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Current Biology

Systematic Revision of Symbiodiniaceae Highlights the Antiquity and Diversity of Coral Endosymbionts

Highlights

- The micro-algal genus *Symbiodinium* is split into multiple genera
- Seven of these genera are formally described based on genetics and ecology
- Dinoflagellates in the family Symbiodiniaceae originated in the Jurassic Period
- Symbiodiniaceae diversification coincided with the radiation of reef-building corals

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In Brief

Symbiodinium are micro-algal symbionts of reef-building corals. LaJeunesse et al. report new estimates from molecular dating, indicating that corals and *Symbiodinium* originated and diversified together ~140–200 mya. Divergent *Symbiodinium* "clades" are now partitioned into multiple genera, better reflecting their long evolutionary history.

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Systematic Revision of Symbiodiniaceae Highlights the Antiquity and Diversity of Coral Endosymbionts

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SUMMARY

The advent of molecular data has transformed the science of organizing and studying life on Earth. Genetics-based evidence provides fundamental insights into the diversity, ecology, and origins of many biological systems, including the mutualisms between metazoan hosts and their micro-algal partners. A well-known example is the dinoflagellate endosymbionts ("zooxanthellae") that power the growth of stony corals and coral reef ecosystems. Once assumed to encompass a single panmictic species, genetic evidence has revealed a divergent and rich diversity within the zooxanthella genus *Symbiodinium*. Despite decades of reporting on the significance of this diversity, the formal systematics of these eukaryotic microbes have not kept pace, and a major revision is long overdue. With the consideration of molecular, morphological, physiological, and ecological data, we propose that evolutionarily divergent *Symbiodinium* "clades" are equivalent to genera in the family Symbiodiniaceae, and we provide formal descriptions for seven of them. Additionally, we recalibrate the molecular clock for the group and amend the date for the earliest diversification of this family to the middle of the Mesozoic Era (~160 mya). This timing corresponds with the adaptive radiation of analogs to modern shallow-water stony corals during the Jurassic Period and connects the rise of these symbiotic dinoflagellates with the emergence and evolutionary success of reef-building corals. This improved framework acknowledges the Symbiodiniaceae's long evolutionary history while filling a pronounced taxonomic gap. Its adoption will facilitate scientific dialog and future research on the physiology, ecology, and evolution of these important micro-algae.

INTRODUCTION

Symbiotic dinoflagellates in the genus *Symbiodinium* have been the subjects of considerable scientific investigation, especially in recent decades. Their mutualisms with many reefal invertebrates, notably stony corals, are fundamental to the existence of tropical and subtropical coral reef ecosystems worldwide. The recent, rapid decline of these marine communities emphasizes how many of Earth's ecosystems are in danger of collapse from anthropogenic global change. However, it is clear from the fossil record that some reef-building corals, and hence their obligate symbioses, have been resilient to major changes in climate [1, 2]. The specific identities of resident *Symbiodinium* influence their host's susceptibility to hot and cold ocean temperatures, contributing to broad disparities in thermal tolerance among individual colonies and host species [3–5]. Therefore, recognizing the diversity, physiology, and ecology of these endosymbionts is paramount for understanding how algal-invertebrate partnerships might respond to rapid shifts in Earth's climate.

Some of the earliest investigations of cultured *Symbiodinium* revealed differences in morphology (cell size, chloroplast and mitochondria volumes), biochemistry (metabolites), physiology (photosynthetic abilities), behavior (motility and host specificity), and genetics (chromosome number and variation in allozyme alleles), which collectively led to the proposition that more than a single panmictic symbiont species existed [6, 7]. Several new species were indeed described [8], but these early reports had little traction in the research community at the time. Subsequent phylogenetic analyses of *Symbiodinium* ribosomal genes (rDNA) sought to broadly characterize the diversity and evolutionary relationships among these symbionts and their hosts [9]. Phylogenetic reconstructions immediately revealed the genus *Symbiodinium* to be composed of many evolutionarily divergent lineages [9, 10]. In recognizing their phylogenetic disparity, these

lineages were provisionally designated by arbitrary letters (e.g., A, B, C) that became referred to as "clades" [11]. Historically, these clade designations often appeared in the literature as species-level equivalents. With the emergence of molecular techniques in recent decades, there has been widespread recognition that most *Symbiodinium* clades contain numerous "sub-clades," "types," or "strains." Notably, most of these within-clade entities exhibit distinct and sometimes substantial differences in their genetics, physiology, and/or ecology—attributes that fulfill the prerequisites of multiple species concepts [12].

Each year, scores of publications report on some aspect of *Symbiodinium* diversity, and most confirm that genetic variation within this dinoflagellate genus is abundant and evolutionarily broad. Given this, a growing number of species (currently 22) have been formally classified and named [12–20]. The same evidence used to describe these species also indicates that hundreds more *Symbiodinium* types are candidates for species binomials [21]. Their continued classification will be required to develop more explicit research hypotheses in fields ranging from cell biology to community ecology [22]. In the past, failing to recognize *Symbiodinium* species boundaries has muddled insights into ecological and evolutionary processes by combining the attributes of numerous disparate entities [14, 21, 23]. Confusion is likely to persist if only a single genus continues to encompass hundreds of both closely and distantly related species. It is therefore necessary to update *Symbiodinium* systematics to incorporate our modern understanding of the evolutionary relationships within the group.

There are abundant reasons, beyond alleviating confusion, to justify replacing historical *Symbiodinium* clades with formal genus names. As Rowan and Powers [10] first recognized, the genetic divergences among clades is similar to, and often much greater than, genetic distances separating other dinoflagellate genera, families, and sometimes orders [24, 25]. This is unsurprising given that provisional molecular clock estimates conservatively place the divergence among clades beginning in the early Cenozoic Era [26, 27]. Additionally, comparisons between different *Symbiodinium* clades reveal low transcript similarity, with typically <20% orthologous gene loci [28, 29]. Transcriptomes provide realistic approximations of gene number and composition for many single-celled eukaryotes; thus, these initial comparative genomic studies also support the notion that clades have undergone significant evolutionary change [30, 31].

Here, we assess genetic, morphological, physiological, ecological, and biogeographic evidence to reorganize the diversity of *Symbiodinium* into a hierarchical framework consisting of multiple genera that better reflects their distant evolutionary relationships. Furthermore, we develop a new molecular clock from the latest phylogeographic and paleontological information to recalculate origination times for each genus and for the group as a whole. These fundamental revisions to *Symbiodinium* systematics should pave the way for improved scientific discourse and future advances in coral-algal symbiosis research.

RESULTS

Times of Divergence among Clades of the Symbiodiniaceae

By using the program MrModeltest v2.3 [32, 33], the most appropriate model of evolution for the nuclear large subunit (*LSU*) rDNA data was identified as the General Time Reversible model with a proportion of invariable sites and rate variation among sites (GTR+I+G; support values: *-ln* L = 23,631.97; *K* = 10; AIC = 47,283.94). Molecular dating using calibrations times (Figure S1) based on the separation of the Pacific and Atlantic Ocean basins in the Pliocene (5.2–3.1 mya) as well as the adaptive radiation of soritid foraminifera in the Eocene (55–42 mya; Figures S2A and S2B) in BEAST 2 [34] placed the age of the Symbiodiniaceae back to the middle Mesozoic at approximately 160 mya (posterior estimates; Figures 1A and 1B). The particular geological chronology shown in Figure 1A was generated from estimates based on "old" (or "early") calibration time "2" involving the divergence point of two distantly related Clade D lineages sharing a most recent common ancestor, but with different host specificities: one associated with foraminifera and the other with cnidarians (Figure S3). The "young" (or "late") calibration time "4" that was used involved sibling Clade C lineages associated with sibling *Porites* corals from the Greater Caribbean and Eastern Pacific, respectively (justifications for all calibration times are detailed in the STAR Methods section Quantification and Statistical Analysis). With these calibrations, a mean clock rate for *LSU* rDNA evolution was estimated at 2.08×10^{-3} ($\pm 2.84 \times 10^{-4}$ Stdev) substitutions per site per million years (based on a strict clock model). Other combinations of old (early) and young (late) calibration times returned similar, and sometimes older, estimates of divergence among Symbiodiniaceae clades (data not shown).

Morphological Variation and Conservatism among Clades of Symbiodiniaceae

Mean cell sizes among Symbiodiniaceae species are small relative to other Suessiales (Figures 2A and 2B; Table S1) and range from ~6 μm among species in Clade B to ~12 μm in non-symbiotic *S. voratum* in Clade E. The number, shape, and arrangement of amphiesmal plates are similar across most clade representatives (Figure 2C). Differences in plate number and shape can be as great between species within a clade as they are among clades (Figure 2C). The only verified morphological autapomorphy diagnostic of any clade is the lack of a pronounced elongate apical vesicle (EAV), also called the "acrobase" or apical furrow apparatus, among members of Clade C (Figure 2C).

Genomic Divergence between and within Symbiodiniaceae Clades

We compared model-corrected inter- and intra-clade (or in some cases, sub-clade) *LSU* rDNA genetic divergence estimates for Symbiodiniaceae with inter- and intra-genus divergence estimates for other members of the class Dinophyceae (Figure 3A). The mean inter-distance values were similar among groups (25.1% ± 7.40% between Symbiodiniaceae clades; 39.4% ± 17.5% between Dinophyceae genera), as were the mean intra-clade values (1.41% ± 1.06% within Symbiodiniaceae clades; 9.12% ± 11.9% within Dinophyceae genera). Notably, all "between" values were >5% (with the exception of the Dinophyceae contrast between *Ansanella*—*Protodinium* at 4.93%). Meanwhile, all "within" values were < 5% (for all Symbiodiniaceae clades; several Dinophyceae genera had higher values). Mitochondrial *cytochrome b* (mt *cob*) analyses produced analogous results, albeit with smaller distance values. Mean inter-clade

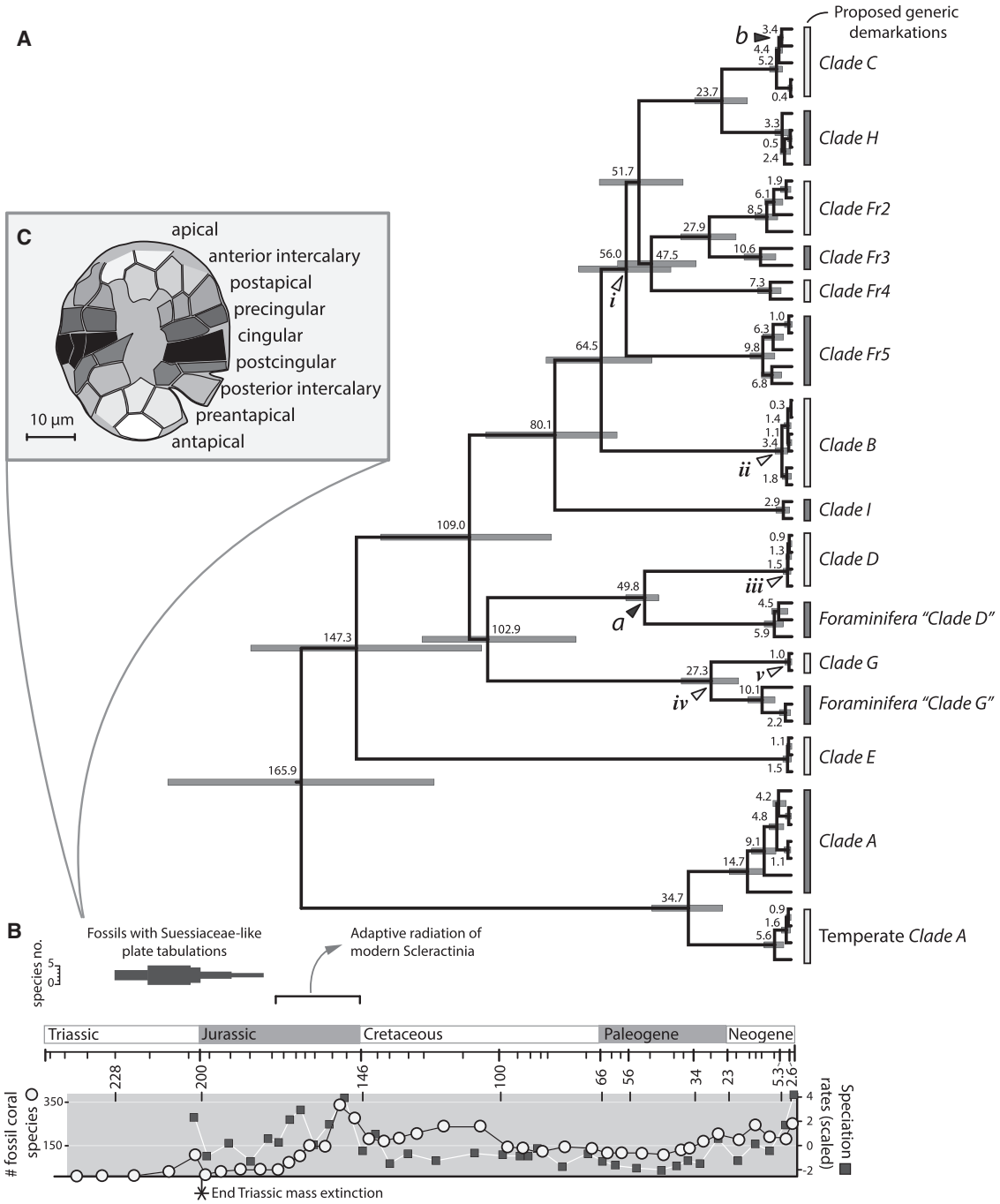

Figure 1. Ancient origins of the family Symbiodiniaceae.

(A) Evolutionary divergence times estimated among Symbiodiniaceae clades using Bayesian evolutionary analysis (employing a strict clock model) on *LSU* rDNA based on early and late calibration events (priors) from host fossil evidence (arrowhead *a*: 45.7–54.5 mya) and plate tectonic changes separating the Pacific and Atlantic Ocean basins (arrowhead *b*: 3.1–5.4 mya). Numbers located next to tree nodes are mean divergence times (millions of years) and gray bars at each branch node delineate intervals of 95% confidence. The mean clock rate was calculated at 2.1×10^{-3} (variance $\pm$ 8.0×10^{-8}) substitutions per site per million years. Prior expected ages for nodes *i* (48–56 mya), *ii* (5–7 mya), *iii* (1–3 mya), *iv* (15–34 mya), and *v* (> 3 mya) are based on available phylogeographic, paleontological, and plate tectonic evidence (see the Quantification and Statistical Analysis section of STAR Methods for further explanations). Vertical white and gray bars to the right of terminal nodes on the phylogram delineate candidate genera.

(B) Timeline indicating the fossil evidence for the origination of dinoflagellates similar in appearance to modern Suessiales; and the Jurassic radiation of modern Scleractinia. Coral paleontological data reproduced from Simpson et al. [35].

(C) The holotype of *Suessia swabiana* from the Late Triassic (201–208 mya), a fossil cyst (~40–50 µm) with 9 rows of plates, including double row of paraplates in the cingular region (adapted from Figure 12a in Morbey [36]); it is morphologically most similar to present dinoflagellates from the order Suessiales. Relates to Figures S1, S2, S3, and S4 and to Quantification and Statistical Analysis.

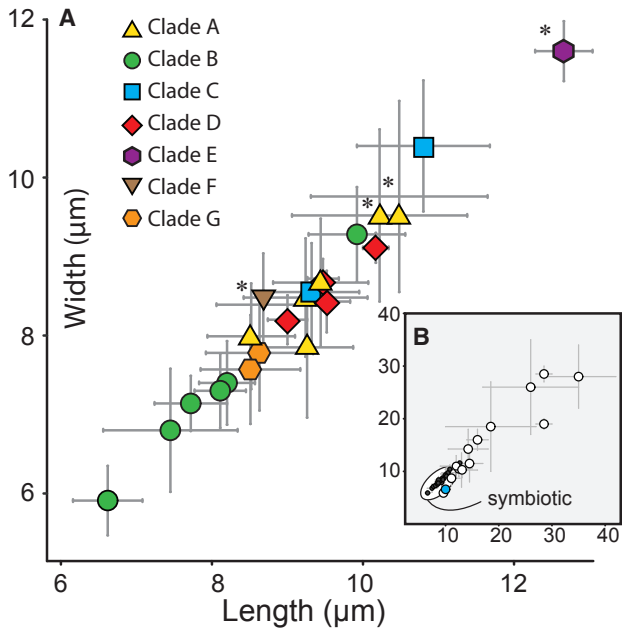

Figure 2. Morphological Differences within and among Symbiodiniaceae Clades

(A) Range of coccoid cell sizes (length and width) among described species of Symbiodiniaceae. Data points correspond to means observed for different species and are color coordinated (see inset). Error bars represent standard deviations. Asterisks denote species unable to colonize aposymbiotic host cnidarians.

(B) The cell sizes of Symbiodiniaceae (black circles) in comparison to other Suessiales (larger open circles). The only other known symbiotic species from this order (*Pelagodinium beii*, which associates with a pelagic foraminifera) is colored in blue.

(C) Amphiesmal plate arrangements and patterns of motile cells currently known for taxa within and between Symbiodiniaceae clades. Plates highlighted in red indicate unique differences in plate numbers among the seven latitudinal series of amphiesmal vesicles. The asterisk indicates absence, or reduction, of the elongate apical vesicle (EAV; "acrobase" or apical furrow apparatus). 1. *Symbiodinium goreaui*, 2. *S. minutum*, 3. *S. psygmophilum*, 4. *S. voratum*, 5. *S. microadriaticum*, 6. *S. necroappetens*, 7. *S. tridacnidorum*, 8. *S. natans*. Relates to Table S1 and to Quantification and Statistical Analysis.

or -genus mt *cob* estimates for Symbiodiniaceae and Dinophyceae were 7.48% ± 2.95% and 10.5% ± 5.10%, respectively, and intra-clade or -genus estimates for Symbiodiniaceae and Dinophyceae were 0.241% ± 0.158% and 1.38% ± 2.32%, respectively (Figure 3B).

To substantiate the sequence divergence observed from *LSU* rDNA and mt *cob* data (Figures 3A and 3B, Tables S2 and S3), transcriptomic comparisons were conducted between representative species from Clades A, B, and F ("sub-clade" Fr5) to identify levels of shared orthologous genes derived from a common ancestor (Figure 3C). We found a much higher proportion of orthologs within a clade (71% for Clade B *Symbiodinium minutum* versus Clade B *S. psygmophilum*) than between clades (10%–22% for pairwise contrasts of Clade A *S. microadriaticum,* Clade B *S. minutum,* and Clade F *S. kawagutii*; Figure 3C). When these values were plotted against divergence times estimated from the *LSU* rDNA data (above), there was a clear pattern of rapidly decreasing orthology with increasing time since divergence (Figure 3C).

DISCUSSION

Geological Age of the Symbiodiniaceae

The earliest comparison of rDNA sequences obtained from the algal symbionts of reef corals and other invertebrates indicated that they were the product of a long evolutionary history [10]. Our revised age for the Symbiodiniaceae coincides with the adaptive radiation of calcifying corals and reef growth during the middle Jurassic Period (Figure 1B) [35]. Therefore, these findings bring into alignment the ancient origin and success of reef-building corals with the evolution of the Symbiodiniaceae. They further imply that Symbiodiniaceae have persisted through tens of millions of years of Earth's history while enduring large and numerous environmental changes.

Independent geochemical and morphological data indicate the emergence of photosymbioses early in scleractinian evolution [37]. Changes in isotopic abundances in $\delta\ ^{18}O$ and $\delta\ ^{13}C$ [38] and characteristics of skeletal banding patterns [39] during the Late Triassic are proxies for the presence of photosynthetic biochemistry. However, early symbiotic coral species became extinct during the End-Triassic mass extinction event (Figure 1B). If our estimates placing the origination and diversification of Symbiodiniaceae in the middle to late Jurassic are accurate, modern-day coral-algal symbioses first emerged during the

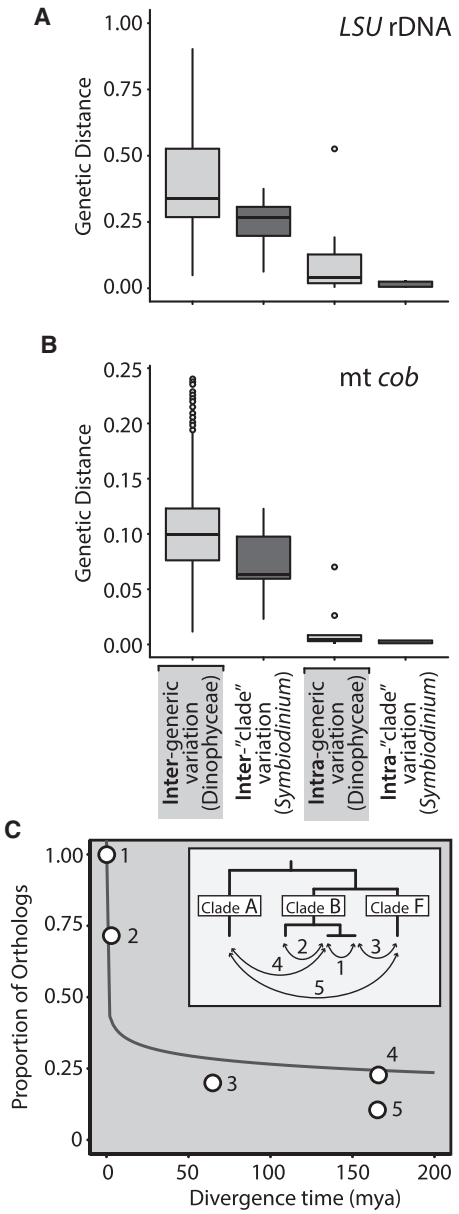

Figure 3. Genetic Distances between Historical Symbiodiniaceae Clades Correspond to Established Dinoflagellate Genera

(A and B) Mean percent divergence (model-corrected genetic distance values) between and within genera of the Dinophyceae for (A) *LSU* rDNA (n = 38 genera) and (B) mitochondrial *cob* (n = 31 genera) genes compared to divergences among and within clades in the family Symbiodineaceae.

(C) Comparison of gene orthology within and between clades based on transcriptome data, showing a decline in similarity with increasing divergence time (= genetic distances between *LSU* rDNA). Transcriptomic comparisons of orthology were made between the following: (1) *Symbiodinium minutum* versus *S. minutum* (different strains of the same Clade B species); (2) *S. minutum* versus *S. psygmophilum* (within Clade B); (3) *S. minutum* versus *S. kawagutii* (Clade B versus Clade F); (4) *S. minutum* versus *S. microadriaticum* (Clade B versus Clade A); and (5) *S. microadriaticum* versus *S. kawagutii* (Clade A versus Clade F). Inset: phylogram of species included in the transcriptomic analyses. Relates to Tables S2 and S3 and to Quantification and Statistical Analysis.

second, and much larger, scleractinian radiation, when communities of these animals began to build reef structures and gave rise to lineages of modern reef corals [35, 40]. These Jurassic corals evolved and rapidly diversified after a long geological dormancy—which lasted tens of millions of years, a consequence of the End-Triassic mass extinction (Figure 1B)—and were distinct from their Triassic cousins [35].

A Mesozoic origin of the Symbiodiniaceae is considerably older than previous approximations, which proposed relatively recent origins of the family in the Paleocene or Eocene epochs, 50–65 mya [26, 27]. However, our assessment of contemporary phylogenetic, geographic, and paleontological evidence identified calibration problems with these earlier molecular clocks, making the likelihood of a Cenozoic Era origination doubtful (see the STAR Methods section Quantification and Statistical Analysis). These issues included the following: (1) using sequences from geographically inappropriate origins to calibrate the separation between the Pacific and Atlantic Oceans (Figure S4); (2) relying on fossil dates from only extant host lineages that are part of a much older group (Figures S2A and S2B); and (3) using a molecular clock calibrated from fossils of a dinoflagellate order whose lineages show accelerated rates of rDNA evolution relative to other genes (Figure 4G). Ultimately, the estimates of evolutionary times presented in Figure 1A reflect a more parsimonious correspondence with the history of modern zooxanthellate shallow-water corals [41, 42]. Additionally, the dates estimated for many internal and recent branch nodes appear consistent with timings of plate tectonic changes that affected biogeographic patterns and drove shifts in paleoclimates [e.g., 43], likely influencing the emergence and success of new lineages.

The reliability of molecular dating is not only dependent on accurate calibration points but also on a relatively robust phylogeny [44]. The future use of additional genes for molecular dating will be important to test their congruence with dates based on *LSU* rDNA, as well as to evaluate the evolutionary hypotheses raised by these newest estimates [e.g., 21]. However, *LSU* rDNA is currently the most versatile gene for analyses of evolutionary history between clades of Symbiodiniaceae. It is the only gene known to provide sufficient resolution to delimit most putative species while being conserved enough to conduct a phylogenetic analysis among distantly related Dinophyceae [45]. Other widely used markers such as ITS rDNA and *cp23S* are best applied in phylogenetic analyses among species within a clade.

The long evolutionary history of the Symbiodiniaceae has generated at least fifteen extant and divergent lineages currently comprising one genus, *Symbiodinium* (Figure 1A). The ecological dominance and host prevalence of each clade is different at local, regional, and global scales and appears to have been influenced by climatic factors prevailing over the past 3–5 million years or more [46]. The apparent species diversity in each clade, mostly unrealized by formal taxonomic standards, varies considerably, but collectively, the species membership in this family likely numbers in the many hundreds [21]. A systematic revision of this complex group is an obvious necessity given the organizational challenges posed by high species diversity distributed across greatly divergent and ecologically diverse lineages.

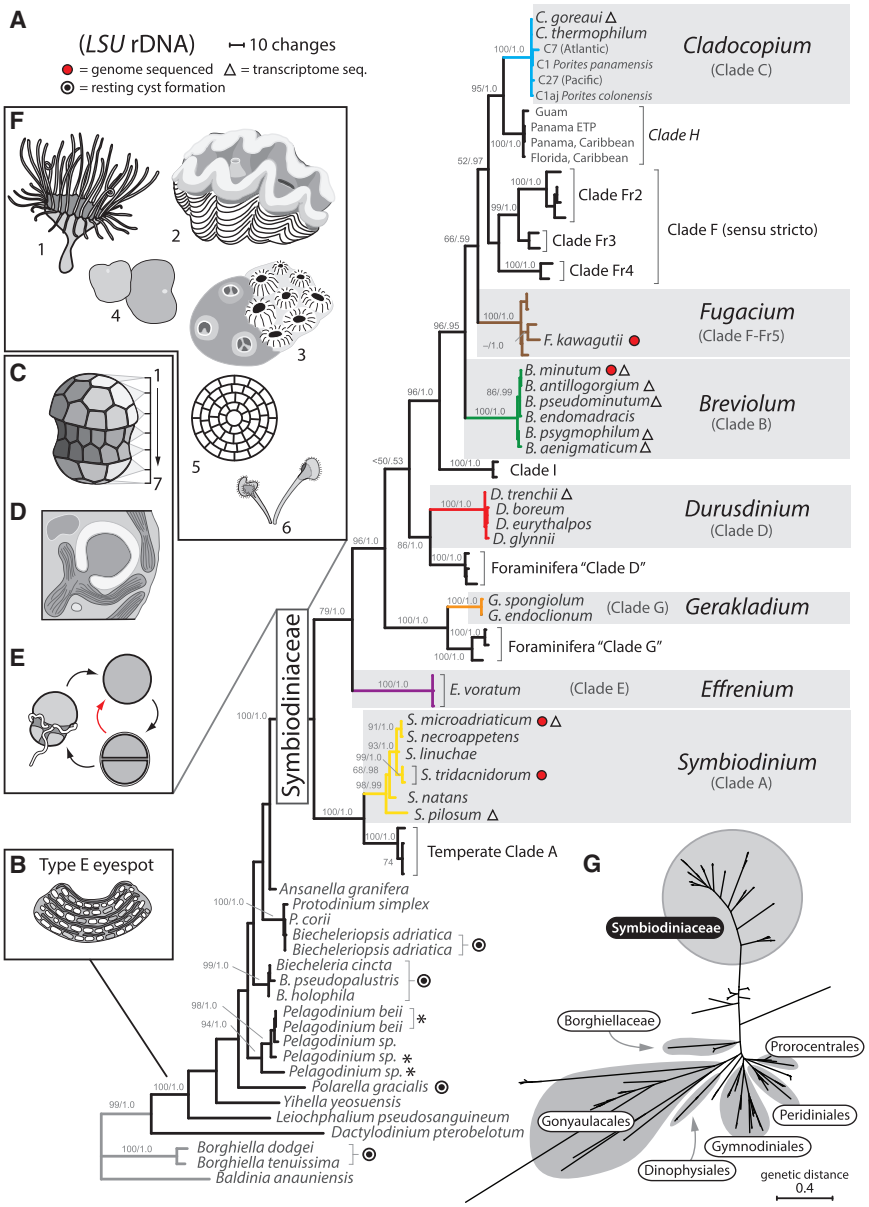

Figure 4. Proposed New Genera in the Family Symbiodiniaceae and *LSU* rDNA Phylogeny Portraying their Evolutionary Relationships, Including Remaining Lineages that Require Generic Names

(A–E) *LSU* rDNA phylogeny of genera in the Order Suessiales. Symbols next to terminal branches indicate species whose genomes or transcriptomes are sequenced (red circle), which taxa form resting cysts (black circle), and non-Symbiodiniaceae taxa that are endosymbionts (asterisks). Bootstrap values based on 1000 iterations and Bayesian posterior probabilities are indicated for each internal branch. The following shared ancestral traits are indicated on the tree: (B) most recent common ancestor possessing an eyespot lens comprising multiple cisternae containing uric acid crystals in a "type E" brick-like arrangement, (C) cells with mastigotes possessing only seven latitudinal series of amphiesmal vesicles, (D) cells having a single pyrenoid surrounded by starch deposits and an internal matrix devoid of thylakoid extensions, and (E) a cell life cycle with a predominant coccoid (metabolically active vegetative cell) phase, which divides *in hospite* producing daughter coccoid cells or transient thecate motile cells.

(F) Genera in the Symbiodiniaceae are most typically symbiotic with various metazoan phyla, including the following: (1) Cnidaria, (2) Mollusca, (3) Porifera, (4) Platyhelminthes, as well as single-celled eukaryotes such as (5) Foraminifera and (6) Ciliates.

(G) Unrooted *LSU* rDNA phylogeny based on maximum likelihood showing genetic distances and evolutionary relationships among major groups of Dinophyceae in comparison to the Symbiodiniaceae. Relates to Table S2 and to Method Details. For the taxonomic summary of the Symbiodiniaceae, see Data S1.

Genetic Insights and Changes in Systematics and Taxonomy

Prior to the availability of genetic evidence, naturalists relied for centuries on morphology to produce their taxonomic descriptions. Thus, until recently, organisms with few morphological characters such as microbial eukaryotes were poorly described and relatively little was known about their diversity, ecology, and evolution. The lack of visual cues among Symbiodiniaceae constrained research on these organisms for many decades, particularly when only a single species was perceived to exist [47]. Being among the smallest of dinoflagellates [48], the group's predominant niche as endosymbionts appears to have constrained the evolution of significant morphological variation within the group and perhaps restricted the size of most species to <11 μm (Figures 2A and 2B) [48]. Presently, only Clade C can be diagnosed by a morphological trait (Figure 2C). However, decades of research on Symbiodiniaceae have relied on genetic delineation of distinct entities for the purposes of biochemical, physiological, and ecological study because genetic data quickly resolve these morphologically cryptic and unambiguously organisms.

The Family Symbiodiniaceae

All Symbiodiniaceae contain a type E eyespot adjacent to the sulcus groove of the motile cell, a distinctive but common trait shared with related members of the order Suessiales (Figure 4B). However, unlike other families in the order, Symbiodiniaceae species do not appear capable of forming resting cysts at any point in their life cycle. Moreover, the Symbiodiniaceae share several key morphological, anatomical, and ecological features that unify and distinguish them (see the Taxonomic Summary in Data S1). These include the following: (1) possession of seven latitudinal series of amphiesmal vesicles in the motile (mastigote) stage (Figure 4C); (2) possession of a single pyrenoid where the internal matrix, the site of carbon fixation in the chloroplast, is

devoid of thylakoid membrane extensions (Figure 4D); (3) a modified cell division *in hospite*, wherein metabolically active coccoid cells reproduce mitotically and the motile stage is bypassed, or significantly reduced (Figure 4E); and (4) the capability to form endosymbioses with metazoan and protistan hosts (Figure 4F).

Phylogenetics and Genomics Indicate "Clades" are Genera

For dinoflagellates, *LSU* rDNA currently serves as the most inclusive and effective marker for assessing phylogenetic relationships and delimiting generic groupings (see the STAR Methods section Quantification and Statistical Analysis). Mitochondrial genes such as *cytochrome oxidase* 1 (*cox* 1) and mt *cob* are also commonly employed in phylogenetic analyses. The tiered pattern of molecular-genetic divergence between and within clades justifies their elevation to genera and avoids ambiguity from morphological evidence. Large differences in the nucleotide sequences of nuclear rDNA (Figure 3A), mitochondrial genes (Figure 3B), and chloroplast genes [e.g., 27], are equivalent to, and sometimes greater than, differences observed among most accepted genera in the class Dinophyceae (Figures 3A and 3B). The phylogenies of several independent nuclear encoding genes [49], as well as low melting points for DNA/DNA hybridizations [50], further substantiate that Symbiodiniaceae clades are equivalent to generic divisions of other dinoflagellates.

Major differences in gene content, involving comparisons of tens of thousands of gene transcripts, further indicate considerable evolutionary divergence between clades (Figure 3C). As expected, strains from the same species and species from the same clade featured a high proportion of orthologs (homologous genes with shared ancestry), whereas species from different clades with much greater divergence times shared far fewer orthologs (Figure 3C). These results confirm the findings of several investigators, who have observed low proportions of orthologs in their comparisons of transcriptomes between members of different clades [28, 29, 31, 51] but high orthology within clades [52].

Analyses of the sequenced genomes of *Symbiodinium microadriaticum* (Clade A) [30], *S. minutum* (Clade B) [53], and *S. kawagutii* (Clade F) [54] revealed that their overall genome organization includes large differences in gene orientation and no gene synteny, along with major variations in gene content (e.g., unique numbers of bicarbonate, amino acid, and ion transporters, as well as stress-associated chaperone domains) [30]. Further comparative work suggested that additional genomic attributes are particular to each clade, which may correspond to (and potentially explain) each lineage's distinct ecological and biogeographic distribution [31]. These multi-gene analyses further support the extent of evolutionary divergence among clades and their phylogenetic positions as revealed through traditional single-gene analysis of *LSU* rDNA.

Demarcation of Symbiodiniaceae Genera Using DNA Divergence

Although we are reluctant to propose any single cutoff value, the establishment of a standard genetic distance reduces ambiguity when demarcating genera. For both *LSU* rDNA and mt *cob* markers, there is a clear separation in sequence divergence between and within established Dinophyceae genera (Figures 3A and 3B). A 5% model-corrected genetic distance at *LSU* rDNA and a 1% distance for mt *cob* appear to be conservative upper boundaries of divergence among many (though not all) of the Dinophyceae genera examined. Current genera that do not fit within these boundaries, such as *Gymnodinium*, may require further systematic and taxonomic study to determine whether they constitute several distinct genera, and in other cases, multiple genera may require consolidation [55]. Ultimately, the gaps in sequence divergence observed within and between most species from distinct Dinophyceae genera provide a natural break for objectively delimiting new Symbiodiniaceae genera.

Thus, for the Symbiodiniaceae, applying thresholds of 5% for *LSU* rDNA and 1% for mt *cob*, we demarcate the historical *Symbiodinium* clades as new genera (Figures 3A, 3B, and 4A). In this context, the largest genetic distance measured within a Symbiodiniaceae clade is 2.9% for *LSU* rDNA (within Clade Fr3) and 0.44% for mt *cob* (within Clade C), values that are well below the proposed thresholds. Additionally, the two most closely related Symbiodiniaceae clades at present are above these values at 6.3% for *LSU* rDNA (between Clades C and H) and 2.3% for mt *cob* (between Clades C and G). While 15 clades and sub-clades within the current genus *Symbiodinium sensu lato* are candidate genera, only seven contain formally recognized type species (see below), so at present, we are restricted to proposing new genera for these lineages.

New Genera of the Symbiodiniaceae

This systematic revision converts the widely used alphabetic "clade" designations into a conventional nomenclatural framework based on the amassed body of available biological information. Here, we define six new genera (Figure 4A) and also redefine the genus *Symbiodinium sensu stricto* to correspond to only former Clade A (which contains the valid type species of the group, *S. natans*, as well as the first "invalidly" described species, *S. microadriaticum*). Aside from large divergences in gene composition, each genus possesses notable differences in species diversity, ecological breadth (e.g., host-range diversity and disparity), and biogeographic distribution. These include differences in prevalence among hosts from environmentally distinct reef habitats, geographic regions, and ocean basins. The new genera are summarized briefly below; formal descriptions, pertinent citations, and more extensive discussion are provided in the Taxonomic Summary in Data S1.

Symbiodinium *Gert Hansen & Daugbjerg—Formerly Clade A; Type Species:* S. natans

This phylogenetically basal genus (Figure 4A) contains the first invalidly described species [47, 56] and the first validly described species [57]. The redefined genus *Symbiodinium* includes species with ecologies ranging from symbiotic to opportunistic or free living. Most are adapted for living in high light or variable light conditions and appear to constitutively secrete UV-adsorbing mycosporine-like amino acids (MAAs) when in culture [58]. Its membership is globally distributed, but individual species are endemic to particular ocean basins. The genus name means "living together" and "whirling."

Breviolum *J.E.Parkinson & LaJeunesse* genus novum—*Formerly Clade B; Type Species:* B. minutum

This genus contains some of the smallest Symbiodiniaceae and its members associate primarily with cnidarians. Phylogenetically

basal lineages of this genus are often found in hosts from high-latitude coastal habitats as well as in hosts that live over wide depth ranges in the tropics. A younger, evolutionarily derived radiation of closely related species is highly prevalent in the Greater Caribbean, where they occur in numerous shallow-dwelling stony and soft coral hosts. The genus name means "short" and "small ones."

Cladocopium *LaJeunesse & H.J.Jeong* gen. nov.—*Formerly Clade C; Type Species:* C. goreaui

The elongate apical vesicle is highly reduced, or lacking, in thecate motile cells (Figure 3C). This is the most species-rich, ecologically abundant, and broadly distributed genus within the Symbiodiniaceae. Members of this group associate with a broad diversity of hosts, including corals and other cnidarians, clams, ciliates, flatworms, foraminifera, and sponges. They are physiologically diverse, with members adapted to a wide range of temperatures and irradiances. Most of its members are highly host specialized, but certain host-generalist species are ecologically abundant in many Indo-Pacific reef coral communities. The name means "branch" and "plenty."

Durusdinium *LaJeunesse* gen. nov.—*Formerly Clade D; Type Species:* D. trenchii

This genus includes species that are known extremophiles with adaptations to survive in regions with large temperature and turbidity fluctuations. Symbioses involving members from this group tend to be resistant to disassociation (e.g., coral bleaching). Its diversity and distribution are centered in the Indo-West Pacific. The name means "tough" and "whirling."

Effrenium *LaJeunesse & H.J. Jeong* gen. nov.—*Formerly Clade E; Type Species:* E. voratum

This genus currently contains just one species, the exclusively free-living (non-symbiotic) *E. voratum*. This species uses its tubule peduncle to graze on bacteria and other unicellular eukaryotes, creates blooms under certain circumstances, uniquely maintains motility during the dark cycle, and has the largest cell volume when compared to other Symbiodiniaceae. It is distributed in the Pacific and Atlantic Oceans and occurs at sub-tropical and temperate latitudes. The name means "living unrestrained."

Fugacium *LaJeunesse* gen. nov.—*Formerly "Sub-clade" Fr5 within Clade F; Type Species:* F. kawagutii

This genus comprises species found in foraminifera and several non-symbiotic species known only from cultured isolates. They probably occur at transient, low-abundance densities in cnidarians, but their ecological attributes remain unknown. It is distinct from other "Clade F" lineages such as Fr2, Fr3, and Fr4; each of these lineages should be considered separate (as yet undescribed) genera. The name means "ephemeral."

Gerakladium *LaJeunesse* gen. nov.—*Formerly Metazoan-Specific "Sub-clade" within Clade G, Type Species:* G. endoclionum

This is one of the more basal lineages of Symbiodiniaceae. It is an ecologically rare genus, and knowledge about this group is comparatively limited. Certain species form specific associations with excavating sponges (Clionaidae) in both the Atlantic and Pacific Oceans, while others associate with Pacific black corals (Antipatharia). *Gerakladium* has been detected at low, background densities in colonies of stony coral and might occur free-living in the water column or benthos. The name means "old" and "branch."

Remaining Lineages within Symbiodiniaceae Requiring Generic Names

The present effort provides a framework for future generic and species classifications in the Symbiodiniaceae. While only nine clades are acknowledged in the literature [59], these assignments are rather arbitrary. The eventual number of defined genera may reach 15 or more (Figures 1A and 4A) because several divergent lineages currently grouped together within one clade can be further separated based on phylogenetic, ecological, and biogeographic evidence. For example, genetic divergences between *Fugacium* and other Clade F lineages well exceed the value between Clades H and C (Table S2) [60]. Another example is the lineage closest to *Durusdinium* (Figure 4), which has been referred to in the literature also as Clade D [27]. However, this divergent lineage occurs in symbioses with foraminifera (Figure S3). Thus, these "orphan" lineages of Symbiodiniaceae will likely be classified as new genera once representative type species are formally described. Finally, additional divergent lineages that may constitute new genera will likely be discovered as ecological surveys continue, especially in understudied ecosystems (e.g., open ocean habitats) or hosts (e.g., foraminifera) [59, 61].

Conclusions

Taxonomic rankings above the level of species are ultimately arbitrary constructs—useful only insofar as they convey meaningful information about the relationships among organisms. Recognizing that clades and some sub-clades of Symbiodiniaceae are akin to genera provides an organized systematic framework for investigating the biodiversity and natural history of these symbionts moving forward. Good systematics improves scientific communication, enables the framing of explicit hypotheses when designing experiments, and reduces confusion by limiting the extent to which conclusions may be drawn about particular groups of organisms. As global climate change decimates coral reefs, we believe this new systematic framework is a necessary step to improve animal-algal symbiosis research at this critical juncture.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
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  - Genetic distances and transcriptome comparisons
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  - Estimated temporal interval for the most recent common ancestor for Clades C-F-H: MRCAs 48–56 mya

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and three tables and can be found with this article online at https://doi.org/10.1016/j.cub.2018.07.008.

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AUTHOR CONTRIBUTIONS

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological Samples		
Symbiodinium iso-clonal cultures, Table S1	This study	N/A
Critical Commercial Assays		
Wizard Genomic DNA Purification Kit	Promega	Catalog number: A1120
Deposited Data		
LSU rDNA sequence matrix	This study	https://doi.org/10.5061/dryad.1717129
cob sequence matrix	This study	https://doi.org/10.5061/dryad.1717129
BEAST2 files	This study	https://doi.org/10.5061/dryad.1717129
R code/data	This study	https://doi.org/10.5061/dryad.1717129
Symbiodinium kawagutii proteome	[54]	http://web.malab.cn/symka_new/data/Symbiodinium_kawagutii.0819.final.gene.zip
Symbiodinium microadriaticum proteome	[30]	http://smic.reefgenomics.org/download/Smic.genome.annotation.pep.longest.fa.gz
Symbiodinium minutum transcriptome	[52]	NCBI Bioproject Accession: PRJNA274852; http://zoox.reefgenomics.org/download/Symbiodinium_minutum.tar.gz
Symbiodinium psygmophilum transcriptome	[52]	NCBI Bioproject Accession: PRJNA274854; http://zoox.reefgenomics.org/download/Symbiodinium_psygmophilum.tar.gz
Oligonucleotides		
28-S forward: CCCGCTGAATTTAAGCATATAAGTAAGCGG	[62]	N/A
28-S reverse: GTTAGACTCCTTGGTCCGTGTTTCAAGA	[62]	N/A
cob1-forward: ATGAAATCTCATTTACAWWCATATCCTTGTCC	[63]	N/A
cob1-reverse: TCTCTTGAGGKAATTGWKMACCTATCCA	[63]	N/A
Software and Algorithms		
PhyML v3.3.20180214	[64]	https://github.com/stephaneguindon/phyml
MrBayes v3.2.6	[65]	http://mrbayes.sourceforge.net/
MrModeltest v2.3	[32, 33]	https://github.com/nylander/MrModeltest2
PAUP* v4.0 b10	[66]	http://paup.phylosolutions.com
InParanoid v4.1	[67]	http://software.sbc.su.se/cgi-bin/request.cgi?project=inparanoid
BEAST2 v2.4.5	[34]	http://www.beast2.org
Geneious v9.1.6	[68]	https://www.geneious.com/
ProgRes Capture Pro v2.8	N/A	https://www.jenoptik.com/
ImageJ v1.50i	[69]	https://imagej.nih.gov/ij/index.html

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Todd C. LaJeunesse (tcl3@psu.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Culturing

Cultures of iso-clonal *Symbiodinium* were grown in ASP-8A medium at 26°C illuminated by fluorescent tubes delivering 80–120 μmol quanta·m^{-2}·s^{-1} photosynthetically active radiation on a 14:10 (light:dark) photoperiod [12]. Cultures were obtained from a variety of cnidarians and giant clams collected from reefs in the Greater Caribbean, Hawaii, and Australia. Specific details about culture origin and location are provided in the original species descriptions.

Host Tissue Collection

Unculturable *Symbiodinium* were obtained by collecting host cnidarian tissue, which was field-preserved in 20% dimethyl sulfoxide buffer (containing 0.25 M ethylenediaminetetraacetic acid and water super-saturated with sodium chloride) and stored at −20°C [19]. Samples originate from around the world, and specific details about host species and location are provided in the original species descriptions.

Ethics statement

This study was performed on marine micro-algae exempt from ethical review. Host cnidarian specimens were collected under appropriate permits (listed in the original species descriptions).

METHOD DETAILS

DNA extraction, PCR and sequencing

Nucleic acids were extracted as described by LaJeunesse et al. [12] using the Wizard DNA prep protocol (Promega Corporation, Madison, WI). The amplifications were performed in 25 μL reaction volumes containing 2.5 μL of 2.5 mM dNTPs, 2.5 μL of 25 mM $MgCl_2$, 2.5 μL standard Taq Buffer (New England Biolabs, Ipswich, MA, USA), 0.13 μL of 5 $U \cdot \mu L^{-1}$ 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. The *LSU* region was amplified with the primers 28S-forward (5'-CCCGCTGAATTTAAGCATATAAGTAAGCGG-3') and 28S-reverse (5'-GTTA GACTCCTTGGTCCGTGTTTCAAGA-3') [62] and the following thermocycler conditions: 2 min at 90°C, followed by 35 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, and then 5 min at 72°C. The mt *cob* region was amplified with the primers cob1-forward (5'-ATGAAATCTCATTTACAWWCATATCCTTGTCC-3') and dinocob1-reverse (5'-TCTCTTGAGGKAATTGWKMACC TATCCA-3') [63] and the following thermocycler conditions: 1 min at 95°C, followed by 40 cycles of 20 s at 94°C, 30 s at 55°C, and 40 s at 72°C. Amplification products were directly sequenced on an Applied Biosciences sequencer (Applied Biosciences, Foster City, CA, USA) at the Pennsylvania State University Genomics Core Facility. Chromatograms were checked and the sequences were aligned using Geneious v9.1.6 [68].

Morphology and phylogenetic analyses

To characterize cell size, either *in vitro* cultures or *in vivo* host tissue samples were imaged as described by LaJeunesse et al. [12]. Cells were photographed during log phase growth under bright-field illumination at a magnification of 400 × – 1000 × using an Olympus BX51 compound microscope (Olympus Corp., Tokyo, Japan) with a Jenoptik ProgRes CF Scan digital camera (Jenoptik, Jena, Germany). Images were captured using the autoexposure setting within ProgRes Capture Pro 2.8 software (Jenoptik) and cell sizes were measured with the program ImageJ [69].The calculation of mean species sizes were based on measurements of *Symbiodinium* (n > 40 cells) from *in vitro* cultures or on cells directly isolated from host tissue samples (n = 1-24 individual samples or strains were measured per species).

To characterize thecal plate patterns, motile cells from iso-clonal cultures were imaged from multiple viewpoints via electron microscopy and plate numbers and shapes tabulated as described by Jeong et al. [13]. For SEM, cells were fixed for 10 min in osmium tetroxide at a final concentration of 0.5% (v/v) in seawater. They were collected on a PC membrane filter and rinsed three times with distilled water. They were dehydrated in a graded ethanol series and dried using a critical point dryer (BAL-TEC, CPD 300, Balzers, Liechtenstein, Germany). The dried filters were mounted on a stub and coated with gold-palladium, then viewed with an FE-SEM (S-4800, HORIBA: EX-250, Hitachi, Hitachinaka, Japan) and SEM (JSM-840A, SEM. JEOL Ltd., Tokyo, Japan). The cells were photographed using a digital camera.

For TEM, cells were fixed in 2.5% (v/v) glutaraldehyde (final concentration) in the culture medium for 1.5–2 h. The cells were then pelleted and rinsed several times in 0.2 M sodium cacodylate buffer at pH 7.4. The cells were postfixed for 90 min in 1% (w/v) osmium tetroxide in deionized water and the pellet was then embedded in agar. Dehydration was performed in a graded ethanol series, and the material was embedded in Spurr's low-viscosity resin. Serial sections were prepared on an RMC MT-XL ultramicrotome (Boeckeler Instruments Inc., Tucson, AZ). They were stained with 3% (w/v) aqueous uranyl acetate followed by lead citrate. Finally, sections were viewed with a JEOL-1010 transmission electron microscope (JEOL Ltd., Tokyo, Japan).

A maximum likelihood phylogenetic tree was inferred from nuclear large subunit (*LSU*) rDNA sequences using the software package PhyML v3.3.20180214 [64] and the posterior probabilities of phylogenetic branching assessed using MrBayes v3.2.6 [65]. Representative sequences from *Symbiodinium* clade and "sub-clade" lineages were aligned with sequences from representative genera in the orders Suessiales, Gymnodiniales, Dinophysiales, Gonyaulacales, Prorocentralies, Peridiniales, and Thoracosphaerales, which were obtained from GenBank, for a total dataset comprising 149 sequences representing 76 species and 38 genera in addition to the Symbiodiniaceae (783 aligned base pairs available at https://doi.org/10.5061/dryad.1717129). A similar, albeit smaller, dataset of 89 mt *cob* sequences from 48 species and 29 genera in addition to the Symbiodiniaceae, and representative of these dinoflagellates orders, was also assembled and used in evaluating genetic distances between and among genera (938 aligned base pairs available at doi: https://doi.org/10.5061/dryad.1717129).

QUANTIFICATION AND STATISTICAL ANALYSIS

Genetic distances and transcriptome comparisons

To quantify within- and between-group genetic distances, MrModeltest v2.3 [32, 33] was used to identify the best model of nucleotide evolution on the same alignment of 143 dinoflagellate *LSU* rDNA sequences utilized in phylogenetic inference (see above). These analyses were also applied to the dataset of 89 mitochondrial *cob* (mt *cob*) sequences. For both genes (*LSU* rDNA and mt *cob*), genetic distances were calculated under the General Time Reversible model of evolution, with a proportion of invariable sites and rate variation among sites (i.e., GTR+I+G) estimated from the data, with PAUP* v4.0 b10 [66].

For transcriptomic/proteomic comparisons, orthologous genes were identified using reciprocal BLASTp searches of open reading frames within InParanoid v4.1 [67] in pairwise comparisons of amino acid data from the public repository reefgenomics.org [70] or the Symka Genome Database. Specifically, datasets for the following species were included: *S. minutum* (*ITS2* type B1) and *S. psygmophilum* (*ITS2* type B2) [52, 53], *S. kawagutii* (*ITS2* type F1) [54], and *S. microadriaticum* (*ITS2* type A1) [30]. Total counts were normalized to the smallest dataset in each comparison to calculate the proportion of orthologs.

Divergence time calculations

Multiple points of calibration for the *LSU* rDNA data were determined from timing geological dates for 1) the oceanographic and physical separation of the Pacific and Atlantic Oceans, and 2) the origination times calculated from the fossil record of symbiotic foraminifera in the family Soritidae, which uniquely harbor dinoflagellates. Detailed explanation and justification for calibrations involving each of seven phylogenetic nodes are provided below.

The program Bayesian Evolutionary Analysis by Sampling Trees (BEAST2 v2.4.5) was employed to estimate divergence times [34]. MrModeltest (see above) calculated the best model of nucleotide evolution used to parameterize the BEAST 2 analyses. Under a calibrated Yule Model, multiple pairwise combinations of priors, using one early and one late node (Figure S1), were analyzed using MCMC of chain lengths of 100 million. Clock models based on a strict clock as well as relaxed clock log normal were conducted. Mean values of node divergence times and clock rates were compared to evaluate consistency across different combinations and repeated MCMC runs.

LSU molecular clock calibrations

Heterogeneity in rates of molecular evolution across lineages can significantly affect the accuracy of divergence time estimates [44]. Given that one part of the gene tree may be evolving faster than another part, different pairwise analyses of early and late calibration times in different combinations were used to test for consistency in age estimates. Paleontological, plate-tectonic, and biogeographic evidence provide multiple independent time spans for calibrating a molecular clock to estimate rates of *LSU* rDNA evolution among the Symbiodiniaceae. Figure S1 identifies calibration times that correspond to the text below.

Calibrations based on host fossil record

The ''early'' (i.e., oldest) nodes for the calibration of the *LSU* rDNA molecular clock rely on the emergence of an important group of single-celled hosts, the soritid foraminifera. Entire lineages of Symbiodiniaceae appear to be associated with only these discoid shell-forming epibenthic organisms. The Paleocene–Eocene Thermal Maximum (PETM) occurred about 55 million years ago (mya), lasted approximately 200,000 years, and led to extinctions of many marine organisms, including large benthic foraminifera [71]. However, after the PETM and during the Eocene Climatic Optimum, a major adaptive radiation of large miliolid soritids occurred [71, 72]. This span of time experienced the most significant adaptive radiations of large benthic foraminifera during the Cenozoic (Figure S2A). The origination of new genera in the family Soritiodea (and in other families of large foraminifera) increased precipitously during the Ypresian and Lutetian epochs of the Eocene (~56–42 mya; Figure S2A).

The Eocene represents the warmest climate in the Cenozoic era, characterized by warm oceans and no polar ice caps, with proxies for atmospheric CO_2 suggesting that concentrations were double or quadruple those of current levels. Thus, sea level was at maximum height and inundated all low-lying terrestrial environments, creating large shallow seas and lagoons. Moreover, high ocean temperatures reduced rates of ocean circulation, leading to widespread and long-standing oligotrophic conditions, ideal for facilitating the evolutionary success of mutualistic symbioses. Of the many Soritoidea genera that emerged during this time, some fossils (e.g., *Orbitolites*) were attached to the surfaces of hard substrates, macro-algae seaweeds, and sea grass blades (i.e., found in fossil sea-grass environments; Figure S2B). This notable ecological shift away from soft sediments appears to correspond with the evolution of photosymbioses in foraminifera and is diagnostic of the possession of dinoflagellates endosymbionts [73].

We posit that the major Cenozoic adaptive radiation of Soritiodea, which included lineages exhibiting photosymbioses (Figures S2A and S2B), initiated a corresponding diversification of Symbiodiniaceae. The current phylogeny of Symbiodiniaceae comprises interleaved lineages, or ''clades,'' that associate with animals, represented mostly by cnidarians, or with soritid foraminifera (Figure S1). Relying on the established concept that host availability is paramount to the evolution and success of Symbiodiniaceae lineages and vice versa [21], and grounded on the principles of cladistics, the ancestral condition among members of a clade can be determined based on prevalence of a particular character trait; in this case, associations with highly distinct groups of host taxa. There are at least 3 nodes in the phylogeny of Symbiodiniaceae that are presumed to represent independent origins of lineages corresponding to the emergence of symbiotic foraminifera during the early Eocene.

Estimated temporal interval for the most recent common ancestor for Clades C-F-H: MRCAs 48–56 mya

Clades C, F, and H are a monophyletic group whose most recent common ancestor is inferred to have originated in response to the adaptive radiation of the Soritoidea and included the emergence of *Orbitolites*, whose earliest appearance is in the Ypresian epoch [27]. The extant symbiotic soritid genus, *Sorites*, first occurs in the Oligocene fossil record, 25–30 mya. However, paleontological evidence can define only the latest possible time of divergence, but not the lower boundary, and for this reason fossil dates are always underestimates [44]. Moreover, the extinct genus *Orbitolites* existed in the early Eocene and appears to have had similar ecological distributions (living attached to phytal substrata) and resembled the morphology of modern descendent soritids bearing dinoflagellate symbionts (Figure S2B). Furthermore, the newest molecular-clock estimates of the origination of the extant soritids harboring Symbiodiniaceae places their origination back to the Eocene, well before the Oligocene [72]. This combined evidence indicates that the early Eocene was the time when large symbiotic miliolid soritids were acquiring photosymbioses and diversifying. Thus, we infer that the common ancestors of several independent lineages of Symbiodiniaceae, including the super-clade comprising Clades C, F, and H, dates back to the first epoch of the Eocene (Figure S2B).

The evolution of "Foraminifera Clade D" lineage: MRCA 48–56 mya

This is a rare and unusual lineage, known only to associate with the foraminifera genera *Amphisorus* and *Marginopora* (Figure S3). Ribosomal DNA sequences indicate that this group contains multiple species. One of these sequences was obtained from a cultured isolate putatively from the Pacific sponge *Haliclona koremella* (designated as isolate P105), but fresh samples obtained from these sponges do not contain Symbiodiniaceae (personal observation). Thus, cultured isolate P105 from a non-symbiotic sponge apparently represents a contaminant from environmental sources of Symbiodiniaceae. Here we presume that the ''Foraminifera Clade D'' lineage (Figure S3) evolved independently in the early Eocene concurrent with the lineage that would give rise to Clades C, F, and H (see above), in response to the adaptive radiation of Soritoidea in the early Eocene (Figure S2A). This lineage is related to, but still divergent from, Metazoan Clade D, which comprises stress-tolerant species symbiotic with cnidarians [14].

Divergence time of a Clade G lineage specialized to associate with *Marginopora*: MRCA 15–34 mya

During the evolutionary history of Clade G, this lineage bifurcated into lineages with distinct ecological distributions. While one lineage associates with metazoans (sponges and cnidarians), the other associates with foraminifera in the genus *Marginopora* [74]; although members of this group may occur, albeit rarely, in other soritids [75]. Thus, the evolution of the foraminifera branch of Clade G may have coincided with the emergence of this newest soritid genus. The oldest fossils of *Marginopora* conservatively date this lineage to at least the mid-Miocene (~15 mya; Figure S2), but molecular clock estimates extend the probable origin of this genus to the Oligocene at 23-34 mya [72].

Calibrations based on oceanic vicariance

The tectonic movements during the separation of the Atlantic and Pacific Oceans, and associated shifts in ocean currents, salinity change, and changes in fossil community assemblages (marine and terrestrial), is one of the most studied and commonly used to calibrate molecular clocks [43].

Clade C symbionts in sibling species *Porites panamensis* (Eastern Pacific) and *Porites colonensis* (Caribbean): MRCA 3.1– 4.7 mya

Porites panamensis shares a most recent common ancestor with *Porites colonensis* and other Atlantic *Porites* [76]. Thus, *P. panamensis* constitutes a relic species isolated during the separation of the Pacific from the Atlantic via uplift of the Central American Isthmus, a geological process that started ~11–13 mya and was complete by ~3 mya [77]. This close genealogical relationship is also supported by the similar identities of their symbionts. High-resolution genetic analyses indicates that their symbionts also share a most recent common ancestor, sibling lineages within Clade C and members of the ''C1 radiation'' [21]. This assumes that each host-symbiont partnership has remained stable for millions of years following closure of the Central American Seaway. By contrast, the more distantly related *Porites* spp. widespread throughout the Indo-Pacific are primarily symbiotic with ''sub-clade'' C15, which does not occur in the Atlantic.

Proxies for ocean salinities indicate the Atlantic and Pacific were separated oceanographically by 4.2–4.7 mya [78], with the physical land barriers established by 3.1 mya [77]. Given this, an offset of 3.1 mya, with maximum probability set around 4.5 mya, was used as a conservative time range spanning the point of divergence between the symbionts of *P. panamensis* and *P. colonensis*.

The divergence between Clade C type C27 (Pacific) and type C7 (Atlantic): MRCA 3.1 to 7.2 mya

This alternate calibration time point involving Clade C was included to evaluate how slight sequence differences in *LSU* rDNA may offset estimates of node ages among Symbiodiniaceae. The non-coding region of the *psbA* chloroplast minicircle ($psbA^{ncr}$) provides considerable genetic and phylogeographic resolution that goes well beyond the limitations of the more conserved nuclear rDNAs [79]; and was used recently to calculate a molecular clock for Clade C [21]. A comparison among putative species of Clade C from the Pacific and Atlantic identified several lineages from each ocean basin sharing a most recent common ancestor. One of these pairings included type C27, a host generalist found associated with scleractinians from the northern hemisphere of the Pacific Ocean, and type C7/C7a, a lineage evolved and specialized to massive reef-building *Orbicella* (= former *Montastraea*) from the Greater Caribbean. Molecular clock estimates based on the $psbA^{ncr}$ sequences place the MRCA of these lineages at 7.7 ± 3.0 mya [21].

Thus, the divergence of these lineages likely initiated in the late Miocene as global temperatures began to cool further and oscillate with increased variation [80], but well before the complete separation of the Pacific and Atlantic Oceans [77].

The separation between lineages of the B1 sub-clade from the B19 sub-clade: MRCA 5 to 7 mya

Phylogeographic evidence of Clade B Symbiodiniaceae indicates a regional diversification, or adaptive radiation, occurred among members of the B1 "sub-clade." This radiation likely initiated during the late Pliocene and continued through the Pleistocene. Few members of the B1 sub-clade occur in the Pacific Ocean. By contrast, multiple members of the B19 sub-clade occur in both ocean basins and are often found in hosts from cold water, high latitude environments. Thus, divergence among older lineages of the B19 sub-clade likely began well before closure of the Central American Seaway and probably during the mid to late Miocene when global climate and ocean temperatures turned significantly colder [81]. These phylogenetic and biogeographic patterns conservatively place the MRCA of the B1 and B19 sub-clade at a time when both oceans were connected, probably at the end of the Miocene from 5 to 7 mya.

Divergence between sibling species in Clade G associated with bioeroding clionaid sponges from the Atlantic and Pacific Oceans: MRCA 3.1–5.3 mya

The "metazoan Clade G" sub-clade, a rare group of endosymbionts, currently contains two sibling species, *Symbiodinium endoclionum* and *S. spongiolum,* that associate with *Cliona orientalis* and *C. varians* from the Pacific and Greater Caribbean, respectively [20]. The common ancestor of these Clade G species probably evolved prior to the separation of the two ocean basins (3.1–5.3 mya). Therefore, DNA sequence divergence between *S. endoclionum* and *S. spongiolum* has resulted from millions of years of isolation. The 3.1–5.3 mya time span since the MRCA is a conservative range because the Pacific species is based solely on collections from the western Pacific. Therefore, the genetic isolation of these lineages could have begun well before closure of the Central American Seaway.

Past estimates of Symbiodiniaceae divergence times

There are two studies which estimate that the evolution of *Symbiodinium* clades has occurred in the Cenozoic < 65 mya. One used calibration times based on plate-tectonic and host origination events [27] similar to those proposed in this study, while a second used rates of rDNA (*LSU*) evolution calculated for a distantly-related group of "armored" dinoflagellates with a fossil record [26].

In their development of a molecular clock, Pochon and coauthors employed early and late calibration points to estimate divergence times among clades of *Symbiodinium sensu lato* [27]. When selecting their early calibration time point, Pochon et al. [27] also targeted the MRCA of Clades C, F, and H (same as time point 1 in Figure S1), but used the range 25–30 mya, a period when species identifiable to the extant soritid genus, *Sorites*, are known to have first occurred in the fossil record. However this time frame was overly-conservative given that fossil data only delimit the upper bounds of an origination, that the presence of *Orbitolites* in the Eocene was not considered (see above), and that new molecular clock estimates places the origination of extant soritid foraminifera back to the Eocene [72].

The late calibration time frame of 3-4 mya used by Pochon et al. [27] was based on the latest physical separation of the Pacific and Atlantic ocean basins by a continuous isthmus [43]. When comparing genetic distances in *LSU* rDNA, they used sequences obtained from Clade H of *Sorites* sp. from Guam in the western Pacific and from *Sorites* sp. of Panama in the Atlantic Ocean [60, 74], but it would have been more suitable to use Clade H found in *Sorites* obtained from the Eastern Pacific side of Panama in calibrating a molecular clock based on this plate-tectonic event [60]. However, Clade H *LSU* rDNA sequences from Eastern Pacific and Western Caribbean *Sorites* are identical (Figure S4), further highlighting that rDNA may not change for million years. The inability of this pairing to calibrate a molecular clock probably explains why Pochon et al. [27] resorted to the sequence from Guam, which differs from the Western Caribbean Clade H sequence by 5 nucleotides (Figure S4). Using sequences from a Symbiodiniaceae obtained from the western Pacific questions the appropriateness of an assigned 3–4 mya calibration time frame.

In summarizing the molecular dating by Pochon et al. [27], the divergence between Clade H lineages in the western Pacific and in the Greater Caribbean could very well have occurred long before the separation of the Atlantic and the Pacific Oceans. Moreover, new and additional evidence indicates an early Eocene, rather than an Oligocene, origin of symbiotic soritids. Thus, the origination and divergence times between clades calculated by Pochon et al. [27] are likely underestimated by at least a factor of two. Our revision of their time estimates using a molecular clock incorporating new paleontological data along with DNA from appropriate sibling species spanning the Pacific and Atlantic Ocean basins places the origin of Symbiodiniaceae well into the Mesozoic.

Tchernov et al. [26] presented a second age estimation of Symbiodiniaceae clades and used an *LSU* molecular clock calibrated from fossil data of the Gonyaulacales. However, sequence divergence of rDNA among genera from this order is high (by a factor of 2-3 times or more) relative to other orders in the Class Dinophyceae (Figure 4F), but such divergence is not observed for other DNA markers such as mitochondrial genes. The application of this molecular clock on other dinoflagellate lineages would thus significantly underestimate times of evolution. Indeed, their chronology places the evolution of Symbiodiniaceae at 65 mya. Use of a rough correction factor ~2x, accounting for differences in rates of evolution, again places the origination of the Symbiodiniaceae back well into the Mesozoic.

Limitations of *LSU* rDNA molecular dating

Variation in rates of molecular evolution, both through time and among lineages, affects the accuracy of estimates for origination times using gene sequence data. Such data from the *LSU* rDNA is currently considered the most versatile for analyses of

Symbiodiniaceae diversity since they provide sufficient resolution to delimit most putative species within a given clade but are conserved enough to permit phylogenetic comparisons to distantly related species in other dinoflagellate orders [45]. While the internal transcribed spacers 1 and 2 (*ITS*) rDNA are used extensively to delimit various types, or species, of Symbiodiniaceae, these rDNA regions are problematic to align with confidence between members of different clades. Furthermore, the hypervariable region (~100 bases) of the chloroplast *cp23S* gene must be excluded before sequences from different clades can be unambiguously aligned. Finally, the mitochondrial *cob* gene is often too conservative to resolve most species within a clade.

By its very nature of containing 100's to 1000's of intragenomic copies, rDNA evolves differently than single, or low-copy, nuclear genes [82]. Genes of the ribosomal operon change via concerted evolution and thus the whole array evolves in tandem as populations become reproductively isolated, more accurately recording the evolutionary history of a lineage [83]. However, concerted evolution also acts to slow, or prevent, sequence divergence in reproductively isolated species over relatively long periods of time. Therefore, despite its exceptional utility as a phylogenetic marker, rDNA exhibits some variation in rates of evolution over time and among lineages of Symbiodiniaceae. The molecular evolution of rDNA can proceed slowly over several millions of years in some lineages [46] and indeed, rDNA has not changed, or changed very little, in some species separated for 3-4 million years or more. For example, rDNA sequences of *Symbiodinium tridacnidorum* (*ITS2* type $A3^{Pacific}$) are identical to lineages of A3 from the Caribbean, as are sequences of Clade H from *Sorites* in the Western Caribbean and Eastern Pacific (Figure S4). However, fixed nucleotide differences in chloroplast (*cp23S*) and mitochondrial (*cob*) genes clearly separate these A3 lineages phylogenetically and differences in microsatellite alleles substantiate their genetic isolation and long evolutionary divergence [84]. Clade B Symbiodiniaceae type B1 also contains multiple cryptic species that have diversified during the Late Pliocene or Pleistocene but show no differences in rDNA [18]. For this reason, multiple possible calibration points were independently employed here across the *LSU* rDNA phylogeny of Symbiodiniaceae and utilized in independent BEAST 2 analyses to test for consistency in age estimates and to compare expected versus observed ages.

Formal descriptions of genera

Data summarized as support for each genus were based on the comparative genetic analyses presented here and on previously published data, particularly from many of the recent species descriptions (cited within each section in the Taxonomic Summary in Data S1). Refer to the primary literature for additional details on sample acquisition, nucleotide sequencing, and specific ecological and biogeographic data.

DATA AND SOFTWARE AVAILABILITY

LSU rDNA and mt *cob* alignments, BEAST2 files, and R scripts for transcriptome comparisons are available from the Dryad Digital Repository: https://doi.10.5061/dryad.1717129.

Additional Resources

The taxonomic summary of the family Symbiodiniaceae is provided as Data S1. The taxonomic revisions therein are based in part on phylogenetic analyses of DNA sequences from nuclear, chloroplast, and mitochondrial markers, as well as available morphological, physiological, biogeographic, and ecological (e.g., host organism) information. An emended description is provided for the family. Six new genera are named and described, while *Symbiodinium* is more narrowly defined to correspond to only one evolutionarily divergent lineage (Clade A). Fifteen new genus-species combinations are proposed. All taxonomic descriptions were conducted following rules established by the International Code of Nomenclature for Algae, Fungi, and Plants [85].