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Molecular Evidence for Cryptic Species of *Aurelia aurita* (Cnidaria, Scyphozoa)

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Morphological taxonomy suggests that marine faunas are species poor compared to terrestrial and freshwater faunas (1). This dichotomy has been attributed to the unique potential of marine plankters for distant dispersal across homogenous oceans with few barriers to gene flow (2). The relative scarcity of opportunities for allopatric divergence has resulted in depauperate marine faunas characterized by a high proportion of widespread or cosmopolitan species. Aurelia aurita *(Linnaeus) has been considered a good example of such a cosmopolite (3, 4, 5, 6). However, recent molecular studies have revealed cryptic species in many marine taxa (7), suggesting that marine biodiversity is higher and opportunities for speciation have been more frequent than generally recognized. Here, we present nuclear and mitochondrial DNA sequence evidence of seven sibling species of* Aurelia aurita *and two additional species,* A. limbata *Brandt and* A. labiata *Chamisso & Eysenhardt. These sequence data indicate speciation events as early as the late Cretaceous or early Tertiary, consistent with the formation of well-recognized biogeographic barriers to gene flow in the seas.*

Traditionally, the genus *Aurelia* comprises two species: *A. limbata,* a polar species, and *A. aurita,* a common inhabitant of nearshore waters circumglobally between about 50 °N and 55 °S (3, 4, 5; Fig. 1). Perhaps due to its ubiquity, *A. aurita* has become a popular research organism for studies as diverse as protein chemistry, development, ecology, ethology, and hydrodynamics (6). *A. aurita* also is economically important because worldwide it preys on or competes with larvae of commercial fisheries and because swarms of medusae may impede trawling or block power-plant intakes (8). Furthermore, this "pest" has been introduced at least into San Francisco Bay (9) and possibly many other places (10). *A. aurita* is also familiar to nonspecialists because it is the most commonly displayed medusa in public aquaria. The systematics of *A. aurita* therefore is of considerable scientific, economic, and general interest.

Aurelia has a typical bipartite scyphozoan life history in which benthic scyphopolyps asexually strobilate ephyrae that grow into sexual medusae, the females of which brood larvae that settle into the shallow coastal benthos within a few days of being released. Of these life stages, the medusa probably is the principal dispersal phase because only the medusa is both long-lived (several months to more than one year; 11) and planktonic (6). The potential of medusae for distant dispersal is consistent with the current classification of *A. aurita* as a circumglobal, almost cosmopolitan, species (4, 5). However, *A. aurita* medusae in Saanich Inlet, British Columbia, and perhaps elsewhere, migrate directionally (12), maintaining breeding aggregations within isolated inlets and probably limiting gene flow among populations. Consistent with limited gene flow, allozyme differences have been found between populations of *A. aurita* in the eastern and western Atlantic Ocean, the Gulf of Mexico, and the eastern and western Pacific Ocean (9, 13). In addition, one species, *A. labiata,* was recently recognized as native to Pacific North America and distinct from *A. aurita* (10).

Novel DNA sequence data from nuclear internal transcribed spacer one (ITS-1) and mitochondrial cytochrome oxidase *c* subunit I (COI) reveal highly structured gene genealogies and at least nine distinct clades of *Aurelia* (Figs. 1, 2). Several lines of argument suggest that these clades warrant recognition as distinct species. First, the length of ITS-1 varies from 240 nucleotides (Charlestown, RI) to 360 nucleotides (Cananéia, Brazil). Such length variation is comparable to that found among congeneric species of

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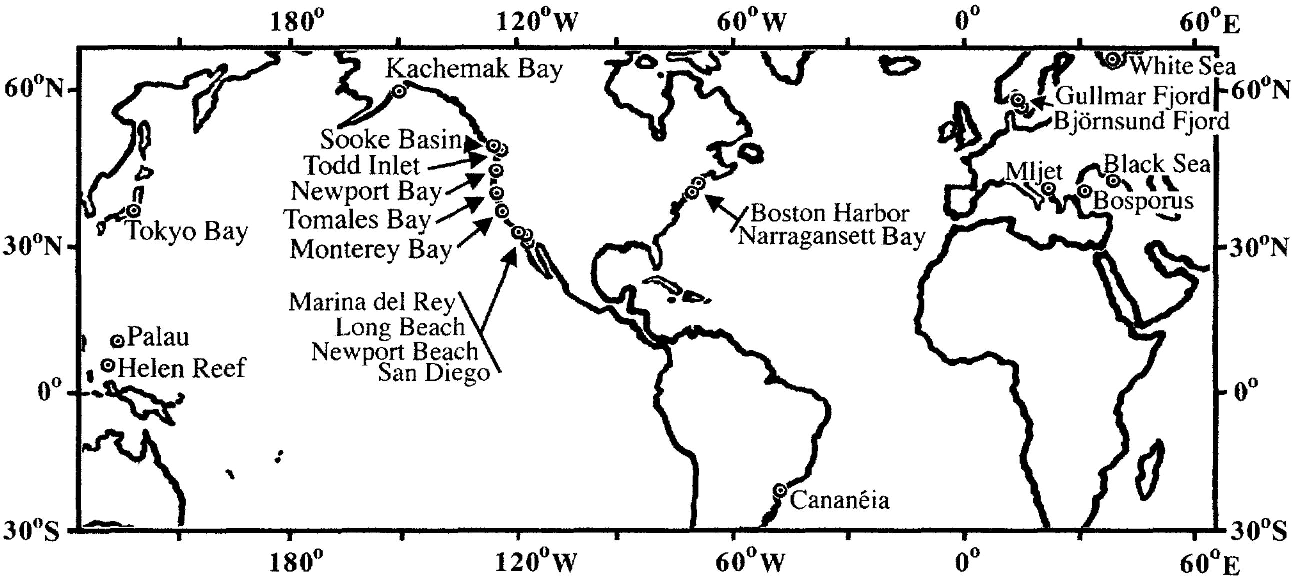

Figure 1. Sample locations of *Aurelia* in this study include sites within the ranges of the three currently recognized morphospecies: *A. labiata* (Pacific North America), *A. limbata* (polar seas below 70 °N from western Greenland to Siberia), and *A. aurita* (Europe [the type locality], northern Atlantic, and all remaining regions). Samples from Narragansett Bay comprise two sites: the semi-enclosed Charlestown Salt Pond and the open Greenwich Cove. Samples from Palau comprise several sites from three different habitats: land-locked, generally meromictic, marine lakes (Big Jellyfish Lake, Ongael Lake, Ongeim'l Tketau, Hotwater Lake); semi-enclosed holomictic 'coves' or similar waters (Tab Kukau, Malakal, Tketau); and the lagoon, which is open to the ocean (Ngell Channel) [see refs. 29, 31]. The population in Mljet inhabits a semi-enclosed meromictic "cove." Gut, bell margin, or oral arm tissues were cleaned, biopsied, and preserved in DMSO + NaCl (29). Total DNA was extracted using a CTAB extraction protocol. PCRs were set up according to the guidelines issued with *Taq* polymerase (Perkin Elmer) in MJ Research MiniCyclers and amplification products cloned using Invitrogen's Topo TA Cloning Kit. Cloned DNA was purified with Pharmacia's Flexiprep kit and sequenced on Applied Biosystems 373 autosequencers according to protocols in the ABI PRISM manual. Sequencing employed the PCR primers (HCO [32], AaCOIi-L = 5′-gcccgtyytaataggrgggtttgg, jfITS1-5f = 5′-ggtttccgtaggtgaacctgcggaaggatc, and jfITS1-3r = 5′-cgcacgagccgagtgatccaccttagaag [29]) and Invitrogen's M13Reverse and M13 Forward primers.

Corallimorpharia (286 to 350 nucleotides; 14) and between genera within the Scleractinia (214 to 297 nucleotides; 15) and Octocorallia (216 to 336 nucleotides; 15). Second, inter-clade percent sequence differences in ITS-1 (excluding regions containing microsatellites), which range from 10% (*A. labiata* [Kachemak, AK] *vs. Aurelia* sp. 1 [Newport Beach, CA]) to 40% (*Aurelia* sp. 2 [Cananéia] *vs. Aurelia* sp. 4 [Ongeim'l Tketau]), also are more similar to inter-specific and inter-generic than intra-specific differences in other taxa. For example, percent sequence difference in ITS-1 among congeneric species ranges from 4.5% to 14.9% in the Corallimorpharia (14) and from 5.8% to 19.5% in mosquitoes (16), and is less than 29% in the scleractinian *Acropora* (17). Inter-generic sequence differences in ITS-1 range from 27% to 79% in the Anthozoa (14, 15). Thus, if a difference of from 5% to 15% between ITS-1 sequences indicates species-level divergence, the sequences reported here are evidence of at least nine species of *Aurelia: A. limbata, A. labiata,* and *A. aurita,* plus six cryptic species previously identified as *A. aurita* (Fig. 2A).

Sequence variation in COI in *Aurelia,* which ranges from 13% (*A. limbata vs. Aurelia* sp. 1 [Marina del Rey, CA]) to 24% (*Aurelia* sp. 3 [Tab Kukau Cove, Palau] *vs. Aurelia* sp. 2 [Cananéia]) also is comparable to inter-specific differences in other metazoans. For example, 18% sequence difference in COI distinguishes congeneric species of copepods (18), 4% to 19% distinguishes species of the snapping shrimp *Alpheus* (19), about 7.5% indicates species of deep-sea clam (Vesicomyidae; 20), and 11.5% to 30.8% characterizes congeneric species of the amphipod *Gammarus* (21). Thus, if 10% to 20% sequence difference is taken as a benchmark of distinct species, these COI data also support recognition of *A. limbata, A. labiata,* and seven sibling species of *A. aurita* (Fig. 2B).

Several other observations suggest that the nine clades of *Aurelia* distinguished by sequence data represent distinct species. For example, *A. labiata* and *A. limbata* are sympatric in Kachemak Bay, providing strong evidence that their molecular differences reflect reproductive isolation. Similarly, Palau cove *Aurelia* occasionally are advected into the lagoon, but have not, according to the molecular data, interbred with lagoon *Aurelia* for millions of years, again implying reproductive isolation. Reproduction by *Aurelia* in Palau coves, lagoon, and lakes is also ecologically, usually geographically, and often temporally isolated (pers. obs.), and brackish, marine lake *Aurelia* may be physiologically inhibited from encountering congeners in seawater (5). For similar reasons, *Aurelia* in the fjord-like Mljet "lake" are probably reproductively isolated from *Aurelia* that occur elsewhere in Europe, including the Black Sea and probably

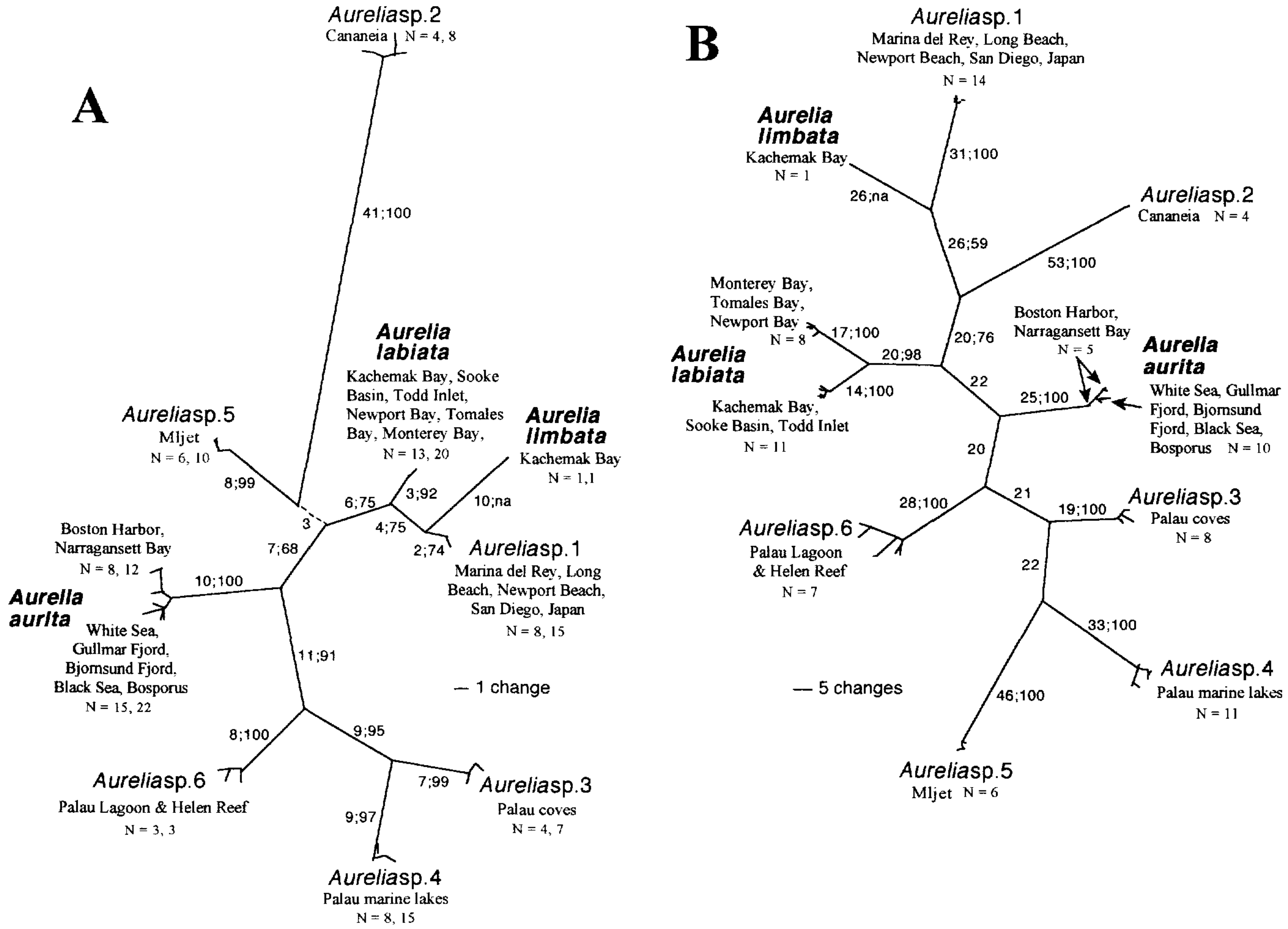

Figure 2. DNA sequence analyses of ITS-1 and COI are consistent with previous allozyme studies and morphological descriptions of *Aurelia* (3, 9, 10, 13) that indicated European and northeastern Atlantic clades within *A. aurita*, northeastern Pacific (*A. labiata*), polar (*A. limbata*), and Japanese or southern California lineages. DNA sequence analyses of ITS-1 and COI also indicate an additional six cryptic species of *A. aurita*. (A) Unrooted ITS-1 gene tree. One of 571 shortest trees (length = 168 steps; CI = 0.7738) recovered by unweighted maximum parsimony analysis (PAUP ver. 4.0b4a; 33) of sequences aligned in CLUSTALW (34; gap opening:extension penalty = 5:1) and amended by eye; gapped positions were excluded from the parsimony analysis, therefore emphasizing the better aligned portions of sequences (*i.e.*, 164 characters of which 83 were parsimony informative). Major branches present in the strict consensus tree are annotated with branch-length and bootstrap support (if >50%); the dashed branch was not present in the strict consensus. Parsimony analyses employed the tree-bisection-reconnection and accelerated transformation options, searching on 5000 trees ≤5 steps longer than the shortest tree encountered during each of 1000 heuristic searches. The same topology was recovered when transitions were downweighted 1:2 cf. transversions, which reflected the relative frequency of these changes in the aligned sequences. Bootstrap analyses (1000 replicates) of unweighted data were completed in PAUP ver. 4.0b4a using the options described above for parsimony analyses (50 heuristic searches per bootstrap replicate). The number of medusae and ITS-1 sequences analyzed (*N* = medusae, sequences) are stated for each region. (B) Unrooted COI gene tree constructed using unweighted maximum parsimony analysis of a 466-bp region in PAUP ver. 4.0b4a (search options as described above). The tree is topologically consistent with the strict consensus of 667 shortest trees (length = 844 steps; CI = 0.5616) recovered by weighted maximum parsimony analyses (1^{st}:2^{nd}:3^{rd} positions were weighted 3:11:1, reflecting the distribution of parsimony informative sites among these positions; weighting transitions:transversions 1:2 did not affect tree topology). Major branches present in the strict consensus tree are annotated with branch-length and bootstrap support. Bootstrap analyses (1000 replicates) of weighted data were completed in PAUP ver. 4.0b4a using the options described above for parsimony analyses (50 heuristic searches per bootstrap replicate). The number of medusae analyzed (*N*) is stated for each region.

the Mediterranean. Finally, at least seven species of *Aurelia*—those from coves, lagoon, and lakes in Palau, the one from Southern California (pers. obs.), and *A. aurita, A. labiata,* and *A. limbata* (3, 4, 5, 10)—are discernible from each other by their morphologies. Unfortunately, however, phylogenetically robust morphological characters are rare in *Aurelia* (4, 9), so new molecular phylogenies may be difficult to reconcile with antiquated morphological varieties or nominal species (*e.g., A. coerulea* Lendenfeld, *A. colpota* Brandt, *A. hyalina* Brandt, *A. japonica* Kishinouye, and *A. maldivensis* Bigelow). Despite this, species of *Aurelia* do conform, at least in part, to biological (isolation), evolutionary, phylogenetic, and recognition species concepts, as well as to the flawed but historically invaluable morphological species concept (22). Considering that many regions of the world remain unsampled—including Africa, Australia, the Caribbean, much of the Pacific, most of South America, and the Indian Ocean—nine is probably a modest estimate of the number of species of *Aurelia.*

Assuming a rule-of-thumb evolutionary rate of 0.5% to 1.4% sequence divergence per million years for COI (19), sequence differences between these nine species (13% to 24% [see above]) suggest divergences on the order of 10 to 50 million years before present (My BP). Similarly, based on the mean evolutionary rate of introns (3.3×10^{-9} substitutions year^{-1} site^{-1}; 23), ITS-1 sequence differences (10% to 40% [see above]) suggest species divergences between 30 and 120 My BP. These divergence times must be treated with caution for several reasons. First, they may be underestimates due to saturation of mutation ("multiple hits") at effectively neutral nucleotide positions. Alternatively, ITS-1 sequence differences may be slightly inflated by difficulties in aligning such highly variable sequences. Third, the clock calibrations are not based on cnidarian sequences and, as in other cnidarians (7, 24), the rates of molecular evolution in *Aurelia* may be slower than those in most other taxa. These factors considered, however, it still seems likely that these species of *Aurelia* diverged tens of millions of years before present.

The modern biogeography of *Aurelia,* therefore, has probably been influenced by ancient vicariant events that have contributed to modern species distributions that are consistent with modern patterns of marine zoogeography (25, 26). Vicariant events that affected the evolution of *Aurelia* may have included the formation of modern temperate faunas approximately 40 My BP (27); segregation of eastern and western Pacific faunas around the Mesozoic-Cenozoic boundary by formation of the East Pacific Barrier (25, 28); formation of the Tethys Sea approximately 95 My BP and subsequent isolation of the Mediterranean Basin from the Indo-Pacific during the Miocene, approximately 18 My BP (26); or possibly earlier events, including species radiations following the breakup of Pangea and the dispersion of continents during and after the late Jurassic, or radiations following the K-T mass extinction (22).

Later dispersal events probably also influenced the modern zoogeography of *Aurelia.* For example, the highly divergent Mljet *Aurelia* may be a relict of the primitive Tethys fauna that survived depletion of the Mediterranean Sea during the Messinian Salinity Crisis and which was subsequently contacted secondarily by *A. aurita* from Northern Europe during the early Pliocene (~5 MyBP) when the Strait of Gibraltar breached (26). Alternatively, both the Mljet *Aurelia* and *A. aurita* may have recolonized the Mediterranean, probably from the Indian and Atlantic oceans respectively, after the Messinian. More recent dispersal events are indicated by the occurrence of two species of *Aurelia* in Palau in habitats that were unavailable 10,000 years ago (29), although the geographic origins and earlier evolutionary histories of these species are as yet unknown. Such dispersal events, new records of *Aurelia* from Pacific islands (*e.g.,* Chuuk; 30), and common haplotypes in Palau and Helen Reef suggest that, although *Aurelia* has generally been considered a neritic genus (3, 5, 6), the medusae of some wider-ranging *Aurelia* species may be largely oceanic.

Aurelia is the best studied of all scyphozoans, yet its taxonomy, biogeography, phylogeography, and evolution are poorly known. This lack of knowledge is attributable largely to the morphological simplicity of *Aurelia* and to homoplasy that, as in other marine taxa, has contributed to drastic underestimation of species diversity (4, 7, 9). The underestimation of marine species diversity seriously compromises our ability to understand the evolution and ecology of marine environments. For example, morphological similarity superficially suggests widespread species, high gene flow, few barriers to dispersal, a dearth of allopatric speciation and, therefore, greater probability that other modes of speciation have contributed significantly to marine biodiversity (2). However, these novel DNA sequence data describing multiple cryptic species in an *Aurelia aurita* species complex suggest that barriers to gene flow—both geographic and ecological—are important sources of diversity in marine taxa. For *Aurelia,* as for other marine organisms including other scyphozoans (7), new morphological studies, more molecular analyses, and better distributional data are required to establish a robust evolutionary framework in which ecological, applied, and other scientific or educational endeavors can be placed in their correct contexts.

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