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Genetic and Morphological Evidence That the Eastern Pacific Damselfish *Abudefduf declivifrons* Is Distinct from *A. concolor* (Pomacentridae)

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No consensus exists in the systematic literature about whether the Mexican night-sergeant, *Abudefduf declivifrons*, from Baja California and the Sea of Cortez, is distinct from the Panamic night-sergeant, *Abudefduf concolor*, from the rest of the tropical eastern Pacific. We present evidence from electrophoretic comparisons of proteins, nucleotide sequence comparisons of the cytochrome oxidase I gene of mitochondrial DNA, and quantitative comparisons of morphology. All agree that the two nominal species are distinct. Based on analysis of isozyme and mitochondrial DNA data from the Caribbean species of night-sergeant, *Abudefduf taurus*, we suggest that *A. declivifrons* split from the lineage leading to *A. concolor* and *A. taurus* before the Pliocene rise of the Isthmus of Panama. The two night-sergeants from the eastern Pacific differ significantly in body, snout, and caudal peduncle depth and can be distinguished by the condition of the suborbital margin (adnate in *A. concolor*, semi-adnate or exposed in *A. declivifrons*).

OPINIONS differ on whether the pomacentrid genus *Abudefduf* is represented in the tropical eastern Pacific by two or by three species. Although it is generally acknowledged that populations of sergeant-majors from Baja California to the Galapagos and Peru belong to the single species *Abudefduf troschelii*, there are disagreements concerning the night-sergeants (sometimes placed in the genus *Nexilarius*). Some authors (e.g., Thomson et al., 1979; Allen, 1991) treat all eastern Pacific populations of night-sergeants as a single species, *Abudefduf concolor*, with a distribution similar to that of *A. troschelii*. Others (e.g., Gill, 1862; Hensley, 1978; A. Edwards, pers. comm. to HAL) maintain that night-sergeants at the northern limit of the range belong to a separate species, *Abudefduf declivifrons*. To resolve this conflict, we compared protein electromorphs, sequences of mitochondrial DNA (mtDNA), and morphology to ascertain whether *A. declivifrons* is distinct from *A. concolor*. We also used biochemical data from the Caribbean species of night-sergeant *Abudefduf taurus* to estimate the time of divergence between *A. declivifrons* and *A. concolor* relative to the Pliocene rise of the Isthmus of Panama. Finally, we used electrophoretic data from populations of *A. troschelii* collected from the same regions in which the night-sergeants were sampled to determine whether northern and southern populations of sergeant-majors also represent distinct species.

Materials and Methods

Localities sampled for each species are shown in Figure 1 and described under Material Examined.

Protein electrophoresis.—Heart, liver, and skeletal muscle from individual fish were frozen in liquid nitrogen and maintained at −80 C in the

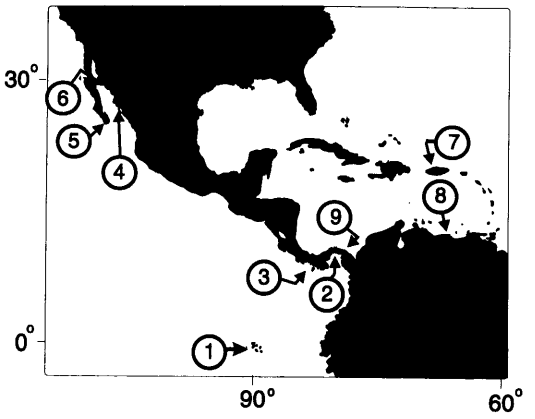

Fig. 1. Collection localities. 1: Galapagos; 2: Bay of Panama; 3: Gulf of Chiriqui, Panama; 4: San Ignacio de Farallón, Mexico; 5: Cabo San Lucas, Baja California; 6: Isla San Pedro, Mexico; 7: Puerto Rico; 8: Las Roques Archipelago; 9: Atlantic coast of Panama.

laboratory. Tissues were homogenized in equal volumes of 10 mM-Tris-0.6 mM-EDTA buffer, pH 7.0, and isozymes were separated using 11% polymerized starch. Gels were stained for 24 enzymes and one structural protein. A total of 46 presumptive loci were scored (Appendix 1). Histochemical staining methods followed Ayala et al. (1972) and Aebersold et al. (1987). Alleles were standardized by electrophoresing tissue extracts from the same individuals in multiple gels.

Nei's (1978) unbiased genetic distances were calculated between all populations, and significance of differences between jackknifed average genetic distances was determined following Mueller and Ayala (1982), using the program in Lessios (1990). Data were summarized by UPGMA cluster analysis using NTSYS (Rohlf, 1992). Phylogenetic relationships among populations were inferred using the MANAD procedure and FREQPARS program of Swofford and Berlocher (1987). The MANAD procedure calculates the shortest possible modified Wagner tree under the limitation that hypothetical ancestors have gene frequencies that sum to 1. Sergeant-majors and night-sergeants have at various times been placed in different genera (Gill, 1862; Jordan and Evermann, 1896; Meek and Hildebrand, 1925). We have, therefore, used *A. troschelii* as the outgroup to root the night-sergeant parsimony tree.

Sequence comparisons of mtDNA.—For DNA isolation, 0.5 g of muscle were homogenized in 100 μl of 2X CTAB buffer (Saghai-Maroof et al., 1984), incubated with 5 μl of proteinase K solution (20 mg proteinase K per ml in 50% glycerol) at 65 C for 1 hr and extracted with chloroform-isoamyl alcohol (24:1) and phenol-chloroform-isoamyl alcohol (24:24:1). DNA was precipitated with isopropanol overnight and centrifuged for 20 min at 14,000 × g. The pellet was rinsed twice with 70% ethyl alcohol in TE (10 mM Tris, 1 mM EDTA, pH 7.5) and resuspended in 200 μl of TE Buffer.

Initial polymerase chain reaction (PCR) amplifications (Saiki et al., 1988) were performed in 50 μl reactions containing 1 μl of genomic DNA solution, 5 μl of 10X buffer (100mM Tris pH 8.3, 20mM $MgCl_2$, 500 mM KCl, 0.1% Gelatin), 2.5 μl each of 10mM stock solutions of the cytochrome oxidase I primers a and f (S. Palumbi, A. Martin, S. Romano, W. O. McMillan, L. Stice, and G. Grabowski, "The Simple Fool's Guide to PRC," 1991, unpubl.), 5 μl of 2.0 mM dNTP mix, and 1.25 units of *Taq* Polymerase. The samples were cycled 30 times on a Perkin-Elmer thermal cycler using the following conditions: 94 C for 45 sec, 50 C for 45 sec, and 72 C for 60 sec. Following amplification, PCR products were run in 1.5% Low Melting Point Agarose gels in TBE (89 mM Tris, 89 mM Boric Acid, 2 mM EDTA) and stained with ethidium bromide. Typically a single band was present. It was cut from the gel and diluted in 400 μl of H_2O. One μl of this sample was amplified a second time using the conditions described above, except that one of the two COI primers was replaced with a phosphorylated COI primer, and the number of amplification cycles was reduced to 20. Two separate reactions were run, one with each phosphorylated primer.

Following the second amplification, the DNA strand initiated with the phosphorylated primer was digested with λ-exonuclease yielding the single-stranded DNA products used as sequencing templates (Higuchi and Ochman, 1989). Forty-five μml of the double-stranded DNA product were mixed with 15 μml of 10X λ-exonuclease supplement (775 mM glycine, 278 mM KOH, 5.8 mM $MgCl_2$, 5.8 mg/ml bovine serum albumin) and incubated for 30 min at 37 C with 2.5 units of λ-exonuclease. The λ-exonuclease was then heat-denatured at 94 C for 5 min. Then the samples were desalted and concentrated in Centricon-30 (Amicon) filters.

Dideoxy sequencing reactions (Sanger et al., 1977) were carried out using the Sequenase 2.0 kit (United States Biochemical Company) and following the vendor's protocol. Products of the sequencing reactions were resolved in 6.0% Long Ranger gels (AT Biochemical, Inc.), dried, and autoradiographed for 12–48 h. On aver-

age, 537 (range 497–625) bases of the mtDNA COI gene were compared across the 22 individuals examined. Roughly 80% of each sequence was verified through comparison of both DNA strands.

Nucleotide sequences of the COI mitochondrial gene of *A. declivifrons, A. concolor, A. taurus,* and *A. troschelii* were deposited in GenBank. Raw and corrected (Kimura, 1980) percentages of nucleotide differences were used as a measure of divergence. NTSYS was used to construct UPGMA trees from the divergence matrix. The branch-and-bound algorithm of PAUP, version 3.1 (Swofford, 1993), with transversions weighted four times as heavily as transitions and with bootstrapping of 500 replicates, was used to identify the most parsimonious tree.

Morphological measurements and counts.—Counts of dorsal and anal-fin rays were made from x-radiographs. The last dorsal and anal soft rays are generally joined at the base and were counted as single elements. Pectoral-ray counts included the splintlike uppermost element. Gill-raker counts included rudiments, which generally numbered 2–4 on the anterior section of the upper limb. Separate counts were made of lower limb rakers. The following measurements were made as described in Allen (1972): standard length (SL), body depth, head length, eye diameter, interorbital width, caudal peduncle length, pectoral-fin length, pelvic-fin length, and caudal-fin length. Preorbital depth was the distance between the lower edge of the eye and upper margin of the upper lip. Caudal peduncle depth was the least depth measured vertically.

Results

Protein comparisons.—Samples of the same species from different localities in the same region (i.e., Galapagos, Panama, Baja California, Las Roques Archipelago) had gene frequencies that were identical or very similar. We, therefore, pooled all such samples in the calculation of gene frequencies (Appendix 2). Samples from Cabo San Lucas and San Ignacio de Farallón, Mexico, were not pooled in order to determine whether the nominal species, *Abudefduf declivifrons,* is limited to Baja California. Samples of *A. troschelii* from Galapagos, Panama, and Mexico were very similar genetically: of 39 loci surveyed in all three regions, 27 were monomorphic, whereas the rest shared the same most common allele (Appendix 2). These data are consistent with the hypotheses that sergeant-majors in the eastern Pacific belong to one species (Thomson et al., 1979; Allen, 1991) and that gene flow between populations of this species is high. Low intraspecific variation also characterized *A. taurus* in the Caribbean. Of 40 loci surveyed in samples from Panama and Las Roques, 33 were monomorphic, and the rest shared common alleles between regions. Samples of *A. concolor* from Panama and Galapagos were fixed for the same allele at 37 loci and shared the same common allele at 9 loci. Inspection of the gene frequencies, however, demonstrates that samples of night-sergeants from Mexican waters are distinct from samples of *A. concolor.* Genetic dissimilarities between *A. declivifrons* and *A. concolor* include completely different alleles at *ADH-2, bGALA, EST-4, EST-6, HBDH, LDH-2,* and *XDH* and diagnostic differences at the 95% confidence level (Ayala and Powell, 1972) at *bGAL* and *EST-1* (Appendix 2). These results indicate clearly that *A. declivifrons* exchanges few, if any, genes with *A. concolor.*

Nei's unbiased genetic distances between regional samples of the same nominal species were small (≤ 0.0023) whether calculated from all loci or the 36 loci common to all comparisons. The average genetic distance between eastern Pacific *A. concolor* and Caribbean *A. taurus* (Table 1) was not significant, whereas the average genetic distance between *A. concolor* and *A. declivifrons* was significantly larger ($0.001 < P < 0.01$, by Mueller and Ayala's test) than the genetic distance between *A. concolor* and *A. taurus.* The largest genetic distances observed were between the morphologically and ecologically divergent sergeant-major *A. troschelii* and the night-sergeants *A. taurus, A. concolor,* and *A. declivifrons* (Table 1). The night-sergeant parsimony tree produced by FREQPARS (Fig. 2) indicates that *A. declivifrons* split from *A. concolor–A. taurus* stock before the latter was split by the rise of the Isthmus of Panama in the Pliocene (Keigwin, 1978, 1982; Coates et al., 1992). UPGMA clustering of Nei's unbiased genetic distance produced a phenogram that agreed with the parsimony tree.

Mitochondrial DNA comparisons.—Within each nominal species, the average proportion of nucleotides of the COI mitochondrial gene varying between individuals was 0.0033 (range 0.0022–0.0060); the corrected average, using Kimura's (1980) two-parameter model and weighing transitions one-fourth as much as transversions, was also 0.0033 (range 0.0026–0.0061). The smallest mtDNA sequence difference within night-sergeants was between *A. taurus* and *A. concolor* (Table 2). The mtDNA sequence difference between *A. declivifrons* and the other two night-sergeants was more than

TABLE 1. JACKKNIFED AVERAGE NEI'S UNBIASED GENETIC DISTANCES, BASED ON AVAILABLE LOCI FOR EACH COMPARISON (RANGE 39–46 LOCI), BETWEEN THREE SPECIES OF *Abudefduf*. Values in parentheses are based on 36 loci common to all samples. Two species differ significantly if average between-species genetic distance is significantly larger than average within-species distance (Mueller and Ayala, 1982).

	A. declivifrons	*A. concolor*	*A. taurus*
A. concolor	0.209**		
	(0.143)*		
A. taurus	0.177**	0.039	
	(0.143)*	(0.028)	
A. troschelii	0.411***	0.292**	0.315**
	(0.402)***	(0.311)**	(0.350)**

*: $0.05 > P > 0.01$; **: $0.01 > P > 0.001$; *** $P < 0.001$.

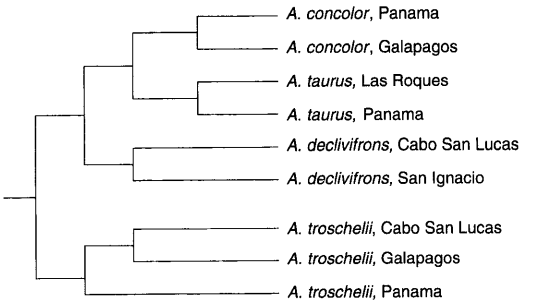

Fig. 2. Modified Wagner tree based on the MANAD procedure of Swofford and Berlocher (1987) and calculated using FREQPARS. The sergeant-major, *Abudefduf troschelii*, was used as the outgroup. Branch lengths are not proportional to the number of character state changes. Only loci assayed in all populations were used.

four times larger than the difference between *A. taurus* and *A. concolor*. *Abudefduf troschelii* was an order of magnitude more divergent from the night-sergeants than were the three night-sergeants from each other (Table 2).

Maximum parsimony analysis involving 75 unambiguous variable characters in the mtDNA sequence data produced a well-supported species-tree topology (Fig. 3) identical to that inferred from isozymes. UPGMA clustering based on the proportion of nucleotide differences agreed with the parsimony analysis. Thus, the mtDNA comparisons also suggest that *A. declivifrons* from the Sea of Cortez is more distantly related to *A. concolor* than *A. concolor* is to the Caribbean *A. taurus*.

Morphological comparisons.—Formal morphological diagnoses of *A. declivifrons* and *A. concolor* are appended. The most consistent feature that separates *A. declivifrons* from *A. concolor* is condition of the lower suborbital margin. Of 18 specimens of *A. declivifrons*, six had partially exposed margins, and 12 had completely exposed margins. All 46 specimens of *A. concolor* had margins obscured by scales. Degree of serration on the preopercle margin is another useful character for distinguishing the two species. The margin is smooth in *A. declivifrons* and either finely serrate or crenulate in *A. concolor*. In addition, there are several morphometric differences: *A. declivifrons* has significantly deeper body, longer snout, and deeper caudal peduncle (Table 3).

Adults of *A. concolor* and *A. declivifrons* have similar color patterns of five to six alternating light and dark bars. The bars are more vivid and distinct in *A. concolor*. The anterior edge of the pelvic fins, the extreme outer edges of the dorsal and anal fins, and the edge of the caudal-fin concavity are whitish in *A. concolor* and dusky in *A. declivifrons*. A feature sometimes useful for distinguishing *A. concolor* is a dark wedge-shaped mark on the uppermost part of the base of the pectoral fin. Frequently, this mark extends across the entire fin base. This mark appears to

TABLE 2. AVERAGE (RANGE IN PARENTHESES) PROPORTION OF NUCLEOTIDE DIFFERENCES BETWEEN SPECIES IN APPROXIMATELY 537 BASES OF THE COI GENE. Values below the diagonal are uncorrected proportions; those above the diagonal are corrected using Kimura's (1980) two-parameter model with transitions receiving one quarter the weight of transversions.

	A. declivifrons	*A. concolor*	*A. taurus*	*A. troschelii*
A. declivifrons	—	0.070	0.078	0.150
	—	(0.066–0.074)	(0.071–0.085)	(0.141–0.158)
A. concolor	0.066	—	0.015	0.152
	(0.062–0.069)	—	(0.011–0.021)	(0.147–0.159)
A. taurus	0.073	0.014	—	0.155
	(0.067–0.079)	(0.011–0.021)	—	(0.144–0.162)
A. troschelii	0.133	0.134	0.136	—
	(0.126–0.139)	(0.130–0.140)	(0.128–0.142)	—

be lacking in adult *A. declivifrons*, although we have not examined specimens less than 130 mm SL for this character.

Small (15–30 mm SL) preserved juveniles of both species have a similar pattern of 6–7 dark bars on each side, but there is a difference in bar shape and width. Bars in *A. concolor* are much wider than the pale interspaces and are poorly defined on the lower half of the body. The bars in *A. declivifrons* are the same width as the pale interspaces and are clearly visible on the lower side. The bars of *A. concolor* also taper in width ventrally, whereas those of *A. declivifrons* exhibit less tapering. The only information on juvenile coloration in life for *A. concolor* is a photograph of a specimen approximately 60 mm total length taken in the Gulf of Chiriqui, Panama. The specimen has the same pattern as adults, with more prominent white bars and more white on the ventrum and on the pelvic and anal fins. Unlike adults, the juvenile specimen had a short, relatively narrow, whitish stripe encompassing the first 8–9 lateral-line scales.

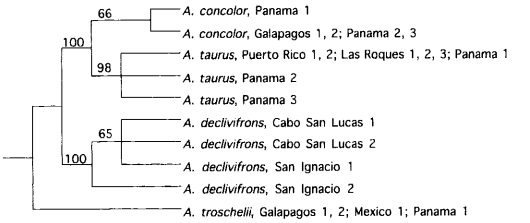

Fig. 3. Strict consensus tree depicting phylogenetic relationships among haplotypes of *Abudefduf concolor*, *A. taurus*, *A. declivifrons*, and *A. troschelii* based on approximately 537 bases of the COI gene. The two shortest trees were 81 steps long (C. I. = 0.938). Bootstrap percentages for each node, based on 500 replications, are shown above branches.

Discussion

Lack of allelic overlap at seven isozyme loci and consistent differences in mtDNA sequence and external morphology between Mexican and Panamic night-sergeants leave little doubt that gene flow between them is restricted or absent. Nei's distances between *A. declivifrons* and *A. concolor* are substantially larger than those usually observed between conspecific populations (Thorpe, 1982, 1983; Nei, 1987) and larger than genetic distances separating them from *A. taurus*. Isozyme genetic distance between Mexican and Panamic night-sergeants is also an order of magnitude larger than that between populations of *A. troschelii* from Mexico and the Galapagos, despite similar mean lengths of larval periods in the three species, as determined from otolith analysis (GMW, unpubl. data). Phylogenetic analysis of both isozymes and mtDNA indicates that *A. declivifrons* is sister to a clade comprised of *A. concolor* and *A. taurus*. The above all support the conclusion that *A. concolor* and *A. declivifrons* are separate species.

Protein and mtDNA comparisons to the Caribbean night-sergeant, *A. taurus*, provide insight into the timing of the speciation event that separated the two eastern Pacific species. *Abudefduf concolor* and *A. taurus* are believed to be "geminate" species, created by the rise of the Isthmus of Panama (Jordan, 1908; Thomson et al., 1977). Data from geminate species of sea urchins have shown that, though proteins may not evolve at constant rates (Lessios, 1979, 1981; Bermingham and Lessios, 1993), mtDNA divergence is sufficiently uniform to allow reasonable inferences regarding the dates of separation between populations. The mtDNA divergence between *A. concolor* and *A. taurus* suggests that the COI gene in night-sergeants

Table 3. Body Depth, Snout Length, and Caudal-Peduncle Depth of *Abudefduf concolor* and *A. declivifrons*. n = 18 for each species. Data were arcsine transformed for statistical comparisons. Values, back-transformed to a linear scale, are expressed as percent of standard length (SL). F values are from one-way analysis of variance.

	SL (mm)	Body depth	Snout length	Peduncle depth
A. concolor				
Range	124.5–158.8	53.9–58.8	10.8–12.9	16.7–18.8
Mean	142.4	56.3	12.1	17.8
SD	9.7	1.8	0.5	0.7
A. declivifrons				
Range	130.2–154.8	57.8–63.1	12.3–15.5	18.3–20.2
Mean	141.8	61.1	13.8	19.0
SD	6.3	2.0	0.8	0.5
F*		89.2	60.2	29.6

* All F values are highly significant ($P < 0.0001$).

has accumulated different mutations in 1.5% of its bases over a minimum of three million years. If one assumes that this gene evolves at roughly constant rates, the split between *A. declivifrons* and the ancestor of *A. concolor* and *A. taurus* must have occurred 4.5 times earlier than the rise of the central American isthmus (Keigwin, 1978, 1982; Coates et al., 1992), or more than 14 million years ago. An assumption of constancy of rates of isozyme evolution would also place this event at 15 million years ago (based on the 36 loci sampled in all populations). Clearly, the interesting question is not whether the two eastern Pacific night-sergeants are different species, but rather why the two species are still so similar in morphology.

Our data on external morphology indicate that *A. declivifrons* and *A. concolor* have evolved differences that can be used to discriminate between them. The distributions of body depth, snout length, and peduncle depth in the two species differ significantly; and any specimen with the suborbital margin completely hidden by scales undoubtedly belongs to *A. concolor*. Thus, the morphological data, when viewed in conjunction with the molecular data, also support the hypothesis that *A. concolor* and *A. declivifrons* are separate species. A. Edwards (pers. comm. to HAL) reached a similar conclusion from a multivariate analysis of morphometric character variation.

Distributional limits of *A. concolor* and *A. declivifrons* remain unknown. It is possible that *A. declivifrons* is restricted to the Gulf of California, Cabo San Lucas, and the west coast of Baja California. Thomson et al. (1979) reported that it was common in the lower Gulf but has been recorded as far north as Guaymas and Bahía San Francisquito in the central Gulf, and in Magdalena Bay on the west coast of Baja California. A. Edwards (pers. com. to HAL) recorded one juvenile specimen of uncertain specific identity from El Salvador and suggested that ranges of *A. declivifrons* and *A. concolor* may be partially overlapping. *Abudefduf concolor* presumably ranges from Mexico to Peru (Chirichigno, 1974; Lopez and Bussing, 1982; Rubio, 1986). Collections from areas between the Gulf of California and Panama are required to establish the geographic limits of these species.

Abudefduf declivifrons (Gill, 1862)

Euschistodus declivifrons Gill, 1862: 146 (Cape San Lucas, Baja California).

Diagnosis.—Dorsal rays XIII, 12–13 (usually 13); anal rays II, 10; pectoral rays 19; tubed lateral-line scales 20–21; vertical scale rows from lateral-line origin to base of caudal fin 28; horizontal scale rows above lateral line at level of dorsal-fin origin 3.5; horizontal scale rows below lateral line at level of anal-fin origin 9; total gill rakers on first branchial arch, including rudiments 19–23, on lower limb only 11–14 (usually 12 or 13). Suborbital usually separated from preopercle and clearly demarcated by free lower edge, but sometimes partly attached (adnate) to suborbital with its lower border partly obscured by scales. Preopercular margin smooth without serrations or rough crenulations. The following proportional ranges are expressed as a percentage of standard length: greatest body depth 59.2–62.6%; head length 30.4–33.9%; snout length 12.3–14.0%; eye diameter 7.5–8.9%; interorbital width 11.7–12.9%; preorbital width 6.6–7.4%; least depth of caudal peduncle 18.3–19.4%; length of caudal peduncle 11.9–14.2%; length of pectoral fin 30.7–33.2%; length of pelvic fin 28.7–30.5%; and length of caudal fin 27.0–31.3%.

Abudefduf concolor (Gill, 1862)

Euschistodus concolor Gill, 1862: 145 (Panama).

Pomacentrus robustus Günther, 1862: 17 (unknown locality).

Diagnosis.—Dorsal rays XIII, 12–13 (usually 13); anal rays II, 10; pectoral rays 19 (rarely 20); tubed lateral-line scales 20–21; vertical scale rows from lateral-line origin to base of caudal fin 28; horizontal scale rows above lateral line at level of dorsal-fin origin 3.5; horizontal scale rows below lateral line at level of anal-fin origin 9; total gill rakers on first branchial arch, including rudiments 19–21, on lower limb only 11–14 (usually 12 or 13). Suborbital attached (adnate) to preopercle, its lower border completely obscured by scales. Preopercular margin finely serrate or with noticeable rough crenulations. The following proportional ranges are expressed as a percentage of standard length: greatest body depth 53.8–59.2%; head length 28.7–32.3%; snout length 10.8–12.9%; eye diameter 7.7–10.1%; interorbital width 10.4–12.8%; preorbital depth 5.5–7.0%; least depth of caudal peduncle 16.7–18.8%; length of caudal peduncle 11.3–14.2%; length of pectoral fin 27.2–32.2%; length of pelvic fin 26.6–33.0%; and length of caudal fin 25.8–33.6%.

Material Examined

Protein electrophoresis.—Abudefduf concolor: Galapagos Islands (Isla Bartolomé, Is. Fernandina, and Is. Pinzón); Bay of Panama (Is. Taboguilla,

Is. Urabá, Is. Contadora, Is. Pachequilla, and Is. Saboga). *A. declivifrons*: Melia near Cabo San Lucas, Baja California; Cabo Pulmo in Baja California; Is. San Ignacio de Farallón, close to the eastern shore of the Gulf of California. *A. troschelii*: Galapagos (Is. Floreana, Is. Santa Cruz, Is. Bartolomé, Is. Santiago, and Is. Genovesa); Bay of Panama (Is. Naos, Is. Taboguilla, and Is. Urabá); Baja California (San Jose del Cabo). *A. taurus*: Atlantic coast of Panama (Is. Margarita, and San Blas Archipelago); Archipelago of Las Roques in Venezuela (Cayo Agua, Cayo Sal, Cayo Dos Mosquitos).

Mitochondrial DNA.—*Abudefduf concolor*: Is. Urabá, Bay of Panama (n = 2); Is. Saboga, Bay of Panama (n = 1); Is. Bartolomé, Galapagos (n = 2). *A. declivifrons*: Cabo San Lucas, Baja California (n = 2); Is. San Ignacio de Farallón, Gulf of California (n = 2). *A. troschelii*: Is. San Pedro, Mexico (n = 1); Is. Urabá, Bay of Panama (n = 1); Is. Bartolomé, Galapagos (n = 2). *A. taurus*: Puerto Rico (n = 2); San Blas Archipelago, Panama (n = 3); Cayo Dos Mosquitos, Las Roques Archipelago, Venezuela (n = 4). Nucleotide sequences of the COI mitochondrial gene of these specimens have been deposited in the GenBank data base (accession nos. L35211-35231).

Morphology.—Specimens used for the morphological study were deposited at the National Museum of Natural History, Smithsonian Institution, Washington, D.C. (USNM) and the Western Australian Museum, Perth, Australia (WAM). *Abudefduf concolor*: USNM 0323790, 19 specimens, 87.8–158.8 mm SL, Is. Bartolomé, off Is. Santiago, Galapagos (approximately 01 15'N, 09 33'W); USNM 323791, 17 specimens, 62.5–115.8 mm SL, Isla Uva, Gulf of Chiriqui, Panama (approximately 07 48'N, 81 45'W); WAM P.30508-001, 4 specimens, 98.5–152.5 mm SL, Is. Bartolomé, Galapagos; WAM P.30507-001, 6 specimens, 76.2–105.7 mm SL, Is. Uva, Bay of Panama; WAM P.30521-001, 11 specimens, 16.5–31.9 mm SL, Islas Galapagos (0. 18'S, 90'W). *Abudefduf declivifrons*: USNM 323792, 14 specimens, 130.2–153.5 mm SL, Isla San Ignacio de Farallón, Gulf of California, Mexico (approximately 25 28'N, 109 24'W); WAM P.30506-001, 4 specimens, 136.7–148.7 mm SL, same locality as USNM specimens. WAM P.30522-001, 15 specimens, 12.8–28.4 mm SL, Cabo San Lucas, Baja California, Mexico. We also examined syntypes of *Euschistodus declivifrons*, 10 specimens, 23.0–71.0 mm SL (USNM 9332) and the holotype of *Pomacentrus robustus*, 109.0 mm SL (BMNH 1855.9.19–273). The holotype of *Euschistodus concolor* was deposited originally at USNM but is now missing from the collection. Five specimens, 28.0–80.0 mm SL, at the British Museum (BMNH 1862.5.21–22), collected at Panama and acquired from the Smithsonian Institution (USNM) in 1862, and which are questionably indicated as paratypes, were examined.

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APPENDIX 1. ENZYMES ASSAYED, TISSUES AND BUFFERS USED, AND NUMBER OF PUTATIVE LOCI SCORED. Abbreviations for tissues: L: Liver, M: Skeletal Muscle, H: Heart. Buffer codes: A: discontinuous 0.076 M Tris-Citrate, pH 8.65; B: 0.087 M Tris-Versene-Borate, pH 9.10; C: 0.009 M Tris-Versene-Citrate, pH 7.00; D: 0.023 M Tris-Citrate, pH 8.00; E: discontinuous 0.046 M Tris-Citrate-Borate-LiOH, pH 8.30; F: 0.214 M Phosphate-Citrate, pH 7.00.

Enzyme	Tissue	E.C. number	Buffer	No. of loci
β-N-Acetylgalactosaminidase	L	3.2.1.53	B	1
Alcohol dehydrogenase	M, H	1.1.1.1	C, F	2
Aspartate aminotransferase	M, L, H	2.6.1.1	D	4
Creatine Kinase	M	2.7.3.2	E	1
Dipeptidase	L	3.4.–.–	D	2
Enolase	M	4.2.1.11	C	1
Esterase	L	3.1.1.–	D	5
Fumarate hydratase	H	4.2.1.2	C	2
β-Galactosidase	L	3.2.1.23	F	1
General protein	M	—	C	3
Glucose dehydrogenase	L	3.5.4.3	E	1
Glycerol-3-phosphate dehydrogenase	L	1.1.1.8	D	1
3-Hydroxybuterate dehydrogenase	M	1.1.130	C	1
Isocitrate dehydrogenase	L	1.1.142	F	1
L-Lactate dehydrogenase	M, H	1.1.1.27	D	2
Malate dehydrogenase	H	1.1.1.37	D	3
Mannose-6-phosphate isomerase	H	5.3.1.8	F	1
Phosphoglucomutase	L	5.4.2.2	F	2
Phosphogluconate dehydrogenase	L	1.1.1.44	D	1
Phosphoglycerate kinase	L	2.7.2.3	D	1
Pyruvate kinase	H	2.7.1.40	F	3
L-Iditol dehydrogenase	L	1.1.1.14	A	1
Superoxide dismutase	L	1.15.1.1	B	2
Triosephosphate isomerase	M	5.3.1.1	C	3
Xanthine dehydrogenase	L	1.1.1.204	B	1

APPENDIX 2. SAMPLE SIZES (NUMBER OF INDIVIDUALS) AND GENE FREQUENCIES OF SAMPLES. Locus abbreviations follow Shaklee et al., 1990. *sAAT-1*, *ADH-1*, and *LDH-1* were assayed in heart tissue, *sAAT-2* and *sAAT-3* in liver, *sAAT-4* and *LDH-2* in skeletal muscle. See Appendix 1 for source tissue of all other loci.

		A. declivifrons		*A. concolor*		*A. taurus*		*A. troschelii*		
Locus	Allele	C. San Lucas	San Ignacio	Panama	Galapagos	Panama	Las Roques	C. San Lucas	Panama	Galapagos
sAAT-1	n	30	21	22	31	21	20	10	29	33
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
sAAT-2	n	30	21	23	31	19	19	1	0	0
	90	0.783	0.833	1.000	1.000	1.000	1.000	1.000		
	100	0.217	0.167	0.000	0.000	0.000	0.000	0.000		
sAAT-3	n	23	21	23	31	19	20	10	33	21
	95	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.015	0.000
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.985	1.000
sAAT-4	n	30	21	23	30	23	20	10	32	34
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
ADH-1	n	30	21	23	31	23	20	10	27	31
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
ADH-2	n	30	21	23	32	23	20	10	32	34
	90	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	100	0.000	0.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
G3PDH	n	30	20	23	31	17	19	9	27	31
	90	1.000	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000
	100	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	1.000
bGAL	n	30	21	23	31	19	20	10	27	34
	90	0.000	0.000	0.913	0.936	0.947	0.875	0.000	0.074	0.015
	99	0.000	0.000	0.044	0.048	0.053	0.000	0.000	0.000	0.000
	100	1.000	0.762	0.044	0.016	0.000	0.125	1.000	0.926	0.956
	108	0.000	0.143	0.000	0.000	0.000	0.000	0.000	0.000	0.029
	120	0.000	0.095	0.000	0.000	0.000	0.000	0.000	0.000	0.000
bGALA	n	30	3	22	21	15	20	10	27	21
	85	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	90	0.000	0.000	0.000	0.000	1.000	1.000	0.000	0.000	0.000
	100	0.000	0.000	1.000	1.000	0.000	0.000	1.000	1.000	1.000
CK	n	30	21	23	31	22	20	10	31	21
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
ENO	n	30	21	23	31	23	20	10	31	34
	100	1.000	1.000	0.957	1.000	1.000	1.000	1.000	1.000	1.000
	110	0.000	0.000	0.044	0.000	0.000	0.000	0.000	0.000	0.000
EST-1	n	29	15	22	30	19	0	0	8	2
	90	0.000	0.000	0.000	0.033	0.053			0.000	0.000
	100	0.000	0.000	0.909	0.967	0.921			1.000	1.000
	110	1.000	1.000	0.091	0.000	0.026			0.000	0.000
EST-4	n	30	20	23	22	17	0	10	17	29
	80	0.033	0.075	0.000	0.000	0.000		0.000	0.000	0.000
	100	0.967	0.925	0.000	0.000	1.000		1.000	0.882	1.000
	110	0.000	0.000	0.870	0.932	0.000		0.000	0.029	0.000
	120	0.000	0.000	0.087	0.068	0.000		0.000	0.088	0.000
	130	0.000	0.000	0.044	0.000	0.000		0.000	0.000	0.000
EST-5	n	30	20	23	29	19	0	0	0	0
	80	0.000	0.000	0.000	0.000	0.026				
	90	0.000	0.000	0.022	0.000	0.605				
	100	1.000	1.000	0.978	1.000	0.368				

APPENDIX 2. CONTINUED.

		A. declivifrons		*A. concolor*		*A. taurus*		*A. troschelii*		
Locus	Allele	C. San Lucas	San Ignacio	Panama	Galapagos	Panama	Las Roques	C. San Lucas	Panama	Galapagos
EST-6	n	30	20	23	30	11	0	10	17	34
	90	0.000	0.000	0.000	0.000	0.000		0.300	0.000	0.015
	100	0.000	0.000	1.000	1.000	1.000		0.650	0.971	0.956
	110	1.000	1.000	0.000	0.000	0.000		0.050	0.029	0.029
EST-7	n	17	12	23	30	19	0	0	0	0
	100	1.000	1.000	1.000	1.000	1.000				
FH-1	n	30	20	23	31	22	19	10	28	21
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
FH-2	n	30	20	23	31	22	20	10	28	32
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
GDH	n	30	20	23	31	19	20	0	25	34
	100	1.000	1.000	1.000	1.000	1.000	1.000		1.000	1.000
GP-1	n	30	21	23	31	23	20	0	31	34
	100	1.000	1.000	1.000	1.000	1.000	1.000		1.000	1.000
GP-2	n	30	3	23	31	23	20	10	31	32
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
GP-4	n	30	21	23	31	15	20	10	26	29
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
HBDH	n	30	21	23	31	15	10	0	25	18
	90	1.000	0.952	0.000	0.000	0.000	0.000		0.000	0.000
	95	0.000	0.048	0.000	0.000	0.000	0.000		0.000	0.000
	100	0.000	0.000	1.000	1.000	1.000	1.000		1.000	1.000
IDDH	n	30	21	23	31	19	19	10	28	34
	90	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.018	0.015
	100	1.000	0.857	0.826	0.936	0.974	0.763	1.000	0.982	0.985
	120	0.000	0.143	0.174	0.065	0.026	0.237	0.000	0.000	0.000
IDH	n	30	21	23	31	19	20	10	25	32
	100	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	1.000
	110	1.000	1.000	1.000	0.968	1.000	0.925	0.000	0.000	0.000
	112	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.000
	130	0.000	0.000	0.000	0.032	0.000	0.050	0.000	0.000	0.000
LDH-1	n	30	21	23	44	22	20	10	25	19
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.974
	110	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.026
LDH-2	n	30	21	23	44	23	20	10	31	34
	90	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	91	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.000	0.000
	100	0.000	0.000	1.000	1.000	1.000	0.975	1.000	1.000	1.000
MPI	n	29	21	19	31	13	20	10	27	32
	90	0.000	0.000	0.000	0.000	0.039	0.100	0.000	0.000	0.000
	100	1.000	1.000	1.000	1.000	0.962	0.900	1.000	1.000	1.000
MDH-1	n	10	21	9	7	0	0	10	7	3
	100	1.000	1.000	1.000	1.000			1.000	1.000	1.000
MDH-2	n	30	21	22	31	23	20	10	27	32
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
MDH-3	n	30	21	22	31	23	20	10	27	33
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
PEPA-1	n	30	21	23	24	18	20	10	28	30
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.983
	105	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017

APPENDIX 2. CONTINUED.

Locus	Allele	*A. declivifrons*		*A. concolor*		*A. taurus*		*A. troschelii*		
		C. San Lucas	San Ignacio	Panama	Galapagos	Panama	Las Roques	C. San Lucas	Panama	Galapagos
PEPA-2	n	30	21	23	24	17	20	10	28	25
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
PGK	n	30	21	23	31	19	20	10	29	34
	90	1.000	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000
	100	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	1.000
PGM-1	n	30	20	22	31	19	19	8	19	32
	96	0.000	0.000	0.000	0.000	0.000	0.000	0.063	0.000	0.000
	100	1.000	1.000	1.000	1.000	1.000	1.000	0.813	1.000	0.984
	105	0.000	0.000	0.000	0.000	0.000	0.000	0.125	0.000	0.016
PGM-2	n	30	21	22	31	19	20	9	21	30
	100	0.000	0.000	0.091	0.048	0.000	0.000	1.000	0.952	0.983
	105	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.017
	118	0.000	0.000	0.000	0.016	0.000	0.000	0.000	0.000	0.000
	120	1.000	1.000	0.909	0.936	1.000	1.000	0.000	0.048	0.000
PGDH	n	30	21	22	31	19	20	9	27	34
	90	0.000	0.000	0.046	0.000	0.000	0.000	0.000	0.000	0.015
	100	1.000	1.000	0.955	1.000	1.000	1.000	0.944	1.000	0.985
	109	0.000	0.000	0.000	0.000	0.000	0.000	0.056	0.000	0.000
PK-1	n	28	21	22	23	19	20	10	26	30
	90	1.000	1.000	1.000	1.000	0.947	1.000	0.000	0.000	0.000
	100	0.000	0.000	0.000	0.000	0.053	0.000	1.000	1.000	1.000
PK-2	n	27	21	22	23	22	20	10	26	30
	90	1.000	1.000	1.000	1.000	0.955	1.000	0.000	0.000	0.000
	100	0.000	0.000	0.000	0.000	0.046	0.000	1.000	1.000	1.000
PK-3	n	28	21	22	23	21	20	10	27	30
	90	1.000	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000
	100	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	1.000
SOD-1	n	30	20	23	31	18	10	10	27	34
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
SOD-2	n	30	20	23	31	16	20	10	27	34
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
TPI-1	n	30	21	23	31	23	20	10	35	34
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
TPI-2	n	30	21	21	28	22	20	10	31	31
	100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
TPI-3	n	29	21	23	31	23	9	10	29	34
	99	0.983	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000
	100	0.017	0.000	0.000	0.000	0.000	0.000	1.000	1.000	1.000
XDH	n	30	21	23	31	19	20	10	28	34
	99	0.000	0.000	0.000	0.000	0.000	0.000	0.100	0.000	0.000
	100	0.000	0.000	0.000	0.000	0.000	0.000	0.900	1.000	1.000
	108	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	110	0.000	0.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000