INTRODUCTION

Reef-building corals contain dense populations of “morphologically cryptic” dinoflagellates assigned to the genus Symbiodinium (Freudenthal 1962). These eukaryotic microbial symbionts comprise numerous genetically divergent lineages, or ‘Clades’, several of which are particularly common to various shallow-water cnidarians (living mostly in the photic zones of tropical and subtropical coastal habitats) including Clades A, B, C and D (Coffroth & Santos 2005). These clades are separated by a level of gene sequence divergence that often defines higher-level taxonomic ranks in other dinoflagellates (Rowan & Powers 1992; LaJeunesse 2001; Stern et al. 2010). Detailed genetic analyses, using more rapidly evolving markers of samples gathered over large geographic scales from various host species, find that each Symbiodinium clade contains diverse groups of phylogenetically distinct lineages referred to by researchers as ‘subclades’, ‘types’, ‘species’, or ‘strains’ (LaJeunesse 2002; Fabricious et al. 2004; LaJeunesse et al. 2010a; LaJeunesse & Thornhill 2011; Thornhill et al. 2014), with the exception of Clade E, which may be monospecific (Jeong et al. 2014). These numerous “subcladal” lineages have different physiological and ecological attributes, further revealing that they are genetically and functionally distinct entities (LaJeunesse 2002; Rodriguez-Lanetty et al. 2004; Frade et al. 2008; Thornhill et al. 2008; Hennige et al. 2009; Sampayo et al. 2008, 2009), and efforts necessary to formally describe them as separate species have been initiated (LaJeunesse et al. 2012).

The nonrecognition, or the incorrect assignment, of species boundaries has hindered progress in our basic understanding of coral–dinoflagellate symbioses, particularly in judging their capacity to respond to climate change (LaJeunesse et al. 2012). Speculations about the present and future significance of thermally tolerant endosymbionts have focused largely on Symbiodinium Clade D (Baker et al. 2004; Berkelmans & van Oppen 2006; Jones et al. 2008; LaJeunesse et al. 2009; 2010b), yet the merit of such discussions depends on the accuracy of interpreting the genetic variation observed among Clade D populations and their corresponding ecology and host specificity. Such differences in perception relate to underlying problems with taxonomic resolution and how to delimit species of “morphologically cryptic” Symbiodinium (Blank & Trench, 1985; LaJeunesse 2001; Correa & Baker 2009; Sampayo et al. 2009; Stat & Gates 2011; LaJeunesse et al. 2012), many of which do not persist in culture media.
Corals with symbionts that tolerate high temperatures and live in extreme environments are less susceptible to the negative effects of acute thermal stress (Rowan 2004; Berkelmanns & van Oppen 2006; Warner et al. 2006), have a higher bleaching threshold (Berkelmanns & van Oppen 2006), and survive warm and cold water anomalies better than colonies with more sensitive symbionts (Jones et al. 2008; Sampayo et al. 2008; LaJeunesse et al. 2009, 2010b). Many cnidarians living in the warmest nearshore turbid habitats of the tropical Indo-Pacific commonly harbor symbionts in Clade D (Fabricius et al. 2004; Mostafavi et al. 2007; Hennige et al. 2010; LaJeunesse et al. 2010a). Clade D is evolutionarily distant from other Symbiodinium spp. (Rowan & Powers 1992; LaJeunesse 2001; Pochon et al. 2006; Stern et al. 2010), but the nucleotide sequences of conserved DNA markers resolve little or no diversity within this group. More rapidly evolving internal transcribed spacer (ITS) ribosomal rDNA markers identify ecologically differentiated entities associated with specific Cnidaria (LaJeunesse et al. 2004a, b, 2010a; Lien et al. 2013). For this reason, a provisional nomenclature that combined letters (signifying the clade of Symbiodinium) and numbers was used to delimit different biological entities, or types (e.g. D1 = Clade D, type 1; see LaJeunesse et al. 2010a for further details on this conditional naming scheme). Preliminary analysis of recently developed microsatellite loci showed that these various Clade D ITS types (e.g. D1–4, D5, D2–4–5, D8) were composed of many individuals identified by distinct multilocus genotypes (MLGs), and that the range of allele sizes were similar within but differed significantly between each type (Pettay and LaJeunesse 2009; LaJeunesse et al. 2010a; Wham et al. 2011). Conclusions from this work suggested that phylogeographic groupings separated by seemingly equivocal differences in nuclear rDNA were reproducibly isolated and, therefore, defined by the biological species concept as distinct species.

Among the genetic types currently comprising Symbiodinium Clade D (n ~ 18), the most common and geographically widespread is type D1a (= D1–4; sensu LaJeunesse et al. 2010a). This D type is provisionally referred to as Symbiodinium ‘trenchi’, but lacks a formal species description (i.e. nomen nudum; see LaJeunesse et al. 2005, 2009, 2010a; Wham et al. 2011). It associates with a wide diversity of mostly scleractinian taxa and is common in coral communities from warm equatorial, turbid environments and rarely occurs at northern and southern latitudes higher than the Tropics of Cancer and Capricorn, respectively (Figs S1–S5; LaJeunesse et al. 2004a, 2010a; Silverstein et al. 2011; but see Mostafavi et al. 2007). This host generalist is the only Clade D found in the Atlantic Ocean where it associates with some coral colonies exposed to acute or chronic physiological stress (Toller et al. 2001; LaJeunesse et al. 2009). It is also one of two Clade D types known to grow successfully in artificial culture media (held at the National Center of Marine Algae and Microbiota and the personal collection of Mary Alice Coffroth, SUNY Buffalo, USA).

Most Clade D types have restricted ecological and geographic distributions and often fail to proliferate outside of the host in culture media. For example, the zebra coral Oulastrea crispata (Fig. S6) is an opportunistic colonizer and one of a small number of coral species that can persist in extreme, marginal, high-sediment intertidal habitats (Lam 2000). Because of this ecology, its symbionts must therefore endure extreme latitudinal, seasonal, or daily changes in temperature and irradiance. Throughout its distribution between the continents of Asia and Australia and extending into high nonreef temperate latitudes around Japan (36°N), O. crispata harbors potentially more than one kind of Clade D Symbiodinium (Chen et al. 2003, 2005; Lien et al. 2007, 2013; Fig. S7). A recent analysis of ITS1 and ITS2 resolved rDNA lineages with different latitudinal distributions (Lien et al. 2013). Type D8 previously identified by LaJeunesse and colleagues (2010a) was exclusive to O. crispata colonies from warm-water, tropical habitats around Southeast Asia, type D13 (and another type characterized by a second codominant ITS2 sequence, designated D12–13) was common in populations from the subtropics, especially at locations along the coast of China, whereas type D15 occurred in O. crispata populations extending from the subtropics to colder-water, northern temperate environments around Japan (Lien et al. 2013). These Oulastrea-associated Clade D types have yet to be detected in other scleractinians and appear to be relatively host specific (Chen et al. 2005; LaJeunesse et al. 2010a; Lien et al. 2012).

To resolve whether the Clade D Symbiodinium associated with Oulastrea are composed of separately evolving species lineages, we tested genetic concordance between multicopy ribosomal ITS1-5.8S-ITS2 and partial large-subunit (LSU) sequence data and ‘single’ copy microsatellite nuclear (D1Sym88, and D1Sym93; Wham et al. 2011), chloroplast 23S ribosomal (cp23S), and mitochondrial cytochrome b (cob) DNA markers by assessing reciprocal monophyly (Avise and Wollenberg 1997). We also examined gene flow and reproductive isolation using population genetic data from eight microsatellite loci (Pettay & LaJeunesse 2009; Wham et al. 2011). We combined these results with complementary evidence obtained from more widely distributed populations of S. ‘trenchi’. These findings were then combined with available ecological and geographic distributions, and average cell size, to formally describe three Clade D species. Using this integrative approach, species are formally described that satisfy the core principles of major species concepts (Sites & Marshall 2004; de Queiroz 2007). Finally, we briefly discuss the underlying selection pressures most likely affecting Symbiodinium spp. diversification.

**MATERIAL AND METHODS**

Tissue collections (n = 4 to 57 per location) of various Scleractinia harbouring Symbiodinium ‘trenchi’ (=type D1a = type D1–4) were obtained from collections throughout the Indo-Pacific region including Zanzibar, in the Republic of Tanzania, Thailand in the Andaman Sea, rock island habitats of Palau, the Red Sea, Western Australia, the central Great Barrier Reef, Taiwan, and the Phoenix Islands (Fig. S7; Table S1). Samples of Oulastrea crispata were acquired from locations across the northeast Indian Ocean and West Pacific region encompassing 3800 km, from low-latitude tropical Thailand (~3°N) to high-latitude temperate Japan (>35°N), and represent samples used in previous
Figs 1–4. Unrooted phylogenetic reconstructions (maximum parsimony) comparing new *Symbiodinium* spp. in Clade D. Numbers above branches indicate bootstrap support for maximum parsimony (500 replicates) and posterior probabilities, respectively.

Fig. 1. ITS1-5.8S-ITS2 rDNA and partial LSU phylogeny based on an alignment matrix of 1186 bases.

Fig. 2. Partial chloroplast 23S-rDNA phylogeny based on an alignment matrix of 658 bases.

Fig. 3. Phylogeny based on flanking region sequence data (an alignment matrix of 279 bases) for microsatellite D1Sym88.

Fig. 4. Phylogeny based on flanking region sequence data (an alignment matrix of 145 bases) for microsatellite D1Sym93.
studies including additional collections from Penghu Island, Taiwan (e.g. Fig. S7; Table S1).

Nine combined years of monthly means (2003–2011) for sea-surface temperatures (SSTs) were calculated for sampling locations in tropical, subtropical and temperate regions, respectively, using data obtained from the Giovanni online data system (http://gdata1.sc.gsf.nasa.gov/daac-bin/G3/gui.cgi?instance_id=mairs_monthly_hres), developed and maintained by National Aeronautics and Space Administration Goddard Earth Sciences Data and Information System. Approximately 24 km² around each sampling location were selected and data retrieved from MODIS-Aqua (SST) databases. The mean annual temperatures and seasonal variation (± standard deviation) at each of eight locations were also calculated.

Nucleic acid extractions were conducted as described by LaJeunesse et al. (2003). Amplifications of ITS1, ITS2, cp23S and cob were performed in 25-μl reaction volumes containing 2.5 μl of 2.5 mM deoxynucleotide triphosphates (dNTPs), 2.5 μl of 25 mM MgCl₂, 2.5 μl of standard Taq buffer (New England Biolabs, Ipswich, Massachusetts, USA), 0.13 μl of 5U 1[^-1] Taq DNA polymerase (New England Biolabs), 1 μl of each forward and reverse primer at 10 μM, and 1 μl of 5–50-ng DNA template (see Sampayo et al. 2009 for details on primer sequence and annealing temperatures). The New Clade D primers were developed to amplify loci D1Sym88 and D1Sym93 (Wham et al. 2011) for sequencing the flanking regions of each microsatellite for phylogenetic analyses. Primers CladD_93for1 (CAAGGAGCTCTAGGGGTAG), CladD_93rev1 (GATTGTGTCCTCTTTTGT), CladD_88for1 (AATTGCTCATTGATGCTAC) and CladD_88rev1 (AAAAAGCTCATGGGATTCT) amplify each locus, respectively, using the following thermal cycle profile: 94°C for 3 min, 40 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 30 s, followed by one final cycle at 72°C for 10 min. Amplified polymerase chain reaction (PCR) products were directly sequenced on an Applied Biosciences sequencer (Applied Biosciences, Foster City, California, USA) at the Pennsylvania State University nucleic acids facility.

Amplifications of the ITS1 and ITS2 were first electrophoresed on denaturing gradient gels (50–90% for ITS1 and 45–80% for ITS2) using a CBSScientific system (Del Mar, California, USA). This screening process is used to identify, excise and sequence the numerically dominant sequence variant(s) comprising the ribosomal array in the genome of the resident symbiont in each sample (for protocol details as well as primer sequences, see LaJeunesse 2002, LaJeunesse et al. 2008). PCR-denaturing gradient gel electrophoresis (DGGE) provides a necessary step that filters out low-copy-number variants that are uninformative for purposes of identification (Thornhill et al. 2007). This protocol rapidly diagnoses whether a genome is represented by a single numerically dominant sequence (one prominent band), or that codominant variants exist within the genome (multiple banding creates a characteristic PCR-DGGE “fingerprint” profile), or that multiple symbionts are present in the host (fingerprints from two or more species detected in one sample; LaJeunesse 2002; LaJeunesse et al. 2008).

Chromatograms were checked and sequences aligned using SeqNavigator v. 1.0 (Applied Biosystems) or Geneious Pro software v. 6.0 (Biomatters Limited, Auckland, New Zealand). Paup 4.0b10 (Swofford 2000) was used with default settings to perform phylogenetic analyses on aligned sequence data under the criteria of maximum parsimony (with indels included as a fifth character state), maximum likelihood (using the HKY85+G substitution model) and distance. We analyzed sequence data for each genetic marker using ModelTest (Posada & Crandall 1998), implemented in the Geneious Pro to calculate the most appropriate model of nucleotide evolution. Both the hierarchical likelihood ratio test (hLRT) and Akaike information criterion (AIC) selected different evolutionary models for loci D1Sym88 and D1Sym93 (hLRT: F81 and AIC: TVM+G for locus D1Sym88 and hLRT: F81 and AIC: GTR for locus D1Sym93); therefore, we evaluated trees based on both models to test concordance. Additionally, Bayesian posterior probabilities were calculated with the software Mr. Bayes v. 3.2 (Huelsenbeck & Ronquist 2001), using substitution models listed above for loci D1Sym88 and D1Sym93. But, because of low sequence divergence in the data sets of ITS, LSU, cp23S and cob, no optimal model nucleotide evolution could be calculated; and, therefore, HKY85+G was used as the default model. Bayesian computations were conducted with Markov chain Monte Carlo chain lengths of 1,000,000 (four chains) sampled every 500 iterations and 500 sampled trees discarded for the burn-in. Phylogenies among independent loci were compared visually to determine reciprocal monophyly.

Eight polymorphic microsatellite loci developed for Clade D Symbiodinium were utilized (D1Sym14, D1Sym17, D1Sym35, D1Sym67, D1Sym87, D1Sym88, D1Sym92 and D1Sym93; Pettay & LaJeunesse 2009; Wham et al. 2011). Each locus was amplified in 10-μl reaction volumes containing 1 μl of 10 mM dNTPs, 0.2 U of Taq DNA polymerase (New England Biolabs), 1 μl of standard Taq buffer (New England Biolabs), 1 μl of 25 mM MgCl₂, 1 μl of each forward and reverse primer at 10 μM, and 1 μl of approximately 50-ng DNA template. PCR amplification conditions consisted of an initial denaturing step of 94°C for 2 min, followed by a touchdown protocol using 10 cycles of 94°C for 15 s, an annealing temperature of 2°C above individually optimized annealing temperatures for each locus (see Pettay & LaJeunesse, 2009; Wham et al. 2011) for 15 s, and an extension step at 72°C for 15 s for each cycle the annealing temperature lowers 0.5°C per cycle. These were followed by 22 cycles as described above at the primer-specific annealing temperature (see Pettay & LaJeunesse, 2009; Wham et al. 2011) and ending in a final extension of 72°C for 5 min. After amplification, fragment sizes were analyzed on an ABI 3730 genetic analyzer (Applied Biosystems) using a 500-base-pair (bp) standard (labeled with a LIZ fluorophore) at the Penn State University Nucleic Acid Facility. Fragment sizes were analyzed visually using GeneMarker v. 1.91 (SoftGenetics, State College, Pennsylvania, USA).

A single MLG was obtained for a majority of samples by combining results from each locus. In samples where multiple genotypes co-occurred (observed in 30–60% of samples collected from regions where Clade D types overlapped, Table S1), alleles whose electropherogram peaks were one-third less than the intensity of the main peak were noted as background and not scored. MLGs could not be
produced from samples containing codominant genotypes because it was unfeasible in these instances to correctly assign alleles to one or another individual clone for each locus. For this reason, samples containing two or more *Symbiodinium* genotypes were removed from the population genetic analyses of MLGs preformed with STRUCTURE. This conservative approach was used to lower the chances of producing MLGs that actually consisted of two divergent lineages while preserving the possibility of observing individuals of mixed ancestry. These samples were instead used to assess the percentage of colonies with mixtures of *Symbiodinium* from the same putative species lineage or of different Clade D species by scoring the diagnostic alleles of Sym14 at sites where species ranges overlapped. Samples with a single diagnostic peak were scored as pure, whereas samples containing both a peak in the size range consistent with type *D15* (165 bp) and a peak in the size range consistent with *D13* (*D12–13*) (177–180 bp) were scored as mixtures regardless of the relative size of the peak.

To analyze MLGs from the set of samples corresponding to *Symbiodinium trenchii* (formalized herein to *S. trenchii*, see below), the dual-allele loci were converted to haploid. This was accomplished by randomly removing one of the two alleles at each locus in the statistics program R v2.15.0 (R Core Team 2013). The resulting haploid genotypes were then analyzed by principle coordinate analysis in the Bayesian clustering program STRUCTURE (Pritchard et al. 2000).
A pair-wise, individual-by-individual genetic distance matrix was generated using the software package GenAlEx v. 6.1 (Peakall & Smouse 2006). For each pair-wise comparison, loci with the same state were assigned a value of 0, whereas loci that differed were given a value of 1. These values were then calculated across multiple loci. Principle coordinate analysis was performed in R, using the ADE4 (Chessel et al. 2004) and ADEGENET (Jombart 2008) packages. The analysis in STRUCTURE was performed without a location prior, under an admixture and uncorrelated alleles model with a burn-in of 100,000 iterations and 1,000,000 iterations of analysis at $K=1–7$. The optimum $K$ value was determined by the Delta-$K$ method (Evanno et al. 2005). For each resulting population, a $U_{PT}$ value (a haploid equivalent to $F_{ST}$) was calculated pair-wise for each population using GenAlEx.

Both field-preserved and cultured coccoid cells were photographed under bright-field illumination at a magnification of $\times400–1000$ using an Olympus BX51 compound microscope (Olympus Corp., Tokyo, Japan) with a Jenoptik ProgRes CF Scan digital camera (Jenoptik, Jena, Germany). An autoexposure setting within ProgRes Capture Pro 2.8 software (Jenoptik) was used to expose and capture the cell images. Measurements taken from live cultures offered a way to assess the effect of preservation on cell size. Two cultured isolates of type $D1-4$, A001 (CCMP3408) and MTB4 (CCMP3409), growing in the artificial culture medium ASP-8A (Ahles 1967) were photographed during log phase of growth under $80–120 \mu$mol quanta $m^{-2}.s^{-1}$ photosynthetically active radiation on a 14:10 (light:dark) photoperiod. To avoid the effect of age on appearance and size, cells were photographed during the middle of logarithmic growth in culture (between days 10 to 15 after reinoculation into fresh media). Cell sizes for at least 40 individuals per culture were calculated with the program ImageJ (Abramoff et al. 2004). Cell volume was calculated on the basis of the dimensions of an ellipsoid ($\text{volume} = \frac{4}{3}\pi abc$, where $a$, $b$, and $c$ are equal to half the length, width, and height. Cell height was assumed to be identical to cell width]. Size and volume differences between putative species were assessed via univariate type III ANOVA (factor: ‘Species’) on a combined data set of all maximum length measurements of cultured and natural material belonging to each group. Data were cube-root transformed to achieve normality and homoscedasticity and statistical significance was assessed with ANOVA at $P = 0.05$ in the software SPSS Statistics v. 19.0 (IBM, Armonk, New York, USA). Cell size averages were plotted in original units for clarity along with 95% confidence intervals.
RESULTS

Symbiodinium trenchii LaJeunesse sp. nov.

Fig. 9

DIAGNOSIS: Coccoid cells, typically 9.8 to 10.4 μm in maximum diameter; nucleotide sequences of the chloroplast cp23S-rDNA (KF220382), mitochondrial cob (KF193523), microsatellite flanking D1Sym88 and D1Sym93 (Figs 3, 4, DRYAD entry doi:10.5061/dryad.3np1m), nuclear ribosomal ITS1/5.8S/ITS2 (KJ019893) and partial LSU (KF740688), and fragment size ranges (allelic variants) of eight microsatellites distinct. Diallele peaks at nearly all microsatellite loci.

HOLOTYPE: USNM 1231333 deposited in the National Museum of Natural History, Smithsonian Institution, Washington, DC, USA.

AUTHENTIC STRAINS: CCMP3408 (= strain A001) and CCMP3409 (= strain MTB4) deposited at the Provasoli-Guillard National Center for Marine Algae and Microbiota, East Boothbay, Maine, USA.

Symbiodinium boreum LaJeunesse & Chen sp. nov.

Fig. 10

DIAGNOSIS: Coccoid cells, typically from 9.2 to 9.7 μm at maximum diameter. Nucleotide sequences of the chloroplast cp23S-rDNA (KF220381), mitochondrial cob (KF193522), microsatellite flanking D1Sym88 and D1Sym93 (Figs 3, 4, DRYAD entry doi:10.5061/dryad.3np1m), nuclear ribosomal ITS1/5.8S/ITS2 (KJ019892) and partial LSU (KF740688), and fragment size ranges (allelic variants) of eight microsatellites distinct.

HOLOTYPE: USNM 1231331 deposited in the National Museum of Natural History, Smithsonian Institution, Washington, DC, USA.

PARATYPES: ASIZC0000966-70, deposited in the Biodiversity Research Center Academia Sinica (BRCAS), Museum, Zoological Collection, Taipei, Taiwan. Samples were collected in the Kochi Prefecture, Otsuki Town, Nishidomari, Japan (32°46′42.82″N, 132°43′57.40″E).

TYPE LOCALITY: Penghu Island, Taiwan (23°31′12″N, 119°33′0″E).

HABITAT: Marine, associated with the scleractinian, Oulastrea crispata.

ETYMOLOGY: From the Greek boreas meaning north and refers to the northern subtropical and temperate environments where this species occurs in association with Oulastrea crispata.

Symbiodinium eurythalpos LaJeunesse & Chen sp. nov.

Fig. 11

DIAGNOSIS: Coccoid cells, typically 8.3 to 9.3 μm in maximum diameter. Nucleotide sequences of the chloroplast cp23S-rDNA (KF220379, KF220380), mitochondrial cob (KF193520/1), microsatellite flanking D1Sym88 and D1Sym93 (Figs 3, 4, DRYAD entry doi:10.5061/dryad.3np1m), nuclear ribosomal ITS1/5.8S/ITS2 (KJ019890, KJ019891, KJ019892), partial LSU (KF740686/7) and fragment size ranges (allelic variants) of eight microsatellites distinct.

HOLOTYPE: USNM 1231332 deposited in the National Museum of Natural History, Smithsonian Institution, Washington, DC, USA.


ETYMOLOGY: Named for Professor Robert K. Trench, a phylogenetic pioneer who first recognized species diversity in Symbiodinium.

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POPULATION GENETIC STRUCTURE

Fig. 7. Population genetic structure exhibited among three genetically distinct lineages of Clade D Symbiodinium. Principle coordinate analysis based on Euclidian distances showing clustering of genotypes corresponding to phylogenetic lineages (see above). Axes 1 and 2 explain 45.5% and 19.7% of variance, respectively.

Fig. 8. Population genetic structure exhibited among three genetically distinct lineages of Clade D Symbiodinium. Bayesian analyses using the software STRUCTURE assigned multilocus data to one of three genetically distinct populations irrespective of geographic origin. A fourth population was delimited (k = 4) corresponding to populations of Symbiodinium eurythalpos in the upper Gulf of Thailand.
PARATYPES: Accession ID numbers S22029–S22036 of specimens deposited at the Ocean Genome Legacy (OGL), Northeastern University, Nahant, Massachusetts, USA. Samples were collected from Khang Kao Island, Thailand, in the Gulf of Thailand (13.11°N, 100.81°E) and from Cape Panwa, Phuket Island, Thailand (7.815°N, 98.404°E).

TYPE LOCALITY: Penghu Island, Taiwan (23°31'12"N, 119°33'0"E).

HABITAT: Marine, associated with the scleractinian, Oulastrea crispata.

ETYMOLOGY: From the Greek eurus (= wide) combined with thalpos (= heat, warmth) and designated for its broad latitudinal distribution spanning tropical and subtropical environments.

Genetic evidence

Sequence data from nuclear-encoded rDNA (ITS1/5.8S/ITS2 and LSU, 1186-base alignment matrix; Fig. 1), partial chloroplast (cp23S-rDNA, 658-base alignment matrix; Fig. 2) and single-copy microsatellite flanking regions (D1Sym88, a 279-base alignment matrix, Fig. 3 and D1Sym93, 145-base alignment matrix, Fig. 4) produced concordant phylogenetic patterns consistent with the existence of at least three genetically cohesive, evolutionarily divergent lineages. Sequence alignments for the two microsatellite markers are available at DRYAD (entry doi:10.5061/dryad.3np1m).

Maximum parsimony, distance and Bayesian approaches produced concordant phylogenies (comparison not shown). Chloroplast cp23S-rDNA sequences for Symbiodinium boreum and S. eurythalpos were identical, but differed from S. trenchii by one deletion and three base substitutions in the hypervariable domain V region (Fig. 2). Noncoding, independently sorting microsatellite flanking sequences provided concordant phylogenetic resolution of all three taxa (Figs 3, 4). A fixed base substitution in the mitochondrial cob sequence (918 bases analyzed) that differentiated only S. boreum (Fig. S8) results in a nonsynonymous amino acid substitution (converting phenylalanine to tyrosine at amino acid position 167) in the functionally conserved gene region containing the Q0 redox center (Fig. S8).

Our analysis of eight microsatellite loci found that most samples of Symbiodinium eurythalpos and S. boreum produced electropherograms with single peaks for each locus with minimal stuttering and nonspecific fragment amplification.

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**Figs 9–11.** Coccoid cell morphology for three Clade D Symbiodinium spp.

**Fig. 9.** Light micrograph of S. trenchii sp. nov. (cultured isolate A001); scale bar = 10 μm.

**Fig. 10.** Light micrograph of S. boreum sp. nov. freshly isolated from host tissues collected off of Penghu Island, Taiwan. Size bar = 10 μm.

**Fig. 11.** Light micrograph of S. eurythalpos sp. nov. freshly isolated from host tissues collected off of Penghu Island, Taiwan. Size bar = 10 μm.

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**Fig. 12.** Coccoid cell size differences among three Clade D Symbiodinium spp. A comparison of mean cell diameters among ovate coccoid cells obtained from various samples and their correspondence to different species of Clade D Symbiodinium. Cultured isolates (light brown squares) and freshly isolated cells of S. trenchii (dark brown squares) acquired from coral genera, including Favia sp. and Goniastrea spp., from Phuket, Thailand and Palau. Freshly isolated cells of S. boreum and S. eurythalpos were obtained from colonies of Oulastrea crispata from Penghu Island, Taiwan, and the Gulf of Thailand. Error bars represent standard error calculated from > 40 measurements.

**Fig. 13.** Coccoid cell volumetric differences among three Clade D Symbiodinium spp. Mean cell volume and 95% confidence intervals for combined samples (from Fig. 12) representing each Clade D species. Letters identify statistically distinct groups (ANOVA, P < 0.05).
obtained from several different hosts, or from culture, were large relative to \textit{S. boreum} and \textit{S. eurythalpos} (Figs. 12, 13).

\textit{Symbiodinium boreum} and \textit{S. eurythalpos} differed in latitudinal distribution among colonies of \textit{O. crispa}ta that relate to major differences in seasonal and average annual temperatures (Figs 14, S12). The distribution of \textit{S. boreum} overlaps with \textit{S. eurythalpos} in subtropical environments (Fig. 14). At sites in this region, approximately 50\% of colonies of \textit{O. crispa}ta were codominated by MLGs representing different symbiont species (Table S1). There were no apparent population genetic subdivisions detected across the geographic range examined for each species (Fig. 8). At locations where these species distributions overlapped, populations of each were genetically distinct (Figs 8, 14).

\section*{DISCUSSION}

Concordant phylogenetic, population genetic, and ecological evidence, as well as differences in geographical distribution and cell size, support the formal designation of these three \textit{Symbiodinium} lineages as separate species in Clade D. Whereas clonal evolution could explain concordant phylogenies from numerous and geographically widespread samples (Figs 1–4; Avise and Wollenberg 1997), our analysis of independently sorting microsatellite loci provided a test for whether genetic exchange occurred within or between lineages identified as phylogenetically distinct (Figs 1–4). Populations of each proposed \textit{Symbiodinium} species contained unique alleles and significant differences in allele frequencies, revealing a lack of genetic exchange between populations representing different species lineages (Figs 6–8; Table 1). Within each species, most clonal populations appeared to be the products of genetic recombination where allele combinations found among individual multilocus microsatellite genotypes were frequently scrambled across many loci (i.e. exhibiting linkage equilibrium among loci; initially calculated in Pettay and LaJeunesse 2009; Wham et al. 2011). These observations support the concept that \textit{Symbiodinium} species comprise numerous clonally propagated cell lines (i.e. individuals) that periodically undergo sex to produce new genetically recombinant clonal lineages (LaJeunesse 2001).

The strong genetic partitioning between sympatric populations of \textit{Symbiodinium boreum} and \textit{S. eurythalpos} validates that they are different species under the biological species concept and not geographically isolated populations of the same species exhibiting genetic structure over a broad latitudinal range (Martin and McKay 2004). When gene flow occurs through hybridization, alleles that are diagnostic of one species should be found in combination with the alleles of another species. Sympatric populations of \textit{S. eurythalpos} and \textit{S. boreum} in Okinawa and Taiwan showed no evidence of hybridization or allele introgression, suggesting that effective cross-hybridization does not occur. (Fig. 8). Even low amounts of genetic exchange in regions of population sympathy, over a few generations, would dissipate the strong genetic partitioning found in our statistical analysis using STRUCTURE; and eliminate concordance among DNA phylogenies produced by inde-
pendently sorting nuclear and plastid loci (Figs 1–4, 8; Wright 1931).

Although intrinsic reproductive barriers maintain genetic boundaries at locations where species populations overlap, population genetic data indicate that gene flow occurs with sufficient frequency to genetically homogenize the populations of each species over hundreds and even thousands of kilometres (Figs 8, 14; Table 1). Widespread gene flow occurs among populations of *S. trenchii* separated by thousands of kilometres from the western Indian Ocean to the Pacific Ocean (Fig. 8). The apparent dispersal capability of *S. trenchii* could be important to this species’ ecological response to climate change and its potential for range expansion (LaJeunesse et al. 2009). Additional populations of *S. trenchii*, especially from the Red Sea, Arabian/Persian Gulf, and Atlantic Ocean (Caribbean), should be analysed to determine whether any genetic structure exists in this widespread species.

ITS nrDNA is commonly used to diagnose the species identity of various organisms (Seifert 2009; Moniz & Kaczmarska 2010; Yao et al. 2010), yet intragenomic and interindividual variation can confound species resolution among *Symbiodinium* (Sampayo et al. 2009; Thornhill et al. 2014). Tropical populations of *S. eurythalpos* were characterized by strains with a single numerically dominant ITS2 sequence (type D8), whereas others possessed a second ancestral codominant variant in the genomes’ ribosomal array, the D12 sequence (type D8–12). In temperate regions, however, most strains (clones) of *S. eurythalpos* possessed a single dominant sequence (type D13), or also in combination with the D12 sequence (type D12–13; Fig. 5; Lien et al. 2013). The geographical patterns of these ITS genotypes suggest that sequences of the nrDNA operon are at different stages of lineage sorting (from ancestral to derived sequence variants) across populations of *S. eurythalpos* (Dover 1982). In contrast, a single sequence (D15) was numerically dominant in the genomes of all samples of *S. boreum* that were analysed and indicates that the rDNA operon is well homogenised across individuals of this species. The genomes of *S. trenchii* are consistently characterised by codominant ITS2 sequences (the 1 and 4, or ‘a’, bands viewed on DGGE gels) that persist intragenomically in similar proportions wherever populations of this species occur.

Analyses of microsatellite markers substantiate the initial conclusions of Blank and Trench (1985) that *Symbiodinium* cells exist predominantly in the haploid phase (Santos & Coffroth 2003; see also Pettay & LaJeunesse 2007, Andras et al. 2011, Pettay et al. 2011; Pinzon et al. 2011); and is a general characteristic feature of the Dinophyceae (Coats 2002). *Symbiodinium eurythalpos* and *S. boreum* possessed only single alleles at each locus (Fig. 5), except in samples where mixed genotypes were clearly present. In contrast, two alleles were routinely scored at most loci for samples of *S. trenchii*, including several independently acquired isoclonal cultures (Fig. 5; Pettay & LaJeunesse 2009; Wham et al. 2011). The dual alleles characteristic of microsatellite
lochi from S. trenchii must be explained by differences in genome ploidy, and not from reoccurring mixtures of two genotypes with allelic compositions that are different at every locus. At this time we reserve speculation on the significance of this finding and whether the apparent duplicated genome of this species explains its ecological breadth and whether it enhances the adaptive potential of S. trenchii populations.

The featureless spherical forms and relatively small sizes of Symbiodinium (typically 7–13 μm in mean diameter) preclude use of a morphological diagnostic trait for visual identification. Although not intended to be diagnostic, cell size analysis provides some morphological evidence for species differentiation (Schoenberg & Trench 1980; Trench & Blank 1987; LaJeunesse et al. 2012). Samples of S. trenchii obtained from a diversity of host genera collected in the Pacific and Indian oceans were similar in average cell diameter and similar to isolates under long-term artificial culture (Fig. 12). Despite some interindividual variation, cell size in this and other Symbiodinium spp. appear strongly influenced by genetic factors (LaJeunesse 2001; LaJeunesse et al. 2012). Cell size, or volume (Fig. 13), from the perspective of functionality, can substantially affect respiration, growth rate, and photosynthesis of microalgae (Banse 1976). The larger cell size of S. boreum, relative to S. eurythalpos, may contribute to the physiological traits that ultimately explain its higher latitudinal distribution (see below).

Natural selection in the struggle for resource acquisition and reproductive success often leads to population subdivision and ecological specialization (Futuyma & Moreno 1988; Schluter 2009). For endosymbiotic dinoflagellates, the cnidarian host represents a stable habitat where growth and reproductive fitness are likely maximized (Knowlton & Rohwer 2003). It is therefore not surprising that host identity determines the distribution of most Symbiodinium spp. (Finney et al. 2010) and that the evolution of host–symbiont specificity, as a form of ecological specialization, drives the population subdivision and diversification of Symbiodinium as mutualistic endosymbionts (LaJeunesse 2005; Thornhill et al. 2014). As sister species in Clade D, S. boreum and S. eurythalpos share a common ecological trait in their symbioses with Oulastrea crispa. This host is one of a small number of Indo-Pacific corals that broods larvae (Lam 2000) and is prone to vertically transmit symbiont cells between generations. Many host-specific lineages of Symbiodinium correspond to animals that vertically transmit (LaJeunesse 2005), and it is possible that the common ancestor to S. boreum and S. eurythalpos first evolved by the process of ecological specialization to Oulastrea (Thornhill et al. 2014).

The geographical partitioning of Symbiodinium boreum and S. eurythalpos suggest that long-standing selection pressure from different latitudinal environments created a secondary axis of niche diversification that initiated lineage bifurcation from a common ancestor (Figs 14, S12). Mean annual temperatures were recently shown by Thomas et al. (2012) to have a large influence in thermal adaptation among phytoplankton obtained at locations encompassing 150° of latitude. Their analyses of the optimum temperatures for growth compiled from over 130 species representing the major phytoplankton groups suggested that populations of microalgae were adapted to local environmental conditions (especially temperature) despite the potential for long-distance dispersal (Thomas et al. 2012). Colonies of Oulastrea crispa and their resident Symbiodinium experience extreme differences in mean temperature and light across the latitudes of their distribution (Fig. 14; Lien et al. 2013). At northernmost locations, average annual water temperatures are approximately 20–22°C (Fig. 14), and seasonal temperatures oscillate 10°C, or more, over the course of a year (Fig. S12). Shallow inshore colonies may experience cold water conditions below 12°C (Yajima et al. 1986). Subtropical locations also experience wide seasonal oscillations in temperature with summer highs similar to the tropics (29–30°C; Fig. S12). Both S. boreum and S. eurythalpos must therefore have physiological adaptations to live with O. crispa in these fluctuating environments, but how they tolerate such cold or warm extremes awaits thorough physiological study.

The presence of a genetically distinct population of Symbiodinium eurythalpos in the upper Gulf of Thailand may be the product of severe selection by regional environmental conditions (Figs 8, 14, S1; e.g. Pettay & LaJeunesse 2013). Surface currents into and out of the Gulf are confluent with the rest of the South China Sea and discounts the possibility that these distinct populations are the product of long-term isolation (Akhir 2012). Instead the strong genetic structure in this most northern portion of the Gulf of Thailand may represent shifts in genetic diversity in response to changes in temperature, sedimentation and salinity created by seasonal water stratification in the Gulf and the discharge of fresh water from numerous rivers in the area (Windom et al. 1984; Yanagi et al. 2001; Cheevaporn & Menasveta 2003). A similar pattern of strong genetic subdivision was recently shown for another Clade D Symbiodinium (S. ‘glynni’ nomen nudum) associated with populations of the coral Pocillopora from the eastern Pacific. Populations of this symbiont were genetically distinct at high latitudes despite no evidence of population subdivision in the host, whose larvae already possess symbionts when planktonic (Pettay & LaJeunesse 2013). The wide annual ranges in temperature and light in the Gulf of California, relative to more equatorial locations, are obvious factors influencing strong genetic differentiation among these S. ‘glynni’ populations. These few examples involving the symbioses of Oulastrea and Pocillopora indicate that environmental gradients can also have a strong effect on the genetic diversification of Symbiodinium.

We considered an alternate hypothesis that host genetic partitioning by latitude, or the presence of cryptic species of Oulastrea, may instead be influencing the distributions of Symbiodinium boreum and S. eurythalpos (Lien et al. 2013). However, our genetic evidence (ITS1 sequence comparison), in contrast to the interpretations of Lien et al. (2013), indicates that O. crispa comprises a single species, albeit with some interindividual nrDNA sequence variation (Fig. S13). Population genetic markers developed and applied to this question may reveal fine-scale genetic partitioning across latitudinal populations of this coral. On the condition that host populations are confluent, we presume that environmental factors, primarily light and temperature, largely explain the evolution of geographically distinct Oulastrea-
specific *Symbiodinium*. In turn, we propose that the maintenance of distinct associations with putatively ‘cold’- and ‘heat-tolerant’ species of Clade D *Symbiodinium*, in part, explains why *O. crispa* persists in marginal habitats over a large latitudinal gradient (Lien et al. 2013).

Finally, preliminary ITS2 rDNA data diagnostic of *Symbiodinium eurythalpos* suggest that this symbiont may occur in populations of *Zoanthus sanssibaricus* (unpubl.) and the aeolid nudibranch *Pteraeolus Ianthina* (Ishikura et al. 2004) at high latitudes (>30°N) around mainland Japan and at similar latitudes where *Oulastrea* harbours *S. boreum*. If these ecological and geographical patterns are substantiated, it indicates that *S. eurythalpos* associates with other non-scleractinian hosts beyond its normal latitudinal distribution with *Oulastrea*. Although apparently present in these higher-latitude regions, *S. eurythalpos* may be competitively inferior to *S. boreum* when growing in *Oulastrea* colonies from colder environments.

In addition to the three species described here, Clade D *Symbiodinium* apparently comprises numerous other separate entities, each exhibiting differences in ecology and physiology. This cautions against making broad assumptions concerning the contribution of this group as a whole in the ecological or evolutionary response of coral–dinoflagellate symbioses to climate change (Baker et al. 2004; Oliver & Palumbi 2009; Stat & Gates 2011). The host specificity exhibited by many in this clade suggests that their ecological significance is limited to few or one host species (LaJeunesse et al. 2010a). In contrast, broadly distributed populations of *S. trenchii* are of potential interest given that this species associates with a wide diversity of coral genera.

This work is the first to utilize population genetics in delimiting species of microalgae (proposed as a general procedure to objectively define species of eukaryote, Hausdorf & Henig 2010; Hey & Pinho 2012). Although no individual can be representative of variation within a species population, the examination of numerous individuals is consistent with evolutionary biology’s view of the species. For most eukaryotic microbes, rarely is it possible to test species hypotheses of microalgae using the biological species concept [Mayr 1942; but see the meticulous work of Coleman (2001), Rynearson & Armburst (2004), and Adams et al. (2009), who worked with numerous cultures]. The clonal cell lines (individual genotypes) that dominated most of these coral samples made feasible the application of population genetic analyses on genotype diversity acquired over different geographic scales encompassing different regional environments. Because of their abundance and high clonality in host tissues, *Symbiodinium* may represent among the few kinds of dinoflagellates where this level of genetic study is feasible, and a model system for examining genotypic diversity, gene flow, and dispersal that relate to the macro- and microevolution of eukaryotic microbes (Andras et al. 2011; Pettay et al. 2011, 2013; Wham et al. 2011; Thornhill et al. 2014).

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**SUPPLEMENTARY DATA**

Supplementary data associated with this article can be found online at http://dx.doi.org/10.2216/13–186.1.s1.

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