity, and habitat availability across the region; these factors constrain the physiological ability of corals to cope with these environmental changes. Therefore, even if coral species could reach high latitudes, they might not have the ability to survive. However, certain coral species (for example, *Acropora* sp., *Stylophora* sp., *Pavona* sp.) do show a wide geographic distribution beyond these environmental limitations (Chen 1999, Rodriguez-Lanetty & Hoegh-Guldberg 2002, Lien et al. 2007). Knowledge of how populations of those widely distributed corals and their associated symbionts are interconnected throughout the distributional range can be important in the understanding of how host–*Symbiodinium* interactions allow colonies to survive under different environmental conditions, and in particular how species might cope with the ongoing climate change (Hoegh-Guldberg et al. 2007, Smale et al. 2011).

Genetic analyses of coral holobionts performed in the last few decades have mainly focused on coral hosts. Van Oppen & Gates (2006) indicated that, depending on species and markers used, connectivity of scleractinian corals could range from tens to hundreds of kilometers. Allozyme assays of *Mycedium elephantotus* from the West Pacific show high gene flow when the populations are found within 50 km of each other, but allozyme assays of *M. elephantotus* from Taiwan show moderate to low gene flow when the populations are separated by 400 km (Dai et al. 2000). On the other hand, *Goniastrea aspera* from the Ryukyu Archipelago in Japan shows moderate to high gene flow when at the range of 500 km (Nishikawa & Sakai 2003). Pairwise F_{ST} values generated from allozyme data from *Acropora hyacinthus* and *A. cytherea* sampled from the east and west coasts of Australia (which are 3500 km apart from each other) were not significantly different from zero, while *A. latistella* sampled from the same geographical locations showed evidence for restricted gene flow (Márquez et al. 2002). The use of fast-evolving microsatellite markers showed no significant genetic structuring over large geographical scales (ranging from 770 to 1800 km) within the eastern or western Caribbean cluster of *A. palmata* populations (Baums et al. 2005). The use of ribosomal internal transcribed spacer (ITS) DNA sequences has identified high genetic similarity in a widely distributed coral, *Plesiastrea versipora,* along the Ryukyu Archipelago (which is 700 km in length); however, there are strong geographical associations among populations along the southeast coast of Australia, which stretches over 4000 km (Rodriguez-Lanetty & Hoegh-Guldberg 2002). High gene flow of another widespread species, *Stylophora pistillata*, has been detected across the populations collected from the Ryukyu-Archipelago in Japan and from the Great Barrier Reef (GBR), which covers a geographic distance of 7000 km (Takabayashi et al. 2003).

Despite the fact that coral hosts can be distributed across a wide geographic region without showing distinct population subdivision, their symbiotic partners *Symbiodinium* spp. might contribute to the acclimatization of coral holobionts to the local environmental condition (Rodriguez-Lanetty et al. 2001, Chen et al. 2003, Lien et al. 2007, Baums et al. 2010, LaJeunesse et al. 2010). So far, 9 phylogenetic clades of *Symbiodinium* (Clades A–I) have been characterized based on nuclear ribosomal DNA and chloroplast DNA, with each clade containing many species (types) (reviewed in Rowan 1998, LaJeunesse 2002, Baker 2003, Pochon & Gates 2010). The genetically diverse nature of *Symbiodinium* may have distinct physiological properties, and the tolerance of reef corals may vary according to the association established. For example, *Symbiodinium* Clade C is the most speciose and cosmopolitan zooxanthellae associated with reef corals in both the Indo-Pacific and Caribbean regions. *Symbiodinium* Clade D is generally found in corals living in marginal habitats, such as very shallow water or turbulent, deep waters, and is probably more tolerant of stressors (LaJeunesse et al. 2010, but see Stat & Gates 2011) such as irradiation, temperature, and mechanical disturbance than other clades living in harsh environments (Toller et al. 2001, Chen et al. 2003, 2005a; reviewed in Baker 2003, Knowlton & Rohwer 2003, Keshavmurthy et al. 2012). Symbiosis of these diverse *Symbiodinium* clades or types may help corals survive across the geographic boundary

where environmental factors fluctuate dramatically (Finney et al. 2010). *Plesiastrea versipora* colonies from subtropical and tropical waters contain *Symbiodinium* C, while *P. verspora* colonies at high-latitude sites contain Clade B (Rodriguez-Lanetty et al. 2001). *Symbiodinium* D is the dominant clade in *Oulastrea crispata,* which occurs from the tropical reefs to the marginal non-reefal coral communities. Several colonies of tropical populations were associated with *Symbiodinium* C alone, and several were associated with both *Symbiodinium* C and D simultaneously (Lien et al. 2007). LaJeunesse et al. (2010) discovered a high frequency of *Symbiodinium* D associations with corals from the turbid and high temperature northeastern Indian Ocean compared to their counterparts in the western Indian Ocean, suggesting that these symbioses are ecologically and evolutionarily responsive and can thrive under various environmental conditions.

In the present study, we examined the genetics of the stress-tolerant coral host species *Oulastrea crispata* and its associated symbiont *Symbiodinum* D across part of their distribution range from tropical peninsular Thailand (~7°N) to the temperate outlying coral communities in Japan (~36°N). *O. crispata* is a simultaneous hermaphrodite with an annual gametogenic cycle and extended spawning period from July to October (Nakano & Yamazato 1992, Lam 2000). This coral is capable of releasing eggs and sexual planulae (without *Symbiodinium*) and asexual planulae (with *Symbiodinium*) and is considered to be both a broadcast spawner and a planula brooder (Nakano & Yamazato 1992). Due to its opportunistic life history trait, this coral is able to colonize wide range of substrata, including those unfavorable to other corals (Lam 2000). *O. crispata* is one of a few coral species that are found from the tropical Indo-West-Pacific to high latitudes around Japan (Veron & Stafford-Smith 2000), indicating its endurance to a wide range of environmental factors over a large geographic area, which might also be attributed to its stable association with *Symbiodinium* D, both spatially and temporally, in the West Pacific (Chen et al. 2003, Lien et al. 2007).

MATERIALS AND METHODS

Study sites, sample collection, and analysis of environmental data

Study sites are described in Fig. 1 and were modified from Lien et al. (2007) by adding one sampling from Maizuru in the Sea of Japan. Our sampling strategy was intended to cover the latitudinal distribution of *Oulastrea crispata* from tropical reefs to the outlying non-reefal coral communities (Fig. 1). Colonies of *O. crispata* were sampled from shallow reef flats and rocky banks (~1–3 m) at 16 localities in the West Pacific and were gathered between 2001 and 2006 (see Table 1). These sampling sites included outlying coral communities in temperate Japan, coral reefs in subtropical Japan, non-reefal

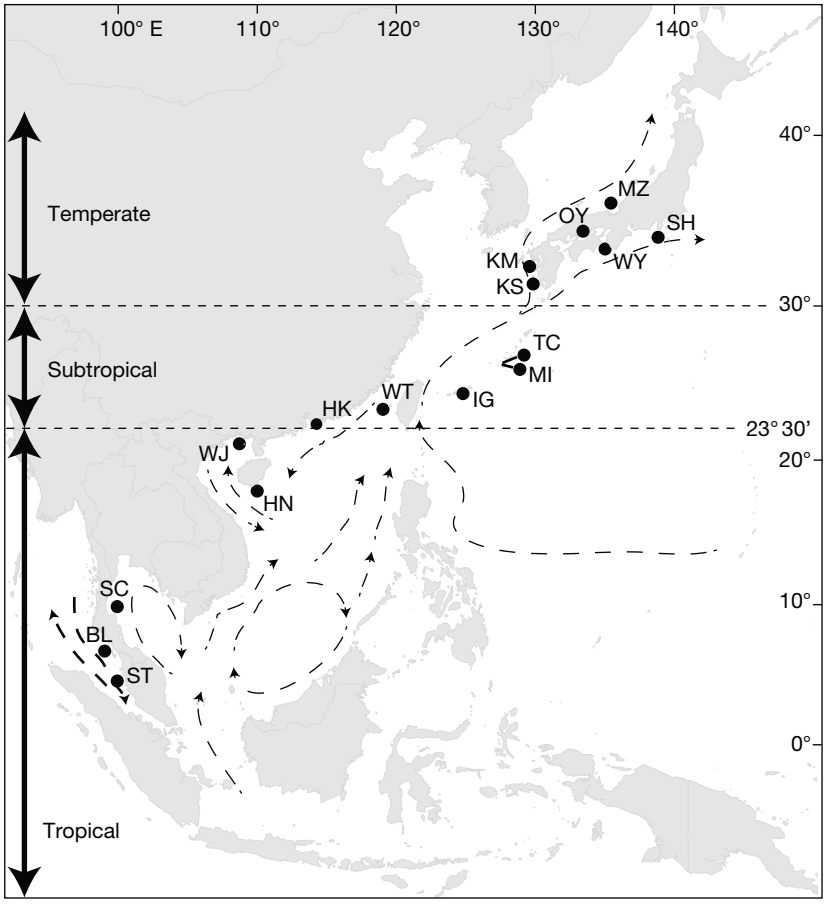

Fig. 1. *Oulastrea crispata* sample locations (●) and summer sea surface currents (dashed arrows; modified from Chen et al. 2004 and Chen & Keshavmurthy 2009). Samples were collected from temperate regions (SH: Shimoda; MZ: Maizuru; OY: Okayama; WY: Wakayama; KM: Kumamoto; KS: Kagoshima), subtropical regions (TC: Toguchi; MI: Miyagi; IG: Ishigaki; WT: Watung; HK: Hong Kong), and tropical regions (WJ: Weijhou Island; HN: Hainan Island; SC: Sichiang; BL: Bulon; ST: Satun)

coral communities in the subtropical South China Sea, coral reefs off tropical South China, and tropical coral reefs in the Andaman Sea in Thailand. Since *O. crispata* is not the dominant coral species in most of the coral communities we visited, we maximized sampling efforts by carrying out at least 3 dives per site, thereby ensuring coverage of the survey areas. Immediately after collection, coral samples were placed in labeled bags, and preserved in 95% (v/w) ethanol.

The monthly means of sea surface temperature (SST), photosynthetically active radiation (PAR), patterns of chlorophyll *a* (chl *a*) concentrations, and colored dissolved organic matter (CDOM) adjacent to the sampling localities for the years 2003 to 2010 were acquired from the Giovanni online data system, which is developed and maintained by the NASA Goddard Earth Sciences Data and Information Center (http://gdata1.sci.gsfc.nasa.gov/daac-bin/G3/gui.cgi?instance_id=ocean_month. Data were accessed and downloaded between 5 and 12 May 2010).

One-way analysis of variance (ANOVA) was conducted to analyze the SST, PAR, chl *a*, and CDOM values between sites, and a Bonferroni test was used to further examine any significant differences in environmental data between latitudinal regions.

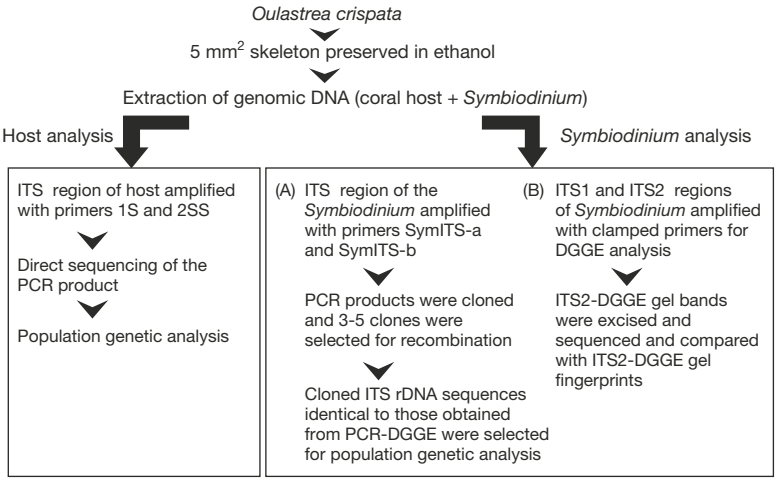

Fig. 2. Flow-chart of the methods used for coral host *Oulastrea crispata* and *Symbiodinium* DNA analysis

DNA extraction, PCR amplification, and sequencing

Flow-chart of the method used for extraction, amplification, and sequencing and analysis of host and *Symbiodinium* DNA is shown in Fig. 2. Approximately 5 mm^2 of ethanol-preserved skeleton with tissue was ground into powder using liquid nitrogen. Total DNA was extracted from the powder with a plant genomic DNA Extraction System (Viogene) according to the manufacturer's instructions. The full length of the internal transcribed spacer (ITS) from ribosomal DNA (rDNA) sequences, including the 5.8S region, was amplified from the coral *Oulastrea crispata* using the metazoan-specific primers 1S (5′-GGT ACC CTT TGT ACA CAC CGC CCG TCG CT-3′) and 2SS (5′-GCT TTG GGC GGC AGT CCC AAG CAA CCC GAC TC-3′) (Odorico & Miller 1997). All polymerase chain reactions (PCR) were carried out as described in Chen et al. (2005a,b), Wei et al. (2006), and Lien et al. (2007). Most coral ITS sequences require cloning and sequencing to avoid the problem of intragenomic variation caused by multiple arrays of rDNA. However, the amplified ITS rDNA of *O. crispata* could be directly sequenced from both directions. A *Symbiodinium*-specific primer set was used to amplify the ITS rDNA of *Symbiodinium* D: SymITS-a (5′-GTT TCC GTA GGT GAA CCT GC-3)′ and SymITS-b (5′-GCG GGT TCA CTT GTC TGA CT-3′). An initial attempt to directly sequence the PCR products was not successful because multiple *Symbiodinium* D types were associated with *O. crispata* (see 'Results'). Thus, the cloning of *Symbiodinium* ITS sequences was performed according to the protocols described in Wei et al. (2006). Three to 5 clones were selected for each recombination to ensure that the diversity of *Symbiodinium* D types was accurately covered. All coral host and *Symbiodinium* ITS DNA sequences obtained in this study were deposited into GenBank under the accession nos. JX415550–JX415818.

Denaturing gradient gel electrophoresis and sequencing of *Symbiodinium* D type diversity

Although cloning techniques can help overcome the problem of multiple divergent DNA sequences of a PCR product, they can also introduce errors during the recombination process that can jeopardize *Symbiodinium* type identification by producing unexpected variation (Thornhill et al. 2007). Thus, denaturing gradient gel electrophoresis (DGGE), as described by LaJeunesse (2002), was used for *Symbiodinium* phylotyping of the subsamples selected from populations of *Oulastrea crispata* representing the tropical, subtropical, and temperate regions of

our sampling range. The ITS1 and ITS2 regions were amplified using clamped primers as described in LaJeunesse & Trench (2000) and Goulet et al. (2008) and with touch-down PCR as described by LaJeunesse (2002). A CBS Scientific system was used to subject the PCR products to 15–16 hours of electrophoresis on denaturing gradient gels (45–80 %). Gels were stained with SYBR Green (Molecular Probes) for 20 min and were photographed for further analysis. PCR bands of each characteristic ITS-DGGE fingerprint were excised, eluted overnight in DNase-free H_2O, re-amplified using the reverse primer without the GC-rich clamp (using a 52°C annealing temperature for 40 cycles), and directly sequenced. Both dominant bands and bands mainly present in the lower portions of the gel were excised for characterization of a *Symbiodinium* type, thus minimizing the sequencing of heteroduplexes that run higher in the gel due to their lower melting characteristics (Myers et al. 1989). To identify the *Symbiodinium* D types, the sequences of diagnostic bands, and the accompanying ITS2-DGGE fingerprints, were compared to each other and to *Symbiodinium* from other regions that had been previously characterized. A specific alpha-numeric designation was given to each new fingerprint found based on methods described in LaJeunesse et al. (2010). The capital letter refers to the clade, and it is followed by a number that pertains to a new unique ITS sequence that is diagnostic of the fingerprint profile.

Sequence alignment, ITS diversity, and minimum spanning network

A combination of ITS-1 and ITS-2 (hereafter ITS) was used for the following analyses of *Oulastrea crispata* and the *Symbiodinium* D types. The 5.8S rDNA was not included in the analyses because it is identical among samples. The sequences were edited using SeqMan software (version 5.05, DNA-Star). Multiple sequence alignments were performed using CLUSTAL XI 2.0 (Thompson et al. 1997) as implemented in the software SeqApp 1.9. To evaluate the polymorphism of the ITS marker, nucleotide content (GC%), substitutions (transitions and transversions), and haplotypic diversity (*h*) were calculated. To examine the average number of nucleotide differences per site between 2 sequences, nucleotide diversity (π) was also calculated in this analysis. Both haplotypic and nucleotide diversity were calculated using DNASP3.35 (Rozas & Rozas 1999). Minimum spanning networks (MSN) were drawn by hand (Rohlf 1973) based on the pairwise distances between all pairs of haplotypes, which were obtained using ARLEQUIN 3.11 (http://cmpg.unibe.ch/software/arlequin3/).

Analysis of coral ITS rDNA

Although ITS rDNA belongs to a multi-copy gene family, direct sequencing shows clear signals in each individual coral, which suggests that intragenomic homogenization is completed across different cistrons of ITS within the *Oulastrea crispata* nuclear genome. Therefore, for the purpose of population genetic analyses, ITS sequences were treated as originating from a single-copy locus. Two statistical analyses were performed using ARLEQUIN. First, the total genetic variation of populations was computed based on the haplotype frequency distribution analysis (pairwise F_{ST}-statistics; Slatkin 1995) using inter-haplotype sequence divergence. The inter-haplotype sequence divergence was corrected for by the Kimura 2-parameter model (Kimura 1980), which incorporates the different ratios of nucleotide transition and transversion. This analysis helps to determine the frequency of genetic drift and inbreeding between populations. The statistical significance of F_{ST} values was estimated by comparing them to the observed distribution generated by randomly distributing individuals into populations to create 10 000 different permutations. The significance threshold of pairwise comparisons ($p < 0.05$) was always adjusted by sequential Bonferroni corrections (Rice 1989). For the second statistical analysis, the extent of differences between populations was examined by analysis of molecular variance (AMOVA; Excoffier et al. 1992). Three hierarchical levels of population structure were computed based on 3 fixation indices: among groups (Φ_{CT}), among populations within groups (Φ_{SC}), and within populations (Φ_{ST}). In this study, 2 hypotheses were defined: (1) climate zone based on geographical regions (i.e. temperate, subtropical, and tropical regions) will correspond to differences in host and/or *Symbiodinium* haplotypes; (2) sea surface currents could be the main factor underlying the genetic separation of these populations. These sea surface current patterns include the Kuroshio Current, the South China Sea Surface Current, and the Andaman Sea Current. The South China Sea Surface Current can be divided into the Gulf of Thailand (SC), the western South China Sea (WJ, HN), and the northern South China Sea (WT, HK). The map of sea surface currents shown in Fig. 1, which is modified from Chen et al. (2004) and Chen & Keshavmurthy (2009), is for the

summer season, when coral larvae are produced. However, defining sea surface currents was complex. In the northern West Pacific, the Kuroshio Current is divided into the main branch, which flows into the West Pacific, and the weak branch, which flows into the Sea of Japan as the Tushima Warm Current (MZ and KM). The weak currents and strong cold fronts cause the SST at the coast of the Sea of Japan to be lower than 12°C in February.

Analysis of *Symbiodinium* D ITS rDNA

To exclude errors introduced by the cloning technique, several steps were adopted to filter the cloned ITS rDNA of *Symbiodinium* D types in preparation for population genetic analysis. First, the ITS rDNA sequences obtained from PCR-DGGE were aligned with those obtained by cloning. Only the cloned ITS rDNA sequences that were identical to those obtained from PCR-DGGE were selected for population genetic analysis, i.e. those ITS rDNA sequences carrying extra mutations were recognized as either due to a very low frequency or as errors derived from the cloning procedure (Thornhill et al. 2007). Although this practice may underestimate the genetic diversity, it does, however, exclude error signals introduced by the cloning technique. The second strategy used to exclude errors was to subject the selected ITS rDNA sequences for each population to *F*-statistics and AMOVA in the manner described for the analysis of coral ITS rDNA. The frequency of every PCR-DGGE identified ITS rDNA sequence from the sampled populations were plotted to visualize the distribution of these *Symbiodinium* D types across different climatic regions.

RESULTS

Regional environmental differences

Average monthly SST, PAR, chl *a*, and CDOM were significantly different among tropical, subtropical, and temperate regions in the West Pacific (Fig. 3). The analysis of monthly mean SSTs clearly indicated

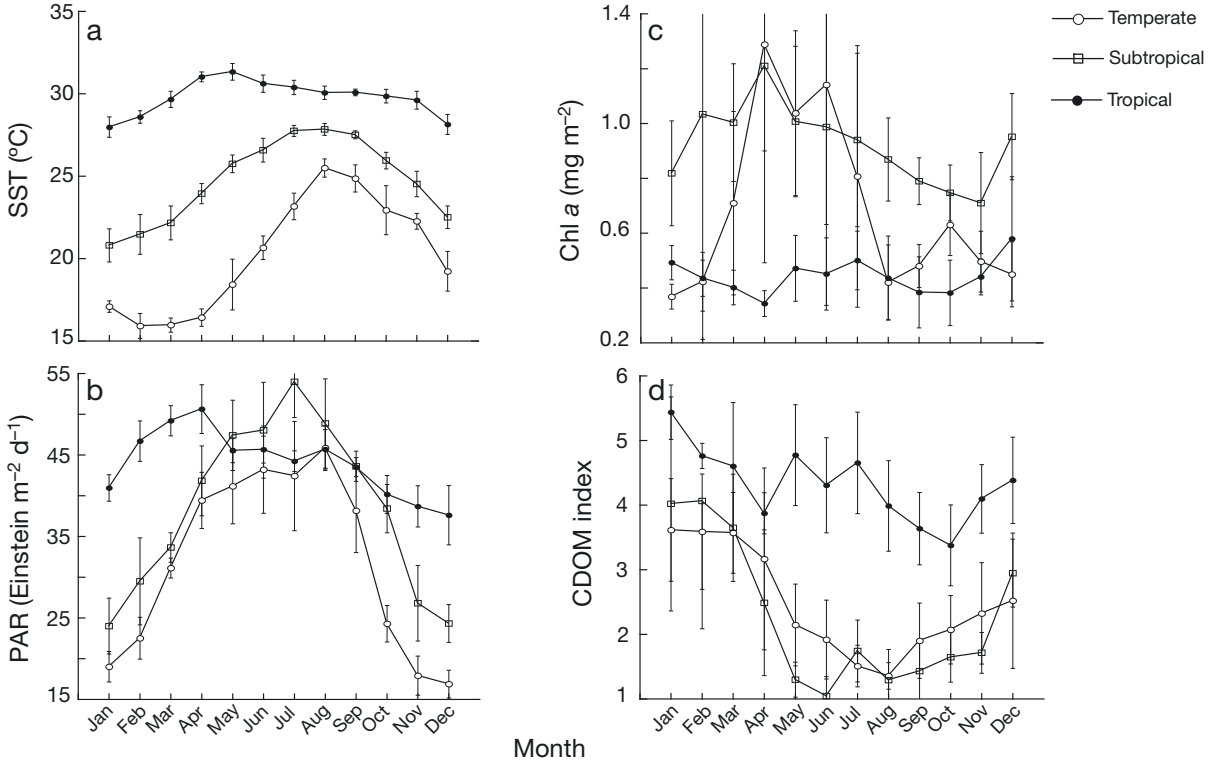

Fig. 3. Monthly averages (±SD) of (a) sea surface temperatures (SST), (b) photosynthetically active radiation (PAR), (c) patterns of chlorophyll *a* (chl *a*) concentrations, and (d) colored dissolved organic matter (CDOM) for temperate (Shimoda, Japan), subtropical (Penghu, Taiwan), and tropical (Sichiang, Thailand) regions. Data are satellite measurements for the years January 2003 to December 2009, acquired from the Giovanni online data system (maintained by the NASA Goddard Earth Sciences Data and Information Center)

a significant seasonal pattern of temperature fluctuations among the regions (1-way ANOVA, $F = 41.646$, $p < 0.001$); the mean SSTs ranged from 20.2°C in Shimoda, Japan, and 24.7°C in Penghu, Taiwan, to 29.8°C in Sichiang, Thailand (Fig. 3a). In high-latitude Japan, the lowest mean SST (15.9°C) was observed in Shimoda in February, and SSTs below 18°C could last for up to 5 mo. SSTs in tropical reefs showed little fluctuation through the seasons and were significantly higher than those of temperate and subtropical regions (Bonferroni test, $p < 0.001$). The PAR and CDOM means were not significantly different between Penghu and Shimoda, but those of Penghu and Shimoda were significantly different from those of Sichiang (1-way ANOVA, PAR $F = 5.409$, CDOM $F = 20.456$, Bonferroni test, both $p < 0.001$). In contrast, chl *a* means from Penghu were significantly higher than those from Sichiang and Shimoda (1-way ANOVA, $F = 16.792$, Bonferroni test, $p < 0.001$).

ITS variability of *Oulastrea crispata* in the West Pacific

A total of 125 complete sequences of ITS-1 and ITS-2 combined (382 bp) were obtained from colonies of *Oulastrea crispata* in the West Pacific. These 125 sequences contained 31 haplotypes. The average GC content of the ITS sequences was 56.6% and the sequences obtained contained 13 transitions and 11 transversions in total. Haplotypic diversity (*h*) ranged from 0.4 for the Sichiang population to 1.0 for the Maizuru population, and the average was 0.887. Nucleotide diversity (π) ranged from 0.00132 for Miyagi to 0.00968 for Weijhou Island, and the average was 0.00548 (Table 1).

MSN analysis showed that 4 haplotypes, A, B, C, and D, accounted for 40.8% of ITS DNA sequences collected, and that those 4 haplotypes were dominant haplotypes in *Oulastrea crispata* (Fig. 4a). Haplotype A was the only haplotype shared by *O. crispata* from the 3 zones, but most (9/13) of the samples containing this haplotype were from Sichang Island in the Gulf of Thailand. Haplotype B was found in populations from temperate and subtropical regions, whereas Haplotypes C and D were found in populations from the tropics and subtropics.

Pairwise F_{ST}-statistics (Table 2) showed significant population differentiation of *Oulastrea crispata* between tropical and temperate populations ($F_{ST} = 0.0784$, $p < 0.05$) and between subtropical and tropical populations ($F_{ST} = 0.0758$, $p < 0.05$), but no significant population differentiation was found between temperate and subtropical populations ($F_{ST} = 0.0124$, $p = 0.150$). AMOVA testing (Table 3) between latitu-

Table 1. *Symbiodinium* D and *Oulastrea crispata*. Collection sites, sample size (n), number of substitutions (*Ti*: transition; *Tv*: transversion), number of haplotypes (n_h), haplotypic diversity (*h*), and nucleotide diversity (π) were calculated using DNASP 3.53 (Rozas & Rozas 1999). –: no data

Population	Latitude, longitude	*Oulastrea crispata*					*Symbiodinium* D				
		n	*Ti*	*Tv*	n_h (*h*)	π	n	*Ti*	*Tv*	n_h (*h*)	π
Temperate											
Shimoda (SH)	34° 40′ N, 138° 57′ E	8	3	1	4 (0.786)	0.00355	12	11	7	10 (0.970)	0.00774
Maizuru (MZ)	35° 29′ N, 135° 23′ E	4	2	1	4 (1.000)	0.00493	6	5	5	6 (1.000)	0.00788
Okayama (OY)	34° 43′ N, 134° 21′ E	9	3	2	4 (0.583)	0.00343	10	11	4	8 (0.972)	0.00722
Wakayama (WY)	34° 04′ N, 135° 02′ E	11	1	3	4 (0.764)	0.00362	16	10	7	10 (0.857)	0.00502
Kumamoto (KM)	32° 55′ N, 130° 25′ E	10	1	6	5 (0.756)	0.00460	12	10	2	10 (0.982)	0.00550
Kagoshima (KS)	31° 25′ N, 129° 43′ E	–	–	–	–	–	6	4	3	6 (1.000)	0.00552
Subtropical											
Miyagi (MI)	26° 62′ N, 128° 18′ E	8	0	2	3 (0.464)	0.00132	12	11	3	9 (1.000)	0.00854
Toguchi (TC)	26° 65′ N, 127° 09′ E	10	1	4	6 (0.844)	0.00341	11	9	6	9 (0.978)	0.00746
Ishigaki (IG)	24° 22′ N, 124° 01′ E	10	2	3	6 (0.844)	0.00430	8	12	1	7 (0.964)	0.00768
Watung (WT)	23° 39′ N, 119° 34′ E	12	1	3	5 (0.742)	0.00379	11	12	6	10 (0.982)	0.00847
Hong Kong (HK)	22° 27′ N, 114° 20′ E	5	1	4	4 (0.900)	0.00591	4	4	1	4 (1.000)	0.00630
Tropical											
Weijhou Island (WJ)	21° 10′ N, 109° 15′ E	6	4	5	4 (0.867)	0.00968	7	7	7	6 (0.952)	0.01058
Hainan Island (HN)	20° 05′ N, 110° 17′ E	5	0	2	3 (0.800)	0.00269	4	2	1	4 (1.000)	0.00355
Sichiang (SC)	13° 08′ N, 100° 51′ E	13	1	2	3 (0.400)	0.00251	8	10	2	6 (0.893)	0.00675
Bulon (BL)	7° 50′ N, 98° 30′ E	3	1	1	2 (0.667)	0.00358	6	6	1	4 (0.800)	0.00473
Satun (ST)	6°43′ N, 100° 04′ E	12	5	1	6 (0.848)	0.00717	10	7	1	7 (0.867)	0.00368
Total		126	13	11	31 (0.887)	0.00548	143	98	43	95 (0.965)	0.00782

dinal groups of populations showed that 75.4% of the variation was within populations, 1.0% was between populations within a region, and 23.6% was between groups, and none of these results were significant (F_{CT} = 0.01, p = 0.337). In contrast, when the sea surface current hypothesis was applied, a significant difference between groups (F_{CT} = 0.1234, p < 0.05) was detected, suggesting that sea surface currents are a better indicator of the connectivity between groups of populations than just latitude. These results support the idea that there is strong genetic homogeneity between subtropical and temperate regions, but that, as revealed by F_{ST}-statistics, there are distinct genetic differences between the subtropical and temperate regions and tropical regions.

ITS variability of *Oulastrea crispata*-associated *Symbiodinium* D in the West Pacific

A total of 143 cloned sequences of ITS (430 bp) were obtained from *Oulastrea crispata*-associated *Symbiodinium* D samples from the West Pacific. These 143 sequences contained 95 haplotypes. The average GC content of the ITS sequences was 53.2%. Transitions were found approximately 2.2 times more frequently than transversions (*Ti* = 98, *Tv* = 43). Haplotypic diversity (*h*) ranged from 0.800 in Bulon, to 1.000 in e.g. Hong Kong, and the average was 0.965. Nucleotide diversity (π) ranged from 0.0355 for Hainan Island to 0.01058 for Weijhou Island, and the average was 0.00782 (Table 1).

To exclude the errors derived from recombinant cloning, ITS-1 and ITS-2 were examined by PCR-DGGE, and bands were cut and eluted for direct sequencing. Four types (D8, D8–12, D12–13 and D15) were identified by the PCR-DGGE band patterns (Table 4). The ITS DNA sequences of D12 and D13 were identical in length and were, therefore,

Table 2. Pairwise F_{ST} values of latitudinal differentiation of ITS in *Oulastrea crispata* (above diagonal) and *Symbiodinium* D (below diagonal) from filtered ITS (D8, D8–12, D12–13 and D15). *Significant exact test (p < 0.05)

Group	Temperate	Subtropical	Tropical
Temperate	–	0.0124	0.0784*
Subtropical	0.5693*	–	0.0758*
Tropical	0.9644*	0.2293*	–

Table 4. *Symbiodinium* type composition in different populations of *Oulastrea crispata*. The frequency of coral colonies containing that particular *Symbiodinium* type is based on the ITS sequences obtained by cloning, which are filtered with the intragenomically dominant ITS1 and ITS2 DNA sequences that were acquired using rDNA-DGGE fingerprinting. These sequences were D8, D8–12, D12–13, and D15

Location	*Symbiodinium* type			
	D15	D8–12	D12–13	D8
Temperate				
Maizuru	2	0	0	0
Shimoda	9	0	0	0
Wakayama	12	0	0	0
Okayama	7	0	0	0
Kumamoto	4	0	0	0
Kagoshima	6	0	0	0
Subtropical				
Miyagi	5	0	0	3
Toguchi	2	3	0	2
Ishigaki	0	0	0	4
Watung	3	0	3	0
Hong Kong	0	1	1	0
Tropical				
Weijou Island	0	2	1	0
Hainan Island	0	0	0	3
Sichiang	0	0	0	4
Satun	0	1	0	2
Bulon	0	2	0	2

Table 3. *Symbiodinium* Clade D with filtered ITS (D8, D8–12, D12–13, and D15) and *Oulastrea crispata*. Hierarchical analysis of molecular variance (AMOVA) within and among 16 populations categorized by latitudinal groups and sea surface current groups

	Latitude			Sea surface currents		
	% variation	Fixation index	p	% variation	Fixation index	p
Oulastrea crispata						
Among groups	1.00	F_{CT} = 0.0100	0.337	12.34	F_{CT} = 0.1234	0.024
Among population within regions	23.63	F_{SC} = 0.2387	0.000	13.94	F_{SC} = 0.1590	0.002
Within population	75.37	F_{ST} = 0.2463	0.000	73.72	F_{ST} = 0.2628	0.000
***Symbiodinium* Clade D**						
Among groups	66.26	F_{CT} = 0.6626	0.000	46.91	F_{CT} = 0.4691	0.004
Among population within regions	6.10	F_{SC} = 0.1809	0.058	23.04	F_{SC} = 0.4339	0.000
Within population	27.64	F_{ST} = 0.7236	0.000	30.06	F_{ST} = 0.6994	0.000

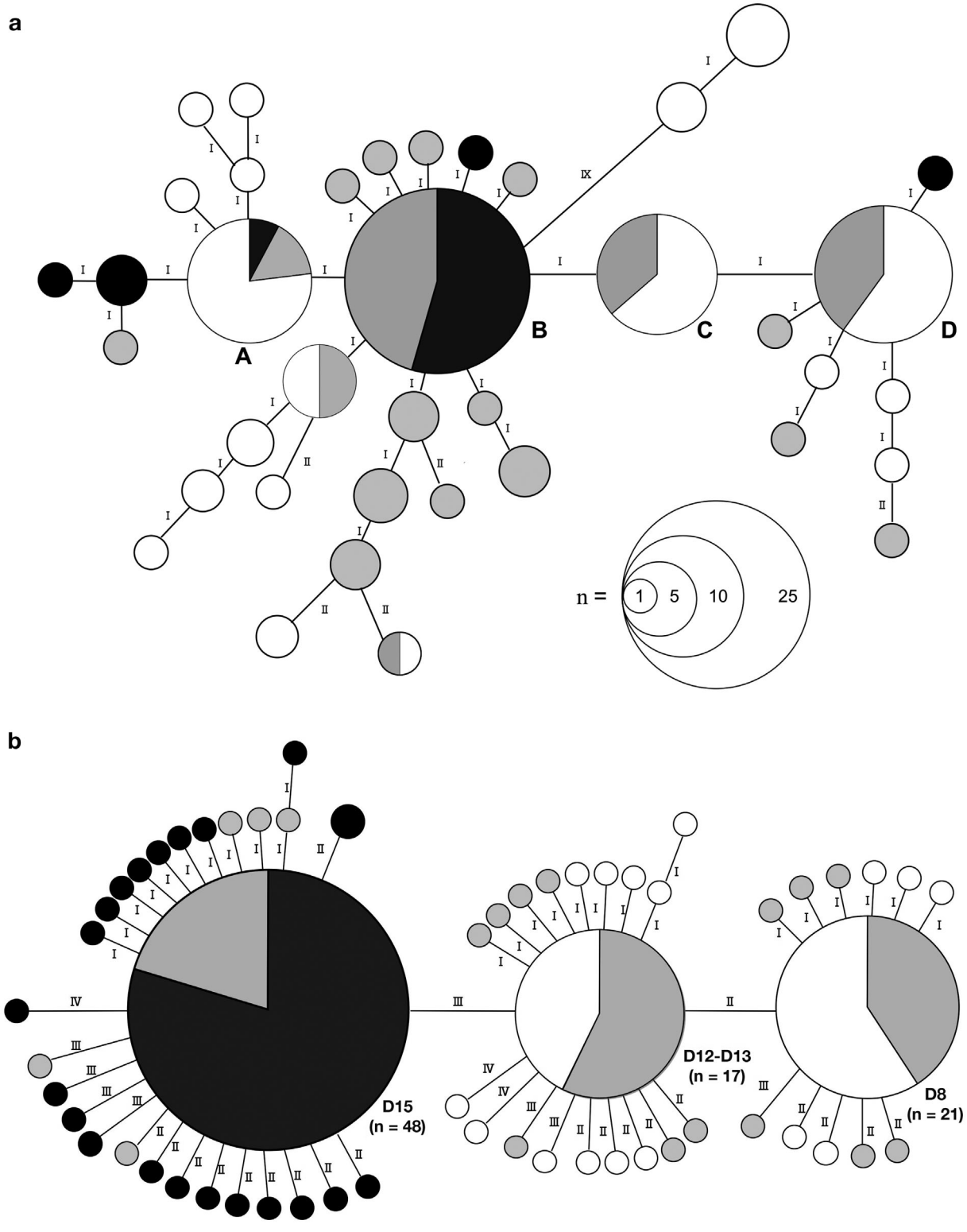

Fig. 4. Minimum spanning network of ITS haplotypes of (a) *Oulastrea crispata* and (b) *Symbiodinium* D. *Symbiodinium* types identified by denaturing gradient gel electrophoresis (DGGE) (see Fig. 5b) are indicated. The size of circles represents the number of individual samples. The number of mutations is indicated by Roman numerals on the branch linking the haplotypes. Climate zones: white: tropical; grey: subtropical; black: temperate

pooled into a D12–13 group for later analysis. D13 differed from D12 by one base pair (C or T) at position 422, confirming the sequence identity, D8 had a CT insertion between positions 331 and 332, and D15 had a GCG insertion at position 252. These 4 directly sequenced ITS sequences were used to filter the cloned ITS sequences. In total, 83 of the 143 (58.04%) cloned ITS DNA sequences were identical to these 4 ITS types and were, thus, used for the following analyses.

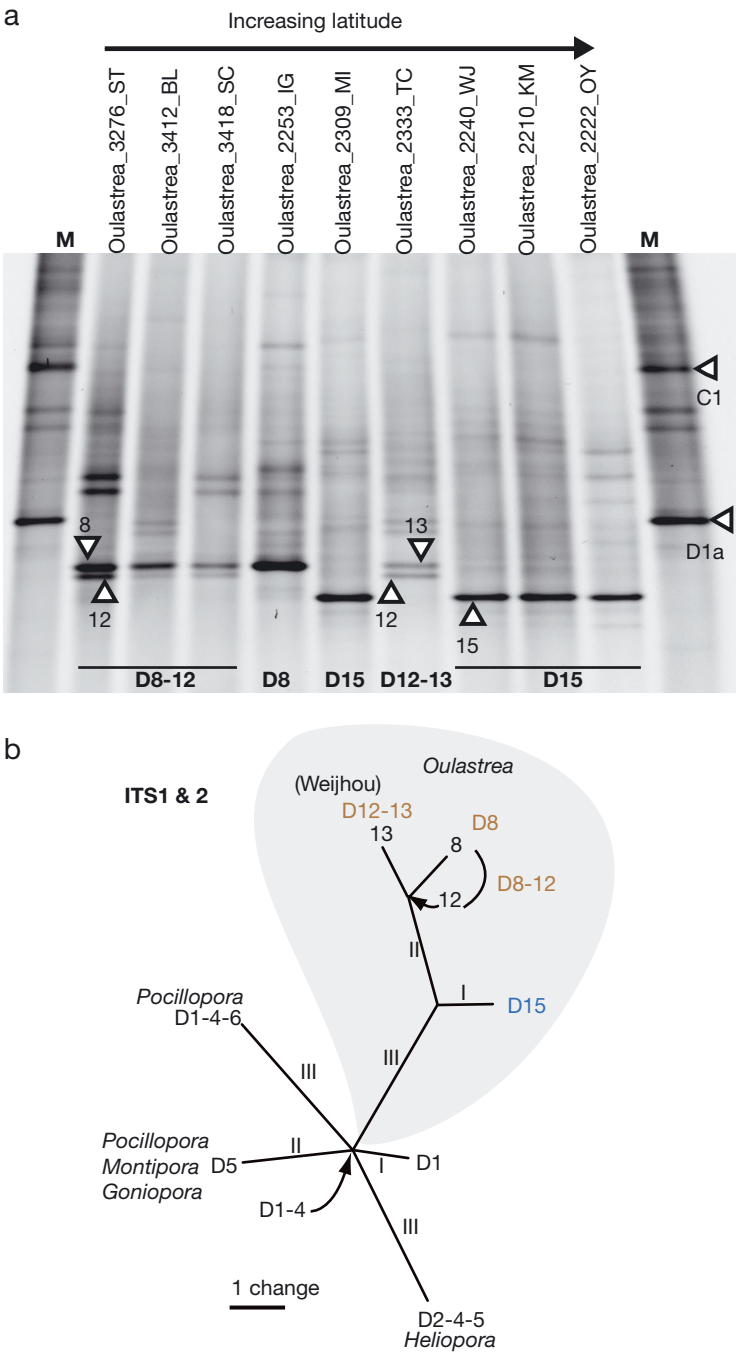

Fig. 5. (a) PCR amplifications of the *Symbiodinium* ITS2 region. PCR products were electrophoresed on denaturing gradient gels to produce diagnostic fingerprints that exhibit its geographic distributions. (b) Phylogenetic relationships among *Symbiodinium* D types based on intragenomically dominant ITS1 and ITS2 sequences acquired using rDNA-DGGE fingerprinting

MSN analysis grouped the above-mentioned 83 *Symbiodinium* D ITS sequences into 3 haplotype groups, which corresponded to the D8, D13/D12–13, and D15 types, and the rest of the cloned ITS sequences could be linked to each of these 3 haplotype groups with only 1 to 4 mutations (Fig. 4b). D15 was the dominant type in the temperate populations and only reached WT levels (Fig. 4b, Table 4). D13/D12–13 types were composed of samples from tropical and subtropical regions (Fig. 4b), and D12–13 had a disjunctive distribution in the Andaman Sea (BL and ST), the northwestern South China Sea (WJ), and the subtropical reefs at IG and TC in Okinawa. Only the D13 type was found in the 'non-reefal' endemic *Symbiodinium* D samples collected from WJ, HK, and WT (Figs. 4b & 5). The D8 type was found mainly in the tropical and subtropical reefs (Fig. 4b, Table 4).

Pairwise F_{ST}-statistics of the 83 filtered ITS sequences of D8, D13, D12–13, and D15 from each population showed significant population differentiations between *Oulastrea crispata*-associated *Symbiodinium* D samples collected from the different climate zones (F_{ST} = 0.2293–0.9644, $p < 0.05$; Table 2). In contrast to the results seen with the host, the hierarchical AMOVA of the latitudinal groups of populations or of the sea surface currents were both significant between groups ($p < 0.05$). However, application of the latitudinal group hypothesis resulted in much more among-group variation (66.2%) than that observed when the sea surface current hypothesis was applied (46.9%) (Table 3).

DISCUSSION

The interactions between the coral hosts and symbiotic algae play an important role in maintaining the coral population and the entire coral reef ecosystem

(reviewed Brown 1997). Thus, the study of coral population diversity must include the investigation of the animal hosts and their symbionts. Our analysis of the genetic similarity of a stress-tolerant coral, *Oulastrea crispata*, showed both genetic homogeneity and similarity across the latitudinal gradient between the tropic, subtropic, and temperate climates indicating a possible effect of environmental factors. In contrast, regional-specific types of *Symbiodinium* D were discovered. These findings have important implications for the adaptation and colonization of *O. crispata* in marginal habitats where most reef-building corals are absent. This also is in concordance with the unique reproductive traits (Nakano & Yamazato 1992, Lam 2000) of being a simultaneous hermaphrodite with an annual gametogenic cycle as well as releasing zooxanthellate sexual and asexual planula.

Genetic differences and homogeneity of the host

Analysis of the host ITS DNA sequences of *Oulastrea crispata* from the West Pacific indicated high genetic similarity between subtropical and temperate populations. However, both subtropical and temperate populations showed distinct population differences as compared to those from tropical regions (Table 2). Environmental factors, such as sea surface temperature, light, and currents (Fig. 1), might play an important role in shaping the genetic composition of *O. crispata* populations across different climate zones in the West Pacific. Indeed, sea surface temperatures were significantly different among temperate, subtropical, and tropical region. However, PAR and CDOM values were similar between the temperate and subtropical regions, and significantly different from those of the tropical regions. Also, the MSN analysis did not group the host ITS sequences into a pattern reflecting the 3 climate zones, suggesting that other factors such as oceanic currents might influence the differences or homogeneity of *O. crispata* host populations. It might be the case that the Kuroshio Current that flows in the West Pacific (reviewed in Chen & Keshavmurthy 2009) may provide the dispersal potential for transportation of *O. crispata* from subtropical to temperate regions. Moreover, in the tropical South China Sea, Gulf of Thailand, and Andaman Sea, current is mainly affected by the monsoon-driven sea surface weather patterns (reviewed in Chen 1999). These patterns could have created the isolation of the tropical populations of *O. crispata* from those found in the subtropical and temperate regions. Similar current-driven connectivity has been suggested to occur in a widespread Indo-Pacific coral, *Plesiastrea versipora* (Rodriguez-Lanetty & Hoegh-Guldberg 2002). *P. versipora* showed no genetic subdivision (through the host ITS DNA sequencing) in the subtropical Ryukyus Archipelago or in populations within the tropical and subtropical GBR, but it did show a strong genetic subdivision between temperate populations from southeastern Australia and populations from the GBR. It has been suggested that restricted genetic connectivity among populations of *P. versipora* from the eastern seaboard of Australia is associated with the surface ocean current that is present (the East Australian Current) along the southwestern Pacific Ocean (Rodriguez-Lanetty & Hoegh-Guldberg 2002).

Our analysis shows that *Oulastrea crispata* might be one contiguous species population, albeit we might have missed the presence of closely related populations due to the low resolution of ITS sequences and lack of suitability of the markers used in the present study for this context owing to the slow evolution of coral genes (reviewed in van Oppen & Gates 2006). ITS nrDNA has been suspected to lack the resolution needed for analysis using population approaches because of their relatively slow mutation rates (Schlötterer et al. 1994, LaJeunesse 2005) and often high levels of intra-individual variation among certain scleractinians (Vollmer & Palumbi 2004, but see LaJeunesse & Pinzón 2007). To determine more definitively whether there are any fine-scale genetic differences in host populations of *O. crispata* along the west Pacific, it might be necessary to use more informative markers such as microsatellites to better resolve genetic structure within and among closely related host populations (Mackenzie et al. 2004, Baums et al. 2005, Magalon et al. 2005). However, studies using fast-evolving markers, such as microsatellites, to investigate corals collected from southwestern Japan (*Acropora digitefera*; Nakajima et al. 2009) or a similar geographic range (*A. cervicornis*; Baums et al. 2005) show that there is high genetic connectivity between populations. This suggests that the lack of genetic structure may be a characteristic of the life history of at least some reef-building corals, regardless of the genetic markers used. We also argue that, although ITS sequences come from ribosomal DNA, which is part of a multi-gene family, intragenomic variation is expected. Extremely high intragenomic variation has been documented in *Acropora* ITS sequences, which employ both hybridization and ancestral polymorphisms as mechanisms to maintain this variation (Vollmer & Palumbi 2004, Wei et al. 2006; reviewed in van Oppen & Gates

2006). However, extensive analysis of ITS DNA sequences from 78 species, representing 28 genera and 12 families of scleractinian corals, shows that ITS sequences are a unique characteristic of *Acropora* that produces high intragenomic variation (Wei et al. 2006). Wei et al. (2006) suggested that with careful evaluation, the analysis of ITS sequences is still applicable in the genetic study of species-specific or intra-species-specific reef-building corals. Also, by screening intragenomic variants, it is possible to retrieve stable genetic signature sequence (LaJeunesse & Pinzón 2007). In *O. crispata,* host ITS DNA sequences were obtained by direct sequencing without further cloning, suggesting that the genome of most individuals was dominated by a single numerically common sequence variants and intragenomic variation was, therefore, not considered in the present study. Similar patterns of ITS sequences were also found in population genetic analysis of *Pavona* spp. (Moothien Pillay et al. 2006).

Symbiodinium D diversity and distribution

Oulastrea crispata was the only coral species found to be specifically associated with the stress-tolerant *Symbiodinum* D types throughout its entire distribution range (Lien et al. 2007). Samples collected from the regions ranging from tropical Thailand to high latitude Japan showed that Clade D was the dominant *Symbiodinium*, although a few colonies from tropical regions were mainly associated with *Symbiodinum* C (Lien et al. 2007). The present study took a step further and examined the fine-scale genetic variation within *Symbiodinum* D. The results indicate that, as proposed by LaJeunesse et al. (2010), *O. crispata*-associated *Symbiodinium* D type exhibits a significant specificity.

Population genetic analysis of the cloned *Symbiodinium* ITS sequences after they had been filtered with the directly sequenced DGGE PCR products showed that there were strong genetic differences within *Symbiodinum* D, and these differences corresponded to the 4 major regional types (D8, D8–12, D12–13, D15). The regional specificity of *Symbiodinum* D types associated with *Oulastrea crispata* resulted in significant differences in the F_{ST}-statistics from the different regions and the intergroup variation of the AMOVA values. The increased intergroup variation that was observed when the latitudinal hypothesis was employed suggested that, as proposed by LaJeunesse et al. (2010), the divergence of D types is driven by the different environments of the different regions. However, the significant Φ_{CT} value observed for the inter-group variation of sea surface currents excluded the possibility of *Symbiodinium* types distribution in the West Pacific by sea surface currents.

High diversity of *Symbiodinium* D has been suggested to result from an adaptive radiation into different types and different host corals that are influenced by environmental differences (LaJeunesse et al. 2010). Studies of *Symbiodinium* diversity in the Andaman Sea have shown that *Symbiodinium 'trenchi'* (D1–4) is ecologically distinguished from other *Symbiodinium* types because it is the only host-generalist symbiont associated with various broadcast-spawning corals; D1–4–6 are associated with *Pocillopora* and *Montipora*; D2–4–5 are associated with the blue coral *Helipora*; D5 is associated with *Gonipora, Montipora,* and *Pocillopora*; and D8 is associated with *Oulastrea*. The combination of persistent high temperatures and variable light conditions as well as ecological specialization to different host taxa has been suggested to facilitate the ecological success and evolutionary radiation of *Symbiodinium* D in the Andaman Sea (LaJeunesse et al. 2010). A similar scenario may be responsible for the divergence of the new type discovered in our study; the D15 type was dominant in the temperate regions potentially due to low temperature and/or light, and the D8 and D8–12 types that were found to extend from tropical Thailand to the subtropical Ryukyus may display this wide range because of their ability to acclimate to a broad range in temperatures and seasonally variable light. On the other hand, the D12–13 types were only found on Weijhou Island, which is likely due to the isolation and restriction of the northern South China Sea currents. Further application of multi-loci genotyping by microsatellites (Pettay & LaJeunesse 2009) is currently underway to investigate these possibilities (Wham et al. 2011).

Symbiodinium D radiation and adaptation of *Oulastrea crispata* holobionts to climate change

Oulastrea crispata is one of a few Indo-Pacific coral species that can extend beyond the biogeographic boundaries of reef-building corals, which implies that *O. crispata* can cope with a wide range of environmental factors over a large geographical area (Veron & Stafford-Smith 2000). Monthly surveys and biogeographic examinations of symbiont communities indicated that *O. crispata* that is associated mainly with *Symbiodinium* D could survive below

temperatures of 18°C without apparent bleaching in subtropical non-reefal and temperate outlying communities (Lien et al. 2007). Ecologically, *O. crispata* is commonly found on shallow reef depressions and on turbid bay bedrock inhabited by only a few other corals (Nakano & Yamazato 1992, Lam 2000). Temperature recordings in Watung indicated that seawater temperatures fluctuated enormously, ranging from 12°C in the winter to 35°C in the summer (Chen et al. 2003). In the high latitudes of Japan, *O. crispata* can be found in habitats where winter water temperatures are commonly ~7–10°C, and air temperatures are several degrees below freezing for approximately 20 days per year (Yajima et al. 1986). Population genetic analysis showed high genetic homogeneity in *O. crispata* between subtropical non-reefal populations and temperate outlying populations, and they were associated with a regional-specific *Symbiodinium* D15, which suggested that D15 is a cold-resistant type that assists coral holobionts to survive in cold marginal habitats. Similar species-specific adaptation to environmental stressors is also found in their counterparts in tropical regions where D8 is the dominant type associated with *O. crispata* (LaJeunesse et al. 2010). Estimates generated by examining the molecular clock of ITS sequences indicate that the ecological success and evolutionary radiation of *Symbiodinium* D that is driven by persistent environmental factors such as the temperature and light levels of different regions might date back to the Pleistocene era approximately 2.6 Myr to 12 000 yr ago (LaJeunesse et al. 2010).

Symbiodinium D was recognized as a 'heat-tolerant' symbiont (Rowan 2004, Oliver & Palumbi 2011). It has been suggested that it may help corals cope with the threat of rising seawater temperatures caused by climate change by 'shuffling' with background populations of symbionts and/or by causing the death of sensitive host–symbiont combinations (Berkelmans & van Oppen 2006, Jones et al. 2008, Sampayo et al. 2008, LaJeunesse et al. 2009, LaJeunesse et al. 2010). However, examining the underlying molecular variation within *Symbiodinium* D indicated that there is a relatively high species-specific and regional-specific diversification of types (or 'species') in this stress-tolerant *Symbiodinium* (LaJeunesse et al. 2009, LaJeunesse et al. 2010). This study provides further evidence that instead of heat-tolerance, some of the D types might have been endemic and adapted to the cold marginal habitats. Current estimates suggest that there will be an increase in temperature of 1.1 to 6.4°C by the end of the century if the atmospheric carbon dioxide level doubles or triples (IPCC 2007). Further examination is needed to determine whether the rising temperature will allow the host-general (D1–4) and host-specific types to survive in tropical regions, cause them to migrate to higher latitude reefs and replace the regional endemic types (D13 and D15), or cause the cold-tolerant D15 type to become extinct.

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