12-2006

The Role of Natural and Sexual Selection in the Origin and Maintenance of Divergence Within and Between the Mexican Sailfin Mollies, *Poecilia velifera* and *P. petenensis*

Shala Hankison
Clemson University, shankis@clemson.edu

THE ROLE OF NATURAL AND SEXUAL SELECTION IN THE ORIGIN AND MAINTENANCE OF DIVERGENCE WITHIN AND BETWEEN THE MEXICAN SAILFIN MOLLIES, *Poecilia velifera* AND *P. petenensis*

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Biological Sciences

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by
Shala J. Hankison
December 2006

Accepted by:
Dr. Margaret Ptacek, Committee Chair
Dr. Richard Blob
Dr. Michael Childress
Dr. Sidney Gauthreaux
Dr. Lesly Temesvari

ABSTRACT

Understanding speciation remains a holy grail of evolutionary biology. One useful approach is studying the evolutionary mechanisms important in population divergence to infer the mechanisms important in speciation. This method is especially useful when closely related species can be compared to determine whether intraspecific differences parallel interspecific differences. I studied population divergence in two species of Mexican sailfin mollies, *Poecilia velifera* and *P. petenensis*. These closely related species are particularly useful for this type of study, as they live in habitats that may differ in the importance of natural selection. In addition, these species may differ in the importance and strength of sexual selection, as males exhibit secondary sexual morphological and behavioral traits. To understand population divergence, I compared morphology among populations in both species. In addition, I observed male mating behaviors to understand the pattern of behavioral differences among populations. Finally, I used microsatellite loci to determine neutral genetic differentiation both within and between the two species. Morphologically, I found that populations in both species were differentiated, and while some morphological trait differences were shared among populations in both species, important differences were also present. For example, caudal peduncle differences among populations in *P. petenensis*, but not *P. velifera*, suggest that habitat differences may shape some morphological differences. Males of *P.*

velifera showed evidence of an alternative male mating strategy, with small males performing only gonopodial thrusts, while large males performed both courtship displays and gonopodial thrusts. Males of all sizes of *P. petenensis* performed both mating behaviors, regardless of body length. In addition, little variation existed between populations of *P. velifera*, however, males of *P. petenensis* showed more population specific rates of mating behaviors. Finally, microsatellite analysis revealed that while most populations were genetically distinct, patterns of genetic variation were not concordant with patterns of phenotypic variation, suggesting that selection, and not genetic drift, is likely promoting population divergence in *P. velifera* and *P. petenensis*. These results, taken together, suggest that differences in population divergence between these species are the results of both natural and sexual selection, which have been important evolutionary mechanisms in sailfin molly speciation.

DEDICATION

For waking me to see Jane Goodall on TV, for looking for deer in the yard, for dinosaur hunting in the woods, for watching the birds. For telling me about grandpas who would not butcher hogs and could talk to sheep. For a belief that an education was to be valued, and that teaching could be noble. For hanging more birdfeeders and birdhouses than anyone I know. This dissertation is dedicated to Joe and Ruby Leffler, who inspired my love of animals, whether they meant to or not.

ACKNOWLEDGEMENTS

My first and fullest thanks are to my advisor, Margaret Ptacek. The progress I have made towards being a good scientist is primarily her doing. Not only did she guide me through this project and all of its stages, she served as a true mentor into how to be a productive (sometimes over-productive) member of an academic department. My graduate experience has gone beyond research, and for all of my preparation for the future, I owe Margaret thanks.

Similarly, I would like to thank my committee members, each of whom has contributed greatly to this project and to my development as a scientist. Michael Childress provided invaluable advice as to statistical design and analysis, presentation tips, and the importance of collaborative efforts. Rick Blob has been a valuable resource on fish morphology and its analyses, and why it is more important than I initially thought it would be to my project. Sidney Gauthreaux has not only been a wonderful committee member, but also an excellent role model for a career in the field of animal behavior. Finally, Lesly Temesvari, has consistently provided insightful advice and assistance throughout my dissertation.

If not for the assistance of the fish crews, both in the lab and in the field, I would not have had a project. Thank you goes to Antonio Aranguren, Jeff Barger, Lisa Durham, Michele Kittell, Alma E. García Morales, Ewen Harrison, Heather Kozak, Christopher Mimms, Jose Ramírez, Angie Rivera, Corey Roelke, Jonathan Rosenfield, Erica Marce Santo, Jennifer Seda, Stephanie Stutts, Maria

Valtierra-Vega. Special thanks go to my co-author Juan Jacabo Schmitter-Soto for the biogeographical insights, and to El Colegio de Frontera Sur (ECOSUR) for the use of facilities.

Thanks are also due to a particularly patient group of DNA women, Megan Harvey, Lisa Durham, Rachael McManamay, and Felicia Hawthorne, for patiently extracting fish DNA and for all of the help with optimizing primers. In addition, Michelle Andrews and Erin O'Leary-Jepsen were gracious instructors and hosts at Idaho State University's Molecular Research Core Facility, and I could not have genotyped everything without their help.

Invaluable assistance on experimental design, statistical analyses, and genetic interpretations were also obtained from Amy Lawton-Rauh, Peter Marko, Saara DeWalt, Janet Loxterman, Holly Nance, Warren Booth, Hoke Hill, Jr., Ernest Keeley, and Joe Travis. Multiple colleagues read early drafts of the manuscripts sent for publication, including Alison Bell, Sean Clark, Tom DeWitt, Brian Langerhans, Molly Morris, and Helen Rodd. Nora Espinoza provided a program I used for Reduced Major Axis analysis. Dave Lewis took excellent pictures of the fish. Melissa Chernick went above and beyond the call of duty with her help in obtaining all GIS related data. Of course, Barbara Piekutowski and all of the BioSci office staff were priceless in their help in numerous areas throughout my graduate career. Gratitude, of course, is also due to the department chair A.P. (Hap) Wheeler for unwavering support of the graduate students and for always making me smile.

My gratitude is also owed to a number of colleagues. First and foremost, to the Ptacek Lab babes, both old and new, especially to Kelly Gunnell and Michele Kittell. Because I do not have Kelly's gift with words, I won't attempt to describe what you have meant to me, but really it is beyond description anyway. Thanks also to the "old school" group, Tildon Jones, Jenny (Michi) Demos, and Rick Laughlin, for being there from beginning to end. Thank you also to those friends who have continued to support me, especially the dinner and a movie regulars, Angie Rivera, Gabe Rivera, Melissa Chernick, and to Adrianna Zitto, Heather Kozak, Laura Hunt, C.J. Geraci, Stephanie Loveless and Jennifer Seda, and to all of the amazing BioSci grads.

For constant support (emotional, financial and otherwise), understanding, and cheerleading, thank you to Mom, Jessie and all of my family, who were always here with me, even if not geographically.

Finally, thank you to Erinn Nicley, who has been more understanding, supportive, and wonderful than I ever could have asked him to be. I can never thank you enough for encouraging me to do whatever I wanted and for always being there, no matter what.

This project was funded by a Clemson University Alumni Graduate Fellowship, A CU Stackhouse Fellowship, Sigma Xi, the Philanthropic Education Organization, the Biological Sciences Graduate Student Association, The CU Graduate Student Government, and a NSF grant to Margaret Ptacek.

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CHAPTER 1

INTRODUCTION TO SAILFIN MOLLIES AND POPULATION DIVERGENCE

Evolutionary studies often center on questions related to speciation. What promotes it and what prevents it? How do multiple evolutionary mechanisms interact; are they in concert, or do they conflict? While certainly there is not a single answer to these questions for all species, the understanding of evolutionary mechanisms in a subset of species may reveal the potential interactions of these mechanisms in a broader context. One important approach to understanding the causes of speciation, especially the processes related to local adaptation and the role of sexual selection in promoting divergence, is the study of population divergence (Foster et al. 1998; Foster and Endler 1999).

Population differentiation is thought to be the initial stage in many models of speciation (Verrell 1998; Coyne and Orr 2004). The study of differentiated populations, especially in a geographic context, may expose the evolutionary mechanisms that are important in promoting or maintaining this divergence. These studies may, in turn, shed light on evolutionary mechanisms that were also potentially significant in promoting differences at higher taxonomic levels (Endler 1989; Foster et al. 1998; Foster and Endler 1999) and may avoid confounding traits that have promoted speciation with those that have arisen since its completion (Coyne and Orr 2004). A further benefit of comparisons of

population divergence is the ability to compare intraspecific population divergence among related taxa and to determine whether intraspecific differences mirror those observed in interspecific comparisons. When such patterns are observed, they provide evidence that inter- and intraspecific differences may be promoted by at least some similar mechanisms (Foster et al. 1998; Coyne and Orr 2004)

While comparative studies of intraspecific population variation in both phenotypic and genotypic traits can allow for a better understanding of the evolutionary processes promoting and maintaining population divergence (Masta and Maddison 2002), most studies are limited to describing divergence in either phenotypic or genetic characters, but not both. Although such studies provide evidence of the high degree of variation that can occur within a single species (Houde 1993), they are unable to compare the levels of divergence in phenotypic and genotypic traits or provide insight into the relative importance of selection and drift in influencing population divergence, as well as the homogenizing effects of gene flow between populations. A comparative study that incorporates both phenotypic and genetic divergence is important, as it can suggest which mechanisms shape divergence (Merilä 1997; Merilä and Crnokrak 2001; Masta and Maddison 2002; McKay and Latta 2002), whether population divergence is likely to be maintained without speciation (Magurran 1998), or whether divergence may ultimately lead to speciation (Lande 1981; Iwasa and Pomiankowski 1995).

Male traits that function as mating signals are one set of phenotypic traits that often show considerable inter-population variation. As targets of sexual selection, these phenotypic traits are important to understanding the role of sexual selection in promoting and maintaining population divergence (Lande 1981; Lande and Kirkpatrick 1988). In addition, genetic drift and chance founder effects may lead to differences in populations (Schluter 2001), which may differentially affect the divergence of male traits. Thus, understanding genetic differentiation and gene flow between populations is crucial to determining the relative importance of selection and drift in shaping population divergence in mating signals. A comparison of these mechanisms can determine whether there are concordant patterns of phenotypic and genetic divergence, the determination of which is necessary to clarifying the relative contribution of these evolutionary mechanisms.

The sailfin mollies *Poecilia velifera* and *P. petenensis* are excellent candidates for a parallel study of population divergence among closely related species (Fig. 1.1). Mollies are livebearing poeciliid fishes. Males of *P. velifera* and *P. petenensis* show high levels of sexual dimorphism (Fig. 1.2; Hubbs 1933), indicating that sexual selection may be important in maintaining intersexual differences (Andersson 1994). This dimorphism is particularly evident in the large size of the male dorsal fin. During courtship displays, used to elicit female cooperation during mating, males erect their dorsal fin, and also generally curve their body into a sigmoid shape and tilt towards the female (Parzefall 1969, 1989; Luckner 1979; Farr and Travis 1986; Ptacek and Travis 1996; Ptacek et al.

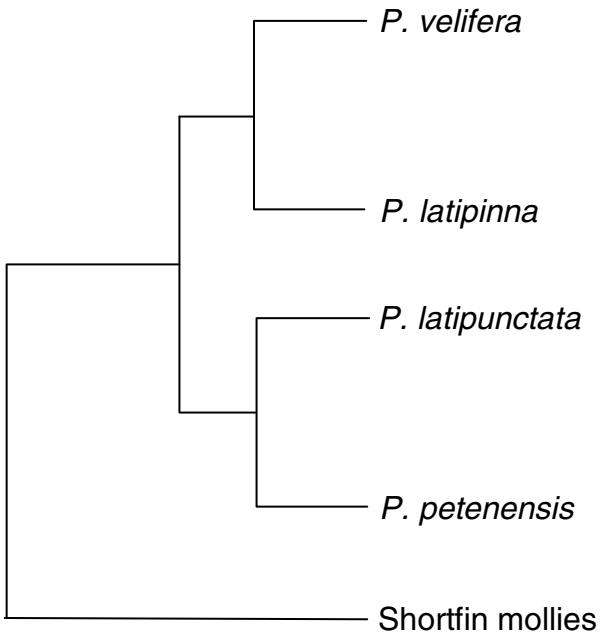

FIG. 1.1. Phylogenetic tree showing the relationships between the four sailfin species and shortfin mollies. Relationships modified from a maximum likelihood phylogeny in Ptacek and Breden (1998).

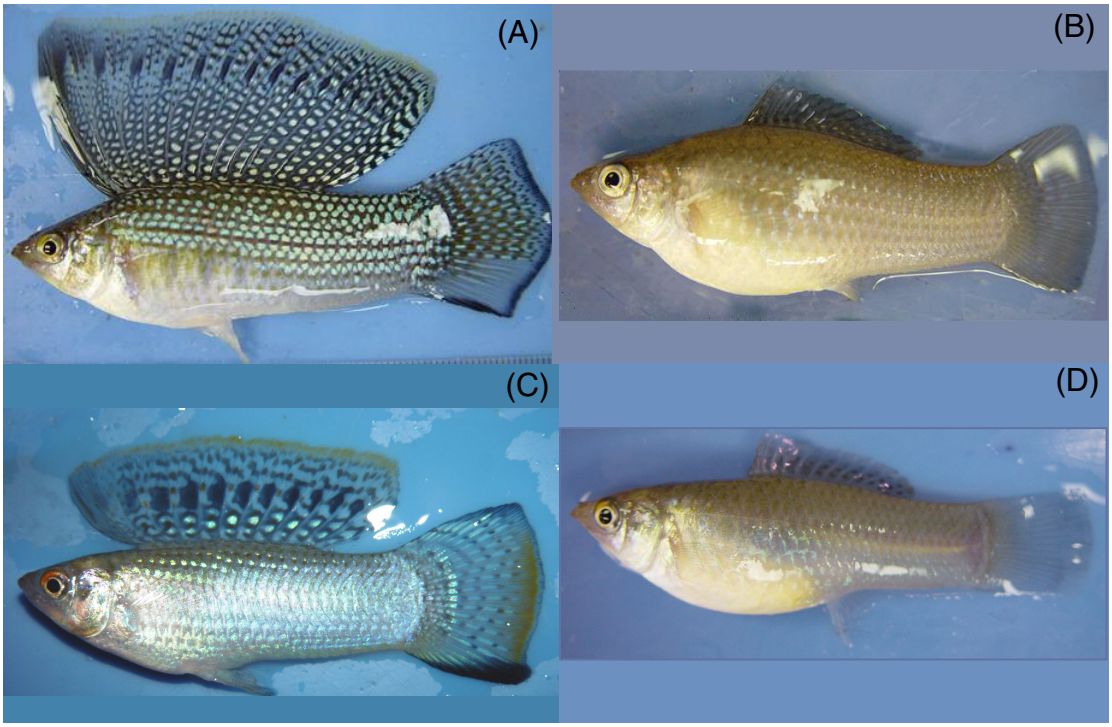

FIG. 1. Digital photographs of males of *Poecilia velifera* male (A) and female (B) and *P. petenensis* male (C) and female (D).

2005). In contrast, males may circumvent female choice through gonopodial thrusting. As in all poeciliid fishes, males posses a modified anal fin, the gonopodium, which is used to transfer sperm packets to females' gonopore (urogenital opening) for internal fertilization (Constantz 1989). Gonopodial thrusting is a type of forced insemination attempt, where the male orients himself behind a female, brings the gonopodium to a forward position, and attempts to insert the tip into the female's gonopore (Rosen and Tucker 1961; Constantz 1989; Farr 1989). A third mating behavior, gonoporal nibbling, occurs when males make nasal or oral contact with the female's gonopore. The function of this behavior is unclear, however it appears to aid a male in determining a female's reproductive status (Farr and Travis 1986; Constantz 1989; Sumner et al. 1994).

Divergence in male mating behaviors may have important implications for how population divergence is maintained (Foster et al. 1998; Foster and Endler 1999). For example, behavioral differences between populations may reflect differences in female preferences, such as population-specific preferences for rates of courtship (Endler and Houde 1995; Ptacek and Travis 1996, 1997). In addition, differences in natural selection may also shape population difference. Predation rates, flow rate, and light intensity, for example, have been shown to strongly influence male courtship displays and rates of gonopodial thrusting in guppies, *P. reticulata* (Endler 1987, 1995; Magurran et al. 1995; Nicoletto 1996; Nicoletto and Kodric-Brown 1999).

In addition to the opportunity to compare mating behaviors within and between *P. velifera* and *P. petenensis*, males of these species also show high levels of morphological variation. Because male size appears to be fixed at maturity (for examples in related species see Kallman 1984, 1989; Zimmerer and Kallman 1989; Travis 1994a, b), comparisons of male morphology can potentially reveal much about the selective influences that have shaped morphological differences. While both *P. velifera* and *P. petenensis* occur in habitats in and immediately surrounding the Yucatán Peninsula in Mexico, they generally inhabit very different habitat types (Fig. 1.3). The species *P. velifera* is generally found in brackish coastal marshes, while *P. petenensis* is also found farther south into Belize and Guatemala, in more inland rivers and streams (Schmitter-Soto 1998).

The differences in geographic range and preferred habitats between *P. velifera* and *P. petenensis* may have important implications for differential levels of migration between populations, and differences in natural selection regimes. The degree of connectedness of saltwater habitats for *P. velifera* is more continuous across coastal marshes, therefore gene flow rates may be higher for this species than for *P. petenensis*. The freshwater species, *P. petenensis*, is more restricted in distribution because of the patchy distribution of freshwater habitats in the Yucatán peninsula and dispersal between drainages is less likely (however, periodic flooding during the rainy season does promote some interpopulation mixing, even between drainages, personal communication, J. J. Schmitter-Soto). The different habitats occupied by *P. velifera* and *P. petenensis* may also result in differential patterns of natural selection between the species.

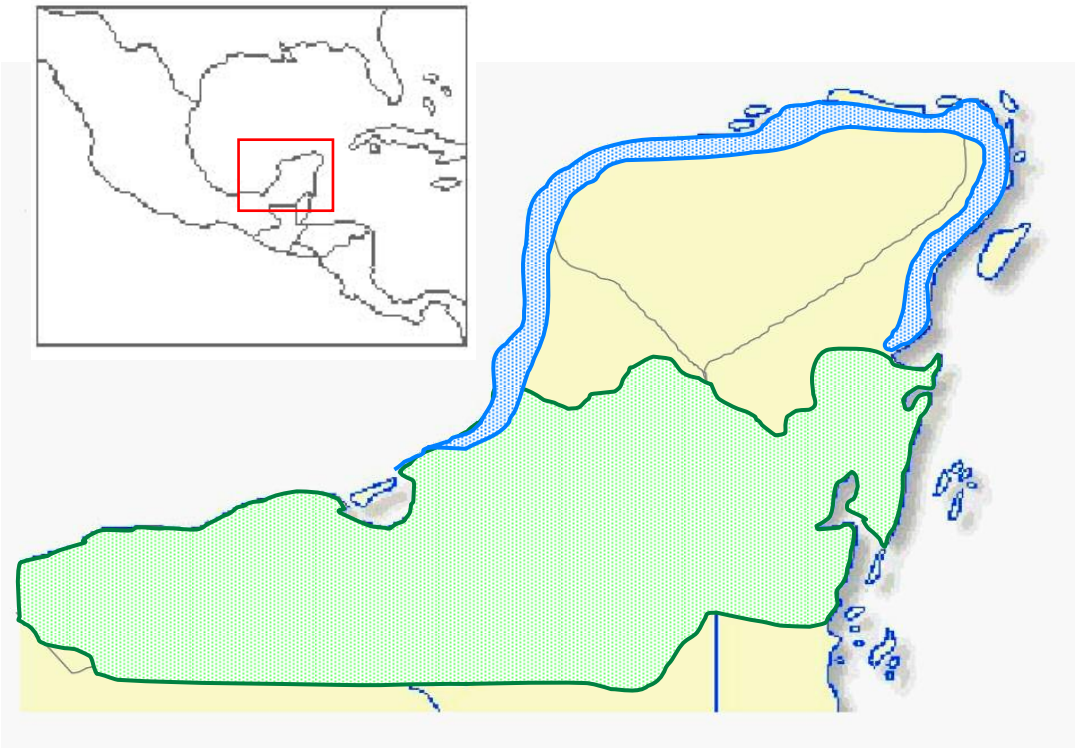

FIG. 1.3. Yucatán Peninsula showing the habitat range of *Poecilia velifera* (blue) and *P. petenensis* (green). Inset map indicates the area that has been enlarged for the range map.

For example, cichlids are a common river predator that may influence morphological and behavioral divergence among populations of *P. petenensis*, but not among populations of *P. velifera*.

An important role for sexual selection in promoting divergence in male morphology and mating behaviors has been demonstrated in a related sailfin species *P. latipinna* (Farr and Travis 1986; Ptacek and Travis 1996, 1997; Ptacek 2005). Population-specific differences persist in the face of high gene flow (Trexler 1988), suggesting that sexual selection is a strong evolutionary force in these populations, overcoming the diluting effects of gene flow. These findings illustrate the potential importance of sexual selection in a closely related species, and provide an opportunity to determine whether similar patterns of divergence are also observed in *P. velifera* and *P. petenensis*.

An additional benefit of using within and between species differences to infer potentially important mechanisms of speciation is the ability to understand the role of selection versus the role of neutral genetic forces, such as genetic drift. Comparing intraspecific divergence in male mating behaviors and morphology provides an opportunity to observe the range of behavioral and morphological variation within a species. When comparisons can further be made between related species, potentially important differences between the species may also be inferred (Verrell 1998; Foster and Endler 1999). However, it is important to distinguish between the role of selection, and its strength in promoting population divergence, and that of historical differences as a result of genetic drift. If divergence in both phenotypic and genetic traits is concordant,

then genetic drift or ongoing gene flow may be important in predicting patterns of population divergence (Foster et al. 1998; Verrell 1998; Coyne and Orr 2004; Nicholls et al. 2006). However, when discordant patterns of neutral genetic and phenotypic divergence are observed, or when there appear to be large differences in the rates of divergence between these types of traits, selection may be indicated to account for the differences (Endler 1977; Foster et al. 1998; Coyne and Orr 2004).

One class of genetic marker particularly well suited to studies of population differentiation is microsatellite DNA (reviewed in Dowling et al. 1996). Microsatellites are short (generally 1-6 base pairs), tandemly repeated units of nuclear DNA. They are codominantly inherited and highly polymorphic, allowing for observation of large numbers of genotypes, and their relatively high mutation rate (generally through polymerase slippage during DNA replication) makes them ideal for population comparisons (Ashley and Dow 1994; Schlötterer and Pemberton 1994; Slatkin 1995; Jarne and Lagoda 1996).

Microsatellite markers are the most useful markers to study neutral genetic variation in mollies for several reasons. First, primers have already been developed for several closely related species (Parker et al. 1998; Becher et al. 2002; Walter et al. 2004), which can easily be optimized for use in mollies. Second, microsatellites are likely selectively neutral (Ashley and Dow 1994), thus, providing a measure of population variation that is immune to the action of selection, and a point from which to compare phenotypic traits for evidence of divergence because of selection. Finally, microsatellites can be used to calculate

both genetic differentiation and genetic distance (Lowe et al. 2004), which provides a quantitative method for comparing phenotypic and morphological divergence.

My dissertation research was designed to compare populations within and between *P. velifera* and *P. petenensis* to gain insight into the relative importance of different evolutionary mechanisms in promoting and maintaining population divergence. I examine population divergence in both phenotypic and neutral genetic traits, as described briefly above, and in more detail in the following chapters. In Chapter 2, I examine morphological divergence within and between *P. velifera* and *P. petenensis* using both linear and geometric morphometric techniques. These analyses not only allow me to determine the patterns of divergence, but to understand whether natural or sexual selection may be shaping morphological divergence. In Chapter 3, I examine male mating behavior variation within and between the two species, and describe an alternative male mating strategy in *P. velifera*, and how sexual and natural selection may have favored its evolution. Finally, in Chapter 4, I use microsatellites to examine neutral genetic structure in *P. velifera* and *P. petenensis*, and compare the patterns of genetic divergence to those observed in phenotypic divergence in order to understand the roles of natural and sexual selection in promoting or maintaining population divergence. Overall, this research provides a better understanding of how and why populations of *P. velifera* and *P. petenensis* differ, and allows for insight into how these same mechanisms may have shaped speciation in sailfin mollies.

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CHAPTER 2

MORPHOLOGICAL DIVERGENCE IN THE MEXICAN SAILFIN MOLLIES

Abstract.—This study examined the patterns of morphological variation both between species, and between sexes and among populations within each species, using geometric morphometrics and linear measures of morphological traits. While sexes within each species differ in characteristics that may be important in sexual selection, such as length of the dorsal fin, species differ in traits, such as body depth, that may also be influenced by natural selection because of differences in habitats. Within each species, many morphological traits are similar among populations, but important differences, including caudal peduncle depth in *P. petenensis* (but not in *P. velifera*), suggest that habitat differences may also be important in shaping population divergence independently within each species. Indeed, the evolutionary vectors of male morphological population divergence for each species differed by an angle of 98.5°, representing nearly orthogonal vectors and suggesting independent shape divergence between these two molly species. Finally, geographic isolation does not explain the morphological differentiation seen among populations, suggesting that natural and sexual selection are strong forces promoting morphological diversification within these two species, despite the potential for a high degree of population connectivity and gene flow.

INTRODUCTION

Examining the pattern of morphological differentiation among populations is one important method of understanding how divergent selective regimes can generate and maintain phenotypic diversification (Langerhans and DeWitt 2004; Ghalambor et al. 2003; Endler 2000; Schluter 2000; Rice and Hostert 1993; Endler 1977; Ehrlich and Raven 1969). Morphology is relevant to nearly all aspects of an organism's biology and, thus, is often subject to strong natural and sexual selection that may vary across a species' range (Bels et al. 2003; Arnold 1983). Because natural and sexual selection may affect morphological traits differently, comparing the kinds and degree of morphological changes may also provide insight into the relative importance of these selective forces in shaping population differences (Kirkpatrick and Ravigne 2002; Kirkpatrick 2001; Panhuis et al. 2001; Schluter 2001). Finally, morphological differences can be quantified and used to measure the amount of divergence among populations and to evaluate the relationship between morphology and other factors such as geographic distance and degree of isolation or biotic and abiotic habitat characteristics.

Comparative studies among closely related species are useful for understanding general patterns and causes of phenotypic diversification among lineages that share common evolutionary histories (McKinnon and Rundle 2002; Holtmeier 2001; Day et al. 1994). Similarities between species may be because of persistent ancestral traits, while differences are likely to reflect varying

selective forces associated with ecological and mating signal divergence (Panhuis et al. 2001; Schluter 2001; Ptacek 2000). Furthermore, by comparing intraspecific variation to interspecific variation, one can make inferences with respect to how forces of evolution promoting population-level divergence may also influence speciation (Kirkpatrick and Ravigne 2002; Magurran 1998; Ptacek and Travis 1998).

This study examined the degree of morphological differentiation within and between two species of poeciliid fishes, the Mexican sailfin mollies *Poecilia velifera* (Regan) and *Poecilia petenensis* (Günther). *Poecilia velifera* is endemic to the Yucatán peninsula region of Mexico, while *P. petenensis* is also found farther south into Belize and Guatemala (Fig. 2.1). Sailfin mollies are an interesting group in which to compare inter- and intraspecific divergence in morphology for several reasons. First, sailfin mollies are highly sexually dimorphic; males possess a greatly enlarged dorsal fin that is presented to the female in a courtship display (Farr 1989; Farr and Travis 1986; Parzefall 1969; Rosen and Tucker 1961; Hubbs 1933; Regan 1913). Furthermore, male mollies, as in all poeciliids, possess a modified anal fin, the gonopodium, which serves as an intromittent organ during internal fertilization in these livebearing fishes (Constantz 1989; Rosen and Tucker 1961). Geographic variation in gonopodium length has been reported for several poeciliid species (Jennions and Kelly 2002; Kelly et al. 2000).

In addition, the two Mexican sailfin species vary in their degree of exaggeration of sexually selected dimorphic fin characteristics; males of *P.*

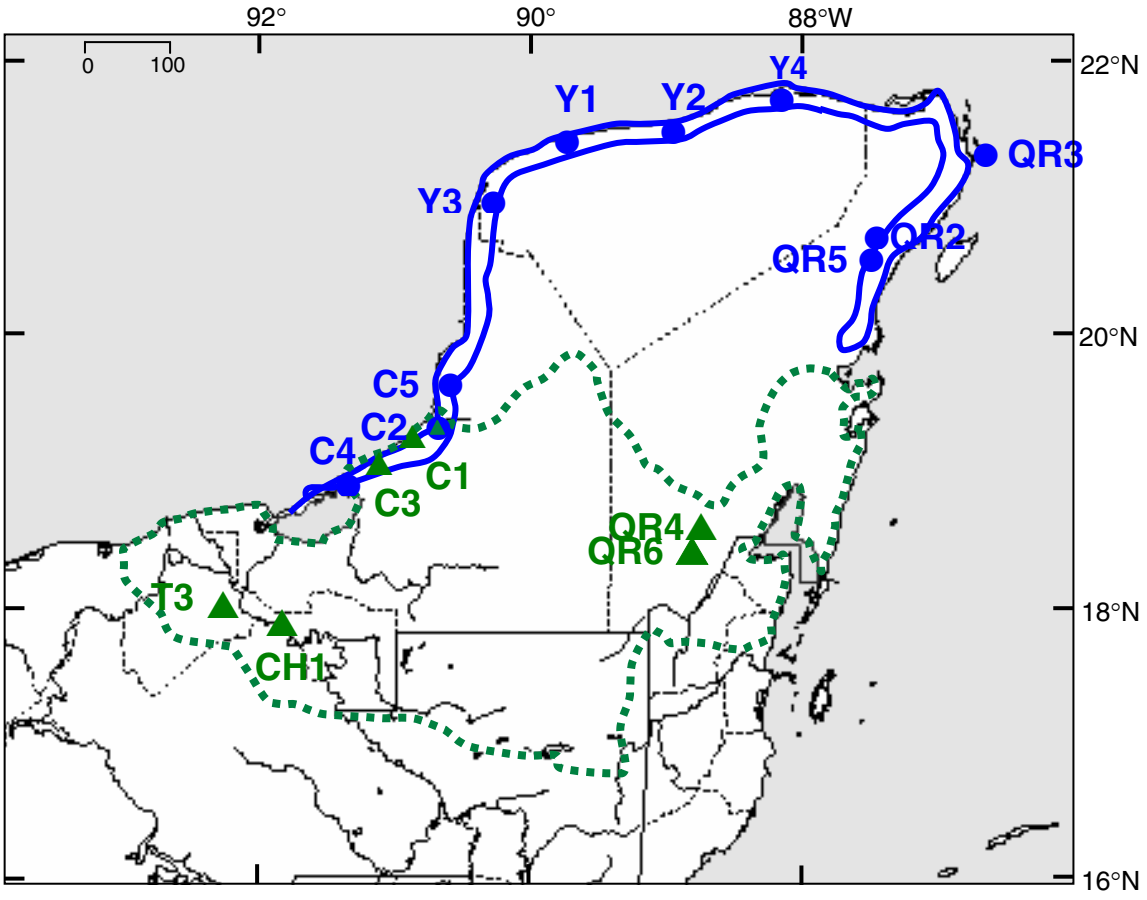


FIG. 2.1. Collecting sites of *Poecilia velifera* (●) and *P. petenensis* (▲) in the Yucatán Peninsula. Letters represent collecting sites in different states: Tabasco (T), Chiapas (CH), Campeche (C), Yucatán (Y), and Quintana Roo (QR). Numbers identify individual sites within states. Both species were collected together at one site (C2). Ranges are indicated by solid (*P. velifera*) and dotted (*P. petenensis*) lines.

velifera have much larger dorsal fin length and area (*P. velifera*: 15 – 19 dorsal fin rays, *P. petenensis*: 12 – 16 dorsal fin rays; Miller, 1983). Larger dorsal and caudal fins (Schmitter-Soto 1998; Basolo 1990; Bischoff et al. 1985; Miller 1983), leading to increased overall apparent size or lateral projection area (the lateral area of the fish including the body and fins), have been shown to be important targets of sexual selection through female mating preferences (MacClaren et al. 2004; Karino and Matsunaga 2002; Rosenthal and Evans 1998), and, thus, sexual selection has led to population divergence in body shape in some poeciliid species (Ptacek 2005; Ptacek and Travis 1997).

Sailfin molly species also vary in their preferred habitats; *P. velifera* is restricted to coastal habitats, such as anchialine cenotes, tidal pools and salt marshes, never higher than *ca.* 20 meters above sea level, while *P. petenensis* is more abundant in interior waters of the Yucatán peninsula, being found in freshwater rivers and impoundments (Schmitter-Soto 1998). Habitat differences lead to variation between the two species in the suite of piscine predators they encounter, as well as environmental differences, such as the degree of salinity and flow regimes. For example, inland populations of *P. petenensis* are more often found in streams that can experience fairly substantial flow regimes, especially during the rainy season (García-Gil et al. 2002; INEGI 1989) and their primary predators include cichlids such as *Petenia splendida* Günther (Schmitter-Soto 1998), juvenile crocodiles (*Crocodylus moreletii*) and kingfishers (*Chloroceryle* spp.). Populations of *P. velifera*, on the other hand, are found in habitats with substantially lower flow regimes, including marshes and cenotes

closer to the coast, where they experience a wider range of piscine predators (not only cichlids, but also many marine fishes, such as *Megalops atlanticus* Valenciennes, *Arius* spp., *Strongylura* spp., *Lutjanus* spp., *Gobiomorus dormitor* Lacepède, *Centropomus* spp., etc.; Schmitter-Soto 1998; Reséndez-Medina, 1981; Hubbs 1936) and bird predation from wading species, such as storks, herons and egrets (Ramo and Busto 1992) as well as crocodiles such as *Crocodylus moreletii* and *C. acutus* (Schmitter-Soto et al. 2002).

In addition to differences in abiotic and biotic features of the habitats characteristic of these sailfin molly species, the degree of spatial isolation and potential gene flow among populations of each species may also contribute to morphological divergence. Coastal, salt marsh habitats characteristic of *P. velifera* offer few barriers to dispersal, and gene flow between contiguous populations may be high (Schmitter-Soto 1998). In contrast, populations of *P. petenensis* occupy geographically separated river drainages in southern Yucatán (Schmitter-Soto 1998) and may experience lower levels of gene exchange, although rivers do connect through flooding during hurricanes and through underground links (Schmitter-Soto et al. 2002). Thus, by comparing the level of morphological divergence between these species, as well as the traits that vary among populations within each species, the relationships between morphological divergence, habitat differences and geographic separation can be assessed.

Three specific questions are addressed in this study. First, which morphological traits best distinguish *P. petenensis* and *P. velifera* and do these traits vary between males and females of each species? Sexual dimorphism in

traits known to be important in sexual selection, such as the dorsal fin, suggests a potential role of sexual selection, while sexual dimorphism in other traits, such as those relating to livebearing roles between the sexes, may indicate that natural selection or historical constraints are stronger in promoting or maintaining both intra- and interspecific morphological differences.

Second, do populations of each species differ in particular morphological traits and is the degree of interpopulation variation comparable between the two species? If natural selection and sexual selection regimes were similar between the two species, one would predict that morphological divergence would proceed along similar lines of evolutionary diversification. Alternatively, if different morphological traits contribute to interpopulation differences between the two species, this would suggest that variation among populations in female mating preferences and environmental features of different habitats result in differences in the strength and direction of sexual and natural selection, leading to independent evolutionary trajectories of morphological change for each species.

Third, to what degree does geographical separation contribute to observed morphological differentiation among populations of each species? Here one would predict that greater geographic isolation (such as between different river drainages for *P. petenensis*) would lead to greater morphological divergence among populations if gene flow were reduced and more isolated populations differ in selective regimes. To answer these questions, the degree of morphological variation between the two species, between sexes within species, and among populations of each species, was assessed using geometric

morphometrics and linear measurements. Using both geometric and linear measurements allows for more detailed analyses of fish morphology, and for comparisons to be made between the results obtained by these different methods and those from previous studies of morphological divergence in mollies (Kittell et al. 2005; Ptacek 1998; Ptacek and Travis 1998).

METHODS

Fish Collection

Live individuals of both species were collected within their native ranges across five states in Mexico (Fig. 2.1, Table 2.1): Campeche (C), Chiapas (CH), Quintana Roo (QR), Tabasco (T), and Yucatán (Y). The sites were chosen to cover a wide range of locales across the distribution of each species and to include sites from each major drainage (Usumacinta-Grijalva, Laguna de Términos systems, Champotón, and Hondo) for *P. petenensis*. Fish were collected using seine nets (6.1 X 1.2 m), cast nets (1.2 m), and minnow traps. Following collection, fish were either photographed and returned to the site, or shipped live to Clemson University where they were photographed and maintained in 568-L stock tanks for additional study. A total of 336 individuals of *P. velifera* (237 males and 99 females) from 10 populations and 259 individuals of *P. petenensis* (152 males and 107 females) from seven populations were used in morphological analyses (Table 2.1).

TABLE 2.1. Sample sizes and site locations for populations of sailfin mollies used in this study. Dashed lines in cells indicate that females from that population were not photographed.

Species	Population	Sex (n)		Site Coordinates
		M	F	
Poecilia velifera	Campeche 2	21	21	N 19°14.230', W 90°50.110'
	Campeche 4	22	---	N 18°53.274', W 91°23.866'
	Campeche 5	15	---	N 19°34.998', W 90°40.002'
	Quintana Roo 2	39	19	N 20°17.305', W 87°22.549'
	Quintana Roo 3	14	17	N 21°13.910', W 86°44.330'
	Quintana Roo 5	28	---	N 20°17.420', W 87°22.666'
	Yucatán 1	21	20	N 21°15.807', W 89°39.648'
	Yucatán 2	27	22	N 21°21.561', W 89°06.072'
	Yucatán 3	27	---	N 20°51.438', W 90°22.983'
	Yucatán 4	24	---	N 21°34.043', W 88°13.780'
P. petenensis	Campeche 1	18	19	N 19°08.620', W 90°57.400'
	Campeche 2	33	21	N 19°14.230', W 90°50.110'
	Campeche 3	30	13	N 18°55.925', W 91°05.350'
	Chiapas 1	26	14	N 17°48.482', W 91°48.779'
	Quintana Roo 4	16	20	N 18°36.678', W 88°48.713'
	Quintana Roo 6	10	---	N 18°30.337', W 88°49.280'
	Tabasco 3	19	20	N 17°58.000', W 92°31.315'

Landmark-Based Morphometrics

To estimate morphological trait values, euthanized or anesthetized live fish (buffered 0.50% MS-222 in the laboratory, or chilled water in the field) were placed on a dissection mat, with the dorsal and caudal fins spread and the gonopodium (for males) pinned away from the body. An image of the left side of each individual was captured using a digital camera (Sony DSC-F707) at 2560 x 1920 resolution. Live fish were revived and either returned to stock tanks at Clemson University (C1, C2, C3, CH1, QR2, QR3, QR4, T3, Y1, Y2, Y4) or to their original collection sites in the field (C4, C5, QR5, QR6, Y3). Individuals from populations collected in the field, but returned to the lab, were held in captivity for varying amounts of time prior to being photographed, with the possibility that progeny were born in the laboratory. A small number of fish included in the study may have been representatives of these lab-raised progeny, rather than wild-caught individuals. It might be expected that laboratory rearing conditions would change the direction of trait differences among these populations, compared to exclusively wild-caught fish. However, most of the fish included in the analyzed populations were field caught, thus the greatest influence on population-specific morphology would be based on these fish. Moreover, any change in the direction of trait divergence, resulting from the inclusion of lab-reared individuals, would lead to greater variability within populations and, thus, a decrease in the ability to distinguish among them. Yet, in this study, it has been possible to significantly distinguish between populations. In addition, I used regression

analysis to test whether time spent in captivity significantly influenced changes in composite shape variables (generated from discriminant scores). I found no such relationships (linear regression, r = 0.000-0.790, df = 5-10, *P* = 0.949-0.088). Growth under laboratory conditions, therefore, did not appear to eliminate the natural shape differences among populations.

Landmark-based geometric morphometric techniques (Adams et al. 2004; Rohlf and Marcus 1993) were used to analyze body shape differences among fish from different populations, species, and sexes. Unlike conventional linear measurements, these morphometric measurements retain information on spatial covariation among landmarks (Rohlf and Marcus 1993) and the position of each landmark relative to all others. TpsDig software (version 1.37) was used to digitize 13 (females) or 14 (males) landmarks onto each image (Fig. 2.2a). Note that while landmarks are difficult to define on most unfixed points, I was able to use landmark three (maximum extent of the caudal fin directly opposite to the maximum curvature of caudal peduncle) because it could be placed on the outer edge of the caudal fin directly opposite the outer curvature of the caudal peduncle. In addition, the insertion of the anal fin (landmark 14) on females was difficult to visualize on digital pictures, this landmark was not digitized on females and thus was not included in comparisons including females. The landmarks were used to calculate geometric shape variables describing uniform and localized variation in landmark positions (uniform components and partial warps) for statistical analysis using tpsRegr software (version 1.26). TpsRegr rotates, translates, and scales landmark coordinates into alignment through generalized

least squares superimposition (Bookstein 1991). The resulting uniform component and geometric shape variables were used as the shape variables in the statistical analyses.

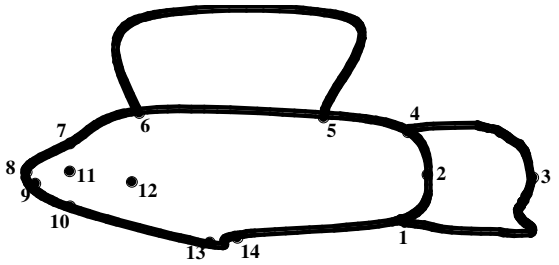
Shape differences described by discriminate function analysis (DFA), while numerically displayed by the uniform component and geometric shape variables, are visually displayed by a deformation grid showing how the shape is changed relative to the consensus (average) fish. The three landmarks (e.g. landmarks 4, 7, and 10) that are most highly correlated with the shape deformation between species or populations are reported here, in addition to the general morphological areas associated with those changes in body shape, as estimated from the deformation diagrams (e.g. body depth).

Linear Morphometrics

Because fin characteristics in male poeciliid fishes are often targets of sexual selection (MacClaren et al. 2004; Basolo and Trainor 2002; Karino and Matsunaga 2002; Rosenthal and Evans 1998; Basolo 1990; Bischoff et al., 1985), a further examination of shape variation in males was made using traditional linear-distance measures. Some fin landmarks in mollies are not fixed points (for example the upper, anterior tip of the dorsal fin is free to rotate about the origin of the first dorsal fin ray, thus the position of this point is variable); therefore, landmarks are difficult to accurately assign to these morphological features or traits (Bookstein 1991). These traits may vary among populations,

however, and provide additional insight into morphological differences. The program NIH Image (version 1.6) was used to measure 16 (*P. velifera*) and 19 (*P. petenensis*) linear characteristics for males of each species (Fig. 2.2b). These measurements included measures of dorsal fin, caudal fin, and body area (left side of fish only, for each measurement), determined by tracing the outline of the body or fins from the digital photograph and using the program's estimate of area. Total lateral projection area of the left side of the fish was determined by adding together dorsal fin, caudal fin, and body areas. Although this method does not correct for body curvature, differences in shape because of curvature are likely similar among male fish and likely are negligible in contributing to differences in relative body area. The additional measures in *P. petenensis* were associated with caudal fin characteristics, as males of this species often possess more elaborate caudal fin shape compared to *P. velifera*. The linear measurement values were transformed into morphological shape values of the form (ln (trait length or area) minus ln (standard length or body area) (Mosimann and James 1979; Ptacek 1998; Ptacek and Travis 1996; Farr et al. 1986) to determine whether intraspecific differences exist in morphological shapes independent of body size.

(a)



(b)

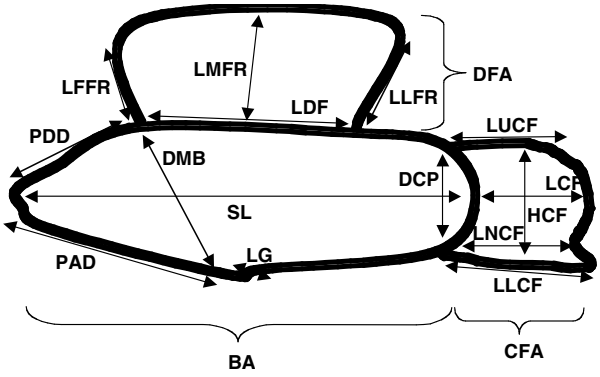


FIG. 2.2. (a) Landmarks used in morphometric analyses. The landmarks represent the following morphological features: (1) insertion point of the ventral most caudal fin ray into the caudal peduncle, (2) insertion point of the caudal fin ray at the maximum curvature of the caudal peduncle, (3) maximum extent of the caudal fin ray directly opposite to the maximum curvature of the caudal peduncle, (4) insertion point of the dorsal most caudal fin ray, (5) posterior insertion point of the dorsal fin, (6) anterior insertion point of the dorsal fin, (7) interorbital margin of body, dorsal to the center of the eye, (8) anterior most point of fish (tip of jaw at the dentary symphysis), (9) origin of the lower jaw, (10) edge of body, ventral from the center of the eye, (11) center of the eye, (12) anterior (dorsal) insertion of pectoral fin, (13) anterior insertion point of the gonopodium, and (14) posterior insertion point of the gonopodium. (b). Linear measurements on male *Poecilia velifera* and *P. petenensis*: PDD- pre-dorsal distance, PAD- pre-anal distance, SL- standard length, LG- gonopodium length, DMB- depth at mid-body measured from the anterior insertion point of the dorsal fin to the anterior insertion point of the gonopodium, LDF- length of dorsal fin, LFFR- length of first fin ray, LMFR- length of middle fin ray, LLFR- length of last fin ray, DCP- depth at caudal peduncle, LCF- length of caudal fin at mid point, HCF- height of caudal fin, BA- body area, DFA- dorsal fin area, CFA- caudal fin area. Fin ray number was also recorded. Linear measurements on *P. petenensis* males only: LUCF-length of the upper part of the caudal fin measured along the dorsal margin, LLCF-length of the lower part of the caudal fin measured along the ventral margin, and LNCF-length to the notch in the caudal fin measured from the insertion point of the ventral most caudal fin ray into the caudal peduncle to most anterior point of the notch in the caudal fin.

Statistical Analysis

Separate canonical discriminant analyses (SYSTAT version 10) were performed using the geometric shape variables generated by TpsRegr and the size-adjusted linear trait measurements. Keeping the geometric and linear analyses separate (e.g. Manier 2004; Valenzuela et al. 2004; Parsons et al. 2003; Larson 2002; Monteiro et al. 2002; Adams and Rohlf 2000) allows for the comparison of results from the two types of analyses and a determination of whether each yields similar morphological features that best separates the species, or populations within each species. Further, this approach allows comparisons to be made with prior studies of the U.S. sailfin molly, *Poecilia latipinna* (LeSueur), where linear measures were used to examine differences between populations and species (Ptacek 1998) and to other studies of variation in fish morphology that have only utilized a single technique (e.g. Neves and Monteiro 2003). Finally, combining the linear and geometric measures into a single analysis would lead to difficulty in interpreting the results, as importance of traits in distinguishing populations would be confounded by the method of measurement.

Discriminant function analysis (DFA) was used to find the combination of either geometric or linear shape variables that best describes differences between groups being compared (either species and sex, or population as the independent variable). This analysis also provides an estimate of the amount of total morphological variation explained by each discriminant axis. Pearson correlation analyses were used to determine the partial warp landmarks and

linear shape variables that most strongly correlate with each discriminant function. The Procrustes coordinates (landmarks) or linear shape variables that most strongly correlate with the discriminant functions were used to estimate which aspects of shape vary along each discriminant axis. Discriminant analyses also provided jackknifed measures of how well individuals could be re-classified back to their actual group, and to which group they were assigned if misclassified. An advantage of thin-plate-spline analysis is the ability to regress superimposed landmark coordinates onto discriminant functions to obtain thin-plate spline diagrams, illustrating body shape differences between the species and sexes and among populations of *P. velifera* and *P. petenensis*. Finally, MANCOVA (geometric, with centroid size as a covariate) and MANOVA (linear) provided an F-score matrix to discern whether populations differed significantly from one another in morphology. All analyses were performed on five different data sets: (1) all individuals, both males and females, both species combined, (2) females of *P. velifera*, all populations combined, (3) males of *P. velifera*, all populations combined, (4) females of *P. petenensis*, all populations combined, and (5) males of *P. petenensis*, all populations combined.

Because within population differences may obscure differences between populations, it is important to determine the overall evolutionary trajectories of the species in morphological space. The angle (θ) between two vectors that represents morphological differences in *P. petenensis* and *P. velifera* males was calculated as follows: if a_1 is the first scaled eigenvector for *P. petenensis* and b_1

is the first scaled eigenvector for *P. velifera* (a_2 is the second scaled eigenvector for *P. petenensis*, etc.), then for *x* eigenvectors,

$$\cos\theta = \frac{a \bullet b}{|a| \times |b|} \text{ (where } a \bullet b = \sum_{x}^{1} ab = a_1b_1 + a_2b_2 + \ldots + a_xb_x,$$

and |a| x |b| = the absolute value (length) of vector "a" multiplied by the absolute value of vector "b") (Hamilton 1989). The angle between these vectors indicates the degree to which these species are morphologically different. Orthogonal, or independent, trajectories of morphological diversification are represented by an angle of 90°.

Mantel tests were used to examine the degree of association between geographic distance and morphological distance from pairwise comparisons of all possible pairs of populations for males of each species separately. To determine morphological distance, Mahalanobis distance was calculated (SAS version 9.0) from discriminant scores (both geometric and linear shape) for both species independently. Mahalanobis distance was then compared to hydrological distance, i.e. a path through wetlands or along water courses between sites, as measured from appropriate hydrological, altitudinal and flood-risk maps (Instituto de Geografía 1990; INEGI 1989). In *P. petenensis* physical barriers may separate some sites, even those that are geographically close. Yearly flooding, however, generally connects these sites (and even rivers within the Yucatán) making movement possible, if not likely (Schmitter-Soto et al. 2002). Significant Mantel correlations would suggest that the morphological distance between

populations is predicted by the geographical distance between them and would provide evidence of a potential role of spatial isolation in contributing to morphological differentiation among populations.

RESULTS

Species Comparisons

As expected, the two species of sailfin mollies show significant morphological shape differences (Table 2.2; discriminant function two (DF2), 18.0% of the total variation in shape among all fish in DFA space, Fig. 2.3). Deeper bodies and differences in caudal peduncle shape (landmarks four, six, and ten; Fig. 2.2a) of both males and females distinguish *P. velifera* from *P. petenensis* (Pearson correlation, r = -0.61 – 0.61, $P < 0.01$ for all; Fig. 2.4). While these landmarks do not fully cover body depth and caudal peduncle shape, they represent the landmarks whose change in position relative to other landmarks captures the most variation between populations. More interestingly, sexes differ along the same discriminant axis (discriminant function one (DF1), 79.0% of the total variation in shape in DFA space, Fig. 2.3) for both species. Anterior body/head shape (differences in the depth indicated by differences in the position of landmarks ten and eleven along the vertical axis, and the relative position of these landmarks to the remaining landmarks; Fig. 2.2a) and position

TABLE 2.2. Morphological differences among populations based on discriminant function analyses (both species and sexes together, *Poecilia velifera* males, *P. velifera* females, *P. petenensis* males, and *P. petenensis* females) and MANCOVA (geometric measures with centroid size as a covariate) or MANOVA (linear measurements). 'Correctly assigned (%)' represents the percent of fish correctly classified back to their native population based on discriminant analysis jackknife results.

Measure	Species	Sex	*d.f.*	*F*	*P*	Correctly Assigned (%)
Geometric	Both	Both	66, 1700	11.95	<0.001	97
	P. velifera	M	216, 1737	10.95	<0.001	86
		F	88, 295	7.88	<0.001	79
	P. petenensis	M	144, 715	6.46	<0.001	75
		F	110, 391	4.05	<0.001	63
Linear	*P. velifera*	M	117, 1680	4.63	<0.001	43
	P. petenensis	M	84, 641	4.63	<0.001	54

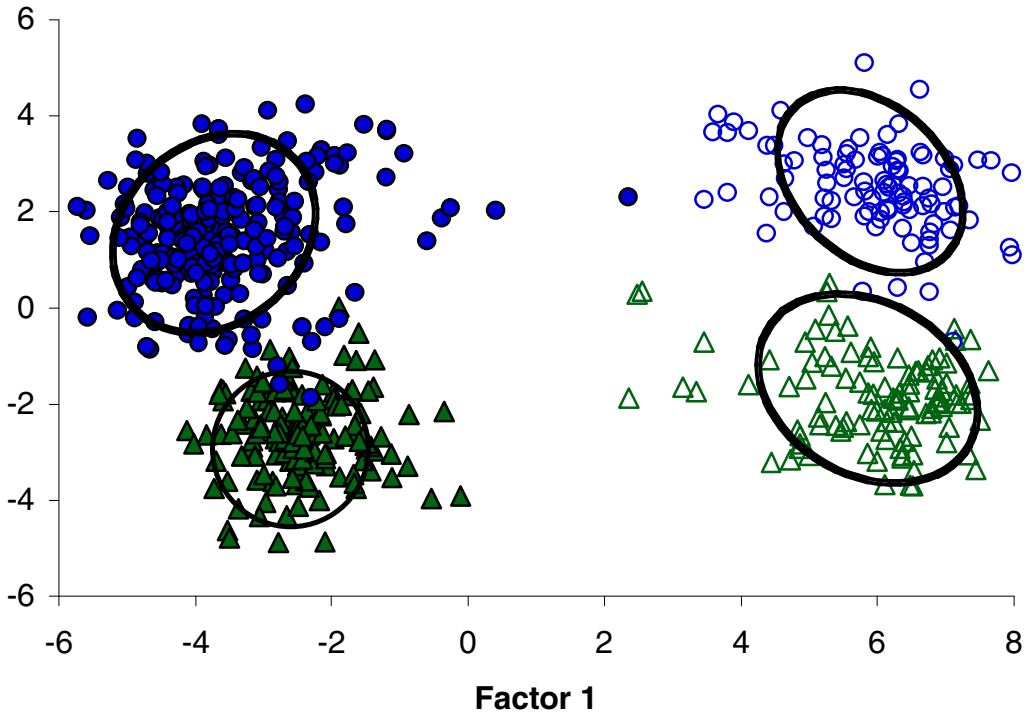


FIG. 2.3. Discriminant scores one and two among *Poecilia velifera* (blue) and *P. petenensis* (green); males (closed symbols) and females (open symbols). Circles represent 95% confidence intervals.

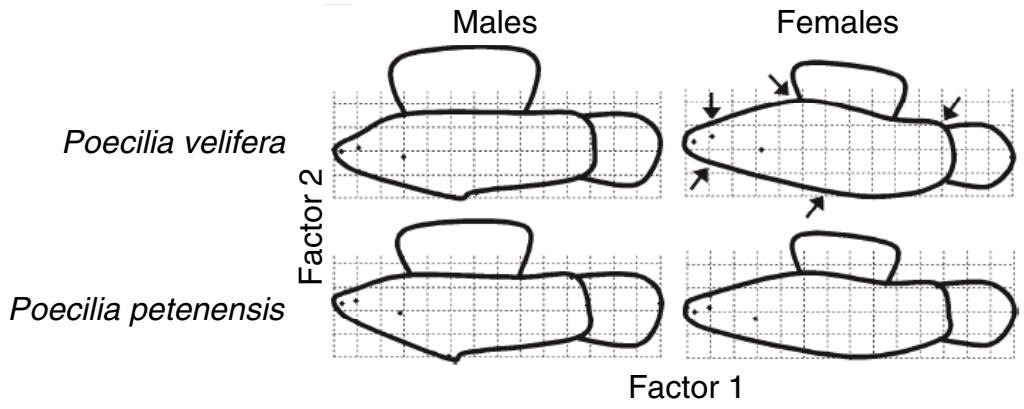


FIG. 2.4. Consensus landmark configurations illustrating morphological differences between species (*Poecilia velifera* and *P. petenensis*) and sexes. Landmarks were used to superimpose body shape onto figures. Arrows point to the landmarks most correlated with difference in sex (10, 11, and 13) and species (4, 6, and 10). Landmarks associated with the origin of the lower jaw (9), eye (11), and pectoral fin (12) are also visible.

of the anal fin (gonopodium, landmark 13; Fig. 2.2a) are the three most important geometric characteristics associated with differences in the sexes (Pearson correlation, r = -0.99 – 0.97, $P < 0.01$ for all), with longer dorsal and caudal fins (landmarks six and three) also important in discriminating males from females in both species (Pearson correlation, r = -0.80 – 0.93, $P < 0.01$ for all). Males of both species have relatively larger heads, more anteriorly positioned anal fins (gonopodium, based on landmark 13 only), and longer dorsal fins than do females (Fig. 2.4). Thus, discriminant function one (DF1) distinguishes the sexes for both species suggesting parallel morphological trait differences.

Population Differences

Discriminant analyses of geometric shape data show significant differences among populations for both males and females of each species, and male populations within each species are also separated by different shape characteristics based on linear measures. In both species, females show less overall variation among populations and are less often correctly classified to their population of origin (Table 2.2). Between the two species, populations of *P. velifera* males are more strongly differentiated (DF1, 41.1% of the total variation in shape among *P. velifera* males in DFA space; DF2, 19.9% of the total variation in shape among *P. velifera* males in DFA space) and individuals are more often correctly classified back to their population of origin than are individuals from different populations of *P. petenensis* (DF1, 29.5% of the total variation in shape

among *P. petenensis* males in DFA space; DF2, 26.9% of the total variation in shape among *P. petenensis* males in DFA space; Fig. 2.5, Table 2.2). In addition, males of both species show more variation in morphological traits that are potential targets of sexual selection (landmarks associated with insertion points of the dorsal fin and gonopodium) compared to females, so further population analyses focused solely on males.

While changes in dorsal fin length (landmark five, Fig. 2.2a) contribute to population separation in DF1 in *P. velifera* (Pearson correlation, r = -0.57, $P < 0.01$), caudal fin shape (landmark two, Fig. 2.2a) is more important in distinguishing males of *P. petenensis* from different populations (Pearson correlation, r = 0.61, $P < 0.01$; Fig. 2.5). Populations of both species differ in the position of the gonopodium (landmark 14, Fig. 2.2a; Pearson correlation, r = -0.61 – 0.42, $P < 0.01$), and anterior body/ head shape (relative position of landmark ten to remaining landmarks, Fig. 2.2a; Pearson correlation, r = -0.37 – 0.60, $P < 0.01$). Overall, changes in body depth (relative vertical positions of landmarks) are important in distinguishing populations of *P. petenensis*, while changes in the relative length of traits (relative horizontal positions of landmarks) tend to distinguish populations of *P. velifera*.

In contrast to results from geometric shape data, discriminant analysis of linear shape variables (Fig. 2.6, Table 2.2) show that males of *P. petenensis* (DF1, 46.2% of the total variation in shape among *P. petenensis* males in DFA space; DF2, 23.9% of the total variation in shape among *P. petenensis* males in

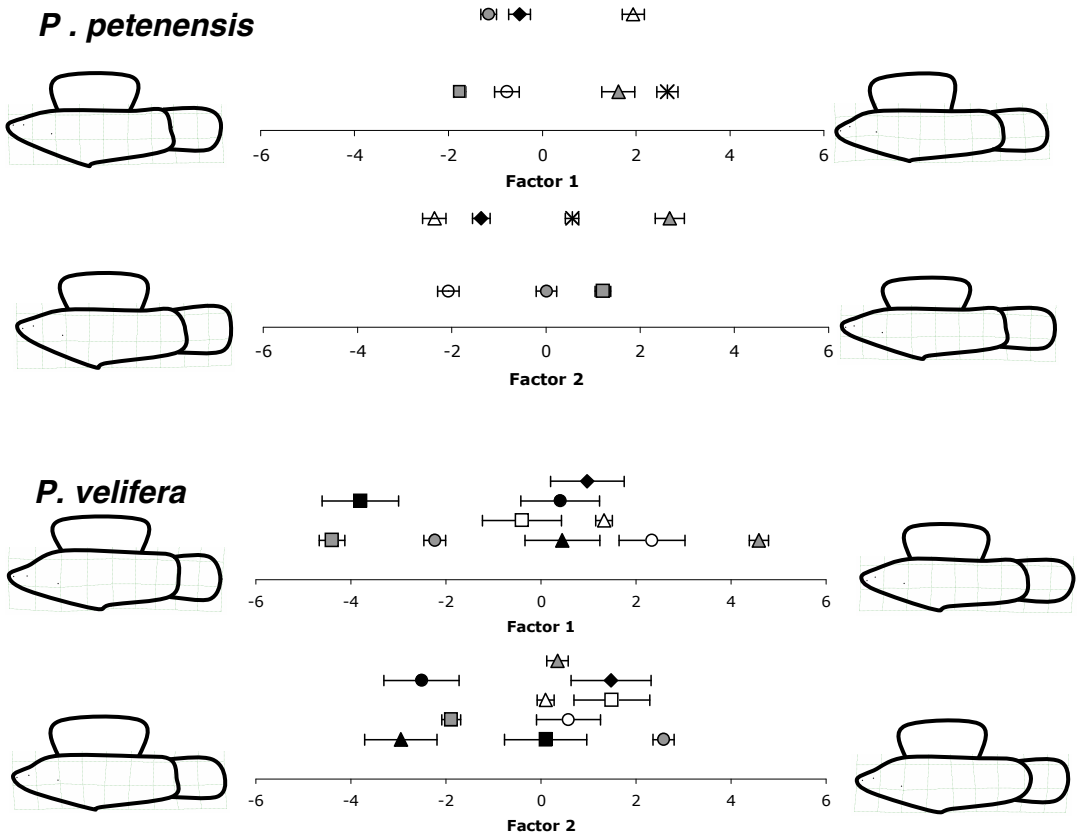


FIG. 2.5. Differences in geometric-based morphology ($\overline{X}$ ± S.E.) across DF1 and DF2 for *Poecilia petenensis* (C1- ▲C2- ●, C3- ■, CP- ◆, QR4- △, QR6- ○, T3- ✳) and *P. velifera* (C2- ▲, C4- ●, C5- ■, QR2- △, QR3- ○, QR5- □, Y1- ▲, Y2- ●, Y3- ■, Y4- ◆). Transformation grids show the differences in morphology across the discriminant function. The fish figures are constructed by drawing curves connecting the landmarks generated from TPSRegr.

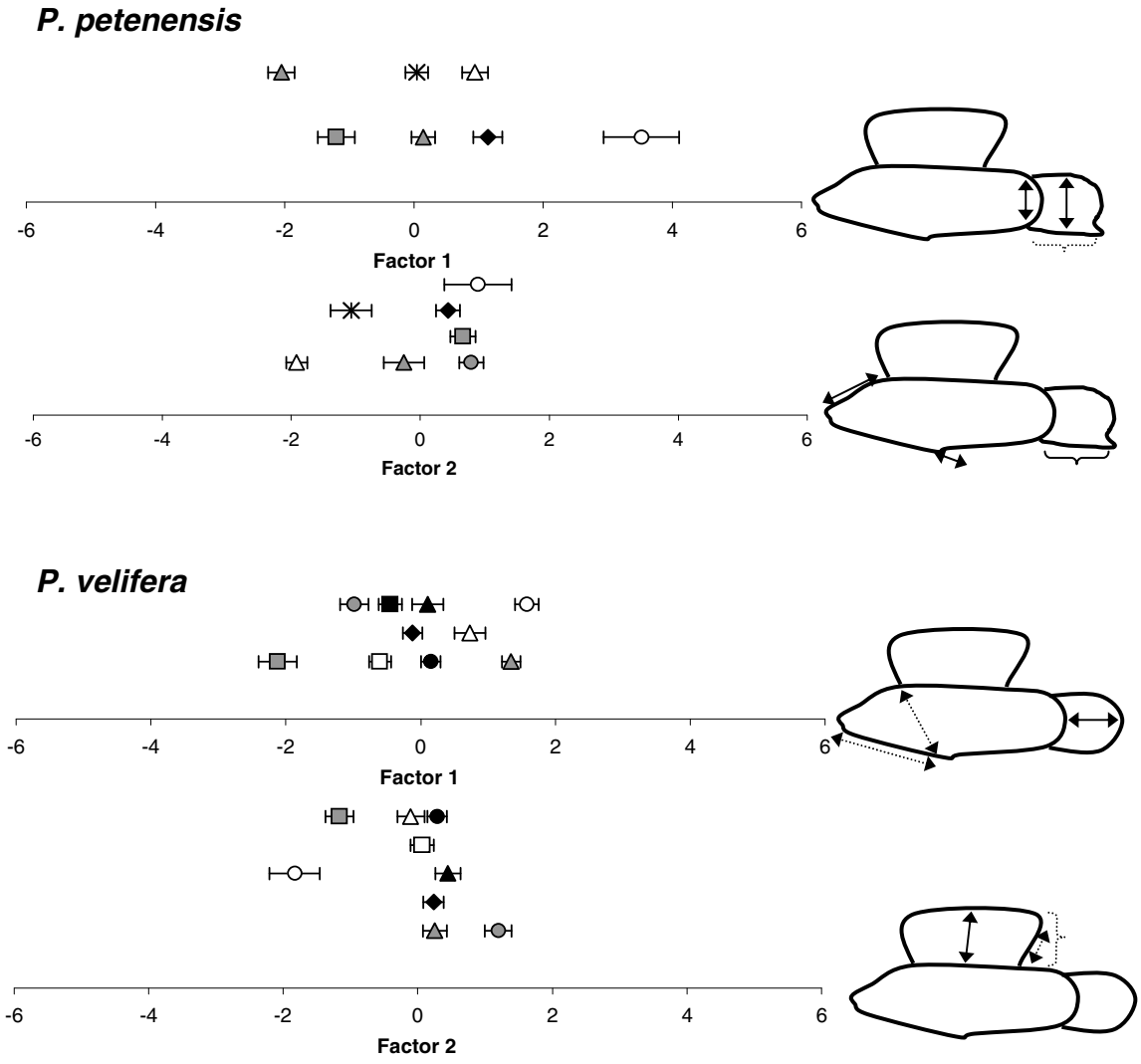


FIG. 2.6. Differences in linear-based morphology ($\bar{X}$ ± S.E.) across DF1 and DF2 for *Poecilia petenensis* (C1- ▲C2- ●, C3- ■, CP- ◆, QR4- △, QR6- ○, T3- ∗) and *P. velifera* (C2- ▲, C4- ●, C5- ■, QR2- △, QR3- ○, QR5- □, Y1- ▲, Y2- ●, Y3- ■, Y4- ◆). Fish diagrams show the three most important linear shape variables in discriminant functions one and two (see text for correlation coefficients and significance levels). Solid lines represent positive correlations and dotted lines signify negative correlations. For example in *P. petenensis*, a higher value for factor one indicates a deeper caudal peduncle and taller caudal fin, but a smaller overall caudal fin area.

DFA space) were more often correctly classified back to their native population than males from different populations of *P. velifera* (DF1, 40.0% of the total variation in shape in deformed DFA space; DF2, 19.6% of the total variation in shape in deformed DFA space). Like geometric measures, however, linear measures reveal significant differences among male populations of each species (with the exception of the Y1 and Y2 populations of *P. velifera*), with different morphological traits best differentiating populations of males of the two species. For example, DF1 primarily differentiates populations of *P. petenensis* based upon height of the caudal fin ($r = -0.725$, $P < 0.01$), depth of the caudal peduncle ($r = -0.507$, $P < 0.01$), and caudal fin area ($r = -0.486$, $P < 0.01$), while DF2 differentiates populations based on gonopodium length ($r = 0.646$, $P < 0.01$), pre-dorsal fin distance ($r = 0.445$, $P < 0.01$), and, again, caudal fin area ($r = 0.565$, $P < 0.01$; Fig. 2.6). In contrast, DF1 in *P. velifera* primarily differentiates populations based upon length of the caudal fin ($r = 0.478$, $P < 0.01$), depth at mid-body ($r = -0.472$, $P < 0.01$), and pre-anal distance ($r = -0.463$, $P < 0.01$), while DF2 differentiates populations based on the length of the middle dorsal fin ray ($r = -0.799$, $P < 0.01$), dorsal fin area ($r = -0.794$, $P < 0.01$), and length of the last dorsal fin ray ($r = -0.768$, $P < 0.01$; Fig. 2.6).

Overall, there is less variation among populations of either species based on linear measurements compared to geometric traits; therefore, populations are better distinguished using geometric shape. Linear measures, however, can be used to identify some important morphological characteristics that vary among populations, such as dorsal fin characteristics in *P. velifera* and caudal fin

characteristics in *P. petenensis*, that were not easily obtainable using only geometric analyses.

Quantitative support for these qualitative patterns, suggesting a different evolutionary trajectory of divergence among populations within each species, was obtained by determining the morphological evolutionary vector of population divergence for each species. The species were found to differ in their evolutionary trajectories by an angle of 98.5° (Fig. 2.7). Ninety degrees represents orthogonal (independent) vectors, so the morphological vectors of population divergence between these two species were nearly independent.

Role of Spatial Isolation

There was no evidence for a strong role of geographic isolation in contributing to population differences in morphology of males of either species. Mantel tests show no correlation between geographical distance between populations and Mahalanobis distance, based on either geometric or linear measures for either *P. velifera* or *P. petenensis* (Mantel tests, $P = 0.06$-0.50). Thus, individuals misclassified in the discriminant analyses are not more likely to be classified to a neighboring population than to a geographically distant population. For example, males of *P. velifera* from a Quintana Roo population (QR5) were not misclassified to neighboring Quintana Roo populations (QR2 or QR3), but rather, to populations in Yucatán (Y2, Y4).

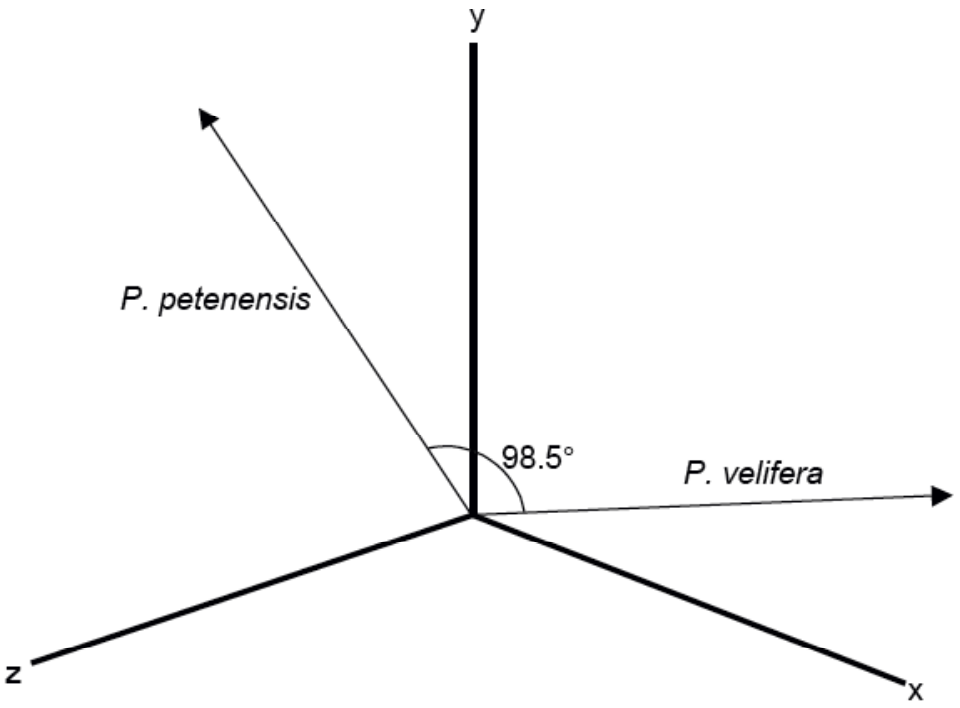


FIG. 2.7. Evolutionary vectors of the angles of morphological evolution in *Poecilia petenensis* and *P. velifera*. Axes X, Y, and Z represent the first three canonical axes based on partial warps in n-dimensional morphometric space. The angle between the vectors is 98.5°, representing nearly independent evolutionary trajectories.

DISCUSSION

Species Comparisons

Poecilia velifera shows similar morphological differences between the sexes compared to males and females of *P. petenensis*. Such a parallel pattern in sexual dimorphism between the species suggests that sexual selection, in addition to developmental differences historically present in livebearing fishes, may play a role in certain morphological differences between the sexes in both species, including the relative position of the dorsal fin and anal fin (gonopodium in males), the length of the dorsal fin, and the shape of the fish anterior to the anal fin as it relates to brooding in females. Previous studies in the sailfin molly, *P. latipinna*, have shown that differences in dorsal fin size are important in female choice, especially as they relate to increasing overall lateral projection area (Ptacek 2005; MacClaren et al. 2004; Ptacek and Travis 1997). Thus, the increased length of the dorsal fin in males of both *P. velifera* and *P. petenensis* may be the result of strong female mating preferences for larger males in both species. Phylogenetic constraints cannot be entirely ruled out as a factor influencing the evolution of dorsal fin dimorphism in *P. velifera* and *P. petenensis*, which are both part of a monophyletic sailfin clade (Ptacek and Breden 1998). These species are not sister taxa, however, and the closest relative to *P. petenensis*, the Tamesí molly, *Poecilia latipunctata* Meek is not sexually dimorphic in dorsal fin characteristics, indicating that sexual dimorphism in this

trait is not under strong phyletic constraints (Ptacek et al. 2005). The difference in the position of the anal fin (gonopodium) between males and females would also be important in males, as it is used to transfer sperm to females during mating and for this reason differs developmentally from the anal fin in females (Rosa-Molinar et al. 1998; Rosa-Molinar et al. 1994). Although late-term females were excluded from the study, the differences between males and females in the ventral shape of the body anterior to the anal fin is likely caused by internal retention of embryos by females (Ghalambor et al. 2003; Neves and Monteiro 2003).

Differences between the two species may reflect differences in the natural selection pressures of their respective environments. Flow regime and vegetative characteristics have been shown to influence a variety of body characteristics including the shape of the caudal region, dorsal fin position, and body depth (Langerhans and DeWitt 2004; Langerhans et al. 2003). For example, fishes that inhabit faster flowing stream environments generally have shallower bodies (less deep, top to bottom) than fishes in lentic environments (Endler 1995; Wood and Bain 1995; Webb 1984; Webb 1982), a characteristic that distinguishes *P. petenensis* (which are generally found in higher flow environments) from *P. velifera*. In addition, piscivorous fish predators have also been shown to influence body and caudal peduncle shape, selecting for morphologies that correlate with better escape performance (Langerhans and DeWitt 2004; Ghalambor et al. 2003; Walker 1997; Poleo et al. 1995). For example, populations of western mosquitofish (*Gambusia affinis* (Baird & Girard))

under high predation have larger caudal peduncles and more elongate bodies compared to populations without predators (Langerhans et al. 2004). Again, a larger caudal peduncle and narrower, more streamlined body also characterizes *P. petenensis*, which primarily are exposed to cichlid predators, compared to *P. velifera*, which face more diverse predator assemblages. The different habitat and predation influences on *P. velifera* and *P. petenensis* may, therefore, be responsible for some of the divergence in morphology between these species.

Population Differences

Evolutionary trajectories of morphological divergence within each species, as examined by vector analyses, indicate that the two species are nearly orthogonal, or independent from one another, in morphological space. Such a dramatic difference in the vectors of interpopulation shape differentiation suggests that the relative roles of natural selection and sexual selection in promoting population divergence in male morphology vary substantially between them.

Differences in certain traits that best separate populations of each species may reflect the different roles of natural and sexual selection in promoting population divergence within each species. Anterior body/head shape (as determined by changes in the relative positions of landmarks 10 and 11) and, in particular, body depth in the ventral region anterior to the gonopodium, vary among populations in both species (Fig. 2.5); however, only populations of *P.*

velifera differ in dorsal fin characteristics (both in geometric and linear analyses). Population divergence in dorsal fin characteristics is more likely because of sexual selection, where females consistently prefer males with larger fins compared to males with smaller fins (MacClaren et al. 2004; Karino and Matsunaga 2002; Ptacek and Travis 1997). In addition, previous studies on other fishes have shown a strong role for natural selection acting on the caudal fin, but not on dorsal fin shape. Webb (Webb 1978), for example, found that complete amputation of the dorsal fins of rainbow trout (*Oncorhynchus mykiss* (Walbaum)) did not result in a decline in fast-start performance. Preliminary studies of fast-start performance in males of *P. velifera* found no difference in either velocity (linear or angular) or acceleration (linear or angular) of fast-starts when compared to these same measures in males of a shortfin molly species, which lack enlarged dorsal fins (*Poecilia orri* Fowler) (M. B. Ptacek & R. W. Blob unpubl. data). Thus, there does not appear to be a large cost (or benefit), at least in escape swimming performance, of the enlarged dorsal fin, suggesting that variation in this trait may be primarily because of the influence of sexual selection. Enlarged dorsal fins may incur a natural selection cost in other types of swimming performance, however, such as endurance swimming in fast-flow environments.

The length of the gonopodium is only important in differentiating among populations of *P. petenensis*. Previous studies have shown that gonopodium length varies among populations in other poeciliid species as well (Langerhans unpublished data; Jennions and Kelly 2002; Kelly et al. 2000). In addition, in two

species of *Gambusia* (*G. affinis* and *Gambusia hubbsi* Breder) males exhibited longer gonopodia in predator-free environments and females of both species preferred males with longer gonopodia (Langerhans et al. 2005). Thus, both natural selection and sexual selection may contribute to population differentiation in gonopodium length in *P. petenensis*.

Caudal fin length is important in separating male populations of *P. petenensis*, but not *P. velifera*, based on both geometric and linear measures. The size and position of the caudal fin and caudal peduncle affect thrust generation and maneuverability in other species of fishes and thus, may vary among different habitats depending upon intensity of predation or water velocity at different sites (Langerhans et al. 2004). For example, varying predation rates on poeciliid fishes such as *G. affinis* and *Poecilia reticulata* Peters have led to population divergence in some of the same morphological traits, such as body depth, caudal fin characteristics, and head shape that separate populations of *P. petenensis* (Langerhans and DeWitt 2004; Endler 1995). Similarly, variation in predation pressure across habitats of varying sizes may also be important in the morphological differences observed here. The lake and river habitats of *P. petenensis* are more spatially and temporally variable than the habitats of *P. velifera* in terms of water velocity: most streams in southern Yucatán are intermittent, turning into a series of isolated ponds during the dry season (García-Gil et al. 2002; Schmitter-Soto 1998; INEGI 1989). Moreover, while *P. velifera* may encounter a wider diversity of predators in salt marsh habitats, it is likely that cichlid abundance, and hence predation intensity, is higher in larger water bodies

(larger rivers and lakes) and hence exerts a stronger selection pressure on some populations of *P. petenensis* (as similarly observed in *Salaria fluviatilis* (Asso) (Neat et al. 2003). Indeed, cichlid piscivores like *Petenia splendida* Günther and *Parachromis friedrichsthalii* (Heckel) attain larger sizes in larger water bodies (Martínez-Palacios and Ross 1994) and these larger predators have the potential to feed on all size classes of mollies, even large males. The rivers in Tabasco and Campeche from which fish were collected were nearer the coast and larger compared to the Chiapas and Quintana Roo interior locales. Population divergence in shape based on geometric morphometrics for *P. petenensis* shows that males from the Tabasco and Campeche populations generally have longer caudal fins and narrower bodies compared to males from the smaller rivers in Quintana Roo and Chiapas (Fig. 2.5, Factor 2). These changes parallel those observed in guppies (as reviewed in Endler 1995) and *Gambusia* (Langerhans et al. 2004).

Role of Spatial Isolation

While males of both *P. velifera* and *P. petenensis* vary in morphology among populations, current results show that geographical separation does not predictably explain the morphological patterns of divergence in either species. This result may be explained by the high degree of connectivity between populations. For example, many of the salt marsh habitats where populations of *P. velifera* are found are relatively contiguous; this pattern is similar to that seen

in *P. latipinna*, which occupy salt marsh habitats along the coastal region of the southeastern United States. Males of *P. latipinna* populations are morphologically differentiated despite a high degree of gene flow among them, suggesting a strong influence of natural and sexual selection in promoting these morphological differences (Ptacek 2005; Trexler 1988). In addition to the continuous nature of coastal salt marsh habitats, rivers in the Yucatán connect through flooding during heavy rains and hurricanes, and as well as through karstic tunnels (Schmitter-Soto et al. 2002), however, no sailfins are present in the ancient cenotes of Yucatán, indicating that sailfins do not disperse underground (J. J. Schmitter-Soto, pers. comm. 2002). For example, the shortest distance between the uppermost tributaries of the Río Hondo (Caribbean versant) and Río Candelaria (Laguna de Términos system) is about 12 km, with no ridges in between, but rather a low zone subject to flooding. The Río Champotón itself is continuous only as far as about 47 km from its mouth; farther inland it becomes a series of *aguadas* (surface water pools), however, seasonally these become connected through *bajos* or valleys between the typical cone-shaped hills of the Río Bec geographic district (Wilson 1980) facilitating a high degree of movement between populations within this drainage.

In conclusion, while this study provides predictions of particular morphological traits that may be important targets of natural and sexual selection in these sailfin molly species, additional work is needed to determine the relative importance of these evolutionary forces in shaping intra- and interspecific differences in both species. Previous studies have shown interpopulation

variation in male courtship behaviors and female mating preferences in the closely related sailfin species, *P. latipinna* (Ptacek and Travis 1997; Ptacek and Travis 1996). Future studies should focus on the role of divergent female mating preferences among populations of *P. velifera* and *P. petenensis* to test for a similar contribution to population divergence. In addition, much more information is needed with respect to the ecological differences that exist among populations of these two species and the role of ecological selection in promoting morphological divergence. The morphological patterns uncovered in this study provide *a priori* predictions regarding how sites may be expected to differ in water velocity or predator regimes; future studies should focus on whether or not such predictions are supported. Finally, estimating the levels of gene flow among populations of each species, and comparing the degree of neutral genetic divergence to that seen in morphology would provide additional evidence to better understand the relative strength of selective forces versus genetic drift in shaping the observed levels of population differentiation in morphological traits.

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CHAPTER 3

BEHAVIORAL DIVERGENCE IN THE MEXICAN SAILFIN MOLLIES

Abstract.— I characterized the mating behavior profiles both within and between *Poecilia velifera* and *P. petenensis* in order to better understand mating signal evolution in the sailfin molly lineage. In addition, I examined whether differences between these species in the size range of mature males and the strength of allometry between dorsal fin size and body length could explain the variation observed in their expression of different mating behaviors. I determined each male's mating behavior profile by observing the behavior of a single male in the presence of a receptive female. I found that *P. velifera* showed evidence of an alternative male mating strategy, with small males generally performing only gonopodial thrusts (forced insemination attempts) towards receptive females, while large males performed courtship displays as well as gonopodial thrusts. Males of *P. petenensis* performed similar rates of courtship displays and gonopodial thrusts regardless of body length. Little variation existed between different populations of *P. velifera* in mating behaviors, while males from different populations of *P. petenensis* showed population-specific average rates of each mating behavior. Variation among individuals in the mating repertoire of *P. velifera*, but not *P. petenensis*, suggests that the greater range of variation in male size at maturity, as well as considerably stronger allometry between dorsal

fin size and body length, may explain why males of *P. velifera* show the greatest degree of expression of alternative male mating behaviors when compared to other sailfin species.

INTRODUCTION

A common theme in the evolution of male mating behaviors is the occurrence of polymorphism in behavioral phenotypes. Numerous examples of alternative male phenotypes have been described and occur throughout a range of taxa (Taborsky 1994; Gross 1996; Brockmann 2001; Lee 2005). Fixed variation in male mating behaviors can arise as a consequence of genetic polymorphisms for alternative mating behaviors, and may be maintained by frequency dependant selection when these alternative strategies have equal fitness at equilibrium frequencies (Maynard Smith 1982; Ryan et al. 1992; Gross 1996). Alternatively, environmentally based behavioral variation may be frequency or status dependant, and male behavior may depend on such conditions as social environment, nutritional state, or maternal effects (e.g. Travis & Woodward 1989; Andersson 1994; Scheuber et al. 2004; Hedrick 2005; Kodric-Brown & Nicoletto 2005; Leary et al. 2006).

Polymorphism in mating behavioral phenotypes is often associated with dimorphic morphological variation where one phenotype exhibits exaggerated morphological features (e.g. larger body size, elongated fins or plumes, brighter coloration) while the other phenotype shows reduced forms of these morphological traits, and may even mimic female or juvenile phenotypes (e.g. marine isopods: Shuster & Wade 1991; fishes: Warner 1984; Gross 1982, 1985, 1991a, b; ruff: Lank & Smith 1987). Such dimorphism in male morphology is often correlated with expression of the alternative behavioral tactics of

courtship/territorial defense versus cuckoldry or satellite male behavior (sneaker male strategy; Gross 1996; Moczek & Emlen 2000). While a number of studies have examined the environmental factors that can influence the expression of alternative male mating behaviors (Gross 1996; Brockmann 2001; Lee 2005), fewer have addressed the relationship between behavioral polymorphisms and morphological polymorphisms. Does morphological variation actually predict the level of behavioral polymorphism in a population, or are behavioral tactics independent of the range of morphological variation that exists within and between species?

The poeciliid fishes commonly known as sailfin mollies (*Poecilia velifera*, *P. petenensis*, *P. latipinna*, and *P. latipunctata*) provide a unique system in which to explore how variation in male morphology and male mating behaviors are related. Variation among males in the expression of certain mating behaviors is both environmentally dependent (e.g. influenced by relative size and social conditions such as operational sex ratio) and correlated with an underlying genetic polymorphism for male size at maturity (Farr et al. 1986; Travis 1989, 1994a; Ptacek & Travis 1996). Males of all sizes in two species of sailfin mollies, *P. latipinna* and *P. latipunctata*, have been found to perform a similar suite of mating behaviors, however, variation among individual males exists in the degree to which social interactions and male size influence the expression of these behaviors (Travis & Woodward 1989; Ptacek & Travis 1996; Ptacek et al. 2005).

My objectives in this study were (1) to characterize and compare mating behaviors both within and between two species of sailfin mollies, *P. velifera* and

P. petenensis, and (2) to determine whether differences in morphology within and between these species predict the degree of behavioral polymorphism that exists for each species. These two sailfin species are ideal for making comparisons of this type for several reasons. First, quantifying the mating behavior repertoire of *P. velifera* and *P. petenensis* allows for a comparison with the other sailfin species, *P. latipinna* (Farr et al. 1986; Ptacek & Travis 1996) and *P. latipunctata* (Ptacek et al. 2005). Second, *P. velifera* and *P. petenensis* vary in several morphological characteristics that could potentially influence their expression of behavioral polymorphisms, including differences in the range of male sizes at maturity and the size and shape of their exaggerated dorsal fin, i.e. sailfin, based on differences in the level of positive allometry between male standard length (SL) (tip of the snout to the end of the last vertebra) at maturity and dorsal fin size (Miller 1983; Schmitter-Soto 1998; Hankison et al. 2006; Chapter 2). I hypothesized that the degree of differentiation in male mating behaviors would correlate positively with morphological variation. Therefore, mating behavior variation would be greatest in *P. velifera*, the species with the widest range of variation in male size at maturity and the strongest allometry between dorsal fin area and male SL.

METHODS

Mating Behaviors and Associated Morphological Traits

Three characteristic mating behaviors are performed by males of all four sailfin molly species: courtship displays, gonopodial thrusts and gonoporal nibbles (Parzefall 1969, 1989; Farr et al. 1986; Ptacek & Travis 1996; Niemeitz et al. 2002; Ptacek et al. 2005). A courtship display, a behavior used by males to elicit cooperation from females during internal fertilization, occurs when a male orients in front of or alongside of a female and erects the dorsal fin, often accompanied by a sigmoid curving of the body and tilting towards the female (Parzefall 1969, 1989; Farr et al. 1986; Ptacek & Travis 1996; Niemeitz et al. 2002; Ptacek et al. 2005). In three of the four sailfin molly species (*P. latipinna, P. velifera and P. petenensis*), the dorsal fin (i.e. sailfin) in males is greatly enlarged, which accentuates the courtship display, potentially making it more visible to females (Regan 1913; Hubbs 1933; Parzefall 1969; MacClaren et al. 2004). A second mating behavior, gonopodial thrusting, is a type of forced insemination attempt, where the male orients himself behind a female, brings the gonopodium (fused anal fin that serves as an intromittent organ for internal fertilization) to a forward position, and swimming forward, attempts to insert the tip into the female's gonopore for sperm transfer. A third mating behavior, gonoporal nibbling, occurs when males make nasal or oral contact with the female's gonopore. The function of this behavior is unclear, however it appears

to aid a male in determining a female's reproductive status (Farr & Travis 1986; Sumner et al. 1994).

Males of all sailfin molly species vary in the range of male sizes at maturity (Farr et al. 1986; Ptacek & Travis 1996; Ptacek et al. 2005; Hankison et al. 2006). Size at maturity for males is a fixed phenotype in mollies; once the anal fin has fused to form the gonopodium, little to no further growth in body length occurs. A pattern of Y-linkage is known to influence the inheritance of male size at maturity in at least one species of sailfin molly, *P. latipinna,* (Travis 1994b) and in other poeciliid fishes such as the swordtails *Xiphophorus nigrensis* and *X. multilineatus*, (Kallman 1984, 1989; Zimmerer & Kallman 1989). In several species of *Xiphophorus*, size at maturity is controlled by a Y-linked multiple-allelic series (up to six different size at maturity alleles) at the *P* (pituitary) locus, which controls the onset of sexual maturity (Kallman 1984, 1989). Males with small body-size *P* alleles mature much sooner (weeks to months) than males with large body-size *P* alleles. A similar pattern of male size at maturity is exhibited by males of sailfin molly species and for one species (*P. latipinna*) the slope of the relationship between a sire's size at maturity and that of his sons is nearly 1.0 (Travis 1994b).

Male size at maturity is phenotypically correlated with the degree of exaggeration of the sailfin; larger males have relatively larger sailfins (Ptacek 2002; Hankison et al. 2006; Chapter 2). In addition male size can influence the relative rates of two of the three male mating behaviors. In *P. latipinna*, for example, while males of all sizes perform all three mating behaviors, there is a

tendency for larger males to perform higher rates of courtship displays, while smaller males perform higher rates of gonopodial thrusts (Farr et al. 1986; Ptacek & Travis 1996). In *Xiphophorus*, such mating behavior polymorphisms have been shown to be under the influence of additional Y-chromosome loci, linked with alleles at the *P* locus, leading to the expression of alternative mating behaviors (courters versus sneakers) in *X. nigrensis* (Zimmerer & Kallman 1989). A similar pattern of Y-linked inheritance for courtship displays has been demonstrated in two species of sailfin mollies (*P. velifera*: Parzefall 1989; *P. latipinna*: Ptacek 2002). These results imply an underlying genetic polymorphism, which may contribute to the expression of alternative mating behaviors observed in male sailfin mollies.

Experimental Animals

Fish used in this study (Table 3.1) were wild caught with the exception of *P. velifera* from the Tulum site in the state of Quintana Roo (PvQRT), which has been maintained in the laboratory since 1993 (the wild population at this collection site has been extirpated). Single populations of approximately 150 adults were kept in mixed sex, 150-gallon Rubbermaid stock tanks with a recirculating filtration system. Stock tanks were maintained at 6ppt seawater, 25-28°C in a research greenhouse, and thus, were exposed to natural lighting conditions. Prior to behavior testing, individual sexually mature females or male/female pairs were acclimated to 19 l glass aquaria at 6ppt seawater, at

approximately 28° C for at least one week. Light was provided by Sylvania Gro-lux fluorescent bulbs (20-W, full spectrum 350–750 nm, with spectral peaks at 400, 440 and 540 nm; Danvers, Massachusetts, USA.) and kept at a controlled 14 : 10 h light : dark cycle, which is similar to summer light conditions in natural habitats. All fish were fed a mixture of freshwater (60%), brine shrimp (38%), and spirolina (2%) flakes (Ocean Star International, Burlingame, CA, USA) once per day. Following use in the experiments, fish were returned to greenhouse stock tanks.

Male Behavior Trials

To record male sexual behaviors, I observed single male-female pairs in direct contact (free swimming) mating trials. Males were generally within 10 mm SL of females (*P. velifera*: 11.5 ± 0.9; *P. petenensis*: 10.8 ± 1.2). To standardize female receptivity, I used receptive females (<48 h postpartum; e.g. Farr & Travis 1986) as stimuli. I also isolated males 24 h prior to tests to standardize male sexual responses. This protocol produces species-typical behavior rates in the closely related sailfin species *P. latipinna* (Ptacek & Travis 1996). Fish were tested in 19 l aquaria that were covered on three sides with black paper and on the fourth side with one-way film (Gila brand privacy window film, model PRS361, Martinsville, VA, USA) to minimize disturbance from the observer during the trial.

TABLE 3.1. Sampling locales and sample sizes for behavior trials.

Population	Location	*n*	Size range of tested males (mm)
P. petenensis			
Campeche #1	N 19°08.620', W 90°57.400'	15	41 – 74
Campeche #2	N 19°14.230', W 90°50.110'	19	52 – 92
Quintana Roo #4	N 18°36.678', W 88°48.713'	13	44 – 90
Tabasco #3	N 17°58.000', W 92°31.315'	2	54 – 64
P. velifera			
Campeche #2	N 19°14.230', W 90°50.110'	19	20 – 80
Quintana Roo #2	N 20°17.305', W 87°22.549'	21	25 – 71
Quintana Roo Tulum	Laboratory Population	15	38 – 70
Yucatan #1	N 21°15.807', W 89°39.648'	15	42 – 75
Yucatan #2	N 21°21.561', W 89°06.072'	15	41 – 75

The male fish was acclimated in the test tank for 15 min, followed by the introduction of the receptive female for an additional 15 min acclimation. I observed male sexual behaviors for a 10 min trial and recorded behaviors using a Tandy (model 102) event recorder.

I recorded the following behaviors (described above): number of courtship displays, gonoporal nibbles, and gonopodial thrusts, and courtship display duration in seconds. The start of the courtship display was recorded when the male's dorsal fin was completely erect, and the display ended when the dorsal fin was lowered. To standardize observations across all trials, a single observer (SJH) recorded all observations.

I tested a total of 85 males from five populations of *P. velifera*: Campeche #2, Quintana Roo #2, Quintana Roo Tulum, Yucatan 1, and Yucatan 2 (Table 3.1). For *P. petenensis*, a total of 49 males from four populations (Campeche #1, Campeche #2, Quintana Roo #4 and Tabasco #3) were included as part of the species comparisons, however the Tabasco #3 population was excluded from population comparisons because of small sample size (n = 2, Table 3.1). Populations were compared within each species, then combined to compare between the species. Although populations may differ in trait values or variance, this method allows the species to be compared, and the determination of whether difference between samples (between species), despite potentially high levels of variation, is greater than within samples (between populations).

Morphology and Allometry

To estimate and compare allometry between the sailfin species, I measured standard length and dorsal fin areas of males. Photographs of euthanized or anesthetized live fish (buffered 0.50% MS-222 in the laboratory, or chilled water in the field) were captured using a digital camera (Sony DSC-F707) at 2560 x 1920 resolution. Live fish were revived and either returned to stock tanks at Clemson University or to their original collection sites in the field if individuals were not collected for return to the laboratory. I used the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) (version 1.6) to measure standard length and dorsal fin area for *P. velifera* and *P. petenensis* (left side of fish only). For standard length, I measured the straight-line distance from the tip of the snout to the end of the last vertebra (base of the caudal fin) (Trautman 1981) of mature males from each species and population within each species. The main distinguishing characteristic of the smallest males compared to juvenile fish or small females was the presence of a fully fused gonopodium, indicating that these small males were mature (Constantz 1989). I determined dorsal fin area for *P. velifera* and *P. petenensis* by tracing the outline of the fully-extended fin from the digital photograph and using the NIH Image estimate of area (males fully erect their fins during courtship displays). Morphological measurements were made on all fish in the collection (not only those used in behavior testing) to encompass the full range of sizes present in each population.

The morphology of these fish was then compared to previously recorded measurements of the same morphological characters in *P. latipinna* (M. B. Ptacek & J. Travis, unpublished data) and *P. latipunctata* (Ptacek et al. 2005).

Data Analyses

To ensure that only sexually motivated fish were included in behavior trial analyses, I excluded trials where there were fewer than five thrusts and/or seconds of display. I square root transformed all count data to correct for normality. A key difference in behavior between *P. petenensis* and *P. velifera* was the presence of two distinct behavioral classes of *P. velifera* males; males that performed courtship displays and those that did not. Thus, for *P. velifera*, I used logistic regression to determine the point of inflection of the logistic regression line, based on standard length, between males that did and did not display. This inflection point was found to be between males that were ≥45 mm (generally performed courtship displays; hereafter referred to as large) and males with a standard length <45mm (generally did not display; hereafter referred to as small). Based on this behavioral difference, I analyzed mating behavior of large and small males of *P. velifera* separately. I used ANOVA to test for differences in behavior rates both within species and between *P. petenensis* and both large and small *P. velifera*, and Fisher's least-square difference of means tests for post-hoc comparisons. For population comparisons of display rate and times, I included only large *P. velifera*. Pearson correlations were used to look for

relationships between behaviors, and between body size and behaviors. With the exception of the logistic regression, for which I used a web-based logistic regression program (Pezzullo, version 05.07.20), all analyses were done using the program Systat (version 10, 2000).

I used model II reduced major axis (RMA) regression to estimate the allometric relationship between standard length and dorsal fin area (ln-transformed). RMA is the most appropriate analysis to compare the relationship between variables when both variables are subject to error (McArdle 1988; LaBarbera 1989; Blob 2000). The slopes of the RMA regressions equal typical least-squares linear regression slopes divided by *r*, the correlation coefficient. I compared body-dorsal fin allometry between the sailfin mollies by determining whether (asymmetric) 95% confidence intervals (calculated using custom computer routines by N. Espinoza and M. LaBarbera) around the slopes of the regression lines overlapped (Blob 2000).

RESULTS

Behavior: Species Comparisons

The most striking difference between the two Mexican sailfin species was the presence of both displaying and non-displaying males in *P. velifera*, but not in *P. petenensis* (Fig. 3.1). Among males of *P. velifera* smaller than 45 mm (our cut-off based on logistic regression analysis), only 6 of the twenty performed any

courtship displays. Males smaller than 40 mm appeared to show little or no sexual dimorphism of the dorsal fin or coloration often observed in larger males and only 2 out of 14 of these males performed any displays. I found differences between *P. velifera* (large and small) and *P. petenensis* in number of displays (Table 3.2, Fig. 3.1) and in the total time spent displaying during trials (large *P. velifera*: 18.7 ± 1.0, small *P. velifera*: 6.6 ± 5.1, *P. petenensis*: 30.0 ± 4.9). The average display duration (total amount of time spent displaying divided by the number of displays) of large *P. velifera* and *P. petenensis* did not differ, however both performed longer displays compared to small *P. velifera* (*P. velifera* large: 1.5 ± 0.2, *P. velifera* small: 0.4 ± 0.2, *P. petenensis*: 1.4 ± 0.1; Table 3.2). In contrast, males of all sizes of *P. petenensis* performed courtship displays at similar rates and retained the sexually dimorphic dorsal fin morphology and male coloration observed in larger males.

Despite the marked lack of courtship displays in small males, the rate of gonopodial thrusting towards females in males <45 mm was the same as in larger *P. velifera* (Fisher's LSD post-hoc = 0.65) and higher than that seen in *P. petenensis* (Fisher's LSD post-hoc = 0.02; Table 3.2, Fig. 3.1). In addition, I found differences between *P. velifera* (large and small) and *P. petenensis* in the number of gonoporal nibbles (Table 3.2, Fig. 3.1).

There were no relationships between the rate of displays, thrusts, or nibbles and male S.L. in *P. petenensis*, large *P. velifera* or small *P. velifera* (Table 3.2). However, there were significant positive correlations between rates

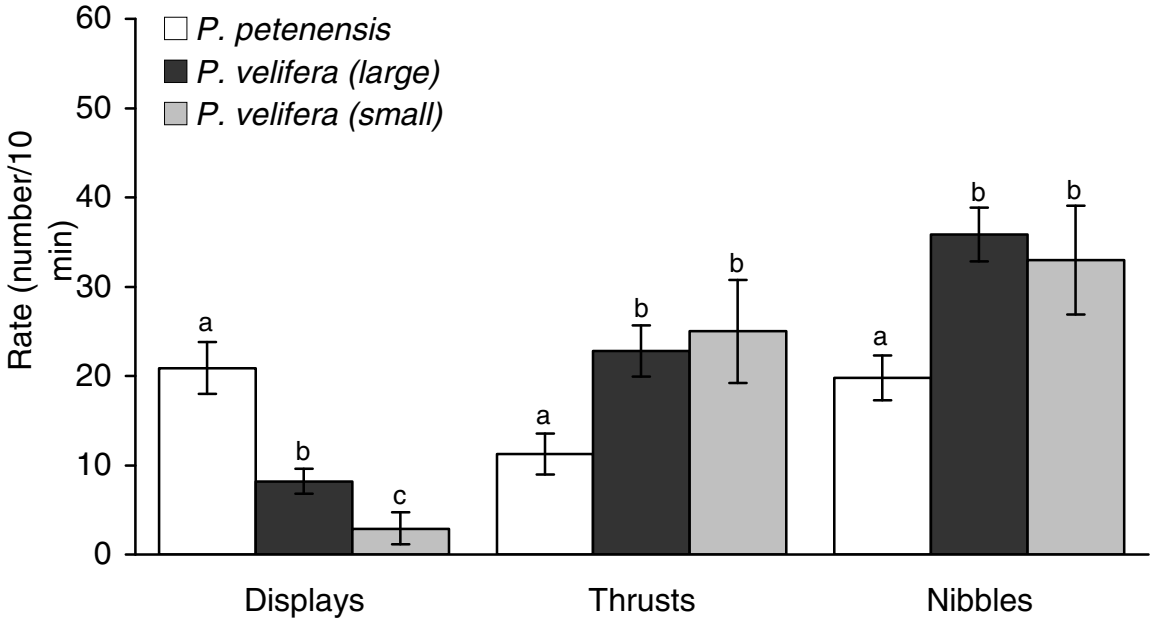

FIG. 3.1: Mean mating behavior rates (± SE) of *Poecilia petenensis* and *P. velifera*. Different letters represent significantly different behavioral rates within a behavior type.

TABLE 3.2. Comparisons of mating behavior between species (*Poecilia petenensis* and large and small *P. velifera*), linear regression analysis comparing standard length to mating behaviors, and Pearson correlations of behavior rates. Significant values are indicated by an asterisk.

Comparison	df	*F*	p	r^2
ANOVA				
display rate	2, 125	22.38	<0.01*	
total display time	2, 125	12.81	<0.01*	
ave. display duration	2, 125	14.61	<0.01*	
thrust rate	2, 125	4.73	0.01*	
nibble rate	2, 125	6.90	0.01*	
Linear regression: *P. petenensis*				
standard length & ave. display time	1, 40	1.37	0.25	0.03
standard length & display rate	1, 40	2.10	0.16	0.05
standard length & thrust rate	1, 40	0.02	0.89	0.01
standard length & nibble rate	1, 40	0.32	0.57	0.01
Linear regression: *P. velifera* (large)				
standard length & ave. display time	1, 64	1.70	0.20	0.03
standard length & display rate	1, 64	1.50	0.23	0.02
standard length & thrust rate	1, 64	3.02	0.09	0.05
standard length & nibble rate	1, 64	2.42	0.12	0.04
Linear regression: *P. velifera* (small)				
standard length & ave. display time	1, 18	3.49	0.08	0.16
standard length & display rate	1, 18	3.46	0.08	0.16
standard length & thrust rate	1, 18	0.96	0.34	0.05
standard length & nibble rate	1, 18	0.63	0.44	0.02
Pearson Correlations	*n*	*r*	*P*	
P. petenensis thrust & nibble rate	42	0.57	<0.01*	
P. velifera (large) thrust & nibble rate	54	0.77	<0.01*	
P. velifera (small) thrust & nibble rate	20	0.85	<0.01*	
P. petenensis display & thrust rate	42	-0.15	>0.05	
P. velifera (large) display & thrust rate	66	-0.53	<0.01*	
P. velifera (large) display & nibble rate	20	-0.48	<0.01*	

of thrusts and nibbles in *P. petenensis*, and in both large and small *P. velifera* (Table 3.2). There was a significant negative correlation between rate of displays and rate of thrusts or nibbles in large *P. velifera*, indicating that males that performed more thrusts (or nibbles) displayed less (Table 3.2). I did not observe this trade-off between displays and thrusts in *P. petenens* (Table 3.2).

Behavior: Population Comparisons

The Mexican sailfin molly species *P. velifera* and *P. petenensis* differed considerably in their degree of population variation in mating behaviors. Populations of *P. velifera* differed only in the rate of nibbles, but no differences were found between populations in rates of thrusting, or in any courtship characteristics (only large males were included in courtship comparisons; Table 3.3, Fig. 3.2). For the population comparison of display rate, the assumption of equality of variances was violated. Thus, ANOVA results were confirmed with a Kruskal-Wallis nonparametric one-way analysis of variance test and found to correspond to those found using ANOVA (Kruskal-Wallis: test statistic = 1.92, d.f. = 4, p = 0.75). In contrast, populations of *P. petenensis* differed in total display time, display rate, thrust rate, and nibble rate (Table 3.3, Fig. 3.2). For the population comparison of thrust rate, the assumption of equality of variances was violated, thus ANOVA results were confirmed with a Kruskal-Wallis nonparametric one-way analysis of variance test and found to correspond to those found using ANOVA (Kruskal-Wallis: test statistic = 9.00, d.f. = 2, p = 0.01).

Morphology and allometry

Males of *P. velifera* and *P. petenensis* exhibited a striking difference in the lower limits of the range of male size at maturity (Fig. 3.3). While males of *P. petenensis* matured at standard lengths of 39 mm and greater, some *P. velifera* males matured at lengths as small as 21 mm. Both species possessed males that matured at greater than 85 mm, showing a broad range of overlap in size at maturity between these species, with *P. petenensis* merely lacking the smallest male size classes observed in *P. velifera*.

All four sailfin species (*P. velifera*, *P. petenensis*, *P. latipinna*, and *P. latipunctata*) exhibited significant positive allometry between standard length and dorsal fin area (Fig. 3.4). Males of *P. velifera* had the highest slope for this relationship, which differed from the other three species based on non-overlapping confidence intervals (Table 3.4). The second highest slope was found in *P. latipinna*, which also differed from the other species. Slopes between *P. petenensis* and *P. latipunctata* did not differ, however these slopes did differ from those of the other two sailfin species. Although the slopes of *P. petenensis* and *P. latipunctata* did not differ, some caution must be used when comparing these species, as the size distributions of the males of these two species do not overlap. Similar results for all regressions were obtained using standard regression analysis.

TABLE 3.3. Population comparisons of behavior within *Poecilia petenensis and P. velifera* based on ANOVA.

Population comparison	*d.f.*	*F*	*p*
P. petenensis			
total display time	2, 43	4.08	0.02*
display rate	2, 43	3.64	0.03*
average display duration	2, 42	2.00	0.15
thrust rate	2, 43	4.12	0.02*
nibble rate	2, 43	3.72	0.03*
P. velifera			
total display time (large males)	4, 58	0.19	0.94
display rate (large males)	4, 58	0.25	0.91
average display duration (large males)	4, 44	1.94	0.12
thrust rate	4, 78	1.24	0.30
nibble rate	4, 78	6.19	<0.01*

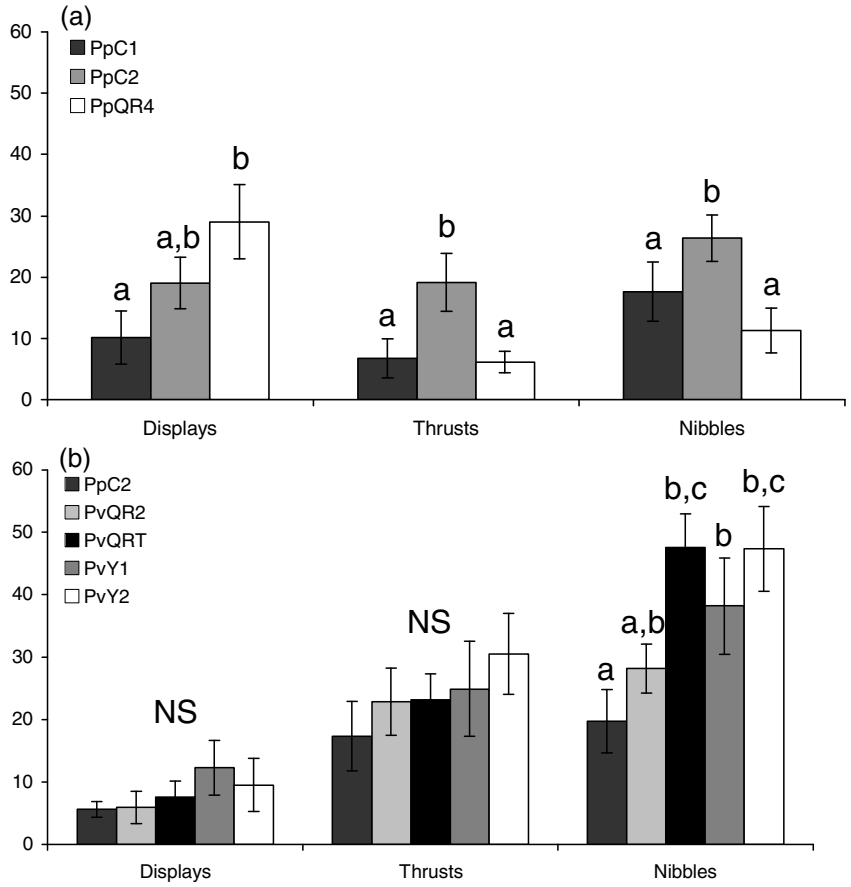

FIG. 3.2. Mean mating behavior rates (± SE) among populations for (a) *Poecilia petenensis* and (b) *P. velifera*. Different letters represent significantly different behavioral rates within a species.

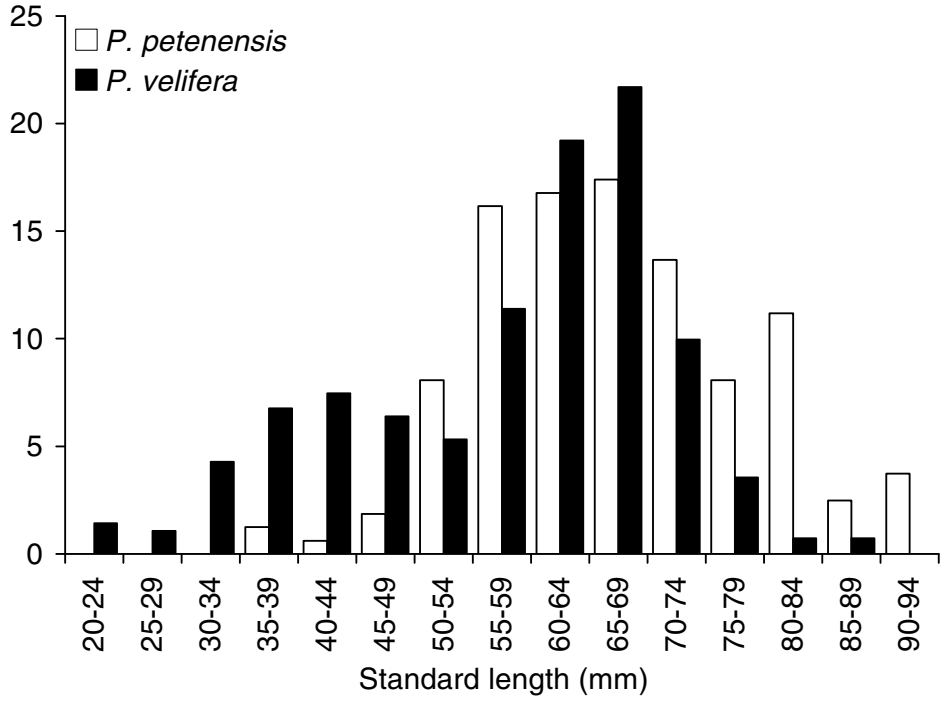

FIG. 3.3. Size frequency histogram for *Poecilia velifera* (11 populations, n = 281) and *P. petenensis* (7 populations, n = 162).

DISCUSSION

Males of the sailfin species *P. velifera* differed markedly from *P. petenensis* and other sailfin molly species in possessing a class of small males that relied almost exclusively on gonopodial thrusting, and a larger class of males that, like those observed in the other sailfin species, performed both courtship displays and gonopodial thrusting. In marked contrast, males of *P. petenensis* generally lacked males of the smallest sizes present in *P. velifera*, and even the smallest males of *P. petenensis* (39 mm SL) performed courtship displays as well as gonopodial thrusts in response to receptive females. Similarly, in the sailfin molly *P. latipinna*, males of all sizes (even as small as 22 mm SL) perform courtship displays, although in some populations there is a tendency for small males to perform higher rates of gonopodial thrusts and lower rates of courtship displays, with the opposite pattern being characteristic of larger males (Farr et al. 1986; Ptacek & Travis 1996).

In addition to the difference between the two species in the degree of size-specific behavioral variation, the rate and duration of mating behaviors varied between the two species. Males of *P. petenensis* performed higher rates of courtship displays and lower rates of gonopodial thrusts and gonoporal nibbles than did males of both displaying and non-displaying size classes of *P. velifera*. The average rate of courtship displays in *P. petenensis* was similar to that reported for two other sailfin species, *P. latipinna* (Ptacek & Travis 1996) and *P. latipunctata* (Ptacek et al. 2005), while rates of gonopodial thrusts and gonoporal

nibbles were somewhat lower than rates reported for these other sailfin species. Average courtship display rates for males of *P. velifera*, even for the large courting size class, were the lowest reported among all four sailfin species (Table 3.4) (Ptacek & Travis 1996; Ptacek et al. 2005). This low courtship display rate exists despite *P. velifera* being the species which exhibits the highest level of sexual dimorphism in dorsal fin area (for example, a male with a standard length of 50-55 mm has a dorsal fin that is 5 -10X larger than that of a similarly sized female) and the strongest allometry between male body length and dorsal fin size (slope = 4.5). Thus, in this sailfin species, female preferences for male size may be more important in mating decisions than preferences based upon the rate of courtship displays. Indeed, female preference for larger male size and larger dorsal fin size has been shown to be considerably stronger in *P. velifera* than in *P. petenensis*, with females of *P. velifera* consistently preferring larger males (either larger because of body size or dorsal fin size), even when those males were heterospecific sailfin males (Kozak 2005).

In addition to differences in mating behavior rates and male strategies between the Mexican sailfin mollies, *P. velifera* and *P. petenensis* also differ in the degree of divergence among populations within each species. While populations of *P. petenensis* differed in the rate of displays, thrusts, and nibbles, populations of *P. velifera* differed only in rates of nibbles. One explanation for this pattern may be differences in the magnitude of natural selection in these two species based upon their differences in habitat (Hankison et al. 2006; Chapter 2).

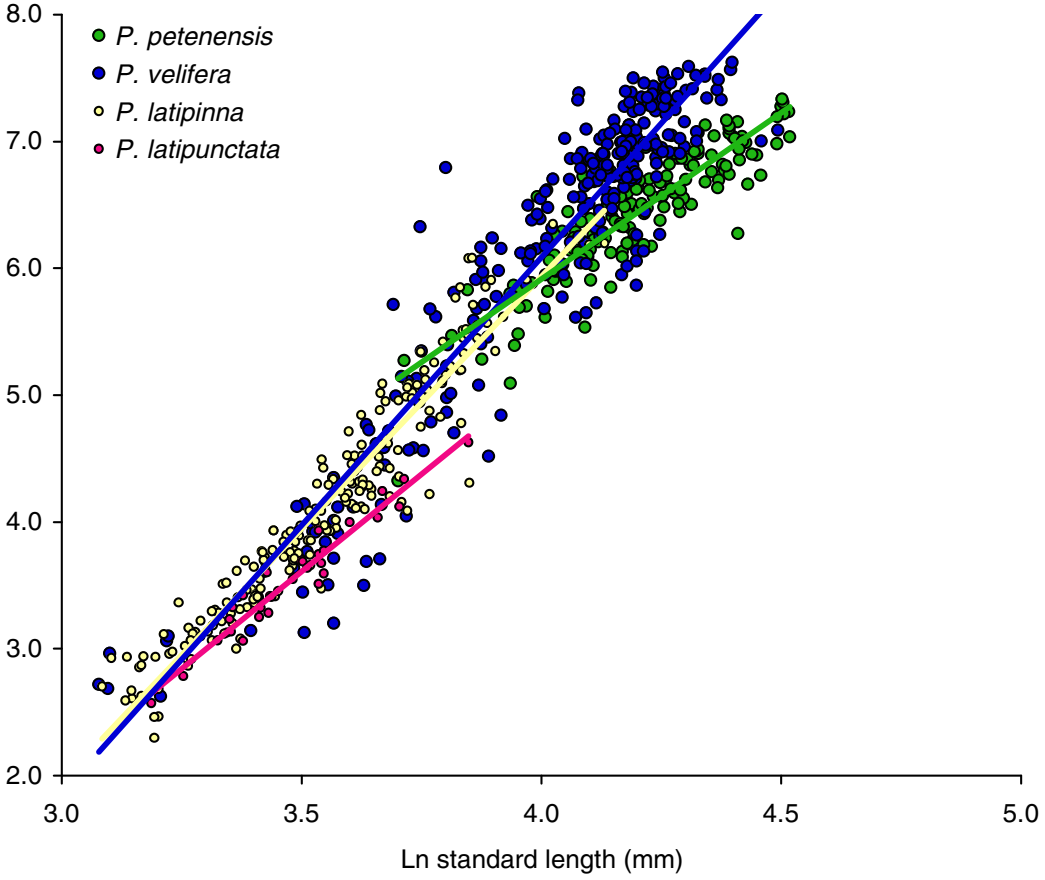

FIG. 3.4. Log-transformed standard lengths and associated dorsal fin areas of the four species of sailfin mollies. The r^2 values for the lines are shown in the legend.

TABLE 3.4. Range of male sizes and the slope representing the allometric relationship between standard length (ln transformed) and dorsal fin area (ln transformed), and the confidence intervals surrounding those regressions. Different superscript letters show slopes that are different based on non-overlapping confidence intervals (all showed significant allometry, $p < 0.001$). Behavior rates for each sailfin molly species are also shown. Samples sizes for morphology are listed under standard length. Samples sizes for behavior studies are listed under display rate.

Species	Standard length in mm (range)	Dorsal fin area in mm^2 (range)	Slope	Confidence interval	display rate (range*)	Thrust rate (range*)	Nibble rate (range*)
P. velifera	21.7 – 89.0 (n = 281)	13.8 – 2384.0	4.5^{A}	4.3 – 4.7	0 – 51 (n = 85)	0 – 98	0 – 113
P. petenensis	40.5 – 91.7 (n = 162)	75.6 – 1532.6	2.9^{B}	2.7 – 3.2	2 – 78 (n = 49)	0 – 59	0 – 66
P. latipinna†	21.8 – 62.3 (n = 208)	9.9 – 571.5	4.1^{C}	4.0 – 4.3	0 – 112 (n = 189)	0 – 132	0 – 119
P. latipunctata‡	24.2 – 46.9 (n = 29)	14.4 – 121.5	3.1^{B}	2.9 – 3.5	6 – 70 (n = 21)	0 – 184	0 – 150

* during a 10 min. trial †behavior rates from Ptacek & Travis (1996) ‡behavior rates from Ptacek et al. (2005)

The saltwater *P. velifera* occupies coastal marshes and tidal pools, while freshwater *P. petenensis* is found in freshwater rivers and impoundments (Schmitter-Soto 1998). Habitat characteristics such as stream flow rate and predation pressure have been shown to influence courtship rates in other poeciliid species (Farr 1975; Magurran & Seghers 1994a, b; Nicoletto 1996) and greater variability in flow regimes and predation intensity between river and lake habitats in Yucatán may lead to greater variability in mating behavior rates in *P. petenensis*. Such habitat variability has been hypothesized to lead to interpopulation variation in male morphology in this species as well (Hankison et al. 2006; Chapter 2).

Aside from behavioral differences, small and large males of *P. velifera* exhibited striking morphological differences correlated with their size. Small males lacked the high degree of sexual dimorphism exhibited by larger males in dorsal fin area and body and fin coloration. Indeed, large males had body-size normalized dorsal fin areas up to 51 times larger than small males (comparison of the largest to smallest ratios of dorsal fin area to standard length squared). The phenotypic result is that small, mature males of *P. velifera* are much more similar in shape and coloration to juveniles than they are to large males (Fig. 3.5). Such strong phenotypic differentiation has led to behavioral polymorphisms in other fish species, where small males adopt a sneak strategy (Gross 1982, 1985, 1991a, b; Warner 1984; Zimmerer & Kallman 1989; Ryan et al. 1990). In contrast, even the smallest males of *P. petenensis* (39 mm) had exaggerated

dorsal fin areas and the bright coloration characteristic of large males of this species (Fig. 3.5).

The considerably stronger allometric relationship between body size and dorsal fin area in *P. velifera* means that the difference between a small male and a large male is vast compared to that for the other three sailfin species. A smaller male has a much smaller dorsal fin relative to his body size compared to larger males and thus is at a considerable disadvantage in attracting females (MacClaren et al. 2004; Kozak 2005). A similar situation occurs in some *Xiphophorus* species, where small males also lack courtship displays and primarily employ a sneaking tactic to circumvent female choice (for large, courting males) and gain copulations (Ryan & Causey 1989; Ryan & Rosenthal 2001). Like the dorsal fin in mollies, the sword of swordtails (*Xiphophorus)* scales allometrically with body size (Ryan & Rosenthal 2001), thus, the small swords of small males would contribute relatively less to an apparent increase in body size, compared to swords in larger males. Small males are not generally attractive to female swordtails (Ryan et al. 1990; Basolo 1998a, b; Rosenthal & Evans 1998; Ryan & Rosenthal 2001) or mollies (Ptacek & Travis 1997; MacClaren et al. 2004), thus any investment in courtship would be wasted, and a more effective strategy would likely be to attempt sneak-copulations.

Behavioral polymorphisms in sailfin mollies appear to span the range from alternative mating tactics in small versus large males of *P. velifera* to environmental-dependent strategies found in *P. petenensis* and large *P. velifera* where reliance on courtship versus sneaking may depend on social environment,

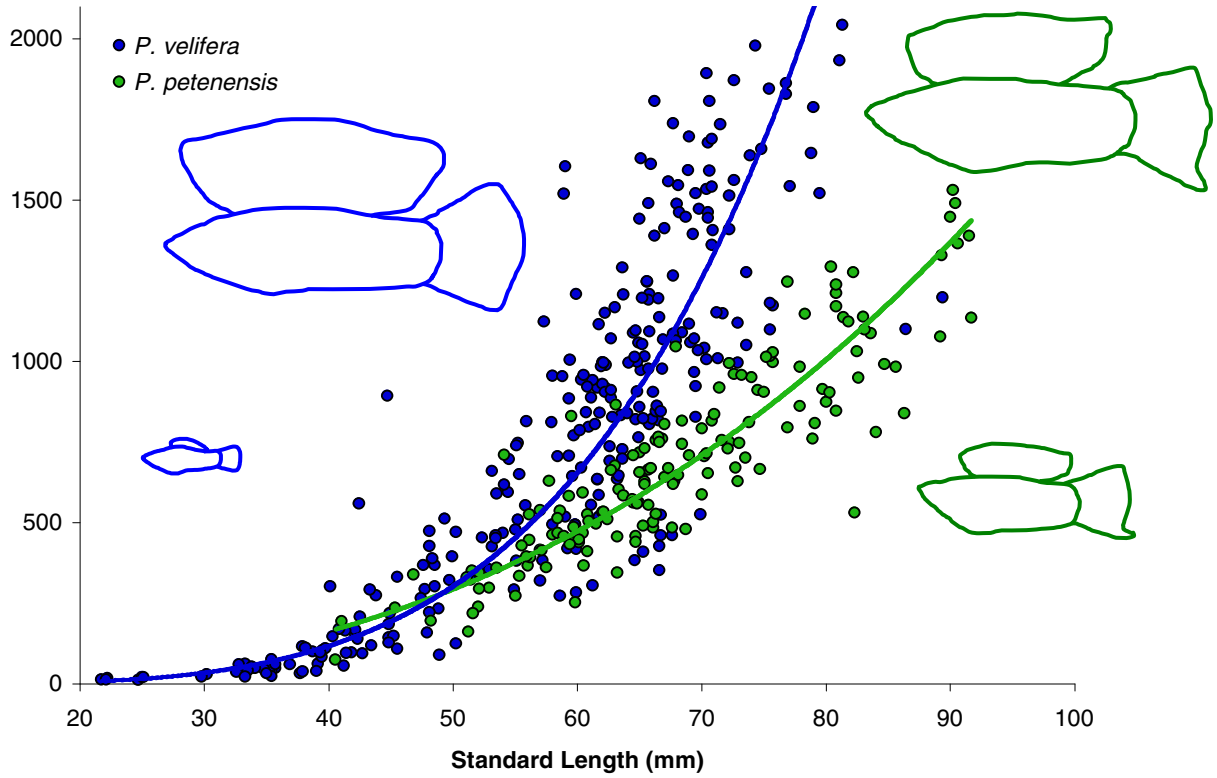

FIG. 3.5. Untransformed standard lengths and fin areas of *P. velifera* and *P. petenensis* demonstrating the difference in allometry for these two species. Inset outlines are size-scaled relative to one another and demonstrate the difference in lateral projection area between large (70 mm SL, *P. velifera* and *P. petenensis*) and small (20 mm SL, *P. velifera*; 40 mm SL, *P. petenensis*) fish in each species. Outlines of *P. velifera* are shown in blue, and *P. petenensis* in green.

relative male size, female receptivity, and abiotic environmental conditions (Farr & Travis 1986; Travis & Woodward 1989; Sumner et al. 1994; Ptacek et al. 2005). Throughout the entire molly clade (Ptacek & Breden 1998), mating signal evolution appears to involve a switch from the total reliance of males on sneaking (such as in the shortfin molly ancestor (Farr 1989)) to courtship displays that elicit female cooperation, characteristic of all species of sailfin mollies (Farr 1989; Niemeitz et al. 2002). Yet, within a single species, the degree of variation in male size at maturity and the degree of phenotypic difference between small, nondescript males and large, courting males may drive the level of variation between reliance on different mating behaviors, especially in *P. velifera* where both alternative mating strategies based on a genetic polymorphism in male size and environmentally-dependent male strategies in larger males appear to have evolved.

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CHAPTER 4

GENETIC DIVERGENCE IN THE MEXICAN SAILFIN MOLLIES

Abstract. – Comparing population divergence using both neutral genetic and phenotypic traits provides a method to examine the relative importance of various evolutionary mechanisms in shaping population differences. Concordant patterns of variation in both types of traits suggest a strong role of genetic drift or ongoing gene flow, while dissimilar divergence patterns suggest that diversifying selection may be important in phenotypic trait divergence. I used eight microsatellite markers to examine genetic population structure in two species of Mexican sailfin mollies, *Poecilia velifera* (N = 9 populations) and *P. petenensis* (N = 9 populations), sampled from across their entire geographic distribution in the Yucatán Peninsula of Mexico. I then compared patterns of genetic structure and divergence to patterns of interpopulation divergence observed in two types of phenotypic traits, morphological characters and rates of two different mating behaviors. Populations of each species were genetically distinct, and conformed to a model of isolation by distance. Based on genetic markers, populations within different geographic regions (which may serve as barriers to gene flow) were more similar to one another than were populations from different regions. Both Bayesian clustering and barrier analysis provided additional support for population separation, especially between geographic regions. In contrast, none of the phenotypic traits showed any type of geographic pattern and population

divergence in these traits was uncorrelated with that in neutral markers. In addition, there appeared to be a weaker pattern of regional differences among geographic regions than was observed based on neutral genetic divergence. These results suggest that while divergence in neutral traits is likely a product of population history and genetic drift, phenotypic divergence is governed by different mechanisms, such as natural and sexual selection, and arises at spatial scales independent from those of neutral markers.

INTRODUCTION

Geographic variation among conspecific populations in particular types of phenotypic traits provides key insights into the action of selection and its role in speciation. The divergence of characters in response to local adaptation to either natural or sexual selection in different populations is constrained, however, by the historical degree of population separation and the relative influences of genetic drift and ongoing gene exchange between contemporary populations. In order to disentangle the relative roles of selection, drift and gene flow in shaping patterns of population divergence, one useful approach is to characterize phenotypic variation among populations in traits that are known targets of selection and compare such phenotypic divergence to that observed in neutral genetic markers (e.g. Ryan et al. 1996; Masta and Maddison 2002; Nicholls et al. 2006; Pröhl et al. 2006). Such an approach uses divergence in neutral molecular markers to uncover the evolutionary history of populations, which can then provide the framework for testing hypotheses with respect to the roles of natural and sexual selection in the origin and maintenance of population-specific traits.

Divergence among populations in traits associated with mating signals can be rapid in response to sexual selection favoring features of a signal that best propagate in a particular environment (Ryan et al. 1990; Boughman 2002), as by-products of adaptive divergence in response to natural selection (Schluter 2001; Nosil et al. 2005), or as arbitrary targets of divergent female mating preferences (Lande 1981; Ptacek 2000; Panhuis et al. 2001). Natural selection can promote

population divergence in traits that are indirectly linked to mating signals, such as morphological characters that increase crypsis or aposematic coloration, or traits that improve performance (e.g., body and fin shape in fishes) that are then co-opted to serve as or enhance behavioral mating signals (Rundle and Schluter 1998; Hatfield and Schluter 1999; Jiggins et al. 2004; Boughman et al. 2005; Nosil et al. 2005). These selective forces, however, may be constrained by the degree of historical separation of populations, which can promote phenotypic differentiation among populations because of drift or selection (Schluter 2001), and the degree of ongoing gene flow between geographically proximate populations that should homogenize phenotypic differences and retard the degree of local adaptation.

Neutral molecular markers can be used to examine the underlying genetic structure of populations, such as the amount of genetic divergence and gene flow between populations, and such data can then be used to test for genetic correlations with contemporary differences in mating signals. Divergence in mating signals and genetic markers may covary for example, if populations are diverging randomly, as a result of genetic drift. Alternatively, incongruent patterns of genetic and phenotypic trait divergence suggest that different mechanisms, such as sexual or natural selection, may be important in promoting population differences in mating signals. Previous studies have shown that such a comparison between genetic and phenotypic traits may illuminate the role of multiple evolutionary processes in shaping population divergence (e.g. Merilä 1997; Merilä and Crnokrak 2001; Thorpe and Murielle 2001; Waldmann et al.

2005). Populations of the túngara frog (*Physalaemus pustulosus*), for example, have been shown to have diverged both genetically and acoustically, and some evidence for prezygotic isolation between regionally isolated populations exists (Ryan et al. 1996; Pröhl et al. 2006). Comparison of genetic and phenotypic traits in the satin bowerbird (*Ptilonophynchus violaceus*) has shown how habitat differences may have shaped call characteristics across the range of this species independent from the historical pattern of divergence in neutral genetic markers (Nicholls et al. 2006).

In this study, I test whether phenotypic divergence in morphological traits and mating behaviors is correlated with genetic divergence in order to infer the relative importance of selection, gene flow, and drift in the maintenance of population differences in the Mexican sailfin mollies, *Poecilia velifera* and *P. petenensis*. Sailfin mollies are an interesting group in which to examine the factors promoting and maintaining geographic variation in phenotypic traits because variation among populations occurs primarily in morphological and behavioral traits of males that are associated with mating signals and swimming performance and therefore are likely targets of both natural and sexual selection (Hankison et al. 2006; Hankison and Ptacek in review; Chapters 2 and 3). In addition, sailfin species in the Yucatán region of Mexico have a broad geographic range, occupying different geographic regions (sensu Wilson 1980; Schmitter-Soto et al. 2002) and habitat types (Schmitter-Soto 1998; Schmitter-Soto et al. 2002). Thus, both genetic and phenotypic divergence may be shaped by geographic barriers to gene flow and historical differences in regional patterns of

colonization. I use this correlational approach to address the following questions regarding population divergence in each species of sailfin molly: 1) Do populations show significant genetic divergence based on neutral microsatellite loci? 2) Do geographic regions serve as barriers that may constrain gene flow between populations leading to a pattern of isolation by distance? 3) Do the geographical patterns of genetic differences predict the patterns of phenotypic differences in morphological traits or mating behaviors? 4) What is the role of historical versus ongoing evolutionary processes, such as gene flow and selection, in shaping levels of contemporary population divergence?

Study System

Biogeography

The Mexican sailfin mollies *P. velifera* and *P. petenensis* are livebearing fishes (family Poeciliidae) found throughout the Yucatán Peninsula and surrounding areas in Mexico (Fig. 4.1). The two species are primarily allopatric in distribution, separated by differences in their preferred habitats. *P. velifera* is generally restricted to coastal habitats, such as anchialine cenotes (coastal salt water cenotes with no surface connection to the sea), tidal pools and salt marshes, whereas *P. petenensis* is more abundant in interior freshwater rivers and impoundments (Schmitter-Soto 1998). The coastal habitats of *P. velifera*

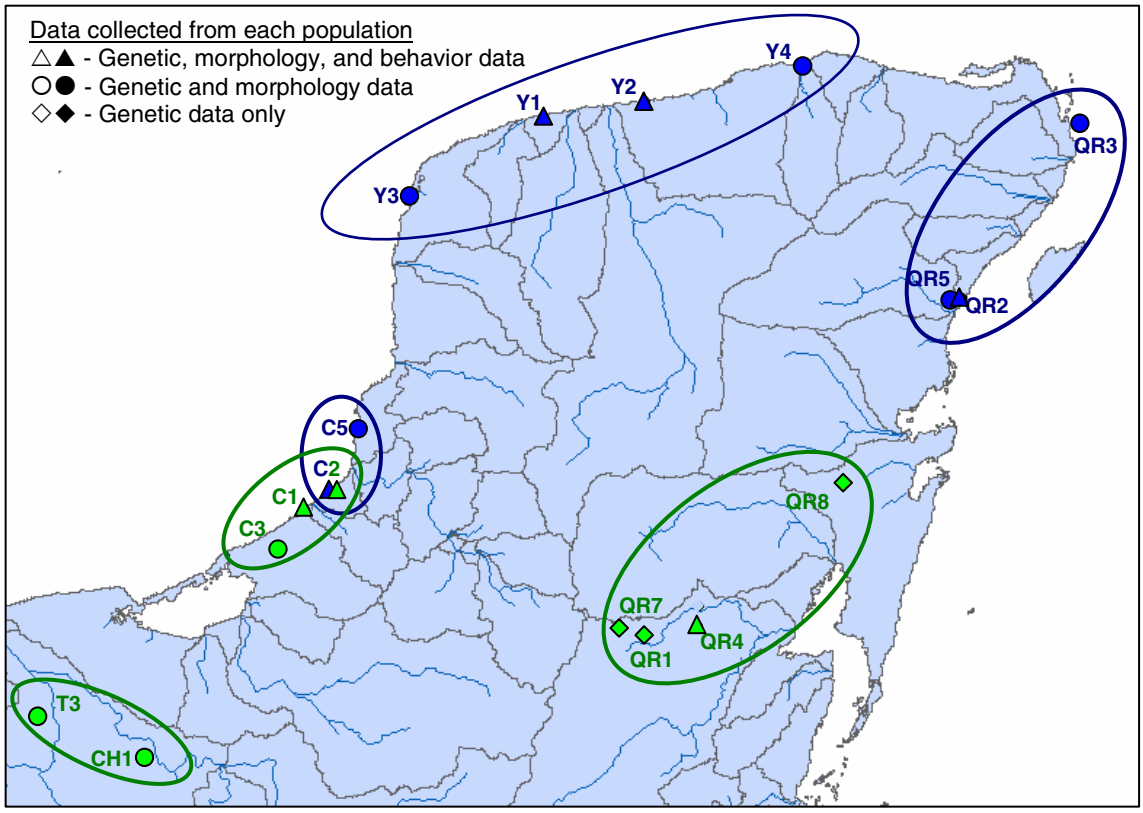

FIG. 4.1. Map of the Yucatán Peninsula showing the collection sites of *P. velifera* (blue) and *P. petenensis* (green). Names next to the sites show the sampling IDs of each site; Campeche (C), Yucatán (Y), Quintana Roo (QR), Chiapas (CH), and Tabasco (T). Genetic data were collected from all sites. Morphological data were collected from fish at a subset of sites. Behavioral and morphological data were collected from a smaller subset of sites. Sites within the same geographic region are circled. Blue lines are major rivers, while grey lines surround drainage basins. Note that in this and subsequent maps, the distance between QR5 and QR2 is exaggerated on the map so that they can be compared (accurate points overlap completely).

offer few obvious barriers to dispersal, although the species range crosses three geographic districts (Wilson 1980; Schmitter-Soto et al. 2002) that potentially differ in ocean current dynamics, which may limit gene flow between them (Fig. 4.1). In contrast, populations of *P. petenensis* occupy geographically separate river drainages in southern Yucatán (Schmitter-Soto 1998) that may serve as potential barriers to gene flow, although these rivers do connect during flooding and through underground links (Schmitter-Soto 2002). Like *P. velifera*, the range of *P. petenensis* crosses three geographic districts (Wilson 1980; Schmitter-Soto et al. 2002), which also correspond to the three major drainages in which *P. petenensis* is found in the southern interior regions of the Yucatán Peninsula (Fig. 4.1).

Phenotypic traits

Previous studies have found differences in morphological traits and rates of different mating behaviors in males between populations of both *P. velifera* and *P. petenensis*. For example, Hankison et al. (2006; Chapter 2) found that populations in *P. velifera* differed primarily in characteristics related to the size and shape of the enlarged dorsal fin, a sexually selected character in males (Kozak et al. in review), while populations of *P. petenensis* differed in characteristics related to the size and shape of the caudal fin, a trait where changes in shape enhance swimming performance in other species of fish (e.g. Langerhans et al. 2003; Langerhans and DeWitt 2004). Vector analysis showed

that populations of these two species were diverging along independent lines of morphological evolution (Hankison et al. 2006; Chapter 2), supporting the idea that sexual selection may be promoting differences in fin size among populations of *P. velifera*, while natural selection may be more important in *P. petenensis*, shaping the caudal fin to a match the flow environment. In addition, populations of *P. petenensis* were found to differ in rates of mating behaviors (Hankison and Ptacek in review; Chapter 3). These included courtship displays, a mating behavior used to elicit female cooperation during insemination (mollies have internal fertilization) (Parzefall 1969, 1989; Farr and Travis 1986; Ptacek and Travis 1996; Niemeitz et al. 2002; Ptacek et al. 2005), and gonopodial thrusts, a type of forced insemination attempt, where the male orients himself behind a female, brings the gonopodium (fused anal fin that serves as an intromittent organ for internal fertilization) to a forward position, and swimming forward, attempts to insert the tip into the female's gonopore for sperm transfer. Populations of *P. velifera*, however, did not differ in these mating behaviors (Hankison and Ptacek in review; Chapter 3).

METHODS

Molecular Sampling

During the early summer months (April – June) of 2002 – 2003, live individuals of *P. velifera* and *P. petenensis* were collected within their native

TABLE 4.1. Site coordinates and population sample sizes for each analysis. Dashed columns indicate that data was not collected for that population.

Population	Site Coordinates	Analysis (*n*) Genetics	Morphology	Behavior
P. velifera				
C2	N 19˚14.232', W 90˚50.110'	20	21	19
C5	N 19˚34.998', W 90˚40.002'	20	15	—
Y1	N 21˚15.807', W 89˚39.648'	20	21	15
Y2	N 21˚21.561', W 89˚06.072'	20	27	15
Y3	N 21˚51.438', W 90˚22.983'	17	27	—
Y4	N 21˚34.043', W 88˚13.780'	13	24	—
QR2	N 20˚17.305', W 87˚22.548'	20	39	21
QR3	N 21˚13.910', W 86˚44.330'	20	14	—
QR5	N 20˚17.420', W 87˚22.666'	13	28	—
P. petenensis				
C1	N 19˚08.620', W 90˚57.400'	20	18	15
C2	N 19˚14.232', W 90˚50.110'	20	33	19
C3	N 18˚55.925', W 91˚05.350'	20	30	—
T3	N 17˚58.000', W 92˚31.315'	20	19	—
CH1	N 17˚48.482', W 91˚48.779'	20	26	—
QR1	N 18˚26.7000', W 89˚6.102'	11	16	13
QR4	N 18˚36.678', W 88˚48.713'	10	10	—
QR7	N 18˚29.412', W 89˚15.000'	8	—	—
QR8	N 19˚16.734', E 88˚1.548'	5	—	—

ranges across five states in Mexico (Table 4.1): Campeche (C), Chiapas (CH), Quintana Roo (QR), Tabasco (T) and Yucatán (Y). The sites were chosen to cover a wide range of locales across the distribution of each species and to include sites from each major river drainage for *P. petenensis* (Fig. 4.1). Fish were collected using seine nets (6.1 X 1.2 m), cast nets (1.2 m), and minnow traps. Following collection, live fish were shipped to Clemson University where they were maintained in 568-L stock tanks for additional study. Individuals used for DNA study were euthanized with an overdose of buffered MS-222 and placed in 95% ethanol. Samples were stored in ethanol at -20˚C. Both males and females were used in genetic analyses based on microsatellites.

Genotyping

DNA was extracted from muscle tissue by incubating ~5 mg of tissue with 160 µl of a 5% Chelex solution and 20 µl protinease K at 65˚C (Walsh et al. 1991). After incubating overnight, the sample was centrifuged and 70 µl of supernatent was transferred to a new tube (the remainder was discarded), diluted to 25 ng/µl and stored at -20˚C until amplified. Using primers developed for *Poecilia reticulata* and *Xiphophorus* spp., conditions were optimized for mollies (by adjusting magnesium concentration and annealing temperatures) and individuals were genotyped at eight microsatellite loci (Table 4.2). Primers were purchased fluorescently labeled (reverse primer only) and PCR products were sized using an ABI 3130 capillary analyzer and scored using GeneMapper version 3.0.

TABLE 4.2. Details of primers used in this study.

Primer name	Genbank ID	Annealing temperature	Mg^{2+} concentration	Size range		Reference
				P. petenensis	*P. velifera*	
G10	AF026454	56.0°C	2.5 mM	189-191	191-193	(Parker et al. 1998)
CA25	AY258696	58.2°C	2.5 mM	113	109-115	(Walter et al. 2004)
CA34	AY258652	58.8°C	1.8 mM	116-126	118-130	(Walter et al. 2004)
G49	AF026459	56.0°C	2.0 mM	164-192	144-200	(Parker et al. 1998)
Pr80	AF467905	56.0°C	2.5 mM	96-102	90-106	(Becher et al. 2002)
Pr92	AF467906	56.0°C	3.0 mM	143-151	135-151	(Becher et al. 2002)
CA120	AY258788	59.3°C	2.5 mM	102-108	100-108	(Walter et al. 2004)
Pr172	AF467908	58.0°C	2.5 mM	176	176	(Becher et al. 2002)

Microsatellite Analysis

Genetic diversity was estimated for each population by determining the mean number of alleles, observed heterozygosity, and the expected heterozygosity using Arlequin version 2.0 (Schneider et al. 2000). Loci were tested for Hardy-Weinberg equilibrium and for linkage disequilibrium using the program GenePop version 3.4 (Raymond and Rousset 1995) using a Markov Chain approximation with 100,000 iterations with 1000 steps. Sequential Bonferroni corrections (Rice 1989) were performed on the probability values of each test, using a testwide significance value of 0.05.

Population differentiation measures from the microsatellite data were estimated using both Wright's *F*-statistic (Wright 1951), which examines the identity of state between alleles, and *R*-statistics (Slatkin 1995), which uses the number of repeat units in the microsatellites as additional information (assuming a stepwise mutation model). The program Arlequin was used to calculate both *F*- and *R*- statistics between all pairwise population comparisons. Pairwise comparisons were also performed between geographic regions (combined populations sharing a geographic region). Values from all pairwise comparisons were checked against sequential Bonferroni corrections (Rice 1989) to ascertain significance. The allele permutation test in the program SPAGeDi version 1.2 (Hardy and Vekemans 2002; Hardy et al. 2003) was used to determine whether R_{ST} was more informative than F_{ST} based on a comparison of observed R_{ST} to a distribution of permutated R_{ST} values. If observed R_{ST} falls within 5% of

permutated R_{ST} values, it should be used; non-significant tests indicate that F_{ST} should be used. Molecular analysis of variance (AMOVA as implemented in Arlequin) was used to describe how genetic variance was partitioned between and within geographic regions, and to compare variance in microsatellite allele frequencies to that observed in morphological traits.

Geographic Information System (GIS) analysis was used to calculate fluvial distance between populations (map layers from http://edc.usgs.gov/profucts/elevation/gtopo30/hydro/namerica.html). For *P. velifera*, this distance is the distance around the Peninsula, as these populations inhabit coastal marshes (Appendix C). For *P. petenensis*, this is the distance of the most direct water route, or, for populations unconnected by water, the straight line distance between populations that, during floods, may allow for passage between sites (Schmitter-Soto et al. 2002) (Appendix D). The habitats of both species are approximately linear (movement was along rivers or coasts), thus fluvial distances were not log transformed in later comparisons (Rousset 1997). Genetic distances determined by F_{ST} and R_{ST} were transformed to $F_{ST}/(1- F_{ST})$ (or $R_{ST}/(1-R_{ST})$ to obtain linear relationships between geographic and genetic distances (Rousset 1997). Transformed values were then compared to fluvial distance using Mantel tests (Mantel version 1.01; Bohonak 2002) with 10,000 permutations to determine the presence of significant associations between genetic and geographical distance. Pairwise D_A values were also used in a barrier analysis (Barriers version 2.2; Manni et al. 2004). The barriers analysis constructs a network of adjacent populations and then uses Monmonier's

maximum-difference algorithm (Monmonier 1973) to place barriers between populations indicating reduced gene flow (for an additional example of the use of this program see Nicholls et al. 2006).

I used the program BAPS (Bayesian Analysis of Population Structure) version 2.0 (Corander et al. 2003, 2004) to provide an additional method of examining population structure, and to compare the population structure from BAPS to that hypothesized by separate geographic groups (Fig. 4.1). The program BAPS employs a Bayesian clustering method to group populations that have statistically similar allele frequencies, as well as calculating the marginal likelihood of the clusterings, thus providing an estimate of how well the data fit the proposed model.

Comparison of Genetic and Phenotypic Divergence

The correlation between neutral genetic variance and phenotypic traits (morphology and behavior; Table 4.1) was examined using Mantel tests (Mantel version 1.01; Bohonak 2002). Mahalanobis distance was calculated both for overall body shape measurements (size-transformed linear measures) and for dorsal fin characteristics in *P. velifera* and caudal fin characteristics in *P. petenensis* which were found previously to be important targets of selection in the two species (Hankison et al. 2006; Chapter 2). Morphological measures were taken from males from all populations of *P. velifera* (Appendix E) and from a subset of six populations (C1, C2, C3, T3, CH1, and QR4) for *P. petenensis*

(Appendix F). In addition, Mahalanobis (or Euclidean) distance was calculated for two different mating behaviors: courtship displays and gonopodial thrusts. Because individuals or populations may differ in both the average rate and the average duration of courtship displays (Hankison and Ptacek in review; Chapter 3), these variables were both included in the calculation of Mahalanobis distance between four populations of *P. velifera* (C2, QR2, Y1, and Y2) (Appendix G) and three populations of *P. petenensis* (C1, C2, and QR4) (Appendix H). Euclidean distances were likewise calculated for average rates of gonopodial thrusts for each population tested for both species. Pairwise phenotypic trait values were then compared to pairwise estimates of Nei's net genetic distance, D_A (Nei and Li 1979) from the same populations that were sampled for phenotypic data, using Mantel tests with 10,000 permutations to determine the presence of significant associations (Mantel version 1.01; Bohonak 2002). I used D_A instead of Nei's standard genetic distance (D_S; Nei 1978, 1987) because D_A includes the difference in number of repeats between alleles at the same locus.

As additional methods to visualize comparisons between genotypic and phenotypic traits, I also used barrier analysis to visualize barriers between populations based on morphological traits (Manni et al. 2004). In addition, I used non-metric multidimensional scaling to plot populations using both genetic clusters (based on D_A) and morphological clusters (based on linear morphological Mahalanobis distances) in a two-dimensional space. Multidimensional scaling analyses were performed using SAS version 9.0. These two types of analyses were not performed on mating behaviors because

of the limited number of populations tested for each species. Finally, I compared morphological and behavioral distance using Mantel tests to determine whether these phenotypic traits were correlated, or whether natural and sexual selection may potentially act on different suites of phenotypic traits independently.

RESULTS

Genetic Population Structuring

For *P. petenensis*, I found a total of 32 different sized alleles across the eight loci examined (Appendix A). Two loci were monomorphic (CA25, Pr172) and all other loci were polymorphic showing one to seven alleles within a single population. All loci were in Hardy-Weinberg equilibrium in all populations of *P. petenensis*. No loci were in linkage disequilibrium (with Bonferroni corrections) either within populations or among a global comparison. For *P. velifera*, I found a total of 57 different sized alleles across the eight loci examined (Appendix B). All loci, with the exception of Pr172, were polymorphic showing one to twelve alleles within a single population. Four loci exhibited significant departure from Hardy-Weinberg equilibrium in one to five populations. No loci were in linkage disequilibrium (with Bonferroni corrections) either within populations or among a global comparison. Populations of each species appear to be similar in their overall levels of heterozygosity and mean number of alleles per locus (Table 4.2),

TABLE 4.3. Measures of genetic diversity (Nei's unbiased genetic diversity, H_e; observed heterozygosity, H_o; and the mean number of alleles per locus) in nine populations of *P. velifera* and nine populations of *P. petenensis*.

Population ID	H_o	H_e	Mean no. alleles	No. private alleles
P. velifera				
C2	0.344	0.466	2.6	0
C5	0.319	0.222	2.8	0
Y1	0.325	0.298	3.3	1
Y2	0.258	0.215	2.6	1
Y3	0.258	0.215	2.6	2
Y4	0.258	0.215	2.6	4
QR2	0.275	0.152	2.3	2
QR3	0.352	0.251	2.9	1
QR5	0.192	0.141	1.9	0
P. petenensis				
C1	0.242	0.273	2.1	0
C2	0.224	0.229	2.0	0
C3	0.218	0.199	1.8	0
T3	0.310	0.294	2.6	2
CH1	0.230	0.243	2.3	2
QR1	0.220	0.182	2.1	4
QR4	0.234	0.139	1.9	1
QR7	0.181	0.156	1.4	0
QR8	0.058	0.025	1.1	0

indicating that population differences may be because of fixed differences between populations, or the presence of private alleles.

Nearly all populations differed based on pairwise F_{ST} comparisons, even after sequential Bonferroni adjustment (35 of 36 comparisons for *P. velifera*, Table 4.3, and 33 of 36 comparisons for *P. petenensis*, Table 4.4). Fewer populations were significantly different based on pairwise R_{ST} comparisons (29 of 36 comparisons for *P. velifera*, Table 4.3, and 29 of 36 comparisons for *P. petenensis*, Table 4.4), however a general pattern of population differentiation was still apparent. Allele permutation tests revealed that the additional allele size information provided by R_{ST} did not contribute additional population divergence information for *P. petenensis* or for *P. velifera* ($P > 0.05$ in all tests).

A strong pattern of isolation by distance was found in both species, indicating increased genetic divergence with increasing geographic separation, for both F_{ST} (Mantel tests, *P. velifera*: $r = 0.523$, $P < 0.001$; *P. petenensis*: $r = 0.368$, $P = 0.018$) and R_{ST} (Mantel tests, *P. velifera*: $r = 0.741$, $P < 0.001$; *P. petenensis*: $r = 0.351$, $P = 0.036$) (Fig. 4.2). Comparing the degree of divergence between the three geographic regions in each species using AMOVA showed that much of the genetic variance was partitioned between regions (*P. velifera*: 15%, *P. petenensis*: 27%) indicating decreased gene flow between regions compared to the degree of exchange between populations within regions (Table 4.5). In addition, F_{RT}, a measure of between region differentiation, was significant in both *P. velifera* ($F_{RT} = 0.144$, $P < 0.001$) and in *P. petenensis* ($F_{RT} = 0.270$, $P = 0.006$).

TABLE 4.4. Estimates of multilocus pairwise F_{ST} (below diagonal) and R_{ST} (above diagonal) values among populations of *P. velifera* for eight microsatellite loci. Significant values are shown in bold.

	PvC2	PvQR3	PvQR5	PvY1	PvQR2	PvY2	PvY3	PvY4	PvC5
PvC2	-------	**0.661**	**0.787**	**0.097**	**0.818**	**0.610**	0.013	0.052	**0.190**
PvQR3	**0.306**	-------	0.015	**0.315**	**0.103**	0.021	**0.515**	**0.291**	**0.760**
PvQR5	**0.445**	**0.102**	-------	**0.428**	**0.086**	**0.141**	**0.626**	**0.408**	**0.929**
PvY1	**0.087**	**0.168**	**0.252**	-------	**0.518**	**0.244**	0.028	-0.038	0.064
PvQR2	**0.418**	**0.040**	**0.130**	**0.231**	-------	**0.275**	**0.691**	**0.507**	**0.915**
PvY2	**0.233**	**0.197**	**0.199**	**0.072**	**0.245**	-------	**0.458**	**0.234**	**0.737**
PvY3	**0.200**	**0.208**	**0.289**	**0.028**	**0.291**	**0.049**	-------	**0.007**	**-0.1204**
PvY4	**0.214**	**0.099**	**0.237**	0.003	**0.237**	**0.037**	**0.082**	-------	**-0.266**
PvC5	**0.120**	**0.078**	**0.198**	**0.066**	**0.144**	**0.158**	**0.116**	**0.105**	-------

TABLE 4.5. Estimates of multilocus pairwise F_{ST} (below diagonal) and R_{ST} (above diagonal) values among populations of *P. petenensis* for eight microsatellite loci. Significant values are shown in bold.

	PpT3	PpC1	PpC3	PpC2	PpCH1	PpQR4	PpQR7	PpQR1	PpQR8
PpT3	-------	0.018	**0.177**	**0.061**	**0.198**	**0.098**	**0.270**	0.027	**0.195**
PpC1	**0.100**	-------	**0.072**	-0.008	**0.095**	**0.196**	**0.280**	0.045	**0.218**
PpC3	**0.139**	-0.003	-------	**0.140**	-0.019	**0.480**	**0.523**	**0.213**	**0.527**
PpC2	**0.175**	**0.102**	**0.093**	-------	**0.172**	**0.309**	**0.424**	**0.129**	**0.371**
PpCH1	**0.140**	0.002	-0.015	**0.093**	-------	**0.487**	**0.484**	**0.216**	**0.496**
PpQR4	**0.441**	**0.525**	**0.574**	**0.575**	**0.544**	-------	**0.377**	0.110	**0.298**
PpQR7	**0.405**	**0.478**	**0.532**	**0.511**	**0.489**	**0.391**	-------	**0.178**	**0.168**
PpQR1	**0.417**	**0.442**	**0.488**	**0.501**	**0.459**	**0.350**	**0.180**	-------	0.078
PpQR8	**0.469**	**0.528**	**0.589**	**0.584**	**0.543**	**0.357**	**0.226**	**0.294**	-------

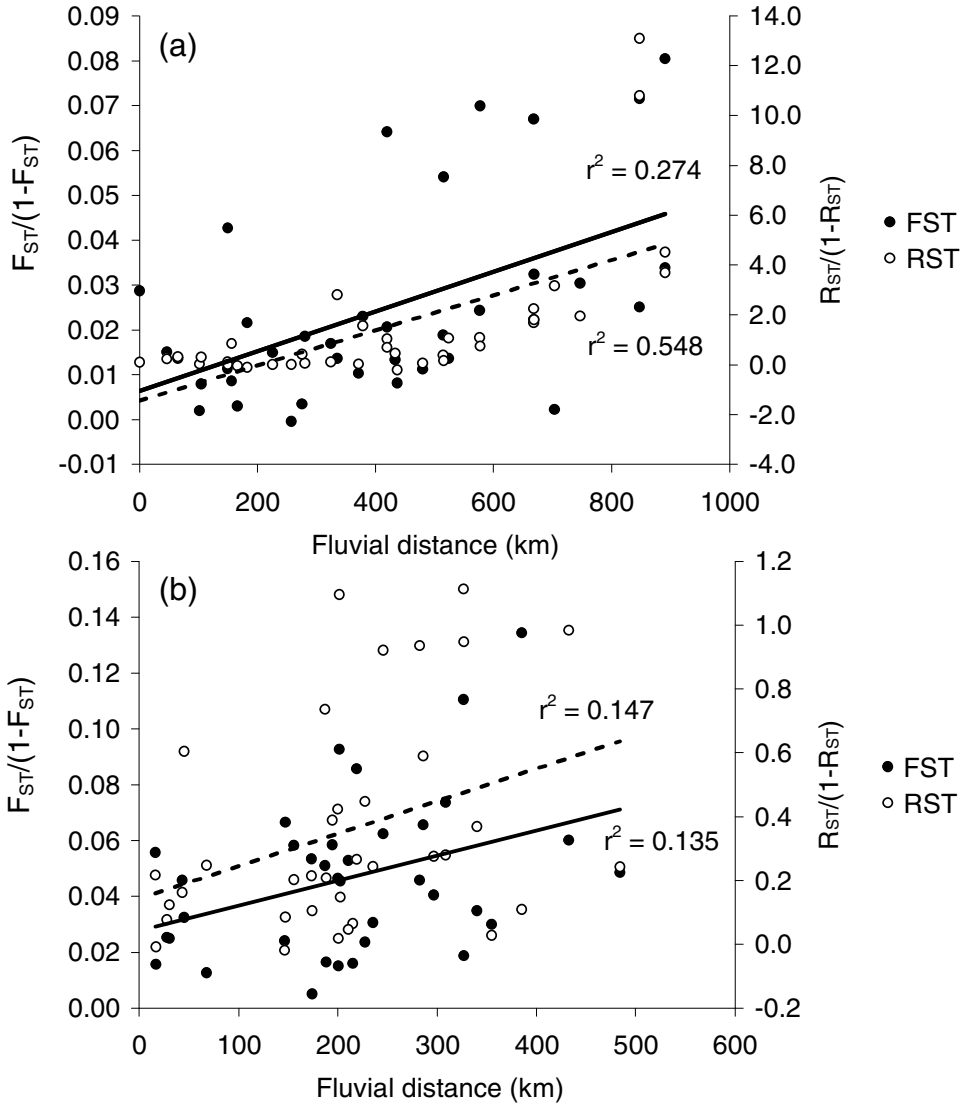

FIG. 4.2. Isolation-by-distance patterns among populations of (a) *P. velifera* and (b) *P. petenensis*, derived from eight microsatellite loci. Plots show genetic distance, as calculated by F_{ST} and R_{ST} against geographic fluvial distance across populations. Lines show the best linear fit to points (F_{ST}- solid, R_{ST} – dashed) dashed). Values for r^2 are from Mantel tests.

TABLE 4.6. AMOVA showing the within and between region variation based on microsatellites in *P. velifera* and *P. petenensis*. Regions are defined as populations within the same geographic region.

	Source of variation	d.f.	Sum of squares	Percentage of variation
P. velifera	Between regions	2	44.4	15.0*
	Between populations within regions	6	20.5	5.9*
	Within populations	317	295.3	79.1
	Total	325	360.2	
P. petenensis	Between regions	2	82.0	27.0*
	Between populations within regions	6	34.0	11.3*
	Within populations	259	236.9	61.7
	Total	267	352.9	

* indicates significance at the $P < 0.001$ level

The BAPS analysis supported the geographic region separation in *P. velifera*, but also suggested additional substructure among populations (Fig. 4.3a). The best partition (posterior probability = 0.767) divided the nine *P. velifera* populations into six clusters: 1) C2, 2) C5, 3) Y1/Y2/Y3, 4) Y4, 5) QR3/QR5, and 6) QR2. Likewise, in *P. petenensis*, the best partition (posterior probability = 0.686) divided the nine populations into five clusters: 1) T3, 2) CH1/C1/C3, 3) C2, 4) QR7/QR1/QR8, and 5) QR4. These clusters also correspond to the division of this species into the three geographic regions, with the exception of some connectivity between two of the three Campeche populations (C1 and C3) and the Chiapas (CH1) population. Barrier analysis (outputting the first three barriers) in *P. velifera* indicated a barrier between the C5 and Y3 (corresponding to a break in these geographic regions), however a second barrier separated QR5 and other populations. A final barrier was placed between Y2 and Y4 (Fig. 4.3a). Barrier analyses in *P. petenensis* provided evidence of barriers between the QR populations, and generally supported an east-west divide between populations, although CH1 was included in the eastern group based on BAPS clustering (Fig. 4.3b). Interestingly, there was no barrier between Tabasco (T3) and Campeche populations (C1, C2, C3) near the coast, despite these being from different geographic regions. It is important to note, however, that small sample sizes in some populations of *P. petenensis*, especially in Quintana Roo (e.g., QR7 and QR8), might have biased the results towards a lack of gene flow (increased barriers) between populations because of missing alleles not detected in my samples. In addition, because D_A was used in

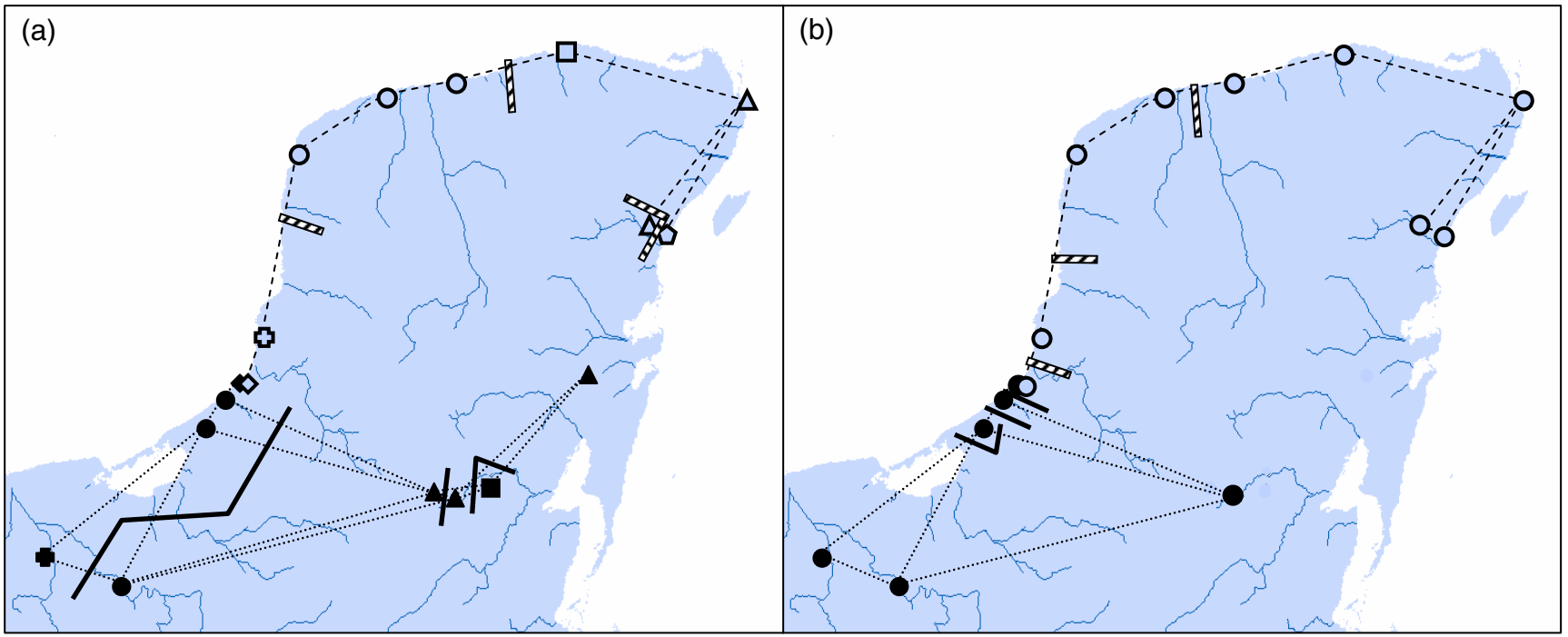

FIG. 4.3. Results of BAPS and barrier analyses examining population structure in *P. velifera* (open symbols) and *P. petenensis* (closed symbols) using genetic (a) and morphological distance (b) (barrier analysis only). Different symbols within a species represent populations classified as separate clusters using BAPS ('a' only). Dashed (*P. velifera*) and dotted (*P. petenensis*) lines show potential connections in barrier analysis. Bars show barriers to gene flow in *P. petenensis* (black) and *P. velifera* (hatched) from barrier analysis.

barrier analysis, while allele frequencies were used to create BAPS clusters, the output from these different types of data may be expected to suggest different clusters. However, this method allows consistent comparisons of D_A and morphology throughout the study.

Comparison of Genetic and Phenotypic Divergence

Despite high levels of morphological divergence between populations in both species (Fig. 4.4; Hankison et al. 2006; see also Chapter 2) and some population differences in mating behaviors in *P. petenensis* (Fig. 4.5; see also Hankison and Ptacek in review; Chapter 3), there was no relationship between any pairwise phenotypic measure (behavior or morphology) and that of the genetic distance measure D_A (Mantel tests, *P. velifera*: $r = -0.696 – 0.374$, $P = 0.257 – 0.841$; *P. petenensis*: $r = -0.364 – 0.557$, $P = 0.164 – 0.366$). In addition, there was no relationship between geographical distance and pairwise phenotypic differences in either morphology or mating behaviors based on the populations included in this study and geographic distances based on GIS measures (Mantel tests, *P. velifera*: $r = -0.391 – -0.021$, $P = 0.447 – 0.676$; *P. petenensis*: $r = -0.493 – 0.670$, $P = 0.169 – 0.506$). These results indicate that the major source of variation among phenotypic traits is not likely the result of the same processes promoting divergence in neutral genetic markers. Finally, in *P. petenensis* there was no correlation between rates of courtship displays or gonopodial thrusts and morphology (Mantel tests, *P. petenensis*: $r = -0.9464 –$.

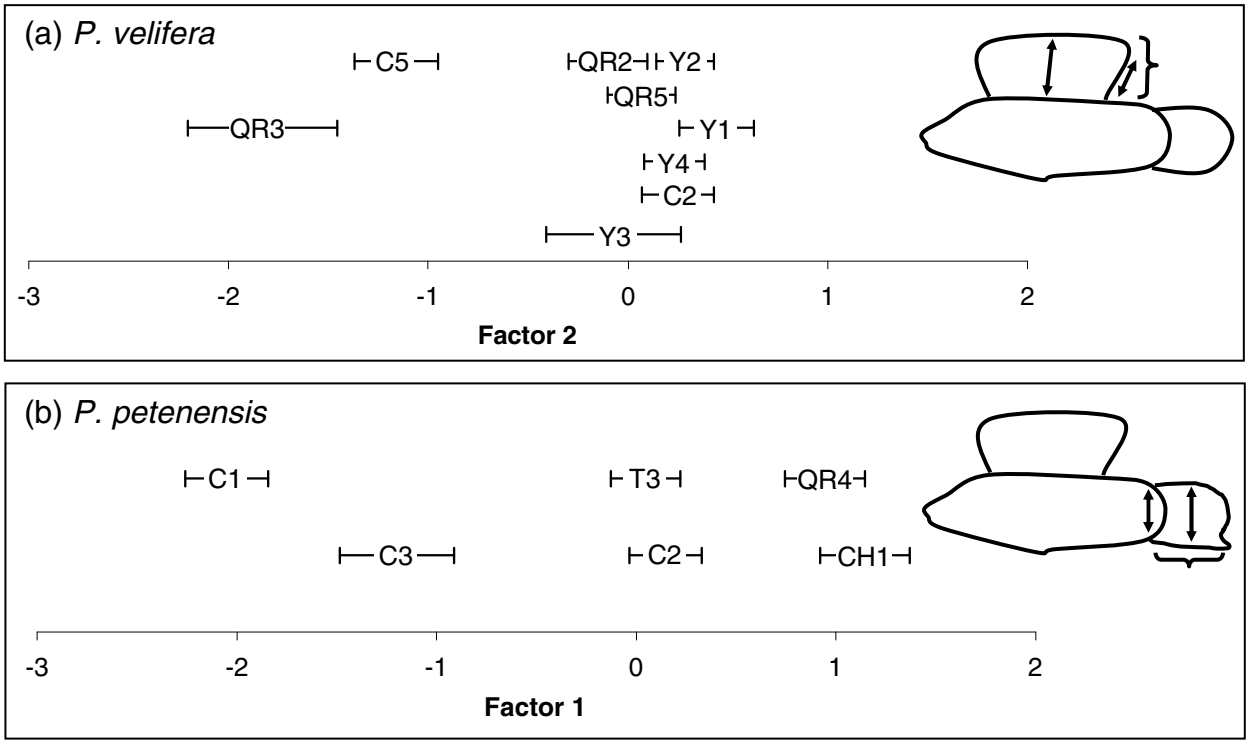

FIG. 4.4. Morphological divergence in *P. velifera* (a) and *P. petenensis* based on the discriminant factor scores of size-corrected linear measurements. Fish diagrams show the most important linear shape variables that distinguish populations (original data in Hankison et al. 2006; Chapter 2).

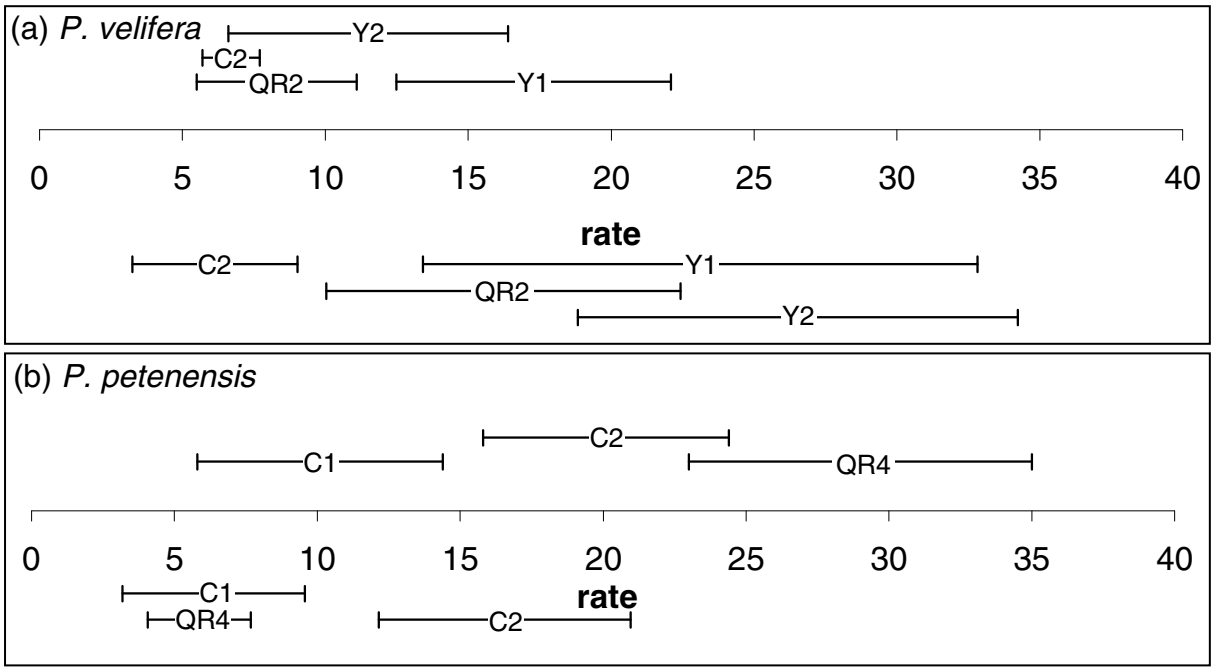

FIG. 4.5. Behavioral differences (± S.E.) within *P. velifera* (a) and *P. petenensis* (b). Symbols above the rate line represent courtship display rate in each population. Symbols below the rate line represent gonopodial thrust rate in each population (original data in Hankison and Ptacek in review; Chapter 3).

0.994, $P = 0.165 – 0.674$). However, in *P. velifera*, morphological distance between populations was positively correlated with courtship display distance (Mantel tests, *P. velifera*: $r = 0.085$, $P = 0.041$). No correlation was found between morphological and behavioral distance for rates of gonopodial thrusts (Mantel tests, *P. velifera*: $r = -0.407$, $P = 0.751$; *P. petenensis*: $r = -0.946$, $P = 0.666$)

Comparisons of MDS plots of genetic and morphological clustering sshow a complete lack of concordance between clusters in morphological space and clusters in genetic space for both species (Fig. 4.6). Finally, although significantly high levels of genetic variance could be explained by differences between regions (AMOVA results, Table 4.5), nested ANOVA showed that considerably less of the variation among populations in morphology could be explained by between-region distinctions in *P. velifera*, and regional differences in morphology were not significant for *P. petenensis* (Table 4.6). Lower levels of regional variation between populations in morphological traits, especially in *P. petenensis*, suggest that morphology is not differentiating between populations purely in response to genetic drift and decreased gene flow between geographic regions.

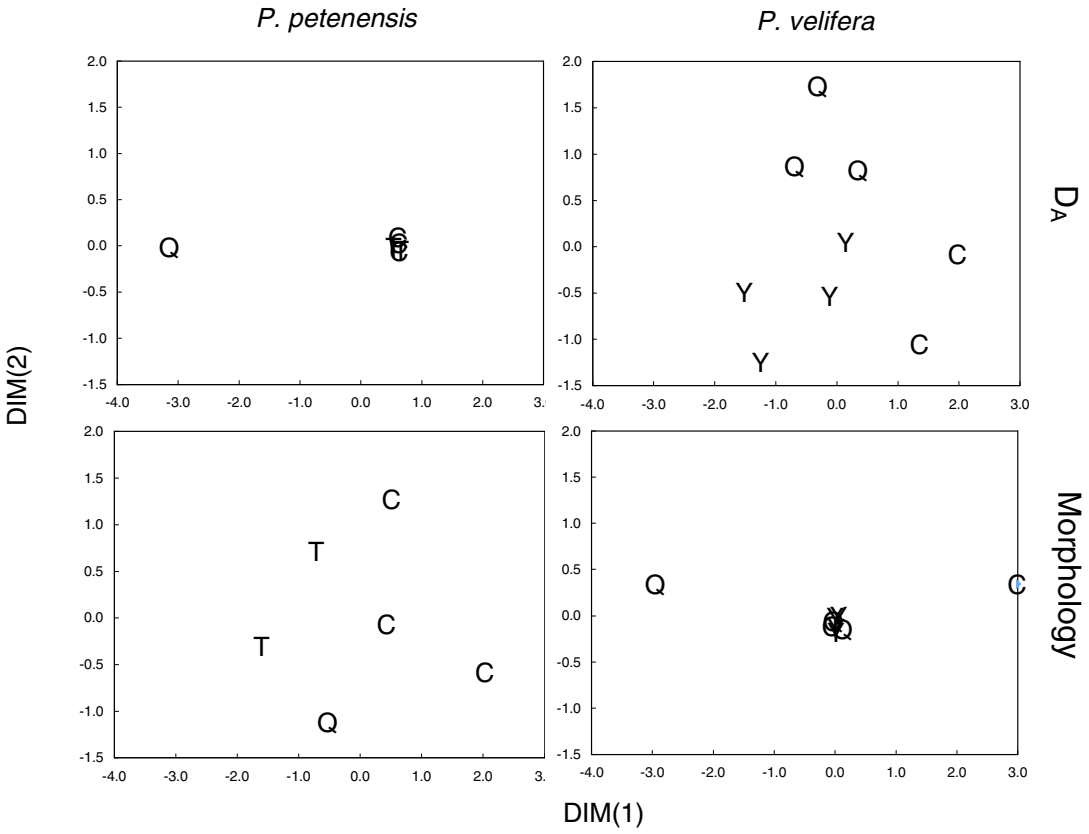

FIG. 4.6. Nonmetric multidimensional scaling plot of genetic divergence (D_A), and morphological distance (size-corrected linear measurements). Populations are coded on the basis of geographic region: C, Campeche; Y, Yucatán; Q: Quintana Roo; T: Tabasco/Chiapas.

TABLE 4.7. Nested ANOVA showing the within and between region variation in morphology (using size corrected linear morphology) in *P. velifera* and *P. petenensis*. Regions are defined as populations within the same geographic regions.

	Source of variation	d.f.	Sum of squares	Percentage of variation
P. velifera	Between regions	2	19.8	9.5*
	Between populations within regions	6	40.6	19.4*
	Within populations	194	148.8	71.1
	Total	202	209.2	
P. petenensis	Among regions	2	1.7	1.3
	Between populations within regions	4	22.2	17.4*
	Within populations	121	103.7	81.3
	Total	127	127.6	

* indicates significance at the $P < 0.001$ level

Discussion

Comparison of patterns of divergence in both neutral genetic and phenotypic (morphological and behavioral) traits indicates that population differences in phenotypic traits, especially between populations of the inland, freshwater species *P. petenensis*, are more likely influenced by selection (both natural and sexual selection) and are not due solely to differentiation as a result of genetic drift or reduced gene flow. While populations differed significantly based on microsatellite variation, these neutral markers showed a strong pattern of isolation by distance and differences in the spatial allocation of variance both within and between geographic regions. Morphological differentiation and, to a lesser degree behavioral variation, was uncorrelated with the level of neutral genetic differentiation and showed much weaker patterns of differentiation based on geographic region. These results suggest that while drift may have played an important role in historical population divergence, selection is likely shaping current patterns of phenotypic differentiation between contemporary molly populations.

Patterns of Genetic Differentiation

Neutral genetic divergence in both *P. velifera* and *P. petenensis* appears to conform to a strong pattern of isolation by distance. Populations were generally genetically distinct, and those within the same geographic region were

more similar to one another than were those from different regions. Such a pattern of regional differentiation suggests that these geographic regions may act as partial barriers to gene flow. Bayesian clustering analysis and a geographic barrier analysis generally supported these results, more often linking populations within a geographic region than those between different regions.

The high level of population differentiation (F_{ST} and R_{ST}) found between populations of *P. velifera* differs from that reported in the U.S. sailfin molly, *P. latipinna*, despite superficial similarities in their habitats. While both species prefer coastal, salt-marsh habitats, *P. latipinna* populations show high levels of gene flow, at least within regions (Trexler 1988; Trexler et al. 1990). There was some evidence for regional variation based on F_{ST} in *P. latipinna*, however, as south Florida populations were genetically differentiated from those in the Panhandle and in Georgia (Trexler 1988). In *P. velifera*, high levels of population differentiation, especially between regions, may be due, in part, to differences in patterns of current flow between the Gulf of Campeche populations and other populations in the Atlantic, corresponding to the different geographic districts in this part of Yucatán (Wilson 1980; Schmitter-Soto et al. 2002).

Populations of *P. petenensis* were also genetically distinct, as may be predicted from their geographically more isolated habitats. While *P. petenensis* primarily lives in freshwater streams and rivers, they may be further isolated to ponds during the dry season because of decreased water levels (Schmitter-Soto et al. 2002). Barriers, BAPS and multi-dimensional scaling all indicated a strong east-west barrier to gene flow in *P. petenensis*, corresponding to geographically

separate river drainages between eastern and western populations that connect only rarely during hurricane events that lead to widespread flooding of the interior of Yucatán (Schmitter-Soto et al. 2002).

In both species, populations from the C2 site were isolated, as determined by Bayesian clustering (but not barrier analysis). A previous study found hybrids at this site between *P. velifera* and a shortfin molly species, *P. mexicana* (Kittell et al. 2005). While levels of hybridization are likely quite low because of prezygotic barriers to gene flow, low levels of introgression between sailfin and shortfin mollies (or potentially between the two species of sailfin mollies) may alter allele frequencies relative to other populations, explaining the separation of the C2 population in both species.

Patterns of Phenotypic and Genetic Diversity

Considerable population differentiation exists between populations of both species for morphological traits, especially those associated with dorsal fins in *P. velifera* and caudal fins in *P. petenensis* (Fig. 4.4; Hankison et al. 2006; Chapter 2). Such a pattern of differentiation is consistent with stronger sexual selection on dorsal fins, which augment the courtship display, in *P. velifera*, where females of this species consistently prefer males or models with larger dorsal fins, even when the signals come from heterospecific sailfin males (*P. petenensis*) (Kozak et al. in review). Potential differences in the strength of female preference for

larger males between different populations might lead to some population differentiation among males in dorsal fin shape.

Population differentiation in *P. petenensis* is more strongly associated with differences in the depth of the caudal peduncle and height and length of the caudal fin (Fig. 4.4). These traits have been shown to be correlated with differences in swimming performance. Fish that live in faster flow environments have deeper caudal peduncles and larger caudal fins, presumably for improved generation of thrust (Langerhans et al. 2003; Langerhans and DeWitt 2004). Divergence in caudal fin characteristics in *P. petenensis* suggests that natural selection may be more important in promoting population differences in morphology in this species. Indeed, males from populations of *P. petenensis* from river habitats (e.g., CH1, T3, QR4) have larger scores for discriminant factor one (longer, taller caudal fins) than those males from karstic sinkhole environments (e.g., C1, C3) (Fig. 4.4). Additionally, female preferences for the largest-sized male, either between species or between different populations of *P. petenensis*, are weaker than in *P. velifera* (Kozak et al. in review; S. Hankison and M. Ptacek unpub. data), suggesting that population-specific shape differences may be more important targets of mating preferences than overall male size in this species.

In contrast to the level of population divergence in male morphology for both species, differentiation among populations in the average rates of male courtship displays and gonopodial thrusts was lower (Fig. 4.5). There was no significant differentiation between populations in either type of mating behavior in

P. velifera, but significant population variation did exist between some populations of *P. petenensis* for both mating behaviors (Hankison and Ptacek in review; Chapter 3). Interestingly, the degree of morphological distance was not correlated to behavioral distance between populations for either behavior in *P. petenensis*. Such a pattern suggests that mating behaviors may evolve to some degree independently of morphological changes. A similar pattern of decoupling of morphology and mating behavior has been described for the sailfin molly, *P. latipinna*, suggesting the opportunity for sexual selection to promote variation in male mating behaviors within the constraints imposed by natural selection on male body shape (Travis 1994; Ptacek and Travis 1997; Ptacek 2005).

In *P. velifera*, morphological distance between populations was positively correlated with courtship display distance, suggesting that male populations with the largest bodies/dorsal fins also exhibit the highest rates of courtship displays. Such a pattern is consistent with variation between populations in the strength of female mating preferences for large courting males, which may arise purely by chance genetic drift (Lande 1981; Kirkpatrick 1982; West-Eberhard 1983; Kirkpatrick and Ravigne 2002).

Overall, there was no correlation between morphological or behavioral phenotypic trait divergences and genetic distance for either species, suggesting that processes other than genetic drift primarily govern phenotypic divergence. This pattern of differences in traits that may be shaped by sexual selection, but are uncorrelated with genetic divergence, fits the pattern observed between phenotypic and genetic traits in some other species (Gleason and Ritchie 1998;

Nicholls et al. 2006; Pröhl et al. 2006). In the satin bowerbird, for example, there is no relationship between vocal divergence (measures of call characteristics) and genetic divergence as measured by microsatellites (Nicholls et al. 2006). In addition, only weak correlations (relative to other comparisons) were found between acoustic distance and genetic distance in the túngara frog, where variation in frog calls was better explained by geographic distance (Pröhl et al. 2006). In both studies, calls, like some phenotypic traits in sailfin mollies, appear to be important in mate choice, and differences in female preferences, at least in the túngara frog, may be important in maintenance of population divergence (Nicholls et al. 2006; Pröhl et al. 2006). In contrast to mollies, however, there is much less differentiation within regions in either satin bowerbirds or túngara frogs (Nicholls et al. 2006; Pröhl et al. 2006), which may relate to the potential constraints of habitat differences (aquatic versus forest, for example), or on the dispersal ability of the organisms being studied.

In contrast to the results based on neutral genetic markers, there was no pattern of isolation by distance in phenotypic traits of either species. For *P. petenensis* the distribution of within and among geographic region variation of morphology did not show a concordant pattern with that obtained from microsatellites. For *P. velifera*, although the morphological variance explained by geographic region was significant, less of the variance was explained by geographic region compared to genetic variance. Populations of *P. velifera* are, however, found in more continuous coastal salt marsh habitats, having weaker barriers to gene flow, and those that do exist may be associated with different

near shore ocean currents that may occur in different geographic regions (Schmitter-Soto et al. 2002). Barriers to gene exchange between populations were more similar based on neutral genetic markers and morphological distance for populations of *P. velifera* than for populations of *P. petenensis*. Habitat differences are greater between populations of *P. petenensis*, which may favor convergent natural selection on male morphology in similar habitats (i.e., faster flowing rivers), even from different geographic regions (e.g., Tabasco and Quintana Roo). Greater homogeneity in habitat characteristics among coastal salt marsh sites may explain lower levels of morphological divergence between populations in this species and a stronger association of morphological divergence with that of geographic regions.

Overall, this study provides evidence that multiple evolutionary mechanisms, including genetic drift, and natural and sexual selection, shape population divergence in the two Mexican sailfin molly species. Unlike in the U.S. sailfin molly, where population differences persist in the face of high gene flow (Trexler 1988; Trexler et al. 1990; Ptacek and Travis 1996, 1997; Ptacek 2005), populations of the Mexican sailfin mollies show higher levels of geographic separation. Differing patterns of divergence between neutral genetic and phenotypic traits, however, suggest that population differences in traits related to mating behaviors and their associated morphological traits (i.e., dorsal fin shape) are not a random result of differentiation as a result of genetic drift, but rather have been shaped by local selection pressures, both natural and sexual selection. There is also some evidence that the selective forces important in

shaping population divergence may have influenced speciation between sailfin mollies in general. For example, morphological differences between populations within *P. velifera* and *P. petenensis* mirror differences important in distinguishing these species, such as dorsal and caudal fin shape (Hankison et al. 2006; Chapter 2). The lack of a relationship between morphology and behavior in *P. petenensis* suggests that both natural and sexual selection are acting on phenotypic divergence, but that their influence may be decoupled. In contrast, strong female preferences for large male size in *P. velifera* (Kozak et al. in review) coupled with population divergence on dorsal fin and body size characteristics (Hankison et al. 2006; Chapter 2) potentially supports an important role for sexual selection in this species.

The separation of the sailfin clade from shortfin ancestors is thought to have been promoted by sexual selection for male courtship and a dimorphic fin that potentially accentuated courtship display (Ptacek and Breden 1998). If similar evolutionary mechanisms shape differences both within and between species (Foster et al. 1998; Foster and Endler 1999; Coyne and Orr 2004), this study provides further evidence of the potential role of sexual selection as an important force in the evolution of mating signals and speciation in the sailfin mollies. In addition, a role for natural selection in shaping morphology *P. petenensis* is also suggested, as morphological divergence in this species was not predicted by genetic divergence. While this study supports the importance of natural and sexual selection in shaping population divergence in *P. velifera* and *P. petenensis*, additional studies investigating the level of divergence in female

mating preferences within each species will provide additional insight into how sexual selection may promote differences in morphology and behavior and potentially lead to speciation in this intriguing group of poeciliid fishes.

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APPENDICES

APPENDIX A. *Poecilia petenensis* microsatellite alleles

Multi-locus genotypes for 8 microsatellite loci from 134 *P. petenensis* sampled from nine populations. Individual genotypes are reported as length of each allele in base pairs. Question marks signify unidentified alleles.

		Microsatellite Locus															
ID	Pop	G10		CA25		CA34		Pr172		G49		Pr80		Pr92		CA120	
342	T3	189	189	113	113	116	116	176	176	168	190	102	102	149	149	108	108
343	T3	189	189	113	113	116	116	176	176	184	188	100	102	149	149	102	108
344	T3	189	189	113	113	116	116	176	176	182	190	100	104	147	147	108	108
345	T3	189	189	113	113	116	116	176	176	180	180	100	102	143	147	102	108
346	T3	189	189	113	113	116	116	176	176	168	190	100	102	147	149	108	108
347	T3	189	189	113	113	116	116	176	176	190	190	100	102	149	149	102	108
348	T3	189	189	113	113	116	116	176	176	190	190	100	100	147	147	108	108
349	T3	189	189	113	113	116	116	176	176	168	188	100	100	147	149	106	108
350	T3	189	189	113	113	116	116	176	176	188	188	100	102	147	149	102	108
351	T3	189	189	113	113	116	116	176	176	168	168	100	102	147	149	102	102
352	T3	189	189	113	113	116	124	176	176	168	182	100	102	149	149	108	108
353	T3	189	189	113	113	116	116	176	176	184	190	100	100	147	149	102	102
354	T3	189	189	113	113	116	116	176	176	188	190	100	102	143	143	102	108
355	T3	189	189	113	113	116	116	176	176	188	190	100	102	143	143	102	108
356	T3	189	189	113	113	116	116	176	176	188	190	98	100	143	?	102	108
357	T3	189	189	113	113	116	116	176	176	184	184	100	100	149	149	102	108
358	T3	189	189	113	113	116	116	176	176	168	188	100	100	143	149	108	108
359	T3	189	189	113	113	116	116	176	176	182	188	100	100	147	149	102	108
360	T3	189	189	113	113	116	116	176	176	182	190	100	100	147	149	102	108
361	T3	189	189	113	113	116	116	176	176	182	188	100	100	149	149	102	108
427	C1	189	189	113	113	116	116	176	176	168	190	100	102	147	149	102	108
428	C1	189	189	113	113	116	116	176	176	190	190	100	102	149	149	102	102
429	C1	189	189	113	113	116	116	176	176	182	182	100	102	149	149	102	108
430	C1	189	189	113	113	116	124	176	176	188	190	100	100	149	149	102	102
431	C1	189	189	113	113	116	116	176	176	168	190	102	102	149	149	102	108

APPENDIX A.
Continued.

ID	Pop	Microsatellite Locus															
		G10		CA25		CA34		Pr172		G49		Pr80		Pr92		CA120	
432	C1	189	189	113	113	116	116	176	176	188	192	?	?	149	149	102	102
433	C1	189	189	113	113	116	116	176	176	168	168	100	102	149	149	102	102
434	C1	189	189	113	113	116	116	176	176	182	190	100	102	149	149	102	102
435	C1	189	189	113	113	116	116	176	176	168	182	100	102	147	149	102	102
436	C1	189	189	113	113	116	116	176	176	190	190	102	102	149	149	102	108
437	C1	189	189	113	113	116	116	176	176	190	190	102	102	147	149	102	108
438	C1	189	189	113	113	116	116	176	176	182	190	100	102	149	149	102	108
439	C1	189	189	113	113	116	116	176	176	188	190	100	102	149	149	102	102
440	C1	189	189	113	113	116	116	176	176	184	190	100	102	147	149	102	108
441	C1	189	189	113	113	116	116	176	176	188	190	102	102	149	149	102	102
442	C1	189	189	113	113	116	116	176	176	182	190	100	102	149	149	102	102
443	C1	189	189	113	113	116	116	?	?	168	182	100	102	149	149	108	108
444	C1	189	189	113	113	116	116	176	176	168	190	100	102	149	149	102	108
445	C1	189	189	113	113	116	116	176	176	182	188	100	102	147	149	102	108
446	C1	189	189	113	113	116	116	176	176	188	190	100	100	149	149	102	102
457	C3	189	189	113	113	116	116	176	176	182	184	102	102	149	149	102	108
458	C3	189	189	113	113	116	116	?	?	188	190	100	100	147	149	102	108
459	C3	189	189	113	113	116	116	?	?	182	190	100	102	147	149	102	102
460	C3	189	189	113	113	116	116	176	176	188	190	100	102	149	149	102	102
461	C3	189	189	113	113	116	116	176	176	188	190	102	102	147	149	102	102
462	C3	189	189	113	113	116	116	176	176	182	190	?	?	149	149	102	102
462	C3	189	189	113	113	116	116	176	176	190	190	100	102	147	149	102	102
463	C3	189	189	113	113	116	116	176	176	190	190	102	102	149	149	102	108
463	C3	189	189	113	113	116	116	176	176	184	188	100	100	149	149	102	102
464	C3	189	189	113	113	116	116	176	176	190	190	?	?	149	149	102	102
464	C3	189	189	113	113	116	116	176	176	182	184	100	102	149	149	102	102
465	C3	189	189	113	113	116	116	176	176	188	190	100	100	149	149	108	108

APPENDIX A.
Continued.

ID	Pop	Microsatellite Locus															
		G10		CA25		CA34		Pr172		G49		Pr80		Pr92		CA120	
465	C3	189	189	113	113	116	116	176	176	188	190	100	100	149	149	102	102
466	C3	189	189	113	113	116	116	176	176	188	190	100	102	149	149	102	102
466	C3	189	189	113	113	116	116	176	176	188	190	102	102	147	149	102	102
550	C3	189	189	113	113	116	116	176	176	182	182	100	100	149	149	102	102
551	C3	189	189	113	113	116	116	176	176	190	190	102	102	149	149	102	102
552	C3	189	189	113	113	116	116	176	176	182	182	100	102	149	149	102	102
553	C3	189	189	113	113	116	116	176	176	188	190	102	102	147	149	102	102
554	C3	189	189	113	113	116	116	176	176	182	184	100	102	149	149	102	108
467	C2	189	191	113	113	116	116	176	176	182	184	100	102	149	149	102	102
468	C2	189	191	113	113	116	116	176	176	168	188	100	100	149	149	102	102
469	C2	189	189	113	113	116	116	176	176	182	188	100	100	149	149	102	102
470	C2	189	189	113	113	116	116	176	176	182	190	100	100	149	149	102	102
471	C2	189	191	113	113	116	116	176	176	182	188	100	102	149	149	102	102
472	C2	189	191	113	113	116	116	176	176	168	188	100	102	147	149	102	102
473	C2	189	191	113	113	116	116	176	176	184	188	100	102	149	149	102	108
474	C2	189	191	113	113	116	116	176	176	182	190	100	100	149	149	102	102
475	C2	189	189	113	113	116	116	176	176	182	182	100	100	147	149	102	102
476	C2	189	189	113	113	116	116	176	176	168	188	100	100	147	149	102	108
477	C2	189	189	113	113	116	116	176	176	188	190	100	102	149	149	102	102
478	C2	189	189	113	113	116	116	176	176	188	188	100	102	149	149	102	102
479	C2	189	189	113	113	116	116	176	176	168	188	102	102	149	149	102	102
480	C2	189	191	113	113	116	116	176	176	188	188	100	100	149	149	102	102
481	C2	191	191	113	113	116	116	?	?	190	190	100	102	147	149	102	102
482	C2	189	191	113	113	116	116	176	176	168	182	100	102	149	149	102	102
483	C2	189	191	113	113	116	116	176	176	182	182	100	100	149	149	102	102
484	C2	191	191	113	113	116	116	176	176	168	190	100	102	149	149	102	102
485	C2	189	191	113	113	116	116	176	176	188	188	100	102	149	149	102	108

APPENDIX A.
Continued.

ID	Pop	Microsatellite Locus															
		G10		CA25		CA34		Pr172		G49		Pr80		Pr92		CA120	
486	C2	189	189	113	113	116	116	176	176	182	190	100	100	149	149	102	108
A	CH1	189	189	113	113	116	116	?	?	188	188	100	102	149	149	102	102
B	CH1	189	189	113	113	116	116	176	176	188	190	100	102	149	149	102	108
C	CH1	189	189	113	113	116	116	176	176	184	190	100	102	149	149	102	108
D	CH1	189	189	113	113	116	116	176	176	184	188	100	100	149	149	102	108
E	CH1	189	189	113	113	116	116	176	176	188	190	100	100	147	149	102	102
F	CH1	189	189	113	113	116	116	176	176	182	190	100	102	147	149	102	108
G	CH1	189	189	113	113	116	116	176	176	182	190	102	102	149	149	102	102
H	CH1	189	189	113	113	116	116	176	176	182	190	102	102	149	149	102	102
I	CH1	189	189	113	113	116	116	176	176	182	188	102	102	149	149	102	102
J	CH1	189	189	113	113	116	116	176	176	190	192	98	102	149	149	102	108
K	CH1	189	189	113	113	116	116	176	176	188	190	100	100	149	149	102	102
L	CH1	189	189	113	113	116	116	176	176	188	190	96	102	149	149	102	102
M	CH1	189	189	113	113	116	116	176	176	188	190	102	102	149	149	102	102
N	CH1	189	189	113	113	116	116	176	176	182	184	102	102	149	151	102	102
O	CH1	189	189	113	113	116	116	176	176	184	192	100	102	149	149	102	102
P	CH1	189	189	113	113	116	116	176	176	182	190	100	100	149	149	102	108
Q	CH1	189	189	113	113	116	116	176	176	184	188	100	102	149	149	102	108
R	CH1	189	189	113	113	116	116	176	176	190	192	100	102	147	149	102	102
S	CH1	189	189	113	113	116	116	176	176	188	190	100	102	149	149	102	102
T	CH1	189	189	113	113	116	116	176	176	188	190	102	102	149	151	102	102
518	QR4	189	189	113	113	118	118	176	176	188	190	102	102	149	149	108	108
519	QR4	189	189	113	113	118	118	176	176	188	190	102	102	143	143	108	108
520	QR4	191	191	113	113	?	?	?	?	?	?	102	102	143	143	106	108
521	QR4	191	191	113	113	118	118	?	?	?	?	102	102	143	143	106	108
522	QR4	189	189	113	113	118	118	?	?	190	190	102	102	143	143	108	108
523	QR4	189	189	113	113	118	118	176	176	184	184	102	102	143	143	106	108

APPENDIX A.
Continued.

ID	Pop	Microsatellite Locus															
		G10		CA25		CA34		Pr172		G49		Pr80		Pr92		CA120	
524	QR4	189	191	113	113	118	126	176	176	188	190	102	102	149	149	108	108
525	QR4	189	189	113	113	118	118	176	176	190	190	102	102	143	149	108	108
526	QR4	189	189	113	113	118	118	?	?	164	164	102	102	143	143	108	108
527	QR4	189	189	113	113	118	118	?	?	188	190	102	102	143	143	108	108
69	QR7	189	189	113	113	118	118	?	?	188	192	100	100	149	149	108	108
70	QR7	189	189	113	113	118	118	?	?	188	192	100	102	149	149	106	108
71	QR7	189	189	113	113	118	118	?	?	188	188	102	102	149	149	108	108
72	QR7	189	189	113	113	118	118	176	176	188	192	100	102	149	149	108	108
73	QR7	189	189	113	113	118	118	?	?	192	192	102	102	149	149	108	108
74	QR7	189	189	113	113	118	118	?	?	188	192	100	102	149	149	108	108
75	QR7	189	189	113	113	118	118	?	?	188	192	100	100	149	149	106	106
76	QR7	189	189	113	113	118	118	176	176	188	188	102	102	149	149	106	108
163	QR1	189	189	113	113	118	118	?	?	188	190	102	102	149	149	106	106
164	QR1	189	189	113	113	118	118	?	?	188	190	102	102	149	149	106	108
165	QR1	189	189	113	113	118	118	176	176	188	190	102	102	149	149	106	108
166	QR1	189	189	113	113	118	118	176	176	190	190	102	102	149	149	106	108
167	QR1	189	189	113	113	118	118	?	?	164	164	102	102	149	149	106	108
168	QR1	189	189	113	113	118	118	176	176	184	190	102	102	149	149	108	108
268	QR1	189	189	113	113	118	118	176	176	184	190	100	100	149	149	106	106
269	QR1	189	189	113	113	118	118	?	?	174	176	?	?	149	149	106	106
270	QR1	189	189	113	113	118	118	?	?	186	190	102	102	149	149	106	108
271	QR1	189	189	113	113	118	118	?	?	188	190	100	100	149	149	104	106
272	QR1	189	189	113	113	118	118	?	?	188	190	100	100	149	149	104	106
380	QR8	189	189	113	113	118	118	176	176	188	188	102	102	149	149	108	108
381	QR8	189	189	113	113	118	118	176	176	188	188	102	102	149	149	108	108

APPENDIX A.
Continued.

ID	Pop	Microsatellite Locus															
		G10		CA25		CA34		Pr172		G49		Pr80		Pr92		CA120	
382	QR8	189	189	113	113	118	118	176	176	190	190	102	102	149	149	108	108
383	QR8	189	189	113	113	118	118	176	176	188	190	102	102	149	149	108	108
384	QR8	189	189	113	113	118	118	176	176	188	188	102	102	149	149	108	108

APPENDIX B. *Poecilia velifera* microsatellite alleles

Multi-locus genotypes for 8 microsatellite loci from 163 *P. velifera* sampled from nine populations. Individual genotypes are reported as length of each allele in base pairs. Question marks signify unidentified alleles.

		Microsatellite Locus															
		G10		CA25		CA34		Pr172		G49		Pr80		Pr92		CA120	
C2	322	191	191	115	115	126	126	176	176	164	164	?	?	143	143	108	108
C2	323	191	191	115	115	126	126	176	176	164	164	?	?	147	147	108	108
C2	324	191	191	115	115	118	126	176	176	164	164	102	102	147	147	108	108
C2	325	191	191	115	115	126	126	176	176	168	168	102	102	145	149	102	108
C2	326	191	191	115	115	126	126	?	?	164	164	?	?	147	149	108	108
C2	327	191	191	115	115	126	126	176	176	164	164	?	?	143	149	108	108
C2	328	191	191	115	115	118	118	?	?	172	178	102	102	143	147	108	108
C2	329	191	191	115	115	126	126	?	?	172	188	?	?	143	147	108	108
C2	330	191	191	115	115	126	126	?	?	164	164	?	?	143	147	108	108
C2	331	191	191	115	115	126	126	?	?	188	188	?	?	143	147	108	108
C2	332	191	191	115	115	126	126	176	176	168	176	102	102	143	143	108	108
C2	333	191	191	115	115	126	126	?	?	164	176	102	102	147	149	108	108
C2	334	191	191	115	115	126	126	176	176	164	184	?	?	143	149	108	108
C2	335	191	191	115	115	118	126	176	176	164	164	100	100	143	143	108	108
C2	336	191	191	115	115	126	126	176	176	164	184	?	?	143	147	108	108
C2	337	191	191	115	115	126	126	176	176	164	184	100	102	143	147	108	108
C2	338	191	191	115	115	118	126	?	?	164	184	?	?	143	149	108	108
C2	339	191	191	115	115	118	126	?	?	164	190	102	102	147	147	108	108
C2	340	191	191	115	115	126	126	176	176	176	188	100	102	143	149	108	108
C2	341	191	191	115	115	126	126	176	176	168	176	?	?	143	147	108	108

APPENDIX B.
Continued.

		Microsatellite Locus															
		G10		CA25		CA34		Pr172		G49		Pr80		Pr92		CA120	
QR3	385	191	191	115	115	126	126	?	?	150	150	?	?	143	?	106	108
QR3	386	191	191	115	115	118	126	?	?	148	148	?	?	143	143	106	106
QR3	387	191	191	115	115	126	128	?	?	150	150	?	?	139	143	106	106
QR3	388	191	191	115	115	126	128	?	?	?	?	?	?	143	143	106	106
QR3	389	191	191	115	115	126	126	?	?	148	148	?	?	143	147	106	108
QR3	390	191	191	115	115	126	126	?	?	154	154	?	?	143	143	106	108
QR3	391	191	191	115	115	126	126	?	?	148	148	?	?	143	143	100	106
QR3	392	191	191	115	115	126	126	176	176	148	154	98	100	149	149	106	108
QR3	393	191	191	115	115	126	126	?	?	150	150	?	?	143	145	106	106
QR3	394	191	191	115	115	126	126	?	?	144	144	?	?	143	143	106	108
QR3	395	191	191	115	115	126	126	?	?	150	154	?	?	139	143	106	108
QR3	396	191	191	115	115	126	126	?	?	154	154	102	102	143	143	106	108
QR3	397	191	191	115	115	126	126	?	?	188	188	?	?	143	145	106	106
QR3	398	191	191	115	115	118	126	?	?	150	150	?	?	145	145	106	106
QR3	399	191	191	115	115	124	126	?	?	150	150	?	?	139	143	106	106
QR3	400	191	191	115	115	126	126	?	?	154	154	?	?	139	145	106	108
QR3	401	191	191	115	115	126	126	?	?	150	150	?	?	143	145	106	106
QR3	402	191	191	115	115	126	126	?	?	148	154	?	?	143	145	106	108
QR3	403	191	191	115	115	126	126	?	?	144	148	?	?	143	143	106	108
QR3	404	191	191	115	115	126	126	?	?	150	150	?	?	143	143	106	108
QR5	539	191	191	115	115	126	126	176	176	150	150	102	102	143	143	106	108
QR5	540	191	191	115	115	126	126	?	?	150	150	?	?	143	143	106	106

APPENDIX B.
Continued.

		Microsatellite Locus															
		G10		CA25		CA34		Pr172		G49		Pr80		Pr92		CA120	
QR5	541	191	191	115	115	126	126	?	?	150	150	90	90	143	143	106	108
QR5	542	191	191	115	115	126	126	?	?	148	150	?	?	143	143	106	108
QR5	543	191	191	115	115	126	126	?	?	150	150	?	?	143	143	106	106
QR5	544	191	191	115	115	126	126	?	?	150	150	?	?	143	143	106	108
QR5	545	191	191	115	115	126	126	?	?	150	150	?	?	143	143	106	108
QR5	546	191	191	115	115	126	126	?	?	150	150	102	102	143	143	106	108
QR5	547	191	191	115	115	126	126	?	?	150	150	?	?	143	143	106	108
QR5	549	191	191	115	115	126	126	?	?	148	148	96	100	143	143	106	108
QR5	702	191	191	115	115	126	126	?	?	150	150	96	96	143	143	106	108
QR5	703	191	191	115	115	126	126	?	?	150	150	94	94	143	143	106	108
QR5	704	191	191	115	115	126	126	?	?	150	150	102	102	143	143	106	106
Y1	405	191	191	115	115	126	126	?	?	144	144	?	?	147	149	102	108
Y1	406	191	191	115	115	126	126	?	?	150	150	?	?	143	143	108	108
Y1	407	191	191	115	115	126	126	?	?	184	190	?	?	143	145	108	108
Y1	408	191	191	115	115	126	126	?	?	184	190	?	?	139	143	108	108
Y1	409	193	193	115	115	126	126	?	?	160	164	?	?	147	147	108	108
Y1	410	191	191	115	115	126	126	176	176	184	190	?	?	143	149	106	108
Y1	411	191	191	115	115	126	126	?	?	162	178	?	?	145	149	102	108
Y1	412	191	193	115	115	126	126	?	?	162	164	?	?	143	149	108	108
Y1	413	191	191	115	115	126	126	176	176	150	150	?	?	143	149	106	108
Y1	414	191	191	115	115	126	126	176	176	164	184	?	?	139	139	108	108
Y1	415	191	193	115	115	126	126	?	?	184	190	?	?	145	149	106	108

APPENDIX B.
Continued.

		Microsatellite Locus															
		G10		CA25		CA34		Pr172		G49		Pr80		Pr92		CA120	
Y1	416	191	191	115	115	126	126	?	?	150	150	?	?	135	149	106	108
Y1	417	191	191	115	115	126	126	?	?	158	158	?	?	143	145	106	108
Y1	418	191	193	115	115	126	126	?	?	150	164	?	?	?	?	108	108
Y1	419	191	191	115	115	126	126	?	?	150	164	?	?	143	149	108	108
Y1	420	191	193	115	115	126	126	?	?	162	164	?	?	143	149	108	108
Y1	421	191	191	115	115	126	126	?	?	158	184	?	?	145	149	106	108
Y1	422	191	191	115	115	126	126	?	?	158	184	?	?	145	149	106	108
Y1	423	191	191	115	115	126	126	?	?	150	150	?	?	143	145	108	108
Y1	424	191	191	115	115	126	126	?	?	160	160	?	?	143	143	108	108
QR2	302	191	191	115	115	126	126	176	176	150	150	?	?	143	143	106	108
QR2	303	191	191	115	115	126	126	?	?	144	144	92	92	143	143	106	106
QR2	304	191	191	115	115	126	126	?	?	144	144	94	94	143	143	106	106
QR2	305	191	191	115	115	126	126	?	?	154	154	?	?	143	143	106	108
QR2	306	191	191	115	115	126	126	?	?	144	150	?	?	143	143	106	106
QR2	307	191	191	115	115	126	126	176	176	144	144	100	102	139	143	106	106
QR2	308	191	191	115	115	126	126	?	?	150	150	94	94	143	143	106	108
QR2	309	191	191	115	115	126	126	?	?	150	150	?	?	143	143	102	102
QR2	310	191	191	113	115	126	126	174	176	154	154	102	102	143	143	106	106
QR2	311	191	191	115	115	126	126	?	?	150	150	102	102	143	143	106	106
QR2	312	191	191	115	115	126	126	?	?	150	150	102	102	143	143	106	106
QR2	314	191	191	115	115	126	126	?	?	144	144	?	?	143	143	106	106
QR2	315	191	191	115	115	126	126	?	?	144	150	94	94	143	143	102	102

APPENDIX B.
Continued.

		Microsatellite Locus															
		G10		CA25		CA34		Pr172		G49		Pr80		Pr92		CA120	
QR2	316	191	191	115	115	126	126	?	?	150	150	94	94	143	143	106	106
QR2	317	191	191	115	115	126	126	176	176	150	150	100	102	139	143	102	102
QR2	318	191	191	115	115	126	126	?	?	150	150	94	94	143	143	106	108
QR2	319	191	191	115	115	126	126	?	?	144	150	?	?	143	143	106	108
QR2	320	191	191	115	115	126	126	?	?	144	144	?	?	139	143	106	106
QR2	321	191	191	115	115	126	126	?	?	144	150	?	?	143	143	106	108
QR2	322	191	191	115	115	126	126	?	?	144	150	94	94	139	143	106	106
Y2	487	191	193	115	115	126	128	?	?	144	174	?	?	143	145	108	108
Y2	488	191	191	115	115	126	126	?	?	150	160	?	?	143	143	108	108
Y2	489	191	191	115	115	126	126	?	?	150	160	?	?	143	143	106	108
Y2	490	191	193	115	115	126	126	?	?	174	174	?	?	143	145	102	108
Y2	491	193	193	115	115	126	126	176	176	150	160	?	?	143	145	108	108
Y2	492	191	191	115	115	126	126	176	176	150	150	100	100	143	143	106	108
Y2	493	191	193	115	115	126	126	176	176	150	160	?	?	143	145	108	108
Y2	494	191	191	115	115	126	126	?	?	150	150	?	?	143	145	108	108
Y2	495	191	191	115	115	126	126	176	176	150	150	?	?	143	145	106	108
Y2	496	191	191	115	115	126	126	?	?	144	150	?	?	143	143	108	108
Y2	497	191	191	115	115	126	126	?	?	150	164	?	?	149	151	106	108
Y2	498	193	193	115	115	126	126	?	?	150	150	?	?	143	145	108	108
Y2	499	191	193	115	115	126	126	176	176	150	164	?	?	143	143	102	108
Y2	500	193	193	115	115	126	126	176	176	150	150	?	?	145	145	108	108
Y2	501	191	191	115	115	126	126	176	176	150	150	?	?	145	145	108	108

APPENDIX B.
Continued.

		Microsatellite Locus															
		G10		CA25		CA34		Pr172		G49		Pr80		Pr92		CA120	
Y2	502	191	191	115	115	126	126	?	?	150	150	?	?	143	143	106	108
Y2	503	191	191	115	115	126	126	?	?	150	174	?	?	143	143	108	108
Y2	504	191	191	115	115	126	126	?	?	150	150	106	106	143	149	108	108
Y2	505	191	191	115	115	126	126	?	?	150	150	?	?	145	145	108	108
Y2	506	191	191	115	115	126	126	?	?	150	174	?	?	145	147	102	108
Y3	681	191	191	115	115	126	126	?	?	150	160	?	?	143	145	106	108
Y3	682	191	191	115	115	126	126	?	?	150	178	?	?	145	145	108	108
Y3	683	193	193	115	115	126	126	?	?	150	178	?	?	143	143	108	108
Y3	684	191	193	115	115	126	126	?	?	166	184	92	94	143	143	108	108
Y3	685	193	193	115	115	126	126	?	?	158	160	?	?	143	149	108	108
Y3	686	193	193	115	115	126	126	?	?	170	170	?	?	143	145	106	108
Y3	687	193	193	115	115	126	126	?	?	184	184	?	?	143	149	102	108
Y3	688	191	191	115	115	126	126	?	?	150	160	100	100	143	143	106	108
Y3	689	191	193	109	115	126	126	?	?	184	170	96	96	143	143	108	108
Y3	690	191	191	115	115	126	126	?	?	176	184	90	90	143	145	106	108
Y3	691	193	193	115	115	126	126	?	?	150	190	?	?	143	151	108	108
Y3	692	191	193	115	115	126	126	?	?	184	186	96	96	143	143	108	108
Y3	693	191	193	115	115	126	126	?	?	172	174	?	?	143	149	108	108
Y3	694	191	191	115	115	126	126	176	176	176	186	?	?	143	145	108	108
Y3	695	191	191	115	115	126	126	176	176	150	174	?	?	143	145	108	108
Y3	697	191	191	115	115	126	126	?	?	150	184	?	?	145	149	102	108
Y3	698	191	191	115	115	126	126	?	?	150	184	?	?	143	145	108	108

APPENDIX B.
Continued.

		Microsatellite Locus															
		G10		CA25		CA34		Pr172		G49		Pr80		Pr92		CA120	
Y4	731	191	191	115	115	126	128	?	?	150	150	102	102	143	149	108	108
Y4	732	191	191	?	?	126	126	?	?	176	176	?	?	?	?	106	108
Y4	733	191	191	111	115	126	126	?	?	184	194	?	?	145	149	106	108
Y4	734	191	191	115	115	126	126	?	?	150	150	94	94	139	149	106	108
Y4	735	191	191	115	115	126	126	?	?	190	198	102	102	143	143	106	108
Y4	736	191	193	115	115	126	126	?	?	150	150	?	?	143	145	106	108
Y4	737	191	191	115	115	126	126	176	176	150	160	94	102	139	145	106	106
Y4	738	191	191	115	115	126	126	?	?	150	150	102	102	143	145	106	108
Y4	739	193	193	115	115	126	126	?	?	180	188	?	?	145	149	106	108
Y4	740	191	191	115	115	126	126	?	?	184	200	102	102	145	145	108	108
Y4	741	191	193	115	115	126	126	?	?	160	170	?	?	143	145	108	108
Y4	742	191	191	115	115	126	126	?	?	150	150	102	102	143	145	106	108
Y4	743	193	193	115	115	126	126	?	?	150	150	102	102	139	149	108	108
C5	711	191	191	109	115	126	130	?	?	186	190	?	?	143	147	106	108
C5	712	191	191	109	115	126	130	?	?	188	190	?	?	143	143	106	108
C5	713	191	191	109	115	126	126	?	?	190	?	92	92	143	143	108	108
C5	714	191	191	115	115	126	126	?	?	164	?	102	102	143	143	106	108
C5	715	191	191	115	115	126	126	?	?	176	?	102	102	143	143	106	108
C5	716	191	191	115	115	126	126	?	?	164	?	102	102	143	147	106	108
C5	717	191	191	115	115	126	126	?	?	186	?	102	102	143	143	106	108
C5	718	191	191	109	115	126	126	?	?	164	?	102	102	143	147	106	108
C5	719	191	191	115	115	126	128	?	?	188	190	102	102	143	143	108	108

APPENDIX B.
Continued.

		Microsatellite Locus															
		G10		CA25		CA34		Pr172		G49		Pr80		Pr92		CA120	
C5	720	191	191	115	115	126	128	176	176	188	190	?	?	143	143	106	106
C5	721	191	191	115	115	124	126	?	?	?	?	?	?	143	143	102	108
C5	722	191	191	115	115	124	126	?	?	164	?	?	?	143	143	108	108
C5	723	191	191	115	115	126	126	?	?	164	?	?	?	143	143	106	108
C5	724	191	191	115	115	126	128	176	176	164	?	?	?	143	147	106	108
C5	725	191	191	113	113	126	126	?	?	164	?	?	?	143	143	106	108
C5	726	191	191	113	113	126	126	?	?	190	?	?	?	143	147	108	108
C5	727	191	191	115	115	126	126	?	?	186	186	?	?	143	143	106	108
C5	728	191	191	113	115	126	126	?	?	170	170	?	?	143	143	108	108
C5	729	191	191	115	115	124	126	?	?	164	164	?	?	143	143	106	106
C5	730	191	191	115	115	126	126	?	?	188	190	?	?	?	?	108	108

APPENDIX C. Geographic distances for *P. velifera*

Distances (km) around the coast between populations of *P. velifera* on the Yucatán Peninsula of Mexico. Abbreviations: C: Campeche; Y: Yucatán; QR: Quintana Roo.

	Geographical Distance									
	C4	C2	C5	Y3	Y1	Y2	Y4	QR3	QR2	QR5
C4	0									
C2	73.09	0								
C5	118.00	46.32	0							
Y3	298.06	226.38	183.12	0						
Y1	396.06	324.38	281.12	101.73	0					
Y2	450.32	378.65	335.38	155.99	64.91	0				
Y4	551.70	480.02	436.76	257.37	166.29	104.59	0			
QR3	818.62	746.94	703.68	524.29	433.21	371.51	275.91	0		
QR2	962.26	890.58	847.32	667.92	576.85	515.15	419.55	149.18	0	
QR5	962.52	890.84	847.58	668.18	577.11	515.41	419.81	149.44	0.26	0

APPENDIX D. Geographic distances for *P. petenensis*

The shortest strait line distances (km) between populations of *P. petenensis* on the Yucatán Peninsula of Mexico. Abbreviations: C: Campeche; QR: Quintana Roo; T: Tabasco; CH: Chiapas.

	Geographical Distance									
	PpC2	PpC1	PpC3	T3	CH	QR7	QR1	QR4	QR6	QR8
PpC2	0									
PpC1	16.77	0								
PpC3	43.67	27.93	0							
T3	214.98	200.35	173.15	0						
CH	188.21	173.97	145.99	67.96	0					
QR7	186.67	194.21	201.60	339.82	282.15	0				
QR1	202.62	210.25	218.67	354.86	296.59	16.64	0			
QR4	227.24	235.34	245.51	385.15	326.86	45.29	30.38	0		
QR6	223.82	234.25	244.88	387.60	330.73	48.59	35.76	11.85	0	
QR8	286.20	308.48	326.79	484.41	432.45	155.36	146.90	199.77	110.81	0

APPENDIX E. Morphological distances for *P. velifera*

Pairwise morphological distances for *P. velifera* calculated as both body shape (using relative warp analysis) (above the diagonal) and dorsal fin shape (first principal component of size corrected linear measures of the dorsal fin (fin length, fin area, height of the first, second, and last fin rays) (below diagonal).

	Morphological Distance								
	C2	C5	QR2	QR3	QR5	Y1	Y2	Y3	Y4
C2	——	45.78	14.70	10.53	19.49	32.88	23.52	24.11	22.26
C5	0.81	——	32.24	49.36	38.00	31.92	25.91	14.25	54.14
QR2	0.12	0.30	——	14.64	22.34	28.44	15.91	23.34	18.76
QR3	0.97	0.68	1.88	——	24.79	39.12	28.40	34.91	33.35
QR5	0.12	1.55	0.49	4.28	——	34.12	27.55	19.29	24.83
Y1	<0.01	0.97	0.19	3.27	0.07	——	9.74	39.97	35.47
Y2	0.18	0.23	0.01	1.70	0.58	0.26	——	25.10	32.24
Y3	0.07	1.39	0.40	4.01	<0.01	0.04	0.49	——	28.61
Y4	0.27	2.03	0.77	5.05	0.03	0.19	0.89	0.06	——

APPENDIX F. Morphological distances for *P. petenensis*

Pairwise morphological distances for *P. petenensis* calculated as both body shape (using relative warp analysis) (above the diagonal) and caudal fin shape (first principal component of size corrected linear measures of the caudal fin (fin length, fin height, fin area, length of the upper fin edge, length of the lower fin edge) (below diagonal).

	Morphological Distance					
	C1	C2	C3	CH1	QR4	T3
C1	——	20.66	23.96	25.34	34.55	25.66
C2	0.29	——	8.25	9.06	20.60	22.90
C3	0.42	0.11	——	17.62	30.26	25.54
CH1	0.51	0.03	0.11	——	19.56	24.82
QR4	<0.01	0.36	0.50	0.60	——	24.08
T3	0.32	1.23	1.47	1.65	0.26	——

APPENDIX G. Behavioral distances for *P. velifera*

Pairwise behavioral distances for *P. velifera* calculated for both display characteristics (first principal component of rate and duration) (above the diagonal) and gonopodial thrusts (below diagonal).

	Behavioral Distance			
	C2	QR2	Y1	Y2
C2	——	0.01	0.37	0.09
QR2	0.16	——	0.33	0.11
Y1	0.14	<0.01	——	0.18
Y2	0.19	0.09	0.11	——

APPENDIX H. Behavioral distances for *P. petenensis*

Pairwise behavioral distances for *P. petenensis* calculated for both courtship display characteristics (first principal component of rate and duration) (above the diagonal) and gonopodial thrusts (below diagonal).

	Behavioral Distance		
	C1	C2	QR4
C1	——	0.41	1.97
C2	0.54	——	0.71
QR4	<0.01	0.56	——