

Molecular phylogeny of *Diadema*: Systematic implications

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ABSTRACT: The extreme morphological similarity between species of *Diadema* has resulted in confused systematics and biogeography. Lessios et al. (in review) constructed a phylogeny based on mitochondrial DNA (mtDNA) from specimens collected around the world. The systematic implications of this phylogeny are discussed here. The molecular data indicate that all *Diadema* species listed in Mortensen's monograph as valid, except for *D. ascensionis*, correspond to reciprocally monophyletic mtDNA clades. *D. setosum* and *D. savignyi*, far from being subspecies as has been previously suggested, are the most anciently separated extant species of *Diadema*. There is an undescribed species around the Arabian Peninsula, most closely aligned with *D. setosum* from the W. Pacific and the Indian Oceans, but quite distinct from it. mtDNA of *Diadema* in Honshu and Kyushu, Japan does not just belong to *D. savignyi* or *D. setosum*, but also to a separate clade, which could be that of *D. clarki*, a species described by Ikeda, but dismissed by Mortensen. mtDNA of *D. antillarum* from the two shores of the Atlantic is as different as mtDNA from *D. savignyi*, *D. paucispinum* and *D. antillarum*. If the latter are maintained as separate species, then *D. antillarum* should also be split in two. mtDNA of *Diadema* populations at Ascension and St. Helena forms a monophyletic entity, but one nested within the W. Atlantic clade, supporting Pawson's (1978) demotion of Mortensen's (1909) *D. ascensionis* to a subspecies of *D. antillarum*. mtDNA of *D. paucispinum* is geographically much more widespread than the previously published range of the species, occurring sympatrically with *D. savignyi* from the central Pacific to the W. Indian Ocean. However, because the two species hybridize, it is not known whether individuals that contain *D. paucispinum* mtDNA have *D. savignyi* nuclear DNA.

1. INTRODUCTION

Diadema is one of the most abundant, widespread, and ecologically important genera of tropical sea urchins (reviews in Lawrence & Sammarco 1982; Lessios 1988a; Birkeland 1989; Carpenter 1997); yet its systematics and biogeography are enmeshed in confusion. Ever since Humphreys erected the genus in 1797 (see Mortensen 1940:244 regarding the debate about the priority of the genus name), various species have been referred to it (review in Mortensen 1940:254). The validity of all but one of these species has been questioned. The systematics of *Diadema* appeared to stabilize when Mortensen (1940) recognized the following species: *D. mexicanum* A. Agassiz from the tropical eastern Pacific and Easter Island; *D. antillarum* Philippi from both coasts of the tropical Atlantic; *D. ascensionis* Mortensen from Ascension, St. Helena and Fernando de Noronha; *D. paucispinum* A. Agassiz from Hawaii; and *D. setosum* (Leske) and *D. savignyi* (Audouin) Michelin with coincident ranges, extending from mid-Pacific to the E. African coast. Referring to *D. mexicanum*,

Mortensen (1940:277) wrote that "...[it] is very closely related to *D. antillarum*, so closely, indeed, that were it not for the geographical reason of their areas of distribution being separated by the Isthmus of Panama, scarcely anybody would have thought of regarding them as two distinct species". Quoting this passage, Mayr (1954) pointed out that separate geographic areas is not a valid reason for recognizing different species. Lessios (1984), however, found that *D. antillarum* and *D. mexicanum* spawn at different phases of the moon, a difference that—if genetically fixed—means that the speciation process has been completed, because it would prevent them from exchanging genes, even if they were to become sympatric.

Because of the morphological similarity of all species of *Diadema*, the working practice of systematists has been to identify specimens based on the locality from which they were collected (H.L. Clark 1925). This practice was, of course, not useful in the case of the sympatric *D. setosum* and *D. savignyi*, and the resultant frequent misidentifications have led to confused biogeography, with the

true geographical extent of each species remaining unknown until a careful study by Pearse (1998) established that *D. savignyi* extended from the central Pacific to the Indian Ocean, while *D. setosum* was tied to continental margins, and probably absent east of Tonga. The uncertain geographical distribution of these species even affected questions of nomenclature. A.M. Clark (1966) suggested that *D. setosum* and *D. savignyi* should be considered as subspecies of *D. setosum*. Because Audouin gave the name *D. savignyi* to specimens figured by Savigny from the Red Sea, and because Clark—unlike Mortensen (1940:268)—considered *D. savignyi* to be absent from this region, she petitioned the International Commission on Zoological Nomenclature to recognize

Michelin (who described the species from specimens collected at Mauritius) as the author of *D. savignyi* (A.M. Clark & Owen 1965), and placed *D. savignyi* (Audouin) in synonymy with *D. setosum*. Dollfus and Roman (1981:39) agreed that *D. savignyi* was absent from the Red Sea, but also found that the only surviving *Diadema* specimen of Michelin's collection from Mauritius belonged to *D. setosum*. Accordingly, they synonymized all references of *D. savignyi* with *D. setosum*.

The difficulties arising from uncertain distributions and the lack of generally agreed diagnostic characters are by no means limited to the two Indo-West Pacific species. Easter Island was said by Mortensen (1940:277) to contain *D. savignyi*, but

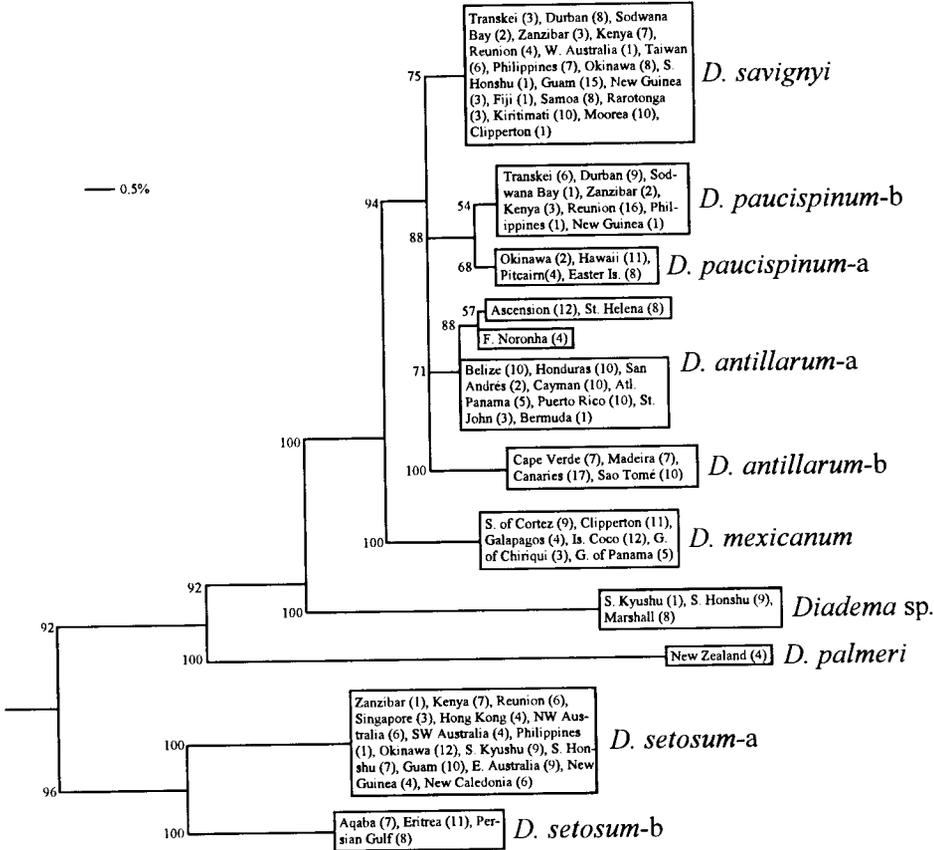


Figure 1. Summary of neighbor-joining phylogenetic tree (Saitou and Nei 1987) based on 497 to 614 nucleotides from the Lysine t-RNA-ATPase-6 and ATPase-8 region of *Diadema* mtDNA. Each major terminal clade has been labeled with a box that contains the names of the localities in which its representatives were collected with the number of individuals in parentheses. For the phylogeny of individual haplotypes, see Lessios et al. (in review). Numbers next to nodes indicate support from bootstrapping the tree in 1000 iterations. Branches with less than 50 % support have been collapsed. The tree was rooted by using homologous sequences of *Echinothrix diadema*, *E. calamaris*, *Astropyga radiata*, and *A. pulvinata* as outgroups.

also *D. mexicanum*. Fell (1974), on the other hand, concluded that the reports of *D. mexicanum* were due to misidentifications. *D. paucispinum* was considered by Mortensen (1940:279) as endemic to Hawaii, but A.H. Clark (1954) reported it from Kiribati on the basis of specimens that most likely belonged to *D. savignyi* (see Lessios & Pearse 1996). Lessios & Pearse (1996) found electrophoretically determined alleles characteristic of this species in Okinawa, and suggested that it may be much more widespread than previously thought. Similarly, Lessios et al. (1996), in addition to many specimens that clearly belonged to *D. mexicanum*, also found a single individual of *Diadema* at Clipperton Atoll, which had mitochondrial DNA (mtDNA) characteristic of *D. savignyi*. Mortensen (1940) gave the bathymetric range of *D. antillarum* as extending down to 400 m. Pawson & Miller (1983), on the other hand, concluded that all reported instances of *Diadema* from depths greater than 40 m along the coast of the United States were probably due to misidentification of *Centrostephanus longispinus*.

In addition to A.M. Clark's 1966 paper, recent systematic additions and modifications to the genus were Baker's (1967) description of a new species, *D. palmeri*, from the north coast of New Zealand, and Pawson's (1978) demotion of *D. ascensionis* to a subspecies of *D. antillarum*.

The difficulty of finding reliable morphological characters for distinguishing between species of *Diadema* points to the need for molecular comparisons. Lessios et al. (in review) reconstructed the phylogeny of the genus based on mtDNA, extracted from specimens collected around the world. They also supported the mtDNA phylogeny with isozyme comparisons between most of the species. Their primary aim was to identify patterns of gene flow and barriers that resulted in cladogenesis. Their data, however, also have systematic implications. The present paper attempts to answer the following questions: (1) Which of the described morphospecies are supported by mtDNA data? (2) Are there sibling species within the described ones? (3) How far does the range of each species extend?

2. MATERIALS AND METHODS

2.1. Collections

Mitochondrial DNA was sampled in a total of 462 individuals of *Diadema* from 54 localities around the world. All species of *Diadema* accepted as valid by Mortensen (1940) and Baker (1967) were included. The help of many people who collected samples from localities around the world (see acknowledgments) provided extremely good geographic coverage. Sampled localities and sample sizes are shown in Lessios et al. (in review).

2.2 Methods

Details of the methods are described in Lessios et al. (in review). Briefly, 642 nucleotides from the Lysine-tRNA, ATPase-6 and 8 region were PCR amplified, and 497 to 614 nucleotides were sequenced. 660 additional nucleotides from the Cytochrome Oxidase I (COI) mtDNA region from two individuals from each major clade, as determined from the ATPase region, were also amplified, and 597 to 639 nucleotides were sequenced. The ATPase tree and the combined ATPase+COI tree were congruent, so only the former is shown here.

Homologous sequences of the diadematids *Echinothrix diadema*, *E. calamaris*, *Astropyga radiata*, and *A. pulvinata* were used as outgroups for rooting the phylogenetic trees. Phylogenetic reconstruction was carried out using the neighbor-joining algorithm of Saitou & Nei (1987), based on DNA distances. These distances incorporated maximum likelihood estimates of parameters describing base frequencies and substitution rates applied to four site categories (one for each codon position, plus one for the region coding for Lysine -tRNA). The data were bootstrapped in 1000 iterations.

3 RESULTS

3.1 Phylogenetic lineages

The 492 individuals contained 199 unique ATPase haplotypes. Figure 1 presents a summary of the reconstruction of the phylogeny of these haplotypes. More information can be found in Lessios et al. (in review). The phylogeny shows a deep split between the *D. setosum* clade and all other *Diadema*. The *D. setosum* clade is itself divided into two quite divergent groups. Whereas *D. setosum*-a is found in most of the Indo-West Pacific, subclade *D. setosum*-b is limited to the Red Sea and the Persian Gulf (Figure 2). The sequence dissimilarity between *D. setosum*-a and *D. setosum*-b is 5.99%. There are 24 DNA sites diagnostic between the two subclades.

In the clade sister to *D. setosum*, the first group to split off is *D. palmeri*. Subsequent to this there is a mitochondrial DNA lineage (*Diadema*-sp) composed of 10 individuals from Honshu or Kyushu, and 8 from the Marshall Islands. The morphological characters of most of these individuals were consistent with those of *D. savignyi* (one was originally identified as *D. setosum*). However, this mtDNA clade has a DNA distance of 8.5% from *D. savignyi* and 15.53% from *D. setosum*.

The sister clade to *Diadema*-sp is composed of four species. *D. mexicanum* is an outgroup to the rest of the species in this clade, with a short but well-supported branch. *D. antillarum*, *D. paucispinum*,

and *D. savignyi* form a polytomy. *D. antillarum* is split into two clades, one from the western and central Atlantic (*D. antillarum*-a), the other from the eastern Atlantic (*D. antillarum*-b) (Figure 2). The average difference between these two clades is 2.73 %, more than the difference between the recognized species *D. paucispinum* and *D. savignyi* (2.10 %) and between *D. savignyi* and *D. antillarum* (2.09 %). There are 9 diagnostically different sites between the two *D. antillarum* clades. *D. antillarum ascensionis* from Ascension and St. Helena is a separate clade, nested within the Brazilian clade, which itself is nested within the western Atlantic clade. There are 3 diagnostic sites that distinguish between Caribbean populations of *D. antillarum*-a, on the one hand, and Brazilian or Central Atlantic ones, on the other; an additional site is unique to *D. antillarum ascensionis*.

The mtDNA lineage of *D. paucispinum* is far from being limited to Hawaii. It was not found in Kiribati, but it extends South to Easter Island and West all the way to the African coast (Figure 2). This clade is split in two reciprocally monophyletic subclades. One is mostly (but not exclusively) found in the Central Pacific, the other mostly found in the Indian Ocean (Figure 2). The estimated genetic distance between the two *D. paucispinum* clades is only 1.12 %, but there are 2 diagnostic sites that distinguish them.

Finally, there is a definite genetic break in *D. setosum* that is not apparent from the phylogenetic reconstruction. Populations from Reunión and Kenya are distinguished by one diagnostic site from those sampled everywhere else (see Lessios et al. in review).

4 DISCUSSION

4.1 Relevance of mtDNA phylogeny to systematics.

As Figure 1 shows, every monophyletic clade of mtDNA corresponds to a species accepted as valid by Mortensen (1940). There are, however, additional mtDNA clades that do not correspond to described species. How is one to decide which of these lineages represent undescribed species, and which simply indicate intraspecific genetic variation?

The answer, of course, depends on the species concept one chooses to apply. If we were to follow one of the variants of the phylogenetic species concept, according to which a species is a cluster of organisms "diagnosable from all other species" (Cracraft 1983), then every separate mtDNA lineage, down to *D. paucispinum*-a vs *D. paucispinum*-b, and even *D. setosum*-a from E. Africa vs *D. setosum*-a from W. Australia and the W. Pacific should be accepted as separate species. Without becoming entan-

gled in the debate regarding the relative merits of the biological vs. the phylogenetic species concepts and their variants (reviews in Avise & Ball 1990; O'Hara 1993, 1994; Graybeal 1995), I would like to explore the question of which of these mtDNA clades coincide with biological species, i.e. have diverged sufficiently to make it likely that they have developed reproductive isolation, which would prevent them from interbreeding, even if they were to become sympatric.

Unlike nuclear loci, mtDNA, being clonal and maternally inherited, cannot provide a direct answer to the question of reproductive isolation, even in sympatric populations (only traits intimately involved in reproduction can address the question for allopatric populations). It can, however, be used like any other character to deduce magnitude of divergence, and thus provide an educated guess as to whether monophyletic entities would be able to interbreed. Its advantage over morphology lies in that the differences can be easily quantified and are thus simple to compare. Obviously, an mtDNA tree (like any phylogenetic reconstruction) cannot be an infallible guide to the presence of biological species, because mechanisms of reproductive isolation may not be the products of gradual accumulation of genetic change (Lessios 1998). Indeed, there is an example from sea urchins in which degree of reproductive isolation is not well correlated with time since separation. Among the three neotropical species of *Echinometra*, the two Atlantic ones, *E. lucunter* and *E. viridis*, though morphologically distinct, are the most recently split and most similar in mtDNA and isozymes (Lessios 1979; 1981, 1998; McCartney et al. in press). Yet, isozyme data indicate that there is complete reproductive isolation between them. *E. lucunter* also shows partial gametic isolation towards the eastern Pacific *E. vanbrunti*, but *E. viridis*, separated from *E. vanbrunti* for the same amount of time as *E. lucunter*, does not (Lessios and Cunningham 1990; Lessios 1998). Despite such difficulties, a phylogenetic tree, by indicating the relative order in which lineages split from each other, also provides a useful means for delimiting biological species. The longer populations have remained separated, the more likely they are to have evolved reproductive isolation, and a cladogenic event that is substantially more ancient than a split known to have produced good species is probably a good indication that the two branches should be accorded specific status.

How different does mtDNA in *Diadema* have to be to suggest the existence of a separate biological species? There are two standards of divergence indicative of specific level, against which other divergences can be compared: (1) Divergence between accepted morphospecies (2) Divergence between good species, as revealed by the ascertainment of reproductive isolation. Obviously, the latter is more

reliable than the former, but the information it requires is valid in only one direction. When reproductive isolation is found between two populations, they are certain to belong to different species. But mechanisms of reproductive isolation can take so many forms, that failure to find them cannot be used as evidence that the populations are conspecific. In *Diadema*, we have two pairs of species that we know from other information to be good species: (1) *D. setosum* vs *D. savignyi*, known from the isozyme study of Lessios and Pearse (1996) to not exchange genes despite the production of occasional hybrids. (2) *D. mexicanum* vs *D. antillarum*-a, known from the study of Lessios (1984) to spawn 15 days out of phase.

4.2. How many species of *Diadema* are there?

Clearly, even if we did not know that the extent of hybridization between *D. setosum* and *D. savignyi* was very low, the evolutionary tree in Figure 1 would have been sufficient to reject A.M.Clark's (1966) suggestion that they are subspecies of the same species. Demoting them to this rank would require that the entire genus be considered as monotypic. Even Jackson (1912), who synonymized nearly all species of *Diadema* under the name of *D. setosum*, maintained *D. mexicanum* as a separate species. A similar argument applies to the two clades of the *D. setosum* lineage. The divergence between these clades is smaller than that between *D. setosum* and *D. savignyi*, but a great deal larger than differentiation between *D. mexicanum* and *D. antillarum*. There is also the additional point, that there is no obvious geographical barrier separating populations that carry these lineages (Lessios et al. in review), so their distinctiveness may be maintained through inability to interbreed freely at their zone of contact (though why they have not invaded each other's ranges, remains an unanswered question).

On the clade leading to the other extant species of *Diadema*, the first species to diverge is *D. palmeri*. Given that this is the only species of *Diadema* found in the temperate zone, one might have wondered whether a new genus should have been erected to accommodate it. That its mtDNA lineage is nested within *Diadema*, indicates that Baker (1967) was correct in referring it to this genus. The next branch to split off consists of *Diadema*-sp found in Japan and the Marshall Islands. There is no way to accommodate this clade in any known species of *Diadema* without also synonymizing all more recently split species on the same clade. This clade is also sympatric with both *D. setosum* and *D. savignyi* (Figure 2), so (unless it is incorporated into the genome of one of these species) its independent evolutionary history is a good indication of reproductive isolation.

It is, therefore, possible that this is a separate species. However, it may not be a "new" species. H.L. Clark (1925:44) had noticed that some specimens of *Diadema* from Japan were distinctive, and Ikeda (1939) described them as a new species, *D. clarki*. Mortensen (1940: 264), however, stated that he did not "see any possibility of maintaining *Diadema Clarki* Ikeda as a distinct species beside *D. setosum*" and, thus, the name has remained unused ever since. Ikeda's specimens came from N. Kyushu and S. Shikoku, so the geographic distribution of *D. clarki* partly coincides with that of the *Diadema*-sp. mtDNA clade. It is possible, therefore, that *D. clarki* should be resurrected in the same manner that another described and subsequently synonymized, species of sea urchin, *Eucidaris galapagensis* Döderlein, was rediscovered on the basis of molecular characters (Lessios et al. 1999).

Closer to the tip of the same lineage, there is *D. mexicanum*, which we know to be a separate species from *D. antillarum*-a. Whether it also reproductively isolated from *D. paucispinum* and *D. savignyi* is not known, and cannot be deduced from the phylogeny. The branch length separating *D. mexicanum* from the polyfurcation of *D. paucispinum*, *D. savignyi* and *D. antillarum* is well-supported but short, so we can no longer place much confidence on relative amounts of mtDNA divergence (and the evolutionary time it implies) to predict whether speciation is complete. However, the hierarchical nature of the evolutionary tree would not permit the taxonomic acceptance of *D. mexicanum* and *D. antillarum* as separate species while merging *D. mexicanum* with the other two lineages forming the final polyfurcation along with *D. antillarum*.

The final polytomy of this branch of the tree, composed of *D. savignyi*, *D. paucispinum*, *D. antillarum*-a, and *D. antillarum*-b, is the most problematic. On the basis of the mtDNA data, one can proclaim each of the four clades contained in it as a separate species, or accept them all as a single species that contains reciprocally monophyletic mtDNA clades. Fortunately, a study by Muthiga (in review) indicates that *D. savignyi* in Kenya spawns 18 days after new moon, whereas *D. antillarum* in the W. Atlantic spawns 1-3 days after new moon (Iliffe and Pearse 1982; Lessios 1984, 1988b, 1991). If these cycles are fixed, then there is good reason to maintain *D. antillarum*-a and *D. savignyi* as separate species, because they would be reproductively isolated even if they were sympatric. An indirect argument also suggests that *D. antillarum*-b represents a species separate from *D. antillarum*-a. High similarity of mtDNA between western and eastern Atlantic populations of *Eucidaris tribuloides* (Lessios et al. 1999) and of *Echinometra lucunter* (McCartney et al. in press) indicates that larvae of these echinoids are able to traverse the width of the tropical Atlantic.

The larval life span of *E. lucunter* in the laboratory is as short as 19 days (Mortensen 1921). It would, therefore, be surprising if larvae of *Diadema*, with a larval life span in the laboratory that ranges from 34 to 90 days (Carpenter 1997; Eckert 1998), were unable to cross the same oceanic expanse. That these larvae do not exchange mtDNA between populations indicates either that some ecological factor prevents propagules of the "wrong" species in each area from growing to sexual maturity, or that they are unable to mate with residents or produce viable hybrid offspring. Whether eastern and western Atlantic *Diadema* belong to separate species, is a question raised by Koehler (1914), H.L. Clark (1925:42) and Mortensen (1940:274). They all decided that the morphological differences were too slight to justify designating *E. Atlantic* populations as a species different from *D. antillarum*, but Mortensen wondered if it should be a separate "variety". In mtDNA, it appears to be much more than that.

Despite my insistence on the biological species concept, it can be argued with some justification that the question of whether *D. antillarum*-a, *D. antillarum*-b and *D. savignyi* have acquired reproductive isolation is operationally moot. These clades are allopatric, so from one point of view it matters little whether they are called by different specific names or not, except as a prediction of whether they will genetically merge should they invade each other's ranges. This, however, is not true for *D. savignyi* and *D. paucispinum*. One of the unexpected findings from the molecular data, first suggested by isozymes (Lessios and Pearse 1996), and now confirmed by mtDNA, was the wide geographical extent of the two *D. paucispinum* lineages (Figure 2). This has obvious biogeographical implications, more prominently regarding Easter Island, previously believed to be inhabited by *D. savignyi* (Mortensen 1940; Fell 1974) or *D. mexicanum*. (Mortensen 1940), whereas all 8 specimens included in the present study have *D. paucispinum* mtDNA. More important for biologists working with *Diadema* in the Indo-Pacific, *D. paucispinum* mtDNA lineages are now shown to be sympatric with *D. savignyi*. The question of their separate specific status, which could have also been considered moot while *D. paucispinum* was thought to be endemic to Hawaii, has become important for anyone attempting to identify *Diadema* specimens from the Pacific or the Indian Ocean, because there are very few and very unreliable morphological characters for distinguishing *D. paucispinum* from *D. savignyi*. There are also no diagnostic isozyme loci, though some alleles in one locus are characteristic of *D. paucispinum*. Given that *D. paucispinum* and *D. savignyi* hybridize (Lessios and Pearse 1996), and that no loci fixed for alternate alleles could be found, it is not impossible that the *D. paucispinum* mtDNA lineages in Indo-

West Pacific may all exist in descendants of hybrids that carry *D. savignyi* nuclear DNA. Depending on the extent of introgression, *D. paucispinum* and *D. savignyi* may be one species that contains two divergent mtDNA lineages. Clearly, a study of nuclear DNA from individuals carrying *D. savignyi* and *D. paucispinum* mtDNA is needed to settle this question.

Given the uncertain status of *D. savignyi* and *D. paucispinum*, it is pointless to speculate whether the least divergent reciprocally monophyletic clades in the tree, those of *D. paucispinum*-a and *D. paucispinum*-b might also represent different species. The same is not true in the central Atlantic. *Diadema* populations of Ascension and St. Helena may comprise a monophyletic entity, but this entity is nested within *D. antillarum*-a. This derived status fully supports Pawson's (1978) decision to demote *D. ascensionis* to a subspecies of *D. antillarum*, contrary to Mortensen's (1909, 1940) opinion.

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