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# Speciation in the Central American Seaway: the importance of taxon sampling in the identification of trans-isthmian geminate pairs

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## ABSTRACT

**Aim** To create a molecular phylogenetic hypothesis for the closely related serranid genera *Alphestes* Bloch and Schneider and *Dermatolepis* Gill and assess the role of the Panamanian Isthmus in speciation within these reef fishes.

**Location** Tropical eastern Pacific, Caribbean, and Indian Oceans.

**Methods** Sequence data from one nuclear (TMO-4C4) and three mitochondrial genes (16S, 12S, and cytochrome *b*) were used in maximum parsimony and maximum likelihood analyses.

**Results** Here we show that previously hypothesized trans-isthmian geminate species are not each other's closest living relatives. Species of *Alphestes* Bloch and Schneider in the eastern Pacific are sister taxa indicating post-closure speciation. Within *Dermatolepis* Gill, we identify a sister group relationship between the Caribbean and western Indian Ocean species, a rarely reported biogeographic pattern. Based on sequence divergence, speciation among the three species of *Dermatolepis* was, however, nearly simultaneous around the time of the isthmian closure event.

**Main conclusions** Our molecular phylogenetic analysis of two closely related genera of reef fishes, each with presumed trans-isthmian geminates, cautions against the uncritical use of morphological similarity in identification of geminates, as well as the assumption that trans-isthmian sister groups date to the isthmian closure event. These findings suggest that in some instances incomplete sampling of species within a clade including putative geminates may lead to improper conclusions regarding the pattern and timing of speciation, as well as incorrect estimation of the rate at which evolution has proceeded.

## Keywords

*Alphestes*, *Dermatolepis*, Serranidae, Isthmus of Panama, speciation, biogeography.

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## INTRODUCTION

The well-dated final closure of the Central American seaway provides a key focal point for testing the tempo of allopatric speciation in the marine realm (Coates & Obando, 1996; Haug & Tiedeman, 1998) and justifiably has received an extraordinary amount of attention (e.g. Knowlton *et al.*, 1993; Collins, 1996; Marko & Jackson, 2001; Marko, 2002). The so-called 'geminate' species pairs, defined by David Starr Jordan as '... twin species – each one representing the other on opposite sides of some form of barrier' (Jordan, 1908, p. 75), have provided model study systems for testing hypotheses regarding the effects of the

disruption of gene flow in an allopatric setting. This is particularly true for species pairs on either side of the Panamanian Isthmus that are assumed to be descendant from a common population at the time of partial or complete closure of the Central American Seaway not less than 3.5 million years ago (Ma) (Coates & Obando, 1996). The availability of an independent estimate of the minimum time since last genetic contact (Coates & Obando, 1996; Haug & Tiedeman, 1998) allows for critical analysis of sequence divergence of trans-isthmian geminates. This process has become a prominent tool used to study the rate at which evolution proceeds, using the closure of the seaway as a reference point for calibrating a

'molecular clock' in many marine organisms (e.g. Bermingham *et al.*, 1997; Arbogast *et al.*, 2002; Marko, 2002). Knowlton and Weight (1998) cautioned that the generally accepted time of 3.5 Ma for the final closure of the seaway should be viewed as a minimum time since divergence because the dispersal of some species was apparently disrupted much earlier by the rising, but still incomplete, Isthmus of Panama. Results from our study add other cautions: that apparent trans-isthmian geminates may not, in all instances, be each other's closest living relatives and that their divergence may be unassociated with the rise of the isthmus.

Geminates are typically identified based on morphological similarity, but rarely is a phylogeny available for larger clades including the hypothesized geminate pair. We present data for all heretofore described species of two closely related genera of reef fishes, both containing morphologically similar members in the eastern Pacific and western Atlantic, which show very different biogeographic relationships. This suggests that the assumption that morphologically similar species occurring in these oceans are geminates can be flawed and that the paradigm of trans-isthmian speciation may not hold true in all otherwise apparent cases.

## BACKGROUND

The epinepheline serranids, commonly known as groupers, are nearshore reef fishes found worldwide in tropical and subtropical reef habitats (Heemstra & Randall, 1993). They include some of the most economically valuable species of reef fishes, contributing a substantial percentage of the world's fish harvest, particularly within developing nations. Groupers are thought to be effective dispersers both in early life as broadcast spawners with pelagic eggs and larvae that may remain in the plankton for as long as 60 days, and in adult life as predators with large home ranges, sometimes undertaking long spawning migrations, and reaching ages in excess of 25 years (Johnson & Keener, 1984; Leis, 1986; Craig *et al.*, 1999; Liao *et al.*, 2001; Sala *et al.*, 2001; White *et al.*, 2002).

The grouper genera *Alphestes* Bloch and Schneider and *Dermatolepis* Gill each contain three species and have representatives on both sides of the Panamanian Isthmus. *Alphestes* comprises two species, *Alphestes multiguttatus* Günther and *Alphestes immaculatus* Breder, in the tropical eastern Pacific (TEP) and one species, *Alphestes afer* Bloch, in the tropical western Atlantic (WA) and Caribbean (C). *Dermatolepis* includes one TEP species, *Dermatolepis dermatolepis* Boulenger, one WA/C species, *Dermatolepis inermis* Valenciennes, and a third Indian Ocean (IO) endemic, *Dermatolepis striolata* Playfair. A recent study based on molecular sequence data provided evidence that *Alphestes* and *Dermatolepis* together form a monophyletic clade nested within the polyphyletic genus *Epinephelus* Bloch (Craig *et al.*, 2001). In his 1908 paper, Jordan provided several examples of species pairs of fishes separated by the Panamanian Isthmus that he thought were geminates. His list included members of both *Alphestes* and *Dermatolepis*: *A. afer* and *A. multiguttatus* (Jordan's paper was

published prior to the description of *A. immaculatus*) and *D. inermis* and *D. dermatolepis* (the latter under the nominal species *Dermatolepis punctata*). Since this initial assumption and in the absence of further study on their intra-relationships testing his hypothesis, these species continue to be regarded as geminate pairs (e.g. Thomson *et al.*, 2001).

## MATERIALS AND METHODS

Fishes were collected in the field by hook and line or spear pole, or were purchased from commercial fish markets. Tissue samples (gill filaments, fin clips, or muscle tissue) were removed and preserved in either 5× Net solution (Craig *et al.*, 2001) or 95% ethanol and stored at ambient temperature in the field prior to freezing at  $-80^{\circ}\text{C}$  in the laboratory. When available, voucher specimens were deposited in the Scripps Institution of Oceanography Marine Vertebrates Collection. Museum numbers for specimens used in this study and GenBank accession numbers for sequence data are listed in Table 1. One to three individuals per species were used for both ingroup and outgroup taxa depending on availability. The speckled hind, *Epinephelus drummondhayi* Goode and Bean was included because it is the putative sister species to the *Alphestes/Dermatolepis* complex (Craig *et al.*, 2001). The flag cabrilla, *Epinephelus labriformis* Jenyns, and the white-bloched grouper, *Epinephelus multinotatus* (Peters), were also included to increase taxon sampling within this lineage of nearly 100 species. The barred sand bass, *Paralabrax nebulifer* (Girard) was used as an outgroup taxon to root the serranid tree as this genus has been hypothesized to be a basal member of the family Serranidae (Pondella *et al.*, 2003).

Total genomic DNA was isolated using the DNeasy kit (Qiagen, Inc, Valencia, CA, USA) and protocols followed manufacturer's recommendations. Polymerase chain reaction (PCR) was used to amplify portions of three mitochondrial (mt) genes (16S, 12S, and cytochrome *b*) as well as one putative protein coding nuclear gene (TMO-4C4). Fifty microlitre PCR reactions were prepared using Red-Taq Ready Mix (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's instructions with the addition of approximately 10–100 ng of template DNA and 10 pmol of both forward and reverse primers. Qiaquick spin columns (Qiagen, Inc., Valencia, CA, USA) were used to remove primers and unincorporated dyes from PCR reactions. PCR reactions were carried out on an MJ Research PTC-200 thermal cycler. PCR reactions were subjected to 35 cycles of the following cycling protocol following a 1-min denaturation at  $94^{\circ}\text{C}$  for 1 min:  $94^{\circ}\text{C}$  for 30 s,  $50^{\circ}\text{C}$  for 45 s,  $72^{\circ}\text{C}$  for 30 s. Sequencing was performed on a Megabace 1000 capillary sequencer using ET-Chemistry (Amersham Biosciences, Piscataway, NJ, USA) following manufacturer's protocols. Sequencing and PCR primers used are listed in Table 2.

Sequences were aligned using CLUSTAL-X (Thomson *et al.*, 1997) with default settings and alignments were visually optimized using MACCLADE 4.0 (Sinauer Associates, Sunderland, MA, USA). A 19 bp segment of the 16S gene

**Table 1** Museum numbers for specimens deposited at Scripps Institution of Oceanography Marine Vertebrates Collection (SIO) and GenBank accession numbers for sequence data in this study. Photo vouchers are retained in the personal collection of MTC.

Species	Museum number	GenBank accession number			
		Cyt-B	16S	12S	TMO4C4
<i>Alphestes multiguttatus</i>	SIO 00-95	AY313995	AF297305	AY313981	AY313991
<i>Alphestes immaculatus</i>	SIO 00-92	AY314002	AF297290	AY313980	AY313994
<i>Alphestes afer</i>	SIO 03-49	AY313996	AY314003	AY313982	AY313992
<i>Dermatolepis dermatolepis</i>	Photo voucher	AY314000	AF297317	AY313984	AY313988
<i>Dermatolepis inermis</i>	Photo voucher	AY314001	AY314005	AY313979	AY313987
<i>Dermatolepis striolata</i>	Photo voucher	AY313999	AY314004	AY313989	AY313989
<i>Epinephelus multinitatus</i>	Photo voucher	AY426254	AY426594	AY426252	AY425675
<i>Epinephelus labriformis</i>	SIO 00-137	AY426255	AF297296	AY426252	AY425676
<i>Epinephelus drummondhayi</i>	SIO 00-50	AY313997	AF297317	AY313985	AY313993
<i>Paralabrax nebulifer</i>	SIO 00-97	AY313998	AF297328	AY072662	AY313990

**Table 2** Sequencing and PCR primers.

Primer name	Gene	Sequence	Reference
16Sar-L	16S	5'-cgctgtttatcaaaaacat-3'	Palumbi (1996)
16Sbr-H	16S	5'-ccggtctgaactcagatcacgt-3'	Palumbi (1996)
12Sa	12S	5'-aaactgggattatagaccactat-3'	Palumbi (1996)
12Sb	12S	5'-gagggtgacggcggtctct-3'	Palumbi (1996)
28For	Cyt-B	5'-cgaactgtgatgaaaaaccatcgtg-3'	Gilles <i>et al.</i> (2000)
34Rev	Cyt-B	5'-aaactgcagcccctcagaatgatattctctca-3'	Gilles <i>et al.</i> (2000)
TMO-F1-5'	TMO4C4	5'-cctccggccttctctaaaacctctc-3'	Streelman and Karl (1997)
TMO-R1-5'	TMO4C4	5'-catcgtctcctgggtgacaaaagt-3'	Streelman and Karl (1997)

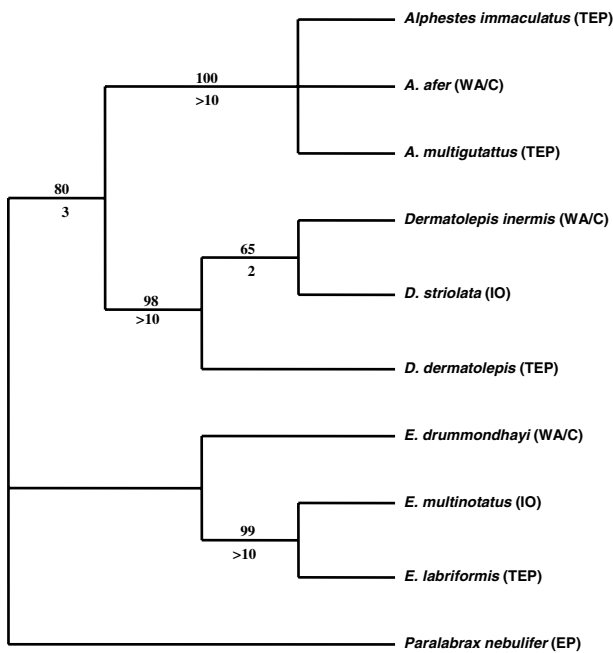
was removed due to ambiguity of the aligned base pairs. Indels in protein coding genes (cytochrome *b* and TMO4C4) were treated as single events in all analyses. Exhaustive search criteria for maximum parsimony analysis and heuristic search criteria for likelihood analysis were employed in PAUP\*4b10 (Sinauer Associates, Sunderland, MA, USA). For the maximum likelihood analysis, the default settings in PAUP\*4b10 were used except that a gamma distribution of rates at variable sites was employed. For the maximum parsimony analysis, default settings in PAUP\*4b10 were used with the exception that the Delayed Transformation Series (DELTRAN) was used, and gaps in the data were treated as a fifth base. A partition homogeneity test was performed in PAUP\*4b10 to assess the validity of combining the four molecular datasets. Bootstrap support values were calculated using default settings in PAUP\*4b10 with 1000 replicates, and Bremer support indices were calculated using NONA (V. 2.0; Goloboff, unpubl. program) in conjunction with the program WINCLADA (1.00.08; Nixon, unpubl. program). Bremer supports were calculated using a heuristic search with a maximum of 100,000 trees, holding 10 trees per replicate, with 100 random addition sequences each with Tree Bisection Reconnection (TBR) branch swapping.

## RESULTS

Using partial DNA sequences for one nuclear (TMO4C4; 535 bp) and three mitochondrial genes (16S, 12S, and

cytochrome *b*; 1447 bp), we explored the phylogenetic relationships of species within *Alphestes* and *Dermatolepis*. The partition homogeneity test supported the combination of all genetic data sets ( $P = 0.44$ ), and the combined data were used in all further analyses. Maximum parsimony and maximum-likelihood analyses of the combined data set recovered similar trees with only minor differences in topology (Figs 1 & 2). In both topologies, relationships within *Dermatolepis* are identical: *D. inermis* of the WA is sister to *D. striolata* of the IO, while *Dermatolepis dermatolepis* of the TEP is sister to these two. Within *Alphestes*, the parsimony analysis includes an unresolved trichotomy, while the likelihood analysis supports the sister group relationship of *A. immaculatus* and *A. multiguttatus*, both of the TEP, with *A. afer* of the WA sister to this pair. This suggests that the ancestor of the two eastern Pacific species and *A. afer* evolved via a trans-isthmian speciation event.

Genetic distances were calculated for all species pairs and ranged from 2.2 to 5.7% among ingroup taxa (Table 3). When calibrated using the average genetic distance and likelihood branch lengths between the species pairs *A. immaculatus* + *A. afer*, and *A. multiguttatus* + *A. afer* (thus accounting for the amount of sequence divergence between the ancestor of *A. immaculatus* + *A. multiguttatus* and *A. afer*, the hypothesized trans-isthmian speciation event) and a disruption of gene flow at 3.5–4.5 Ma (Coates & Obando, 1996; Knowlton & Weight, 1998), speciation within

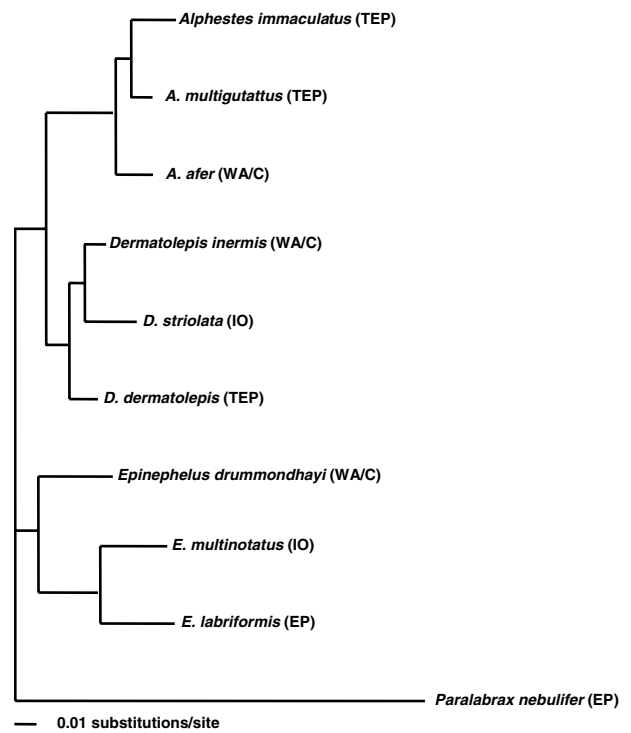


**Figure 1** Consensus of two most parsimonious trees (669 steps) for the serranid genera *Alphestes* Bloch and Schneider and *Dermatolepis* Gill. Numbers above and below nodes are Bootstrap (1000 replicates) and Bremer support values, respectively. Geographic range is indicated after species names as follows: WA, Western Atlantic; C, Caribbean; TEP, Tropical Eastern Pacific; EP, Eastern Pacific; and IO, Indian Ocean. Consistency index (CI) and retention index (RI) were identical for both trees (CI = 0.728, RI = 0.546).

this group was most likely initiated during the middle-late Miocene, approximately 7–9 Ma.

**DISCUSSION**

The analyses presented here confirm the previously hypothesized monophyly of *Alphestes* and *Dermatolepis* and confirm the validity of generic status for these clades (Craig *et al.*, 2001). Our molecular phylogenetic hypotheses for these two closely



**Figure 2** Maximum likelihood tree (-ln likelihood = 6246.8547) obtained for the serranid genera *Alphestes* Bloch and Schneider and *Dermatolepis* Gill. Abbreviations of geographic ranges are as in Fig. 1.

related genera of reef fishes reveal some quite unexpected biogeographic patterns. In both analyses, there is no evidence for an extant trans-isthmian species pair in either genus. The timing of the speciation events in each genus implies that partial or complete closure of the Panamanian isthmus did not play as crucial a role in precipitating the most recent speciation events in these reef fishes as was previously hypothesized (Jordan, 1908).

The topologies presented for each genus in both analyses are not biogeographically incongruent. The closure of the Central American Seaway apparently precipitated allopatric speciation

**Table 3** Percent sequence divergence between species of *Alphestes* Bloch and Schneider and *Dermatolepis* Gill and selected outgroups. Abbreviations of geographic ranges are as in Fig. 1

Species	1	2	3	4	5	6	7	8	9
1. <i>Alphestes immaculatus</i> (TEP)	*	*	*	*	*	*	*	*	*
2. <i>Alphestes afer</i> (WA/C)	0.033	*	*	*	*	*	*	*	*
3. <i>Alphestes multiguttatus</i> (TEP)	0.022	0.023	*	*	*	*	*	*	*
4. <i>Dermatolepis inermis</i> (WA/C)	0.054	0.057	0.048	*	*	*	*	*	*
5. <i>Dermatolepis dermatolepis</i> (TEP)	0.055	0.054	0.053	0.022	*	*	*	*	*
6. <i>Dermatolepis striolata</i> (IO)	0.054	0.057	0.055	0.026	0.029	*	*	*	*
7. <i>Epinephelus drummondhayi</i> (WA/C)	0.063	0.067	0.064	0.055	0.054	0.062	*	*	*
8. <i>Epinephelus multinotatus</i> (IO)	0.074	0.079	0.076	0.063	0.064	0.068	0.062	*	*
9. <i>Epinephelus labriformis</i> (TEP)	0.077	0.081	0.075	0.068	0.068	0.071	0.06	0.047	*
10. <i>Paralabrax nebulifer</i> (EP)	0.132	0.142	0.138	0.132	0.131	0.133	0.13	0.138	0.137

in *Alphestes*, with subsequent speciation occurring within the TEP. Within ocean speciation in *Alphestes* is plausible because, although these species overlap in distribution, *A. multiguttatus* generally occurs in deeper water where it co-occurs with *A. immaculatus* (M.T.C., pers. obser.). Post-isthmian speciation within the TEP has been hypothesized for other fishes (e.g. Rosenblatt, 1963).

Similarly, the closure of the Central American Seaway could have precipitated allopatric speciation in *Dermatolepis* with subsequent dispersal to and speciation in the Indian Ocean by the western Atlantic member via a stepping-stone model, first across the Atlantic, and subsequently around the southern tip of Africa into the Indian Ocean. Trans-Atlantic dispersal has been noted in several shore fish species (Joyeux *et al.*, 2001) and genetic data show minimal distance between western Atlantic and mid-Atlantic populations (Rocha *et al.*, 2002). Additionally, sister species status has been hypothesized for eastern and western Atlantic species in the seahorse genus *Hippocampus* Rafinesque (Jones *et al.*, 2003) and several species of fishes occur in both western and eastern Atlantic localities [e.g. the serranids *Paranthias furcifer* Valenciennes and *Epinephelus marginatus* Lowe, the sparid *Sparisoma rubripinne* (Valenciennes), the labrid *Bodianus pulchellus* (Poey), the chaetodontid *Chaetodon sedentarius* (Poey), and the pomacanthid *Pomacanthus paru* (Bloch)] (Afonso *et al.*, 1999; Bernardi *et al.*, 2000; Joyeux *et al.*, 2001).

Species with long-lived larval stages such as the serranids (Liao *et al.*, 2001) would be particularly suited to these trans-Atlantic crossings, and while there are several island groups that may provide stepping stones (e.g. Ascension Island), they may not be required as the south equatorial counter current may be sufficient to disperse larvae across the Atlantic (Joyeux *et al.*, 2001). Three other related grouper species, *Mycteroperca rubra* (Bloch), *Mycteroperca fusca* (Lowe), and *Mycteroperca acutirostris* (Valenciennes) are known from both sides of the Atlantic basin, but are absent from the mid-Atlantic island groups (Heemstra & Randall, 1993) supporting the hypothesis that trans-Atlantic crossings are possible in these fishes. Additionally, a 1.8 m specimen of the warsaw grouper, *Epinephelus nigritus* (Holbrook), was reported from the coast of Brittany (46°47' N, 3°50' W) in summer of 1997, and a specimen identified as the scamp, *Mycteroperca phenax* (Jordan and Swain), was recently reported from the Azores (P.C. Heemstra, pers. comm.).

While the crossing of the Atlantic basin has been demonstrated by several species (Joyeux *et al.*, 2001), dispersal around the southern tip of Africa is poorly documented. However, both the dusky grouper, *Epinephelus marginatus* (Lowe), and the comber, *Serranus cabrilla* (Linnaeus), range from the Mediterranean Sea, along the entire western coast of the African continent, and into a portion of the western Indian Ocean, the area from which *D. striolata* has been recorded (Heemstra & Randall, 1993). *Epinephelus marginatus* also is known from the western Atlantic (Heemstra & Randall, 1993).

The hypothesis of a sister group relationship between western Atlantic and Indian Ocean shore fish species, to our knowledge, has never been reported. While corals of the family Meandrinidae show a similar distribution (Veron, 2000), the timing of their diversification is more consistent with dispersal through the now closed Tethys Sea. This explanation does not apply to the species of *Dermatolepis* as the closure of the Tethys Sea 18–20 Ma (Hallum, 1994) well predated the hypothesized speciation events within this genus. While this area relationship is rare, it is consistent with dispersal into the eastern Atlantic and around the tip of Africa by *Dermatolepis*, with subsequent extinction along the western coast of Africa or persistence of an unnoticed population in this remote and relatively poorly known area. The latter possibility is supported by the known distribution of the dusky grouper, *E. marginatus* (see above).

While the biogeographic pattern hypothesized for the species within *Dermatolepis* is unique, the timing of this process offers another interesting finding for speciation hypotheses associated with the Central American Seaway. Examination of the rates of sequence divergence between these species and corresponding branch lengths in our phylogenetic analysis supports a pre-closure dispersal scenario. Percent sequence divergences between sister species are comparable for *Dermatolepis* and *Alphestes* (Table 3). These data imply that the first speciation within *Dermatolepis* predated the first (trans-isthmian) speciation event in *Alphestes*, while subsequent speciation resulting in *D. striolata* and *D. inermis* was more or less concomitant with the closure of the isthmus (Fig. 2 and Table 3). Additionally, the uncorrected genetic distance between the eastern Pacific *D. dermatolepis* and the Caribbean *D. inermis* is less than that between *D. inermis* and *D. striolata*, despite the sister species status afforded to the latter pair in our phylogenetic analyses. This is consistent with a nearly simultaneous divergence of all three species within *Dermatolepis* (given the short branch lengths between the ancestor of the *D. striolata* and *D. inermis* clade and *D. dermatolepis*).

These findings provide a strong argument for complete taxon sampling of clades including putative trans-isthmian geminates prior to evaluating hypotheses regarding their evolution. Had all species within *Alphestes* and *Dermatolepis* not been sampled, particularly *D. striolata* from the Indian Ocean, several alternative and incorrect hypotheses may have been deduced for the patterns of speciation in these fishes. For example, without this species being included, *D. dermatolepis* and *D. inermis* would necessarily have been sister taxa, leading to incorrect hypothesized rates of molecular evolution within this group. Alternatively, we might have hypothesized that the rising isthmus interrupted gene flow at different times in the evolutionary history of *Dermatolepis* and *Alphestes* as suggested by the model presented by Knowlton & Weight (1998). These and similar findings indicate that the evolutionary history of trans-isthmian geminate species is often far more complex than previously thought.

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#### ADDENDUM ADDED IN PRESS

Recently, a specimen of *Alphestes afer* (Bloch 1873) was reported from a fish market at Sao Tome Island (eastern Atlantic) by Dr Peter Wirtz. Dr Wirtz was able to photograph the specimen and obtain a tissue sample for analysis. Examination of DNA sequence data from the mitochondrially encoded Cytochrome B, 16S, and 12S genes, the nuclear TMO-4C4 locus, and characters in the photograph indicate that the specimen from Sao Tome is conspecific with specimens from the Florida Keyes (uncorrected p distance = 0.004). Originally described from the coast of Guinea, the type locality of *A. afer* has long been regarded as a locality error (report forthcoming by M. T. Craig and P. C. Heemstra). The incorporation of this information provides further support for our biogeographic hypothesis of trans-Atlantic dispersal in *Dermatolepis*. The data suggest that the eastern Atlantic served as a stepping stone for *Dermatolepis* prior to its dispersal around the Cape of Good Hope and subsequent speciation within the western Indian Ocean.

#### BIOSKETCHES

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