ISSN 1930-1448

# Development of microsatellite markers for Permit (*Trachinotus falcatus*), cross-amplification in Florida Pompano (*T. carolinus*) and Palometa (*T. goodei*), and species delineation using microsatellite markers

SEIFU SEYOUM, CECILIA PUCHULUTEGUI, KATHRYN Y. GUINDON, CHRISTOPHER M. GARDINAL, STEVE H. DENISON, AND MICHAEL D. TRINGALI

Florida Fish and Wildlife Conservation Commission
Fish and Wildlife Research Institute
Technical Report No. 20 • 2016
MyFWC.com

# Development of microsatellite markers for Permit (*Trachinotus falcatus*), cross-amplification in Florida Pompano (*T. carolinus*) and Palometa (*T. goodei*), and species delineation using microsatellite markers

SEIFU SEYOUM, CECILIA PUCHULUTEGUI, KATHRYN Y. GUINDON, CHRISTOPHER M. GARDINAL, STEVE H. DENISON, AND MICHAEL D. TRINGALI

Florida Fish and Wildlife Conservation Commission
Fish and Wildlife Research Institute
100 Eighth Avenue Southeast
St. Petersburg, Florida 33701
MyFWC.com

Technical Report 20 • 2016


Rick Scott
Governor of Florida


Nick Wiley, Executive Director
Florida Fish and Wildlife Conservation Commission

The Fish and Wildlife Research Institute is a division of the Florida Fish and Wildlife Conservation Commission, which "[manages] fish and wildlife resources for their long-term well-being and the benefit of people." The Institute conducts applied research pertinent to managing fishery resources and species of special concern in Florida. Programs focus on obtaining the data and information that managers of fish, wildlife, and ecosystems need to sustain Florida's natural resources. Topics include managing recreationally and commercially important fish and wildlife species; preserving, managing, and restoring terrestrial, freshwater, and marine habitats; collecting information related to population status, habitat requirements, life history, and recovery needs of upland and aquatic species; synthesizing ecological, habitat, and socioeconomic information; and developing educational and outreach programs for classroom educators, civic organizations, and the public.

The Institute publishes three series: Memoirs of the *Hourglass* Cruises, Florida Marine Research Publications, and FWRI Technical Reports. FWRI Technical Reports contain information relevant to immediate needs in resource- management.

Gil McRae, FWRI Director
Bland Crowder, Editor and Production

This publication is available online and may be downloaded at http://myfwc.com/research/publications/scientific/technical-reports/. Copies may also be obtained from:

Florida Fish and Wildlife Conservation Commission
Fish and Wildlife Research Institute
100 Eighth Avenue SE
St. Petersburg, FL 33701-5095
Attn: Librarian

We suggest that this document be cited as follows:
Seyoum, Seifu, Cecilia Puchulutegui, Kathryn Y. Guindon, Christopher M. Gardinal, Steve H. Denison, and Michael D. Tringali. 2017. Development of microsatellite markers for Permit (*Trachinotus falcatus*), cross-amplification in Florida Pompano (*T. carolinus*) and Palometa (*T. goodei*), and species delineation using microsatellite markers. Florida Fish and Wildlife Conservation Commission, Fish and Wildlife Research Institute Technical Report **No. 20.**

Cover and text papers used in this publication meet the minimum requirements of the American National Standard for Permanence of Paper for Printed Library Materials Z39.48–1992(R2009).

# Development of microsatellite markers for Permit (*Trachinotus falcatus*), cross-amplification in Florida Pompano (*T. carolinus*), and Palometa (*T. goodei*), and species delineation using microsatellite markers

SEIFU SEYOUM, CECILIA PUCHULUTEGUI, KATHRYN Y. GUINDON, CHRISTOPHER M. GARDINAL, STEVE H. DENISON, AND MICHAEL D. TRINGALI

## Abstract

Three of the 20 species in the genus *Trachinotus*, in the jack family, Carangidae, are found in Florida waters. These are Florida Pompano (*T. carolinus*), Permit (*T. falcatus*), and Palometa (*T. goodei*). Florida Pompano is a coastal pelagic species found in estuarine and marine waters; it spawns in multiple batches in offshore waters. Permit is the largest and longest lived of the three species and also spawns offshore in multiple batches, near reefs. As adults, Permit can be found nearshore and offshore and are often associated with reefs, but as juveniles they are common estuarine inhabitants. Palometa is a marine species, similar in size to Florida Pompano, and has the widest latitudinal distribution of the three species. Palometa spawn in offshore waters throughout the year with two peaks of activity. All three species support commercial or recreational fisheries on both the Gulf of Mexico coast and Atlantic coast of Florida. Very little has been done to evaluate movement patterns of *Trachinotus* species. Based on a few tagging studies, it appears that Pompano do not travel far from coastal waters.

The only preliminary investigation of genetic stock structure for the Florida Pompano population from Tampa Bay, FL, and Puerto Rico was based on microsatellite markers developed for the Pompano. The report's key conclusion was that Pompano from Puerto Rico and from Florida belong to two highly distinct genetic stocks.

This study was conducted to re-examine, using different microsatellite markers, the genetic status of Pompano stocks in Florida and Puerto Rico. The objectives of this study, therefore, were the following: 1) to develop microsatellite markers for Permit; 2) to cross-amplify the markers in Pompano and Palometa; and 3) to use these markers to confirm the status of Puerto Rico Pompano as a novel genetic stock using the methods of Bayesian population assignment, phylogenetic clustering, and factorial correspondence analysis.

We developed microsatellite markers based on an enrichment protocol and identified 54 DNA segments that contained microsatellite loci. These loci were characterized in 63 specimens of Permit collected from Charlotte Harbor, FL, and were cross-amplified and characterized in Pompano specimens from Tampa Bay (N = 29) and Puerto Rico (N = 84) and in Palometa specimens from Puerto Rico (N = 37). Variability of microsatellite markers across these taxa showed that, in general, the Permit samples had more polymorphic loci and were more variable in all standard measures of genetic variation than were the Pompano and Palometa samples. Forty-one markers amplified in all four taxa, but only 35 of these were used to genotype the specimens of the four taxa.

Three methods were used to investigate the relationship among the taxa using the microsatellite genotype data obtained from the samples. The results from the three analytical methods, based on Bayesian population assignment tests, phylogenetic clustering, and factorial correspondence analysis of genetic relationships among the four *Trachinotus* samples, showed that Florida and Puerto Rico Pompano samples belong to two highly distinct gene pools. But other multiple molecular tools, particularly nuclear-DNA sequences from many introns, and nonmolecular tools, such as morphological and meristic data, should be used together to determine species-level categorical designation for the Puerto Rico Pompano.

# Introduction

The genus *Trachinotus* comprises 20 species of the tropical and subtropical regions of the Atlantic, Pacific, and Indian oceans. Commonly called Pompanos, they are members of the jack family, *Carangidae*. Pompanos are targeted recreationally because of their food value and strong fighting ability on light tackle and commercially because of their food value and market value. In fact, Florida Pompano, *Trachinotus carolinus*, has been called Florida's most highly valued fish (McMaster et al. 2003). Five *Trachinotus* species occur in the western Atlantic: *T. carolinus, T. falcatus, T. goodei, T. cayennensis,* and *T. marginatus*. The first three species are found in Florida waters and are the focus of preliminary genetic study in this report.

## Florida Pompano

The Florida Pompano (*Trachinotus carolinus* [Linnaeus, 1766]) is a coastal pelagic species, smaller than Permit (*T. falcatus* [Linnaeus, 1758]). It occurs in marine and estuarine waters from Massachusetts to northern Argentina (Gilbert and Parsons 1986; Díaz de Astarloa et al. 2000). Pompano are rare in Bahamian waters and scattered in the West Indies (Robins and Ray 1986). Randall (1983) reports that they are present in the Bahamas but prefer turbid places, like mangroves and deeper water, and avoid clean water. The only fishery-independent life history study of Florida Pompano estimated size, age, growth, and mortality parameters and took place in Tampa Bay and adjacent Gulf of Mexico waters in 2001 and 2002 (Guindon et al. 2008). Pompano appear to be multiple batch spawners in offshore waters (Fields 1962; Guindon et al. 2008). Larvae have been collected and described from offshore waters ranging in size from 3 to 11 mm (Fields 1962; Finucane 1969). Young-of-the-year juveniles (10–150 mm standard length [SL]) are common constituents of the surf zone community along exposed sandy beaches that serve as the Pompano's primary nursery habitat (Springer and Woodburn 1960; Fields 1962; Finucane 1969; Naughton and Saloman 1978; Saloman and Naughton 1978; Guindon et al. 2008). Based on fish size at the time of shoreline settlement it appears that the spawning season is protracted in Florida with new recruits (>20 mm) arriving into the fall even though peak numbers occur during April or May (Fields 1962; Finucane 1969; Guindon et al. 2008; Solomon and Tremain 2009). In the more tropical waters of Brazil, Florida Pompano recruit to the surf zone nursery habitats year-round, indicating potential year-round spawning capability (Felix et al. 2007; Mazzei et al. 2011).

Very little has been done to evaluate movement patterns in Florida Pompano or any other *Trachinotus* species. Along the Atlantic Seaboard, adults are reported to move north in the summer (Fields 1962; Berry and Smith-Vaniz 1978), but patterns of their exact movement are relatively unknown for Florida, in either Atlantic or Gulf waters (Bellinger and Avault 1970). Based on very few tagging studies, it seems that Florida Pompano does not roam far from coastal waters (Berry and Iverson 1966; Ross and Lancaster 2002).

Florida Pompano are immensely important to Florida's economy as the foundation of a highly valued commercial and extremely popular recreational fish. In general, total statewide landings are evenly divided between Gulf and Atlantic coasts and between recreational and commercial fishery sectors. For example, in 2008, 54% of the Florida Pompano landings was attributed to recreational anglers, and 51% of the catch were on the Gulf coast.

The first stock assessment of the Florida Pompano, Murphy et al. (1996) concluded that *T. carolinus* was growth-overfished on both coasts from 1989 to 1995, especially along the Gulf coast, where most fish were landed. In the most recent assessment, however, Murphy *et al*. (2008) concluded that the fishery had changed. The species is no longer considered overfished because the population biomass estimate for both the Gulf and Atlantic coasts exceeded the estimated size threshold for maintenance of a minimum stock. The assessment indicated overfishing is clearly not occurring along the Gulf coast, although the status of overfishing on the Atlantic coast is less definitive.

## Permit

Permit (*Trachinotus falcatus* [Linnaeus, 1758]), are found from Massachusetts to southeast Brazil, including in the Gulf of Mexico, in the Bahamas, and across the West Indies. They are the largest and longest-lived of the three species, reaching 101 cm FL (fork length) and ages as great as 23 years (Crabtree et al. 2002). Permit, like Florida Pompano, are multiple batch spawners near reefs during spring and summer (Crabtree et al. 2002; Graham and Castellanos 2005). Few Permit larvae (5–11 mm) from offshore waters have been collected and described (Fields 1962). Back-calculations from daily age estimates using young-of-the-year otoliths approximated duration of the larval phase 15–20 days (Adams et al. 2006). An analysis of ten years (1991-2000) of fishery-independent monitoring data from Charlotte Harbor, Florida showed two distinct settlement events occurred in May–June and Sept.–Oct. (Adams and Blewett 2004). New recruits

(<40 mm SL), however are present year-round in seine samples from the Florida Keys (Crabtree et al. 2002; Adams et al.et al. 2006), possibly indicating year-round spawning or larval transport from more tropical locations. Newly recruited Permit in the Florida Keys and Belize are 20 mm SL and are approximately 30 days old (Adams et al. 2006). Nursery areas are typically low-energy (Adams and Blewett, 2004) or medium-energy windward beaches (Adams et al. 2006) that can be found in estuarine systems or high-salinity surf zones (Finucane 1969; Mazzei et al. 2011). Adult Permits may be found inshore, nearshore, or offshore and often are associated with reefs. A citizen-based tagging program called Project Permit is under way to evaluate the range and distribution of Permit in Florida (Kathryn Guindon, personal communication 2015). Permit support a minor commercial fishery relative to Florida Pompano. Landings data show Permit are caught on both the Gulf and Atlantic Coasts, and most commercially harvested Permit are reported bycatch because of the net limitation laws in Florida. It is the recreational fishery that contributes the most to Permit landings. For example, in 2008 recreational anglers accounted for 87% of Florida's catch. In general, total Permit landings (combined from both sectors) show the fishery is much smaller than that of its congener, the Florida Pompano. However, there is growing concern among some public that future recreational fishing pressure on Permit will increase since several other reef fish species are becoming more restricted and Permit can still be harvested. The last statewide stock assessment on Permit concluded that there were not enough life-history data to conduct a biological assessment of the species' status (Armstrong et al. 1996). While more recent formal assessments have not been done, the FWC continuously monitors the species' landings and Fish and Wildlife Research Institute status and trend reports (2012) which all indicate that Permit landings are stable.

## Palometa

Palometa (*Trachinotus goodei,* D.S. Jordan & Evermann 1896) is a marine species and has the widest latitudinal distribution of the three congeners found in Florida. They are found in coastal waters from Massachusetts, east to Bermuda, and south to Argentina, including the northern and southern Gulf of Mexico (McEachran and Fechhelm 2005) and the Caribbean Sea. In Florida and other parts of its range, this fish is predominantly encountered as juveniles, along clear, sandy beaches, as it is in other locations (Nero and Sealey 2005; Felix et al. 2007; Mazzei et al. 2011). Juveniles seem to prefer habitats with a high abundance of microcrustaceans and low coverage of vertical seagrasses or macroalgae (Nero and Sealey 2006). As adults Palometa are associated with reefs and so are more often encountered in deeper waters. Maturity has been reported at 300 g for males and 350 g for females and at ages of 1–1.5 years (Thouard et al. 1989). This is more similar to the data for Florida Pompano than to those for Permit, a much larger species (Crabtree et al. 2002). Spawning habitat is offshore waters, and spawning season has been reported as May–June in Cuba (Adams and Blewett 2004). In Martinique spawning occurs year-round, with two peaks, in August and February (Thouard et al. 1989), and in Brazil juveniles recruit to sandy-beach surf zones year-round (Felix et al. 2007; Mazzei et al. 2011), so we can infer either that spawning is year-round in Brazil or that specimens recruit to Brazilian nursery habitats throughout the year.

The species is considered a game fish throughout much its range, and reef-associated adults off a Brazilian coastal island have been documented in catches using hook and line, spear, and by trolling (Pinheiro et al. 2010). In Florida it is typically bycatch for anglers targeting Pompano along east-coast beaches and is rarely caught along the Gulf coast. The species does support a minor commercial fishery and aquaculture projects (Thouard et al. 1989; Alvarez-Lajonchère and Ibarra-Castro 2012). No fishery stock assessment of the population has been done.

## Phylogenetic relationships among the three *Trachinotus* species

Phylogenetic analysis based on molecular data among seven species of *Trachinotus* (cytochrome b sequences; Reed et al. 2002) and trends for acrocentric chromosome and mapping of multiple sites of 5S and 18S rDNA sites (Jacobina et al. 2012) shows that the three *Trachinotus* species considered here are closely related; *T. goodei* appears as the most derived species (evolutionarily recent), and *T. carolinus* as basal.

The only other genetic study involving these three species is a preliminary report on the genetic population structure of the Pompano, *T. carolinus* (Tringali et. al., 2006), based on 13 microsatellite markers developed for the species (Seyoum et al. 2006). The report concluded that Pompano from Florida and Puerto Rico were highly distinct populations. This distinction could signal a divergence event in process and spurred reinvestigation of this radiation.

The objectives of this study were as follows: 1) to develop microsatellite markers for Permit; 2) to cross-amplify the markers in the Permit's congeners Pompano and Palometa; 3) to illustrate how markedly microsatellite

markers further define the genetic stock separation between Florida and Puerto Rico Pompano using the methods of Bayesian population assignment, phylogenetic clustering and factorial correspondence analysis. The markers developed for the permit could also be used in future studies: (1) whether Permit and Palometa found in Florida and Puerto Rico also have a similar analogous evolutionary radiation with corresponding different gene pools as in the Pompano: (2) population structure of the Permit and (3) population structure of the Florida Pompano.

# Methods

## Sample collection and DNA extraction

Specimens were collected for three species in the genus *Trachinotus*; Permit from Florida in 2013, Pompano from Florida and Puerto Rico in 2005, and Palometa from Puerto Rico in 2005. Fin clips the size of half a dime were snipped and preserved in 70% ethanol. Total DNA was isolated from approximately 500 mg of fin-clip tissue using Puregene DNA isolation kits (Gentra Systems Inc., Minneapolis, MN) and rehydrated in 50 μl of deionized water.

## Development of microsatellite markers

Nuclear DNA from liver tissue of a single Florida Permit collected in Tampa Bay was purified via density-gradient ultracentrifugation using the method of Lansman et al. (1981). This step maximizes efficiency by excluding the mitochondrial DNA (mtDNA) mass from the total DNA precluding interference during the development of microsatellite markers during +enrichment. We generally followed the enrichment protocol of St. John and Quinn (2008) for identifying DNA segments that contained microsatellite loci. A few additional steps were included in this protocol in some of the steps to facilitate efficiency. Ten μg of the purified nDNA was digested with Sau3AI restriction enzyme according to the manufacturer's protocol (New England Biolabs, Ipswich, MA) to fragment nDNA into 500–1500-bp (base pair) fragments with 5′ overhangs. Four μl of the digested DNA was electrophoresed on a 1% agarose gel to verify optimum digestion, which was verified as a smear in the region of 500–1500 bp in size. Digestion was optimized by varying digestion time, concentration, and type of enzyme (we used Sau3AI) before continuing the process. The digested DNA fragments were phenol-chloroform–extracted (Sambrook et al. 1989), precipitated in ethanol, and resuspended in deionized water. The resuspended nDNA was run through a 2% agarose low-EEO (electroendosmosis, flow of solvent in the electric field during electrophoresis) allowing shorter electrophoresis runs and high mechanical resistance for easier handling). The portion of the gel containing fragments between the regions corresponding to a DNA ladder of 300- to 1500-bp sizes was cut and the fragments eluted using Spectra/Por-2 dialysis membrane tube (Thermo Fisher Scientific Inc.) in the following method.

The spectra Por-2 membrane dialysis tube was washed thoroughly with deionized water, tightly clipped at one end and filled with the same buffer as that used as for the electrophoresis. The gel containing the 300- to 1500-bp fragments was cut out in a shape that would fit in the spectra Por-2 membrane tube, leaving a buffer-filled space at one end, which was then tightly clipped. These membrane tubes were placed in an eletrophoretic buffer rig, with the buffer-filled end placed toward the anode, and current run through them for 2–3 hours. Movement of the fragments out of the gel and into the buffer can be checked periodically with a UV light. When this is completed, the buffer is collected in a tube and phenol-chloroform–extracted, ethanol-precipitated, and resuspended in deionized water. Two to 3 μg of DNA fragments would be required for optimal success of the enrichment protocol.

The resuspended DNA fragments were incubated with 10 U mung nuclease (10,000 U/ml; New England Biolab) at 30°C for 45 minutes to remove the 5′ (five-prime end) overhang and purified using the QIAquick purification kit and eluted in 50 μl of sterile distilled water (SDW). The elute was dephosphorylated using 10 U calf intestinal phosphatase (10,000 U/ml; NEB, New England Biolabs) for 1 hour at 37°C, purified using a QIAquick purification kit and eluted in 30 μl of SDW. Sca linkers (ScaF: 5′-CAGTGCTCTAGACGTGCTAGT-3′ and phosphorylated ScaR: 5′-p*ACTAGCACGTCTAGAGCAACTGAAAA-3′) were prepared in large quantity by annealing the single-stranded linkers together to make them double stranded. This was done by mixing equal volumes of ScaF and ScaR to make up a 10-μM concentration and heating the mixture for 5 minutes at 94°C and reducing the temperature by 10°C twice in a half-hour interval to 74°C and then by 3°C every half hour to room temperature overnight in an Eppendorf thermocycler. The prepared double-linker was aliquoted into smaller volumes and stored at −20°C; aliquots were thawed and used as necessary. The eluted nDNA fragments were ligated to the double linker with T4 DNA ligase (NEB) overnight with 10U ScaI restriction endonuclease (10,000 U/ml; NEB) in a 30-μl reaction 15× (16°C for 30 min/37°C for 10 minutes) in a thermocycler. The Sca linkers were specifically designed to ligate to the blunt-ended fragments.

The efficiency of the linker-ligation was tested via PCR using 1 μl of the linker-ligated product with only the forward primer for 25 cycles at 58°C annealing temperature. A run of the PCR product in a 2% gel should give a smear in the 300–1500-bp region similar to that in the run from which the fragments were initially excised. The linker-ligated fragments were then divided into four aliquot volumes denatured at 95°C for 10 minutes and each aliquot hybridized to one of four 3′-biotinylated oligonucleotide (oligo) probes [$(AC)^{11}$, $(AG)^{13}$, $(CAG)^{6}$, $(GATA)^{8}$] (Life Technologies, Carlsbad, CA). The hybridization captured DNA fragments with microsatellite sequences complementary to the oligo (probes).

Hybridization was carried out in an Eppendorf thermocycler using a program that reduces the temperature from 80°C by 1°C every 5 min up to the annealing temperature of the probe and then by 1°C every 5 min to 10°C below the specific annealing temperature of the probe). The hybridization solution contained 2 μl (100 ng of the DNA), 2 μl of 50-μM oligo probe (100 pmol) and 60 μl [(10× standard saline citrate (SSC) = (1.5M NaCl, 0.15M $Na_3C_6H_5O_7 \times H_2O$)]. The resulting hybridized DNA was captured on Streptavidin MagneSphere® Paramagnetic Particles (Promega) that had been washed three times in 6× SSC, mixed gently for 45–60 min on a rocker platform in a blocking solution reagent (0.2% I-BlockTM reagent, 1× TBS buffer pH 7.6, 0.05% Tween®, deionized $H_2O$ to 100 ml) and finally washed three times with 6× SSC after the bead block. The excess unbound probe was removed from the hybridized DNA by a series of low- and high-stringency washing (using 2× SSC and 6× SSC), and the enriched single-stranded DNA was eluted from the beads by incubation at a temperature 10°C greater than the hybridization temperature. This final elution was divided into two aliquots. One aliquot was directly cloned[1], while the other served as template for PCR performed using ScaF linker as a primer using the following profile: 94°C for 5 min., followed by 15 cycles (30 s at 94°C, 30 s at 58°C, and 30 s at 72°C), and a final extension of 5 min at 72°C. The PCR product was purified (PCR purification kit, Qiagen) and ligated into a plasmid vector (Bluescript PBC KS-, Stratagene, La Jolla, California) that had been tailed with dTTP (Marchuk et al. 1991) to facilitate T-A cloning (Zhou and Gomez-Sanchez 2000) and transformed into competent *E. coli* cells (Sambrook et al. 1989) to produce a microsatellite-enriched library. For each recombinant colony, a 12.5-μl PCR reaction was performed as above except that the annealing temperature was 55°C, and the number of cycles was 35, with T3 and T7 vector primers included in the reaction in addition to the complementary sequence of each of the biotinylated oligo probes. PCR products found to have two or more bands when run through 1.5% low-EEO agarose gel were reamplified using only vector primers. The PCR products from these reactions were cycle-sequenced from both directions and then visualized on an ABI Prism™ 3100-Avant Genetic Analyzer (Applied Biosystems). The resulting DNA sequences were visually inspected for the presence of simple sequence repeats, or using the program MSATCOMMANDER (Faircloth 2008; available at http://code.google.com/p/msatcommander/downloads/list). PCR primers were designed for the putative microsatellite loci using OligoPerfect™ Designer (available at http://www.invitrogen.com/) or primer3, which is an integral part of MSATCOMMANDER (Rozen and Skaletsky 2000). Forward primers were 5-end labelled with a fluorescent dye, and loci were screened for polymorphism in 12.5-μl multiplex PCR reactions, each of which included primers for three loci. Loci in a multiplex reaction were first confirmed for no primer dimmer formation and no overlapping fragment sizes. Thermocycling conditions were as above but employed a step-down (high to low) annealing temperature from 58°C for 6 cycles, 57°C for 8 cycles, 56°C for 10, and 55°C for 10 cycles. Fragments were visualized on an ABI Prism™ 3130-Avant genetic analyzer with GeneScan 500 ROX as a size standard and analyzed using GENEMAPPER (version 4.0; Applied Biosystems Inc.).

## Microsatellite genotype analysis

Multiplex PCR reactions containing three sets of labeled microsatellite primers and 100 ng of total DNA were carried out in 12.5-μl volumes using the step-down reaction profile described above. One μl of PCR product was mixed with 12 μl of deionized (Hi-Di) formamide and 0.5-μl of ROX500 size standard, denatured (94°C for 4 m) and snap-frozen on a −20°C cold rack. Fragments were visualized on an ABI Prism™ 3100-Avant Genetic Analyzer and genotyped using GeneMapper (version 4.0; Applied Biosystems Inc.). All microsatellite markers identified were characterized using Permit specimens collected from Charlotte Harbor (N = 63). These markers were also cross-amplified and characterized using specimens of Florida Pompano from Tampa Bay (N = 29), Puerto Rico Pompano (N = 84), and Palometa from Puerto Rico (N = 37).

## Data analysis

A file in GENEPOP data format was generated from fragment sizes recorded using the Microsatellite Marker Toolkit add-on (version 3.1.1; Park 2001; available at http://animalgenomics.ucd.ie/sdepark/ms-toolkit/). GENEPOP data were converted to other formats using the

conversion tool PGDspider (version 2.0.1.9; Lischer and Excoffier 2012). Pairwise genetic distances ($F_{ST}$) between samples (Weir and Cockerham 1984) were estimated with 10,000 permutations in the program GENETIX (Belkhir et al. 2000). Departures from Hardy-Weinberg equilibrium (HWE) were determined using GENEPOP (version 3.4; Raymond and Rousset 1995, 2008). Sequential Bonferroni corrections were applied to multiple tests of hypotheses (Rice 1989). Observed ($H_0$) and expected heterozygosity ($H_e$, with and without a bias correction), averaged over all loci, were obtained from GENETIX (Belkhir et al. 2000). Null allelism was investigated with the randomization test of Guo and Thompson (1992) and the U-test statistics of Raymond and Rousset (1995), using the program ML-NullFreq (available at http://www.montana.edu/kalinowski/software.htm). For each locus in each species data set, microsatellite variation was quantified in terms of genetic diversity, number of alleles, and allelic richness (a diversity measure that corrects for differences in sample size; Leberg 2002) using the program FSTAT (version 2.9.3.2; Goudet 2001)

## Bayesian population assignment test

We used three analytical approaches to examine, using microsatellite loci genotype data, genetic relationships within each of the *Trachinotus* species. Bayesian population assignments using the program STRUCTURE (version 2.3.2; Pritchard et al. 2000, 2009) was used to detect the number of naturally occurring clusters ($K$) in the examined individuals. This method could reveal the presence of distinct nDNA clusters, particularly when two taxa are found to significantly diverge. STRUCTURE uses likelihood statistics to cluster individuals based on linkage disequlibrium, between genotypes at multiple loci. In a Bayesian population assignment, genetically homogeneous groups of individuals within which linkage disequilibrium is minimized are identified as a cluster. The number of clusters would be equal to the number of species if two or more species are admixed. Ten replicate simulations were conducted using a $2.0 \times 10^5$ Markov-Chain Monte Carlo (MCMC) simulation after a $1.0 \times 10^5$ burn-in period for each value of $K$ from 1 to one or two more than the number of potential local geographic populations in the data using the no-admixture model, to detect subtle structure (Pritchard et al. 2009) and the independent-allele-frequencies option in the program to preclude overestimating the number of gene pools (Falush et al. 2003).

We also used the admixture model for comparative purposes. The result file for 10 replicated runs for each cluster from STRUCTURE was archived into a zip file and uploaded to a web-based program, STRUCTURE HARVESTER (version 0.56.3; Earl et al. 2012), which uses posterior probabilities from STRUCTURE to calculate LnP(D) and the magnitude change of LnP(D), that is, the log likelihood for each $K$ relative to the standard deviation, called $\Delta K$ (Evanno et al. 2005). Evanno et al. (2005) suggest that the $\Delta K$ parameter is a reliable measure of the relative support for each level of $K$. Bayesian population assignments were also calculated with GENECLASS2 (Corunet et al. 1999). Specifically, the Ranalla and Mountain (1997) resampling algorithm was employed with a specified assignment threshold of 0.05. Once the most likely number of clusters had been identified, the average proportions of membership from the 10 replicated runs from STRUCTURE HARVESTER were aligned and summarized using CLUMPP (version 1.1.2; Jakobsson and Rosenberg, 2007) under the Greedy algorithm, with 1000 replicates. The average proportion of each cluster and individuals in each of the populations under the optimal level of $K$ was visualized using Excel. The average genotype membership across runs for the sample and individuals from CLUMPP was plotted using DISTRUCT (version 1.1; Rosenberg, 2004) and the postscript visualized using Ghost View (available at http://pages.cs.wisc.edu/_ghost). Although STRUCTURE is specifically designed to explore population structure, the optimal number of groups or number of clusters estimated using the method of Evanno et al. (2005) and STRUCTURE HARVESTER may be expected to be equal to the number of species. The 35-microsatellite genotype data were constructed for STRUCTURE-STRUCTURE HARVESTER ANALYSES with four taxa and with all possible combinations of three of the four taxa.

## Phylogenetic clustering

Phylogenetic clustering results in hierarchical, nonoverlapping groups based on genetic or morphological similarities among taxa. It is a model-based approach that discerns population structure from molecular data without assuming linkage equilibrium. The molecular data is used to estimate phylogenetic relationships of individuals that share a common ancestry. The analysis of minimum dissimilarities in phylogenetic clustering is analogous to minimum linkage disequlibrium in a Bayesian population assignment. Although phylogenetic clustering can have serious limitations when used to analyze conspecific populations, it is generally a useful method for determining, whether taxa are significantly different from their measurable position on the branching tree they occupy, based on similarities and differences in their physical or genetic characteristics. A variety of algorithms allow finding similarities among taxa. In this study we used two algorithms, 1) Cavalli-Sforza and Edwards' (1967)

chord distance ($D_C$) and 2) Nei et al.'s (1983) distance method ($D_A$). We used both $D_C$ and $D_A$ to construct neighbor-joining trees because, when microsatellite markers have been used, they have been found to be appropriate, generally showing a greater probability of resulting in the correct branching pattern of a tree (Takezaki and Nei, 1996). $D_C$ is a statistical method of estimation applied to stochastic models of evolution in which populations are conceptualized as points in an m-dimensional Euclidean space, which are specified by *m* allele frequencies (i.e., *m* is the total number of alleles in both populations). The distance is the angle between these points and is calculated as the chord distance ($D_C$) between pairs of taxa using the microsatellite DNA genotypes. $D_A$ measures the number of net nucleotide substitutions per site between populations, which is more efficient than several of the other available distance methods. These algorithms have been found to be the most appropriate applications for microsatellite DNA loci for studying the evolutionary relationships of closely related populations (Takezaki and Nei 1996). But we also employed the widely used $F_{ST}$ method (Latter 1972) computed, using the software POPTREE 2 (Takezaki et al. 2010), without (Nei 1973) and with (Nei and Roychoudhury 1974; Nei 1987) correction for bias due to sample size. The neighbor-joining algorithm (Saitou and Nei 1987) was used to cluster $D_C$, $D_A$, and $F_{ST}$ distances calculated using TreeFit (1000 bootstrap values; Kalinowski 2009). The TREEVIEW file written from TreeFit was visualized using the software FIGtree (version 1.4.1.; Rambaut 2014) (available at http://tree.bio.ed.ac.uk/software/figtree/). Patterns of groups or clades observed in the phenogram indicate the relationship and the possible number of species.

### Factorial correspondence analysis

The third method of determining the number of taxa in genotype data sets factorial correspondence analysis (FCA) conducted using the software GENETIX (version 4.02; Belkhir et al. 2000). FCA is a means of investigating relationships in tables, in this case, of determining whether a correspondence existed between rows (individuals) and columns (alleles). Each allele is considered an independent variable, and axes are generated based on the combinations of alleles that explain portions of the total inertia of the table. These axes are then used to plot the genotypes of specimens in multidimensional space. The alleles that exhibit the strongest nonrandom association among individuals contribute most strongly to the axes. In this analysis, the individual genotypes were plotted into three-dimensional space based on the genetic relationships observed at the polymorphic microsatellite locus numbers. We calculated the distance between the centers of mass of each pair of the four categories included in the analysis using the three-dimensional distance formula $d = \mathrm{sqrt}[(x_1 - x_2)^2 + (y_1 - y_2)^2 + (z_1 - z_2)^2]$ based on the absolute coordinates from GENETIX. The FCA also gives a visual interpretation of the relationship between the species based on the distance they occupy and number of species based on the projected clusters the in three-dimensional figure. The number of novel genetic classes could be directly countable and equals the number of clusters, occupying different spatial positions. But the relationship between the closer clusters should be further analyzed using other molecular tools to establish their position as representing different categories.

## Results

### Microsatellite DNA assay

Fifty-four microsatellite DNA markers were developed for Permit and characterized in specimens collected from Charlotte Harbor (N = 63; Table 1). These markers were cross-amplified in specimens of Pompano from Tampa Bay (N = 29) and Puerto Rico (N = 84) and in specimens of Palometa from Puerto Rico (N = 37) (Table 2). Among all 54 loci, there were 16 monomorphic loci across all taxa, ranging from 8 to 11 in each taxon with 4 monomorphic loci shared across all 4 taxa. Forty-one microsatellite markers amplified across all taxa (Table 2) but six loci (Tfal-3, -4, -7, -16, - 47 and -63) were excluded from the final analysis because data for these loci were incomplete among individuals in all samples. The numbers of loci that were monomorphic, polymorphic, or failed to amplify among the 4 taxa are given in Table 3. The various average genetic standard measurements over all the 35 loci that amplified across all taxa including genetic diversity, numbers of alleles, allelic richness, and observed and expected heterozygosities are also given in Table 3. Variability of microsatellite DNA markers across taxa showed that in general Permit had more polymorphic loci and greater variability for all the standard measures of genetic variation than did Pompano and Palometa.

### Bayesian population assignment of individuals in the *Trachinotus* species

In the Bayesian population assignment, the number of naturally occurring taxa was expected to be the same as the optimal number of clusters (*K*) within the examined individuals. With all four taxa in the STRUCTURE and STRUCTURE HARVESTER analyses, the expected

Table 1. Characterization of 54 microsatellite DNA loci in 63 specimens of the Florida Permit (*Trachinotus falcatus*) from Charlotte Harbor, Florida.

| Locus No. | Primer Sequence (5′ → 3′) | Repeat Motif | Allele Size Range | K[a] | $H_O$[b] | $H_E$[c] | Gen. Bank Accession |
|---|---|---|---|---|---|---|---|
| Tfal–01 | F:CGTAAAGGAAAGGAATGAAGTTAA<br>R:CCTCTTCCTCTTTCTATCTCTCTTTG | (GT)17 | 220–260 | 13 | 0.72 | 0.80 | KJ1416385 |
| Tfal–02 | F:GTTGGTAATAAAATGTGGAAATGAAA<br>CCCTGAAGTACATAGTTCAAGCTACA | (TG)12 | 137–159 | 10 | 0.74 | 0.79 | KJ416386 |
| Tfal–03 | F:GGAAGGTAGAAACTAAGGAAGGAAAG<br>R:AACATCTGCATCTGCATCTGTATAAC | (CA)27 | 135–167 | 14 | 0.94 | 0.85 | KJ416387 |
| Tfal–04 | F:AAGTTTAACAGTTGAATGGATCAGC<br>R:CAAGACACAGATGTGTGGATAAGTC | (TGA)8 | 236 | 1 | — | — | KJ416399 |
| Tfal–05 | F:ATTAGGATGAAGAAGGAAAAGCAAA<br>R:TCATTTATGGGGAATAATCTGAATG | (CA)13 | 156–218 | 23 | 0.89 | 0.89 | KJ205450 |
| Tfal–07 | F:CGTTTACTTTACTTTGGTCTCTGGT<br>R:AACCAATAAATTAAAGCGGCTCTC | (TTA)7 | 178–212 | 11 | 0.83 | 0.84 | KJ416400 |
| Tfal–10 | F:CTGTCATGTATAGGCTGGAGACAA<br>R:AGTTTGTCAGTGAGCAACATTTCAT | (GCC)7 | 90–108 | 5 | 0.25 | 0.25 | KJ416388 |
| Tfal–11 | F:ATGCTGGTTTATATGGGATTTCTG<br>R:TTTACATTAAGGAGTAATTTTTGTGGA | (TTA)7 | 118–128 | 3 | 0.35 | 0.32 | KJ416389 |
| Tfal–12 | F:CTTTGTGTTCAATACATCCTTGAGA<br>R:GATATTTTACCTAATTGCGTCTCTCC | GT)9/(GT)7 | 184–186 | 2 | 0.02 | 0.02 | KJ205454 |
| Tfal–13 | F:CATCTTGGACAATACCAACCACAG<br>R:GCTGAGGTGTTAAAGAGTCTGATGA | (GCA)8 | 158–164 | 3 | 0.13 | 0.12 | KJ205455 |
| Tfal–14 | F:ACCCAAGTAAACACAACATTACACAC<br>R:ACATCCAAGGCAGACTCCAAAC | (CA)10/(CA)12/(CA)7 | 178–200 | 8 | 0.64 | 0.66 | KJ205456 |
| Tfal–15 | F:ACACTAAGCAATACAAGAGCACTCC<br>R:TAAACCACAGAAATGCAGACAATTT | (GT)10 | 131–135 | 4 | 0.44 | 0.60 | KJ205457 |
| Tfal–16 | F:CTCAGTGATGAAGATGAAGATGATG<br>R:TTATGACTGAGTAAATCCAAAACAGC | (CT)20(CA)20 | 154 | 1 | — | — | KJ416401 |
| Tfal–17 | F:TGGGAAGATTTACTGAACTTGATTC<br>R:TGGGTGGTGTTTTTATTCTTTATTT | (TC)19 | 185–207 | 9 | 0.73 | 0.82 | KJ205458 |
| Tfal–19 | F:TTACAGGCCTATAGTGGCTGGTCT<br>R:TAGCTTTTGATTATATGTGAATTGTTC | (CTT)26 (T)11 | 134–240 | 34 | 0.97 | 0.95 | KJ416390 |
| Tfal–20 | F:AGAGGGGAGAGTAGAGGAGAGTGT<br>R:ATTTGAACTTTGAATGAGTCTCCTG | (GACA)3 (GATA)12 | 189–245 | 11 | 0.56* | 0.86 | KJ416391 |
| Tfal–21 | F:TGTGTGAGAAAAACATGTAGGACTG<br>R:GAACAACATTATTTCCAGTCTGGTG | (GT)16 | 124–156 | 11 | 0.84 | 0.75 | KJ416392 |
| Tfal–23 | F:GAGACACTCCTATGGCTTCGTATC<br>R:AATATTACACAGCCAAATTGTACTGG | (TAA)7 | 199 | 1 | — | — | KJ416402 |
| Tfal–25 | F:TACAATTGTCAACCTCATACTGACTG<br>R:CCTGTACCAAGGACAGCAAATATCTA | (GATA)22 | 157–213 | 15 | 0.89 | 0.90 | KJ416393 |
| Tfal–26 | F:TGTGTTTTACAACTCTCCTCACATT<br>R:TGAGCACCTTTTGTGTGATATTTTA | (CA)7/(CA)10 | 215–223 | 3 | 0.24 | 0.24 | KJ416394 |
| Tfal–28 | F:GCAAGTTAGTTTAAACAGTAGTTGTGG<br>R:CAATAGTGTGAAGCTAAACTTTGTCC | (TAG)6/(TAG)4 | 176 | 1 | — | — | KJ416403 |
| Tfal–30 | F:GACAGGTCTCCTCTCTGAGCTG<br>R:CTCGACTCTAAGTCTGGAGTGTTTC | (GT)21 | 141–143 | 2 | 0.11 | 0.10 | KJ416395 |
| Tfal–31 | F:GTTTCCTTCATTTCATTCTCCAGT<br>R:CAAAAGTATGTGCATGAAAAGGATT | (CA)17 | 131–163 | 7 | 0.68 | 0.66 | KJ205459 |
| Tfal–33 | F:CTGTTCTCTGCAGCACTCATACACT<br>R:AGTCAGGGAAAATCAGTGTGAAAT | (CA)11/(CA)22 | 167–185 | 11 | 0.79 | 0.82 | KJ205460 |
| Tfal–35 | F:AGCTGTCACCACCACCAACTT<br>R:CATTCTCCTCATGGTCCGTATGT | (CAG)8 | 160–162 | 2 | 0.00* | 0.11 | KJ416404 |
| Tfal–36 | F:CTCAATGGCTTTGACTGTAATCTTT<br>R:AAGGACTCAAAACTCTCTGTTTTTG | (CA)19 | 105–137 | 15 | 0.88 | 0.85 | KJ205461 |
| Tfal–37 | F:GGAGGCATAGTATACATACCAgACG<br>R:CTGAAATTTAGAAAAAGAGGAATGG | (TG)17 | 145 | 1 | — | — | KJ416405 |
| Tfal–39 | F:AAACGCATCCTCTCACATACTCAC<br>R:GCAAACACACACTCCACTCTGTTAT | (CT)4(AC)12/(TC)3/(CT)6 | 202–214 | 6 | 0.61 | 0.61 | KJ205462 |

Table 1 (*continued*). Characterization of 54 microsatellite DNA loci in 63 specimens of the Florida Permit (*Trachinotus falcatus*) from Charlotte Harbor, Florida.

| Locus No. | Primer Sequence (5′ → 3′) | Repeat Motif | Allele Size Range | K[a] | $H_O$[b] | $H_E$[c] | Gen. Bank Accession No. |
|---|---|---|---|---|---|---|---|
| Tfal–40 | F:AGTGATGGAGAGCAAAAACGAAC<br>R:ACTGCGACTGAGTGTGTTTATATGG | (GT)10(GA)3 | 200–208 | 2 | 0.02 | 0.02 | KJ416406 |
| Tfal–41 | F:TGTTGAGGAAGGAAGGAACTAATC<br>R:ACATATGTGCGTTTTCCTCTGG | (CA)12 | 199–203 | 3 | 0.03 | 0.03 | KJ416407 |
| Tfal–42 | F:TGCAGTACCTACCAAATTACAAGTG<br>R:CACTCCGCTCTCATGACTGAC | (TG)17 | 174–184 | 4 | 0.09* | 0.25 | KJ416408 |
| Tfal–43 | F:ACAGTGATAGTTCCTGCTACAGTGG<br>R:ACCTTCTCTGCCATCACTCATTTTA | (CA)9 | 155–163 | 4 | 0.28 | 0.30 | KJ205463 |
| Tfal–46 | F:ATCTCAGCAGTCCACTCTCTCTCT<br>R:TGTAAAGGAGAAGGTTGGAGTATGT | (CT)6/(CT)9 | 121 | 1 | — | — | KJ416409 |
| Tfal–47 | F:CCTCCTTTCAGCTATCTTTATCTCTC<br>R:GGACAGCGTATGAAAGGACTTAAA | (TC)7/(TC)3 | 214 | 1 | — | — | KJ416410 |
| Tfal–48 | AGCTGTGCGATAATATCTGATGAAT<br>R:CTGGAGAAATACCCTGTGAGTACAT | (GT)5(GA)3 | 115 | 1 | — | — | KJ416411 |
| Tfal–49 | F:TGGGTGAGTGAGTGAGAGATAAAG<br>R:TTTAAAAGCCATTGTTCTCCTCTG | (TG)17 | 146–158 | 6 | 0.60 | 0.57 | KJ205464 |
| Tfal–50 | F:CCACTTACCCAACACACATACTACA<br>R:TCATTTCTTGATTTTGTTTCTTTCA | (GATA)18 | 128–194 | 13 | 0.77 | 0.88 | KJ205465 |
| Tfal–51 | F:GAGAAGAGAGAAAAGAGCAGAGCA<br>R:AAGCCTTTATACTTCACTCTCCTGT | (GT)19 | 190–210 | 15 | 0.87 | 0.89 | KJ205466 |
| Tfal–52 | F:CGTTGACGAACAATACTAAACTGTG<br>R:GTGACTGTGTGTTAGCCACTGC | (GATA)20 | 135–223 | 19 | 0.84 | 0.92 | KJ205467 |
| Tfal–53 | F:AGCATGATTAGCACATTAGTCAACA<br>R:CATCATGTCTACATTGTTTTCCAAG | (TG)11 | 141–145 | 2 | 0.06 | 0.06 | KJ416412 |
| Tfal–54 | F:TATACAGGCATCTCAAACTGTCTGG<br>R:TTGTGATGGTTAAGGTTAGGGTAAG | (AC)12/(CA)8 | 151–169 | 4 | 0.64 | 0.62 | KJ416396 |
| Tfal–56 | F:TAGAGCAGAAAAACAACTTTCAACC<br>R:R:CTGGCAAGCCAAATATATGATCTAC | (CT)10(TC)31 | 129–193 | 28 | 0.77* | 0.93 | KJ205468 |
| Tfal–58 | F:AAACTTTGAATGCACAAAACATTTA<br>R:TGCATACAACAATATAAAACAGAGACA | (GTTT)2(GT)27 | 141–161 | 11 | 0.80 | 0.86 | KJ416397 |
| Tfal–60 | F:TTTCATCTTTATACCCCGTGTTTT<br>R:GCAGAGGGGTCTATTCTAATGACTT | (TC)13 | 119–139 | 8 | 0.62 | 0.73 | KJ205469 |
| Tfal–61 | F:AAATCAAATAAGTTTACTGGTCAGACA<br>R:TGGCTATCTAATGATGTTTATCTTTC | (CA)13 | 133–171 | 15 | 0.90 | 0.90 | KJ205470 |
| Tfal–62 | F:ATAATTCATCCATTCAGCCTACTTG<br>R:ACTAATCCAATTTCTAGCCGAAGAC | (AC)34 | 151–189 | 13 | 0.95 | 0.87 | KJ205445 |
| Tfal–63 | F:CTGAGTAATACCTGTTCCCCACAT<br>R:CTGAAATAAGCTTTCTTTGGCTGT | (TG)12 | 173–181 | 5 | 0.48 | 0.46 | KJ205446 |
| Tfal–64 | F:ACATTGGCGTTGTTGTTATAGTTCT<br>R:GAGCAGATAACCGTCTAATCATCTG | (GCA)28 | 120–204 | 23 | 0.92 | 0.93 | KJ205447 |
| Tfal–65 | F:CTTTTCCTGCATCCTGCTATAACC<br>R:TGGAGGAATGTGAACAAGTAATACA | (CA)12 | 147–149 | 2 | 0.02* | 0.98 | KJ416413 |
| Tfal–66 | F:CTTTCCATTCACACTCTGAACTCC<br>R:ACTGACTGGCACAGCATAAGAGAC | (CA)10 | 168–174 | 3 | 0.33 | 0.31 | KJ416398 |
| Tfal–67 | F:GAGCAGATAAAAGCAGTTTGTCAGT<br>R:CTGAGCTCAAGCTACCTCAGAATC | (CA)15 | 203–207 | 3 | 0.05 | 0.05 | KJ416414 |
| Tfal–70 | F:GGCATATTAACAACACACTCACAGA<br>R:CATTTGCACAAAGTGATTTAACGTA | (CA)16 | 109–117 | 4 | 0.65 | 0.65 | KJ205448 |
| Tfal–71 | F:AGTAATACCTTCCTCCCCATTACAC<br>R:CTTAACTAAGGGGATAGTGCTCGAC | (GT)20 | 164–310 | 54 | 0.90 | 0.97 | KJ205449 |
| Tfal–72 | F:TAGTTTTGACCCAGAAGGAATAGACT<br>R:CAGTACAAATAGACATGATGCTCCTC | (AC)10 | 169 | 1 | — | — | KJ416415 |

Table 2. Cross amplification of 54 Permit microsatellite DNA loci for the Florida and the Puerto Rico Pompano (*Trachinotus carolinus*) and the Puerto Rico Palometa (*T. goodei*). Forty-one loci amplified across all taxa.

| | Pompano (*T. carolinus*) | | | | | | | | Palometa (*T. goodei*) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Tampa Bay (n = 29) | | | | Puerto Rico (n = 84) | | | | Puerto Rico (n = 37) | | | |
| Locus | Allele size range | K[a] | $H_O$[b] | $H_E$[c] | Allele size range | K[a] | $H_O$[b] | $H_E$[c] | Allele size range | K[a] | $H_O$[b] | $H_E$[c] |
| Tfal–01 | 238–262 | 10 | 0.60 | 0.69 | 226–262 | 5 | 0.50 | 0.56 | 240–260 | 11 | 0.90 | 0.88 |
| Tfal–02 | 133 | 1 | — | — | 133 | 1 | — | — | 112–170 | 17 | 0.48 | 0.92 |
| Tfal–03 | 117–151 | 10 | 0.83 | 0.86 | 113–149 | 6 | 0.59 | 0.61 | 129–147 | 8 | 0.49 | 0.61 |
| Tfal–04 | 167–223 | 3 | 0.00* | 0.5 | 217–223 | 2 | 0.04 | 0.3 | 199–206 | 2 | — | 0.05 |
| Tfal–05 | — | — | — | — | — | — | — | — | 170–175 | 5 | 0.56 | 0.67 |
| Tfal–07 | 170–200 | 13 | 0.92 | 0.87 | 178–192 | 7 | 0.57 | 0.57 | — | — | — | — |
| Tfal–10 | — | — | — | — | — | — | — | — | 96–124 | 9 | 0.58 | 0.64 |
| Tfal–11 | 118 | 1 | — | — | 118 | 1 | — | — | 118 | 1 | — | — |
| Tfal–12 | 192–198 | 3 | 0.40 | 0.38 | 192–198 | 3 | 0.53 | 0.51 | 168–198 | 6 | 0.58 | 0.53 |
| Tfal–13 | — | — | — | — | — | — | — | — | — | — | — | — |
| Tfal–14 | — | — | — | — | — | — | — | — | 166–180 | 7 | 0.73 | 0.77 |
| Tfal–15 | 131–205 | 22 | 0.96 | 0.87 | 145–187 | 12 | 0.85 | 0.8 | 127–133 | 2 | 0.05 | 0.05 |
| Tfal–16 | 150 | 1 | — | — | 146–150 | 3 | 0.04 | 0.06 | 146–169 | 3 | 0.60 | 0.54 |
| Tfal–17 | 189–213 | 6 | 0.71 | 0.63 | 189–193 | 3 | 0.37 | 0.44 | 165–183 | 6 | 0.48 | 0.57 |
| Tfal–19 | 124–136 | 5 | 0.21 | 0.26 | 127–136 | 3 | 0.01 | 0.13 | 115–124 | 6 | 0.48 | 0.44 |
| Tfal–20 | — | — | — | — | — | — | — | — | 221–300 | 15 | 0.97 | 0.89 |
| Tfal–21 | 110–148 | 9 | 0.80 | 0.78 | 118–148 | 8 | 0.71 | 0.80 | 115–117 | 2 | 0.97 | 0.92 |
| Tfal–23 | 185 | 1 | — | — | 185 | 1 | — | — | 199 | 1 | — | — |
| Tfal–25 | 176–264 | 22 | 0.48* | 0.94 | 200–276 | 14 | 0.3 | 0.84 | 242–294 | 12 | 0.94 | 0.90 |
| Tfal–26 | 225–229 | 3 | 0.60 | 0.59 | 225–229 | 3 | 0.17 | 0.17 | 220–286 | 5 | — | — |
| Tfal–28 | 167–198 | 4 | 0.32 | 0.35 | 167–169 | 2 | 0.18 | 0.27 | 165–199 | 5 | 0.51 | 0.47 |
| Tfal–30 | 137–139 | 2 | 0.04 | 0.04 | 137–141 | 2 | 0.22 | 0.20 | 141–151 | 4 | 0.15 | 0.15 |
| Tfal–31 | — | — | — | — | — | — | — | — | 109–117 | 4 | 0.69 | 0.53 |
| Tfal–33 | 133–163 | 12 | 0.96 | 0.88 | 133–181 | 11 | 0.47 | 0.86 | 109-117 | 4 | 0.53 | 0.52 |
| Tfal–35 | 136–144 | 2 | 0.17 | 0.15 | 136 | 1 | — | — | 145–235 | 7 | 0.65 | 0.60 |
| Tfal–36 | 93–131 | 8 | 0.68 | 0.72 | 93–133 | 8 | 0.64 | 0.72 | 109–143 | 13 | 0.95 | 0.85 |
| Tfal–37 | 136 | 1 | –– | –– | 136 | 1 | — | — | 145 | 1 | — | — |
| Tfal–39 | — | — | — | — | — | — | — | — | — | — | — | — |
| Tfal–40 | — | — | — | — | — | — | — | — | — | — | — | — |
| Tfal–41 | — | — | — | — | — | — | — | — | 199–205 | 2 | 0.06 | 0.05 |
| Tfal–42 | 180-210 | 5 | 0.33 | 0.37 | 168-200 | 3 | 0.01 | 0.03 | 192–210 | 8 | 0.79 | 0.77 |
| Tfal–43 | — | — | — | — | — | — | — | — | — | — | — | — |
| Tfal–46 | 123 | 1 | — | — | 123 | 1 | — | — | 121 | 1 | — | — |
| Tfal–47 | 183 | 1 | –– | –– | 183–218 | 2 | 0.00 | 0.22 | 214–216 | 2 | 0.23 | 0.2 |
| Tfal–48 | 183-211 | 3 | 0.043 | 0.043 | 183 | 1 | –– | –– | 197-217 | 8 | 0.85 | 0.78 |
| Tfal–49 | 136–138 | 2 | 0.2 | 0.24 | 136–140 | 3 | 0.56 | 0.55 | 146–150 | 3 | 0.40 | 0.38 |
| Tfal–50 | — | — | — | — | — | — | — | — | — | — | — | — |
| Tfal–51 | 202–210 | 6 | 0.65 | 0.69 | 136–210 | 4 | 0.40 | 0.43 | 186–204 | 8 | 0.55 | 0.54 |
| Tfal–52 | — | — | — | — | — | — | — | — | 232–300 | 13 | 0.77 | 0.88 |
| Tfal–53 | 133–149 | 3 | 0.48 | 0.58 | 133–145 | 2 | 0.46 | 0.41 | 141 | 1 | — | — |
| Tfal–54 | 159–263 | 20 | 0.81 | 0.86 | 165–233 | 9 | 0.30* | 0.77 | 169–253 | 6 | 0.84 | 0.80 |
| Tfal–56 | 113–153 | 17 | 0.88 | 0.90 | 123–177 | 5 | 0.45 | 0.50 | 87–89 | 2 | 0.03 | 0.03 |
| Tfal–58 | 97 | 1 | — | — | 97 | 1 | — | — | 137–161 | 9 | 0.5 | 0.86 |
| Tfal–60 | — | — | — | — | — | — | — | — | 115 | 1 | — | — |
| Tfal–61 | 133 | 1 | — | — | 133 | 1 | — | — | 135–149 | 8 | 0.61 | 0.72 |
| Tfal–62 | 131–231 | 19 | 0.96 | 0.9 | 155–197 | 9 | 0.68 | 0.75 | 123–145 | 9 | 0.56 | 0.49 |
| Tfal–63 | 179–183 | 3 | 0.16 | 0.15 | 179–185 | 2 | 0.02 | 0.02 | 185–191 | 4 | 0.18 | 0.17 |
| Tfal–64 | 120–204 | 20 | 0.88 | 0.93 | 124–224 | 11 | 0.69 | 0.70 | 114–171 | 18 | 0.87 | 0.89 |

Table 2 (*continued*). Cross amplification of 54 Permit microsatellite DNA loci for the Florida and the Puerto Rico Pompano (*Trachinotus carolinus*) and the Puerto Rico Palometa (*T. goodei*). Forty-one loci amplified across all taxa.

| | Pompano (*T. carolinus*) | | | | | | | | Palometa (*T. goodei*) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Tampa Bay (n = 29) | | | | Puerto Rico (n = 84) | | | | Puerto Rico (n = 37) | | | |
| Locus | Allele size range | $K^a$ | $H_O{}^b$ | $H_E{}^c$ | Allele size range | $K^a$ | $H_O{}^b$ | $H_E{}^c$ | Allele size range | $K^a$ | $H_O{}^b$ | $H_E{}^c$ |
| Tfal–65 | 151 | 1 | — | — | 151 | 1 | — | — | 159 | 1 | — | — |
| Tfal–66 | 188–210 | 9 | 0.67 | 0.7 | 198–202 | 3 | 0.42 | 0.40 | 107–188 | 8 | 0.58 | 0.71 |
| Tfal–67 | 136–233 | 6 | 0.50 | 0.65 | 227–231 | 2 | 0.29 | 0.37 | 169–243 | 12 | 0.67 | 0.69 |
| Tfal–70 | 111–127 | 7 | 0.52 | 0.49 | 111–125 | 5 | 0.57 | 0.54 | 101–111 | 4 | 0.57 | 0.56 |
| Tfal–71 | 134–142 | 4 | 0.52 | 0.52 | 134–142 | 4 | 0.58 | 0.58 | 134–140 | 4 | 0.18 | 0.21 |
| Tfal–72 | 161 | 1 | — | — | 161 | 1 | — | — | 169 | 1 | — | — |

n = number of individuals; $K^a$ = number of alleles; $H_O{}^b$ = observed heterozygosity; $H_E{}^c$ = expected heterozygosity

Table 3. Average standard measures of genetic diversity for three species of the genus *Trachinotus* overall 35 Permit microsatellite loci amplified across all taxa. The numbers in brackets are values for all 54 microsatellite DNA loci.

| | Permit (*T. falcatus*) | Pompano (*T. carolinus*) | | Palometa (*T. goodei*) |
|---|---|---|---|---|
| | Tampa Bay | Tampa Bay | Puerto Rico | Puerto Rico |
| MP | 31 [45] 31 | 26 [30] | 24 [30] 24 | 28 [40] |
| MM | 4 [9] | 9 [11] | 11 [11] | 7 [8] |
| MNA | [0] | [13] | [13] | [6] |
| $K^a$ | 12.9 | 11.9 | 5.5 | 7.5 |
| $K^r$ | 12.2 | 10.5 | 4.9 | 5.2 |
| $H_O$ | 0.63 | 0.53 | 0.46 | 0.51 |
| $H_E$ | 0.63 | 0.59 | 0.48 | 0.55 |
| Gd | 0.65 | 0.55 | 0.56 | 0.51 |
| $F_{IS}$ | 0.047 | 0.076 | 0.156 | 0.107 |

MP = number of polymorphic microsatellite markers; MM = number of monomorphic microsatellite markers; MNA = number of microsatellite markers that did not amplify; $K^a$ = average number of alleles over all loci; $K^r$ = average number of allelic richness over all loci; $H_O$ = average expected homogzygosity over all loci; $H_E$ = average expected heterozygosity over all loci; G.D = average genetic diversity over all loci; $F_{IS}$ = fixation index over all loci

optimal number of clusters was $K = 4$. But contrary to this expectation, the observed optimal peak was $K = 2$ ($\Delta K = 302$; Figure 1A), at which Permit and Palometa were combined in one cluster and Pompano from Florida and Puerto Rico were combined in the second cluster (Figure 2A, K = 2). At K = 3 in this analysis ($\Delta K = 3$; Figure 2A, K = 3), the four samples were divided into three clusters, Permit in one, Palometa in one, and Pompano in the third in 9 out 10 replicates, but in 1 out 10 the cluster was the same as K = 2. At K = 4 ($\Delta K = 4$; Figure 2B), four clusters corresponded to the 4 taxa in 9 out 10 replicates, but in 1 out of 10, it was the same as $K = 3$. Therefore, $K = 3$ and $K = 4$ did not adequately explain the data, and the Bayesian population assignment did not differentiate the samples to the expected 4 clusters. In the combination of three taxa that included Permit and the two Pompano taxa, the expected optimal peak was $K = 3$, but the observed optimal peak was at $K = 2$ ($\Delta K = 375$, Figure 1B). The expected peak K =3 appeared at a much lower peak ($\Delta K = 36$). In this three-taxon analysis at the optimal peak $K = 2$, Permit and Pompano were clearly separated in all 10 replicates. At the lower peak $K = 3$, the Pompano from Florida and those from Puerto Rico were in different clusters in all 10 replicates, indicating that these two samples were of highly different genetic stocks. But $K = 3$ was not the best explanation of the data. In the second three-taxon combination with Palometa and the two Pompano samples, again the expected peak was $K = 3$, but the observed optimal peak was $K = 2$ ($\Delta K = 224$; Figure 3A), and the expected $K = 3$ appeared at a lower peak ($\Delta K = 61$; Figure 3A). This result was virtually the same as that for the previous three-taxon combination with Permit and the two Pompano taxa. The only difference was that the expected $K = 3$ was more pronounced in the latter analysis. When only one of the two Pompano samples were combined in a three-taxon combination, STRUCTURE-STRUCTURE HARVESTER analysis produced, a single optimal $K = 3$ ($\Delta K = 427$,

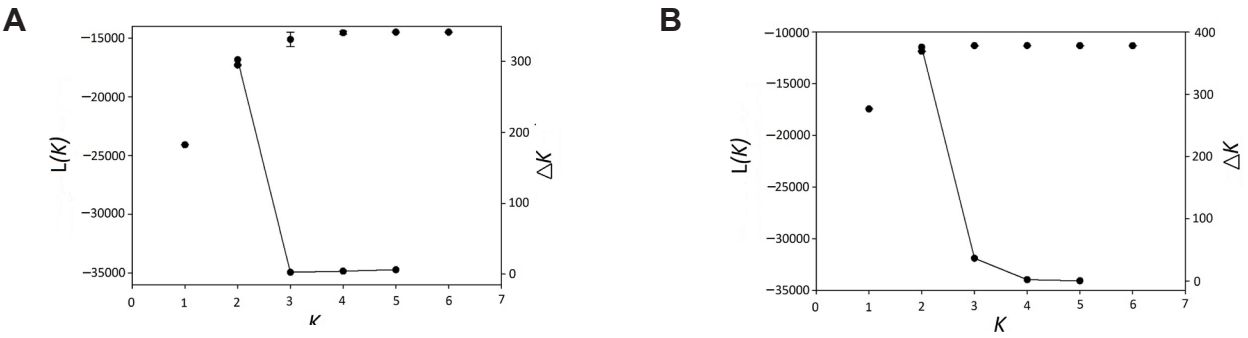


Figure 1. Mean likelihood L (posterior probability) L(K) and ΔK values in relation to the number of clusters (K) from STRUCTURE HARVESTER 10 replicate runs of each value of K (from 1 to 6): (A) for data with four samples each from Permit, Palometa, Florida Pompano and Puerto Rico Pompano showing a single optimum cluster at K = 2 (ΔK = 302); (B) with three samples each from Permit, Florida Pompano and Puerto Rico Pompano showing a hierarchical structure with optimum value at K = 2 (ΔK = 376) and a second smaller peak at K = 3 (ΔK = 36).

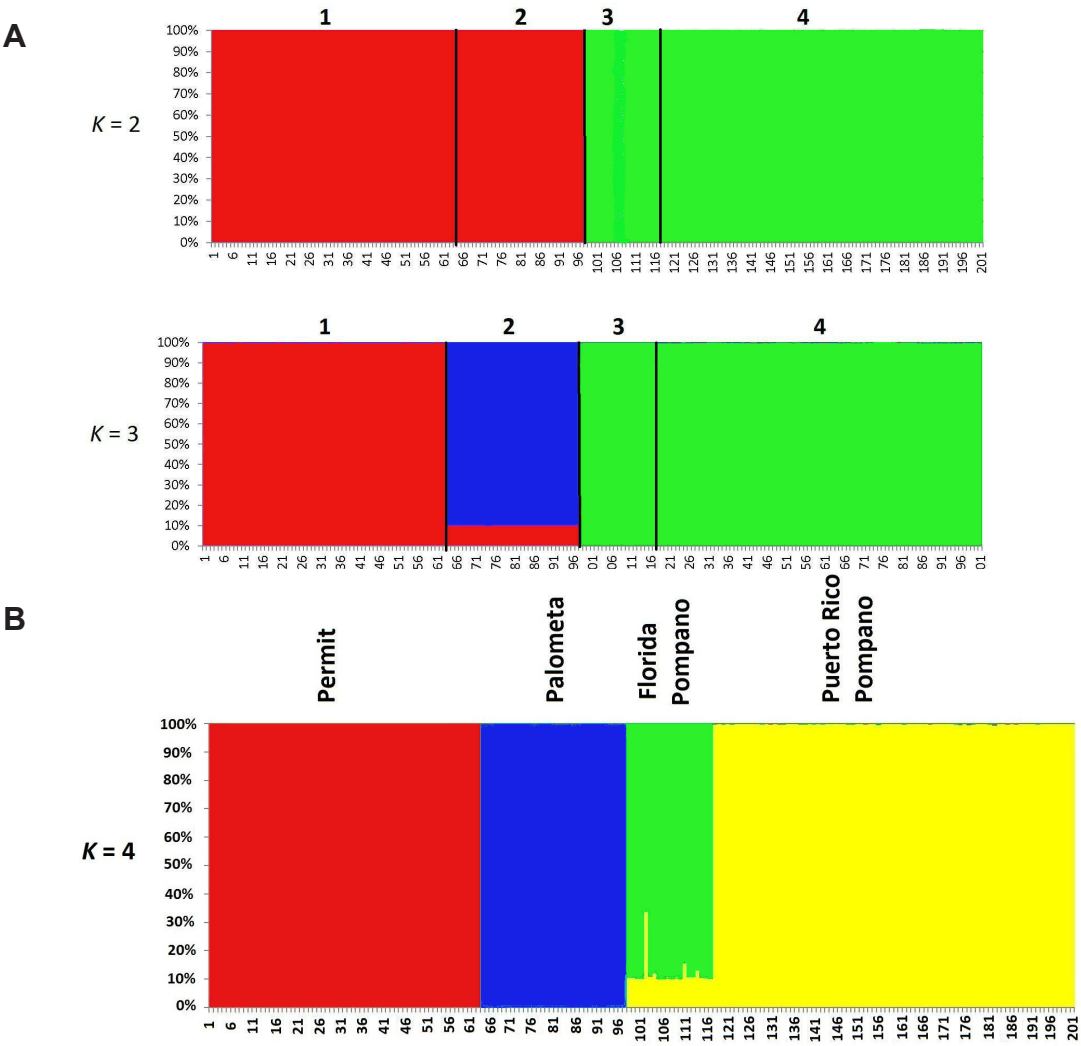


Figure 2. Species delineation of *Trachinotus* taxa using Bayesian model–based assignment tests according to posterior probability produced by STRUCTURE-STRUCTURE HARVESTER of CLUMPP output of 10 replicate runs for only Figure 1A: 2A, K = 2 (ΔK = 302) the single optimum modal value; 2A (K = 3) and 2B (K = 4) though both have ΔK = 0 in Figure 1A, the CLUMPP output indicate the presence of three and four clusters (species) respectively. In Figure 2A, K = 2 and K = 3, indicate taxa: 1 = Permit, 2 = Palometa, 3 = Florida Pompano, 4 = Puerto Rico Pompano.

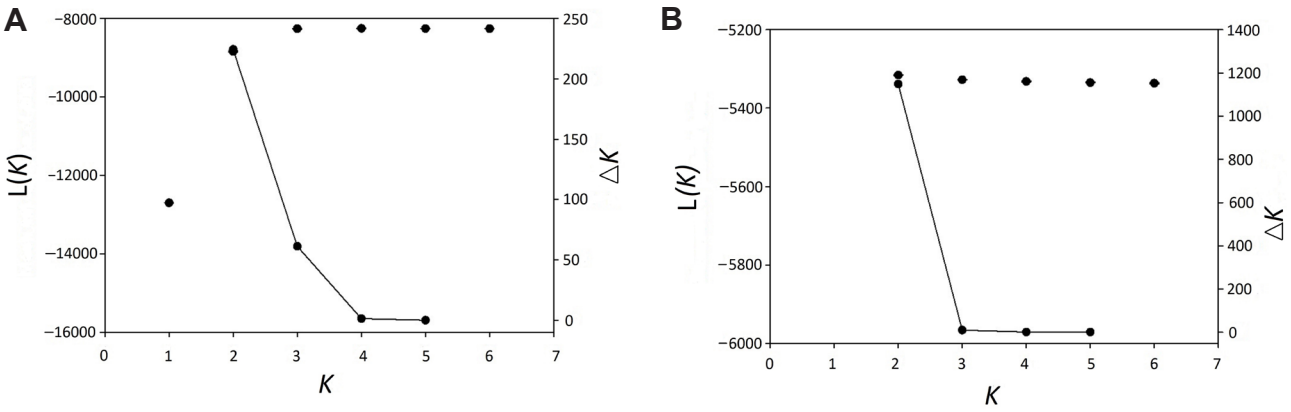


Figure 3. Mean likelihood L (posterior probability) L(K) and ΔK values in relation to the number of clusters (K) from STRUCTURE HARVESTER 10 replicate runs of each value of K (from 1 to 6): (A) for data with three samples each from Palometa, Florida Pompano, and Puerto Rico Pompano showing a hierarchical structure with optimum value at K = 2 (ΔK = 224) and a second smaller peak at K = 3 (ΔK = 61); (B) with only Pompano samples showing a single optimum cluster at K = 2 (ΔK = 1148).

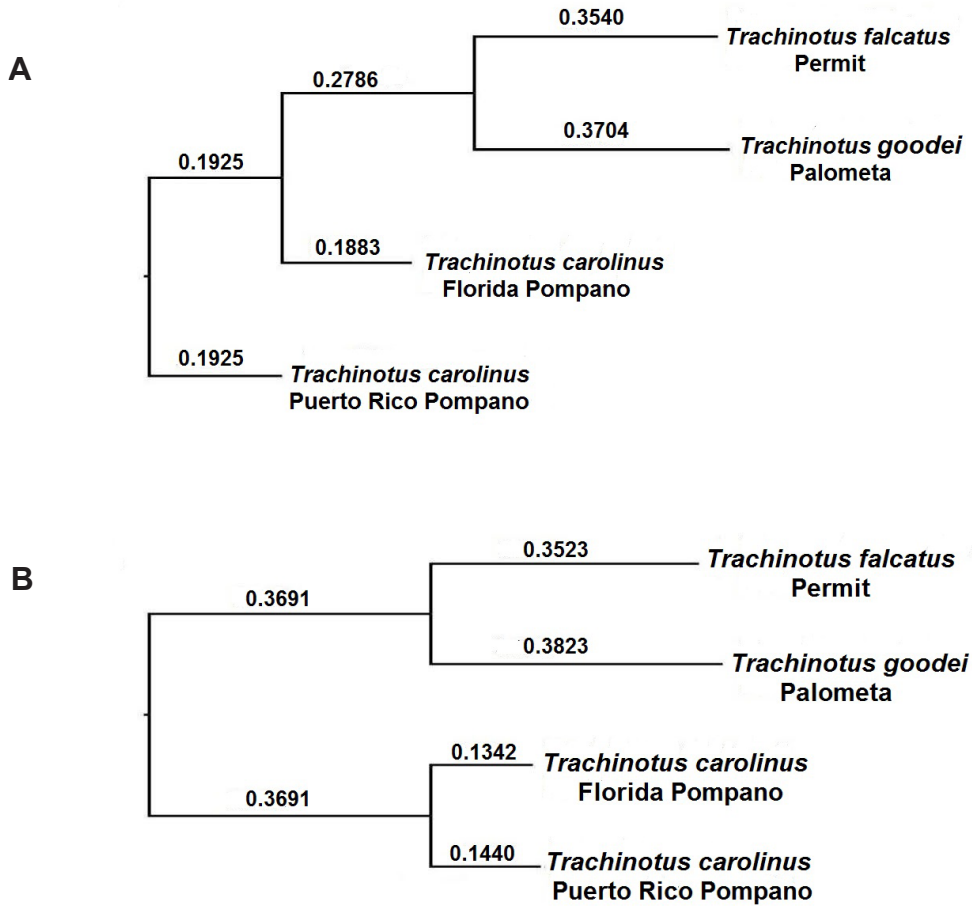


Figure 4. Unrooted neighbor–joining phenogram topologies estimated from distance methods (A) Cavalli-Sforza and Edwards' chord distance (DC) and (B) and Nei et al.'s DA distance from Permit 35 microsatellite DNA loci. Topologies of DA and FST were similar and are not shown here.

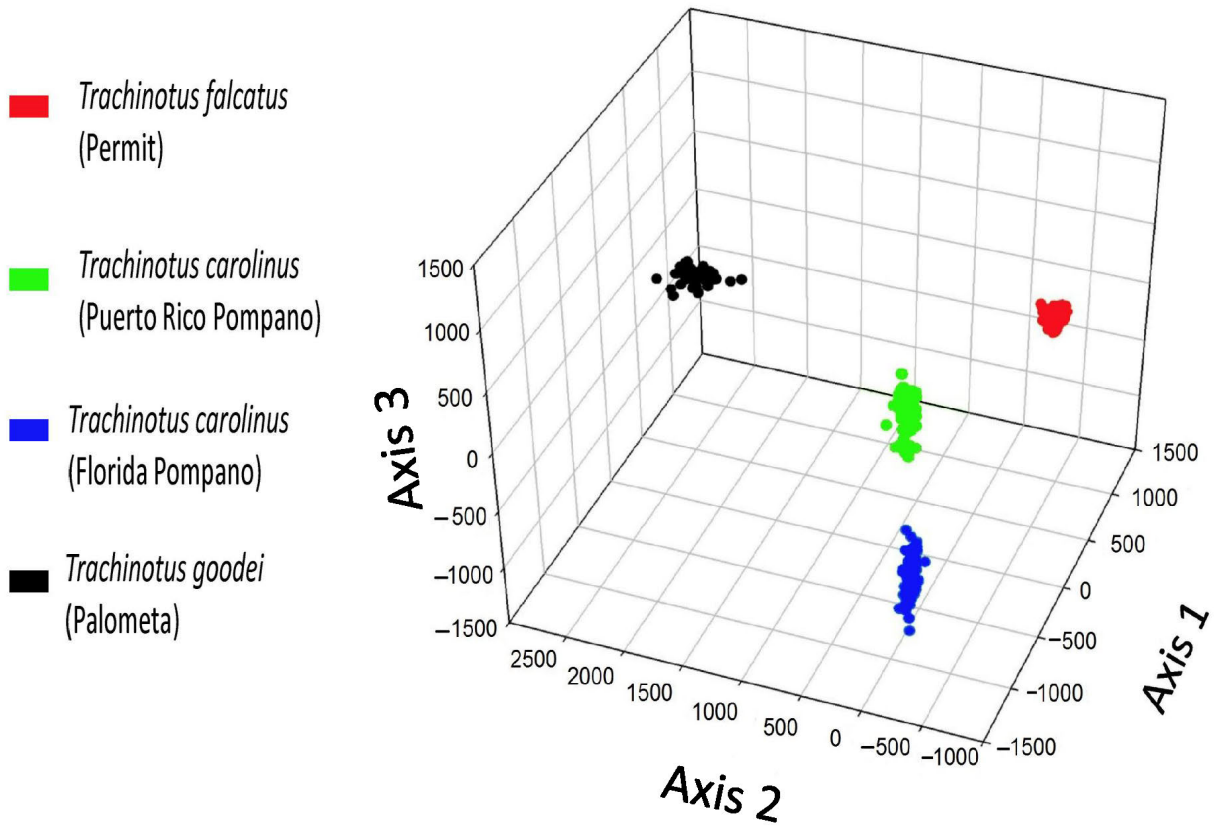


Figure 5. Three-dimensional plots of factorial correspondence analysis results for the four taxa in the genus *Trachinotus* species genotyped with 35 polymorphic Permit microsatellite DNA loci.

with Florida Pompano; and ΔK = 328; with Puerto Rico Pompano) (no figure shown). Here there was complete agreement between observed and expected K values. Finally, when only the two Pompano samples were STRUCTURE-STRUCTURE HARVESTER analyzed, the Florida and Puerto Rico samples were strongly classified in two clusters (K = 2; ΔK = 1148; Figure 3B).

## Phylogenetic clustering

The neighbor-joining phylogenetic trees constructed based on $D_C$ (Figure 4A) and $D_A$ (Figure 4B) showed slightly different topologies of phylogenetic tree network. Both topologies showed four major branches with different branching length including the partitioning of the Florida and Puerto Rico Pompanos, indicating that the Pompano samples are composed of highly differentiated populations. The difference stems from the $D_C$ method, which puts the Florida Pompano closer to Palometa-Permit, and the Puerto Rico Pompano basal (oldest). The estimated distance between these taxa from the $D_C$ and $D_A$ are given in Table 4. Takezaki et al. (2008) stated that the branching pattern of a tree constructed with microsatellite DNA was generally the most reliable for $D_A$. The pattern from the $D_A$ distance (Figure 4B) does not indicate which taxon is the most derived (recent) or basal (oldest), and this information cannot be verified without an outgroup, that is an additional taxon that could serve as a basis to analyze the tree.

## Factorial correspondence analysis (FCA)

In three-dimensional plots produced from the FCA results (Figure 5), the simultaneous expression of the genetic signal contributed by each allele is visualized (as in the plot based on Pompano microsatellite data in Tringali et al. 2006). It is apparent that Florida and Puerto Rico Pompano, both classified in the species *T. carolinus*, were projected in tight clusters well separated from each other and from Permit and Palometa clusters. The pairwise spatial distances calculated between the centers of mass of each of the taxa in the plot (Table 4) indicated that *T. goodei* or *T. falcatus* was the most divergent and that the Florida Pompano was basal. But the FCA does not depict the relationships among the taxa according to evolutionary hierarchy, and the relation of derived-basal could not be determined.

Table 4. Estimates of Cavailli-Sforza and Edwards' (Dc) chord distance, uncorrected $F_{ST}$ genetic distance [in brackets] and values of factorial correspondence analysis representing the distance between the centers of the clusters of each pair of taxa in the 3-dimensional FCA plot (in parentheses) {below diagonal}, and NEI et al.'s DA distance and corrected $F_{ST}$ genetic distance [in brackets] {above diagonal} between four pairs of the four *Trachinotus* samples. Distance values were estimated based on the 35 Permit microsatellite genotypes.

| | Permit | Palometa | Florida Pompano | Puerto Rico Pompano |
|---|---|---|---|---|
| **Permit** | 0 | 0.736 [0.450] | 0.8499 [0.521] | 0.871 [0.561] |
| **Palometa** | 0.7244 [0.465] (293) | 0 [0.536] | 0.8912 [0.576] | 0.8897 |
| **Florida Pompano** | 0.8174 [0.537] (917) | 0.8408 [0.557] (1036) | 0 | 0.2781 [0.205] |
| **Puerto Rico Pompano** | 0.8286 [0.568] (419) | 0.8379 [0.588] (722) | 0.3808 [0.233] (700) | 0 |

All distance values were statistically highly significant ($P < 0.005$).

# Discussion

## Microsatellite variation

A high rate of polymorphism, high abundance, and pervasive distribution throughout the genome of almost all organisms have made microsatellites one of the most popular markers in population genetics. The development costs and technical challenges in the construction of enriched libraries (clones containing short tandem repeats) could be compensated for by applying markers developed for one species to the study of congeners or other species of the same family. But due to the well-documented phenomenon of ascertainment bias (deviations from the expected theoretical result due to sampling processes used to find and estimate population-specific allele frequencies) the maximum information that can be derived from microsatellite markers is limited to the organism from which they were developed. Although markers developed for one species may amplify in others, their variability appears to wane in cross-amplified species. The gradual decrease of variability is probably proportional to relative distance of relationship between the donor and recipient organism; the more distantly related the organisms, the less variable the markers. As expected, Permit from which the markers were developed showed more genetic variation for all the genetic parameters measured than did the congeners. This phenomenon, known as microsatellite bias, has been reported for simple genetic parameters in congeners (Forbes et al. 1995). Thus the use of a small number of markers from one species may be inadequate in the study of the genetic population structure of related species. For example, the use of 6 highly polymorphic markers in the Sheepshead (*Archosargus probatocephalus*), originally developed for other sparids, showed no differentiation between the Gulf and Atlantic samples (Anderson et al. 2008). But markers developed de novo for the Sheepshead showed the Sheepshead is fragmented into three clusters within the Gulf and Atlantic regions (Seyoum et al. in preparation). Perhaps the use of a large number of sparid markers may reveal the presence of fragmented structure in the Sheepshead.

One of the most important uses of amplifying microsatellite markers among congeners is in species and hybrid identification. For example, the four loci that were monomorphic across all taxa, though identical between Permit and Palometa, were of different sizes from those of the Pompano samples. These loci can be effectively used to distinguish Pompano samples from those of Permit or Palometa and also to assess whether interspecific hybridization occurs between these species.

## Bayesian population assignment test

Bayesian population assignment is a powerful tool that can discriminate differentiation among populations between which gene flow has been curtailed. By extension, the model-based individual assignment tests could be used to delineate species (Noble at al. 2010). It should clearly and consistently identify the significant differentiation in Pompano samples. It did so, however, only when the Pompano samples were analyzed separately. When all the congeners were included, however, this method did not operate to give four clusters in either the no-admixture model or the admixture model. (There was no difference in the results obtained from these models.) In these analyses several possible combinations of samples were considered in the STRUCTURE-STRUCTURE HARVESTER analyses, and the expected *K* values were not observed in all combinations that included both Pompano populations and one or two of the other congeners. The observed and expected *K* values were the same only when one of the Pompano populations was excluded. The expected *K* values were not realized in these analyses because the Permit microsatellite markers could not discriminate between the two Pompano populations with absolute clarity, probably a result of microsatellite bias. In the presence of all four populations the Permit micro-

satellite markers could not differentiate not only between the two Pompano populations but even between Permit and Palometa samples such that it combined Permit and Palometa in one cluster and the two Pompano populations in a second cluster as the best explanation of the data. This is possibly again due to ascertainment bias, which is indicated in terms of microsatellite marker sizes. For example, there were four microsatellite loci that were monomorphic in all four taxa. The sizes of these were identical in Permit and Palometa, but are different in the Pompano; three of these were larger in size than in the Pompano samples. Consistent differences in microsatellite marker size have been shown to exist between related species (Amos 2000), and, although it is not immediately clear whether this explains the observed result of $K = 2$ in the four-taxon STRUCTURE-STRUCTURE HARVESTER analyses, the four monomorphic loci indicate why Permit-Palometa are in one cluster and the Pompano taxa in the second cluster. The microsatellite markers selected from Permit specifically represent the genealogical histories of the Permit genome and could not represent the genealogical histories of congener genomes with absolute measure, thus failing to properly classify divergent but closely related taxa. Even though the expected optimal peaks were not observed in the four-taxon Bayesian population test, under the assumption of $K = 4$, the predominant $K$ in the 10 replicate runs was 4. Hence the Bayesian population assignment delineates the number of naturally occurring taxa in the background, in the presence of the taxon from which the markers were selected. We do not know exactly how this bias phenomenon functions or whether ascertainment bias (see Eriksson and Manica 2011; Li and Kimmel 2013) is operating in the assignment test. It appears that markers developed for one species can only adequately represent the genealogical histories of that species much better, losing their efficiency in revealing evolutionary histories in other species, probably proportionally to the distance of the species in relationship to the microsatellite source taxon. This phenomenon probably also operates in a dual fashion, with a combined effect of bias toward one taxon from which the markers were selected and bias against other taxa leading to the masking of secondary peaks in a manner analogous to reverse microsatellite bias (Hogan et al. 2009). Furthermore, the magnitude of the forward and reverse microsatellite biases could be accentuated by the relative distance between the taxon microsatellite marker source and its congeners. But if the marker source is from one of two closely related taxa and if the other taxa in the clade are distantly related, the bias would have no effect in the assignment test.

Differentiation of Pompano samples could not be verified when Permit and Palometa were present in the analyses. But when Permit was coupled with Palometa and only one of the Pompano taxa was present in a three-taxon combination, an optimal $K = 3$ was obtained. In the three-taxon analysis of the Pompano samples with either Permit or Palometa, $K = 3$ was obtained though at a lower peak, in which all 10 replicates defined the Pompano samples in different clusters. $K = 3$ was not the optimal peak in these analysis, apparently because the Permit from which markers were selected was functioning as a distant out group and could not adequately define the closely related Pompano taxa through the Bayesian population test. This occurrence seems to be analogous to the suggestion that phylogenetic analysis is susceptible to random biases introduced by too-distant out-group taxa (Kirchberger et al. 2013). The selected Permit markers could be too centered in the Permit genome, and, external to this center, they may introduce random biases that identification of the two closely related Pompano taxa could not be revealed with absolute clarity in the assignment test. When only two taxa are involved, however, the Bayesian population test is an excellent method of delineating species, particularly if the taxa are sympatric (Noble et al. 2010). In species delineation involving only two taxa, regardless of where the microsatellite markers were selected from, there can be no microsatellite bias, and the Bayesian population test functions properly, as seen when only Florida and Puerto Rico Pompano were treated together with Permit microsatellite markers.

Florida and Puerto Rico Pompano populations are allopatric, so the differentiation between them could be due to isolation by distance and geography, but the Bayesian population assignment test in which Pompano samples were considered separately revealed that Florida and Puerto Rico Pompano individuals were assigned to different clusters with >99% confidence probability, indicating the existence of two distinct gene pools.

## Phylogenetic clustering and FCA

Traditionally, phylogenetic trees among numerous taxa have been constructed from mtDNA sequences from a single region such as 16S rRNA, COI, or Cyt B. Later these mtDNA regions may have been replaced or fortified by sequences from nDNA such as introns or internally transcribed spacer (ITS) sequences. A tree constructed for a group of species based on a single locus from a homologous (orthologous) sequence sampled from different species is called a gene tree. It represents the life history of the gene through time of adaptations as it evolves

through duplication, loss, and nucleotide substitution. Although it is a part and linked, it may not reveal the evolutionary pathway of the species involved. A species tree is the life history of the organism through time of adaptations) (Szöllosi et al. 2013). Gene trees from different regions of the genome may undergo different historical events and may differ from one another and from that of the species tree (Degnan and Rosenberg 2006). The assumption that the species tree can be estimated by the most common tree among several gene trees can be highly misleading. Instead, by combining all the information on the genes, a reliable estimate of the historical pathway of the species can be obtained. A species tree, therefore, is the historical conglomerate of many gene trees blended into one. For this purpose microsatellite markers can provide an abundant number of loci and thus abundant historical information. By combining the results from many loci, one can obtain a more precise and statistically powerful means of comparing populations (species), resulting in a better measure of the evolutionary history of the species. With some limitations, microsatellite markers have been used to determine evolutionary relationships among organisms that diverged several million years ago (Richard and Thorpe, 2001). The number of loci needed for accurate estimation of the evolutionary pathway of populations varies from 30 (Takezaki and Nei 1996) to hundreds (Pollock et al. 1998). The majority of population studies, however, have been done with only six loci (Koskinen et al. 2004). This is far less than the lower limit of 30 and automatically cannot adequately expose inherent genetic properties, particularly fine population-genetic structure. Although for practical purposes a fixed number of loci should not be advanced, in general, the more loci employed, the better the estimate of the evolutionary pathway of populations.

Although microsatellite loci lose their efficiency as a reliable estimation of the species tree because of high mutation rate and the potential of homoplasy, they have been used to resolve phylogenies of taxa that diverged as long as 30 million years ago (Ochieng et al. 2007). In the present study the phenogram constructed from the microsatellite-marker data decidedly showed a highly significant differentiation between Florida and Puerto Rico Pompano samples. A strong indication in the separation of these taxa to a higher level than a stock is also evident from the measure of the $D_C$ tree between Florida and Puerto Rico Pompano. The result we found in the phylogenetic analysis among the *Trachinotus* species from the $D_C$ was similar in terms of most derived (Palometa–Permit) and basal (Pompano) with that of Jacobina et al. (2012) and Reed et al. (2002) phylogenetic relationships among these species. The result of the FCA was probably the most direct and observable evidence that Florida and Puerto Rico Pompano clusters occupy closer but clearly different spatial positions as that of the other species in the three-dimensional space. The number of clusters (sample clouds) visible on the three dimensional figure was four, indicating that there are four taxa with three recognized *Trachinotus* species, one of which, the Pompano, is divided into two, closer clusters (sample clouds).

## Conclusion

The results from the three analytical methods based on the Bayesian population assignment tests, the phylogenetic clustering, and the factorial correspondence analysis of the genetic relationships among the four samples of the three *Trachinotus* species showed that Florida and Puerto Rico Pompano samples belong to distinct genetic populations. Preliminary observation has shown that Pompano exhibit considerable intraspecific variability in body depth, and photographic evidence suggests that specimens from Puerto Rico may possess anterior morphometric differences (especially head size and shape) from those of Pompano in coastal U.S. waters (M. Tringali, pers. comm.).

To warrant a specific designation of the Puerto Rico Pompano above the highly distinct genetic population level, however, other multiple molecular, particularly nDNA sequences from many introns and nonmolecular tools should be used concurrently with detailed study of morphological and meristic differences.

## Acknowledgments

We thank the volunteer recreational anglers of Florida and the staff of the Fisheries Independent Monitoring program and Marine Fisheries Biology subsection of the Florida Fish and Wildlife Conservation Commission's Fish and Wildlife Research Institute for collecting the samples. We are especially grateful to Steve Geiger and Elizabeth Wallace for insightful comments on a draft of this report. This work was supported by the U.S. Department of the Interior, U.S. Fish and Wildlife Service, under the Federal Aid in Sport Fish Restoration Program, Grant F-69. The statements, findings, and conclusions are those of the authors and do not necessarily reflect the views or policies of the U.S. Department of the Interior. Mention of trade names or commercial products does not constitute their endorsement by the U.S. government.

## References


Adams AJ, Blewett DA. 2004. Spatial patterns of estuarine habitat type use and temporal patterns in abundance of juvenile Permit, *Trachinotus falcatus,* in Charlotte Harbor, Florida. Gulf Caribb Res. 16(2):129-139.

Adams AJ, Wolfe RK, Kellison GT, Victor BC. 2006. Patterns of juvenile habitat use and seasonality of settlement by Permit, *Trachinotus falcatus*. Environ Biol Fishes. 75:209-217.

Álvarez-Lajonchère L., Ibarra-Castro L. 2012. Juvenile yield index to highlight intensive culture potential in tropical marine fish. Hidrobiológica. 22(1):42-48

Amos W. 2000. A comparative approach to the study of microsatellite evolution. In: Goldstein DB, Schlötterer C, editors. Microsatellites, evolution and applications. New York (NY). Oxford University Press Inc. p. 66-78

Anderson JD, Karel WJ, Anderson KA, Roper-Foo PA. 2008. Genetic assessment of Sheepshead stock structure in the northern Gulf of Mexico: morphological divergence in the face of gene flow. N Am J Fish Manag. 28(2):592-606. DOI: 10.1577/M07-056.1 http://dx.doi.org/10.1577/M07-056.1

Armstrong MP, Hood PB, Murphy MD, Muller RG. 1996. A stock assessment of Permit, *Trachinotus falcatus,* in Florida waters. St. Petersburg, Florida Marine Research Institute, In-house Report IHR 1996-005.

Belkhir K, Borsa P, Chikhi K, Raufaste N, Bonhomme F. 2000. GENETIX, Logiciel sous Windows$^{TM}$ pour la génétique des samples. Laboratoire Génome, Samples, Interactions CNRS UMR 5000. Montpellier, France, Université de Montpellier II.

Bellinger JW, Avault JW Jr. 1970. Seasonal occurrence, growth, and length-weight relationship of juvenile Pompano, *Trachinotus carolinus,* in Louisiana. Trans Am Fish Soc. 99(2):353-358.

Berry F, Iversen ES. 1966. Pompano: biology, fisheries and farming potential. Proc Gulf Caribb Fish Inst. 19:116-128.

Berry FH, Smith-Vaniz WS. 1978. Carangidae. In: Fischer W, editor. FAO species identification sheets for fishery purposes. West Atlantic (Fishing Area 31). volume 1. Rome, FAO.

Cavalli-Sforza LL, Edwards AWF. 1967. Phylogenetic analysis: models and estimation procedures. Am J Hum Genet. 19:233-257.

Corunet JM, Piry S, Luikart G, Estoup A, Solignac M. 1999. New methods employing multilocus genotypes to select or exclude populations as origins of individuals. Genetics. 153:1989-2000.

Crabtree RE, Hood PB, Snodgrass DJ. 2002. Age, growth, and reproduction of Permit (*Trachinotus falcatus*) in Florida waters. Fish Bull. 100:26-34.

Degnan JH, Rosenberg NA. 2006. Discordance of species trees with their most likely gene trees. PLoS Genet. 2(5): e68. DOI: 10.1371/journal.pgen.0020068

Díaz de Astarloa JM, Figueroa DE, Cousseau MB, Barragán M. 2000. Occurrence of *Trachinotus carolinus* (Carangidae) in Laguna Costera Mar Chiquita, with comments on other occasionally recorded fishes in Argentine waters. Bull Mar Sci. 66(2):399-403.

Earl DA, Vonholdt MB. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv Genet Resour. 4(2):359-361.

Eriksson A, Manica A. 2011. Detecting and removing ascertainment bias in microsatellite from the HGDP-CEPH panel. G3 (Bethesda). 1(6):479-488. doi: 10.1534/g3.111.001016

Evanno G., Regnaut S, Goudet J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol.14:2611-2620.

Faircloth BC. 2008. MSATCOMMANDER: detection of microsatellite repeat arrays and automated, locus-specific primer design. Mol Ecol Resour. 8:92-94. DOI:10.1111/j.1471-8286.2007.01884.x.

Falush D, Stephens M, Pritchard JK. 2003. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics. 164:1567-1587.

Felix FC, Spach HL, Moro PS, Schwarz RJ, Santos C., Hackradt CW, Hostim-Silva M. 2007: Utilization patterns of surf zone inhabiting fish from beaches in southern Brazil. Panam J Aquat Sci. 2:27-39.

Fields HM. 1962. Pompano (*Trachinotus* spp.) of the south Atlantic coast of the United States. Fish Bull. 62:189-222.

Finucane JH. 1969. Ecology of the Pompano (*Trachinotus carolinus*) and the Permit (*T. falcatus*) in Florida. Trans Am Fish Soc, 98(3):478-486.

[FWRI] Florida Fish and Wildlife Conservation Commission, Fish and Wildlife Research Institute. 2012. Status and trends report: 2012 Permit species account. St. Petersburg, Florida, Florida Fish and Wildlife Conservation Commission, Fish and Wildlife Research Institute. In-House Report IHR2012-034.

Forbes SH, Hogg JT, Buchanan FC, Crawford AM, Allendorf FW. 1995. Microsatellite evolution in congeneric mammals: domestic and bighorn sheep. Mol Biol Evol. 12(6):1106-1113

Gilbert C, Parsons J. 1986. Species profiles: life histories and environmental requirements of coastal fishes and invertebrates (South Florida): Florida Pompano. U S Fish Wildl Serv Biol Rep. 82(11.42 ). U S Army Corps Eng. TR EL-82-4.

Goudet J. 2001. FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Available from: http://www.unil.ch/izea/softwares/fstat.html

Graham RT, Castellanos DW. 2005. Courtship and spawning behaviors of carangid species in Belize. Fish Bull. 103:426-432.

Guindon KY, Powell C, Barbieri LR. 2008. Size, age structure, growth and mortality of Florida Pompano in the Tampa Bay area, Florida, USA with notes on reproductive biology. In: Investigations into nearshore and estuarine gamefish behavior, ecology and life history in Florida. St. Petersburg, Florida, Florida Fish and Wildlife Conservation Commission, Fish and Wildlife Research Institute. Grant F-59 Report, File Code: F2678-03-08-F, p. 3-22.

Guo SW, Thompson EA. 1992. Performing the exact test of Hardy-Weinberg proportions for multiple alleles. Biometrics. 48:361-372.

Hogan FE, Cooke R, Norman JA. 2009. Reverse ascertainment bias in microsatellite allelic diversity in owls (Aves, Strigiformes) Conserv Genet. 10(3):635-638. DOI: 10.1007/s10592-008-9595-0.

Jacobina UD, Vicari MR, Bertollo LAC, Molina WF. 2012. Discriminatory profile of rDNA sites and trend for acrocentric chromosome formation in the genus *Trachinotus* Lacépède, 1801 (Perciformes, Carangidae). Comp Cytogenet. 6(4):359-369.

Jakobsson M., Rosenberg N. 2007. CLUMPP (version 1.1.2): a cluster matching and permutation program for dealing with label switching and multimodality in analysis of sample structure. Bioinformatics. 23(14):1801-1806.

Kalinowski ST. 2009. How well do evolutionary trees describe genetic relationships between populations? Heredity. 102:506-513.

Kirchberger PC, Sefc KM, Sturmbauer C, Koblmuller S. 2013. Out-group effects on root position and tree topology in the AFLP phylogeny of a rapidly radiating lineage of cichlid fish. Mol Phylogenet Evol. 70(100):57-62.

Koskinen M, Hirvonen TH, Landry P-A, Primmer CR. 2004. The benefits of increasing the number of microsatellites utilized in genetic population studies: an empirical perspective Hereditas. 141:61-67.

Lansman RA, Shade RO, Shapiro JF, Avise JC. 1981. The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations. III. Techniques and potential applications. J Mol Evol. 17:214-226.

Latter BDH. 1972. Selection in finite populations with multiple alleles. III. Genetic divergence with centripetal selection and mutation. Genetics. 70:475-490.

Leberg PL. 2002. Estimating allelic richness: effects of sample size and bottlenecks. Mol Ecol. 11:2444-2449.

Li B, Kimmel M. 2013. Factors influencing ascertainment bias of microsatellite allele sizes: impact on estimates of mutation rates. Genetics. 195:563-572.

Lischer HEL, Excoffier L. 2012. PGDSpider [version 2.0.1.9]: an automated data conversion tool for connecting sample genetics and genomic programs. Bioinformatics. 28:298-299.

Marchuk D, Drumm M, Saulino A, Collins FS. 1991. Construction of vectors, a rapid and general system for direct cloning of unmodified PCR products. Nucleic Acids Res. 19(5):1154.

Mazzei E, Joyeux JC, Simon T. 2011. Length–weight relationships for juvenile and small-sized adult fishes of the surf zone. J Appl Ichthyol. 27(4):1137-1138.

McMaster MF, Kloth TC, Coburn JF. 2003. Prospects for commercial Pompano mariculture – 2003. Presentation at Aquaculture America 2003, Louisville, Kentucky. February 2003.

McEachran JD, Fechhelm JD. 2005. Fishes of the Gulf of Mexico. Vol. 2. Austin: University of Texas Press.

Murphy MD, Muller RG, Guindon K. 2008. A stock assessment for Pompano, *Trachinotus carolinus,* in Florida waters through 2005. St. Petersburg, FL, Florida Fish and Wildlife Conservation Commission, Fish and Wildlife Research Institute.

Murphy MD, MuAler RG, Hood PB. 1996. A stock assessment of Florida Pompano (*Trachinotus carolinus*). St. Petersburg, Florida Department of Environmental Protection, Florida Marine Research Institute.

Naughton SP, Saloman CH. 1978. Fishes of the nearshore zone of St. Andrew Bay, Florida, and adjacent coast. Northeast Gulf Sci. 2(1):43-55.

Nei M. 1973. Analysis of gene diversity in subdivided populations. Proc Natl Acad Sci USA. 70:3321-3323.

Nei, M. 1987. Molecular evolutionary genetics. New York, Columbia University Press.

Nei M, Roychoudhury A. 1974. Sampling variances of heterozygosity and genetic distance. Genetics. 76:379-390.

Nei, M, Tajima F, Tateno FY. 1983. Accuracy of estimated phylogenetic trees from molecular data. J Mol Evol. 19:153-170.

Nero VL, Sealey KS. 2005. Characterization of tropical near-shore fish communities by coastal habitat sta-

tus on spatially complex island systems. Environ Biol Fishes. 73(4):437-444.

Noble DWA, Qi Y, Fu J. 2010. Species delineation using Bayesian model–based assignment tests: a case study using Chinese Toad-headed Agamas (genus Phrynocephalus). BMC Evol Biol. 10:197-211.

Ochieng JW, Steane DA, Ladiges PY, Baverstock PR, Henry RJ, Shepherd M. 2007. Microsatellites retain phylogenetic signals across genera in eucalypts (Myrtaceae). Genet Mol Biol. 30(4):1125-1134. DOI: http://dx.doi.org/10.1590/S1415-47572007000600016.

Park SDE. 2001. Trypanotolerance in West African cattle and the population genetic effects of selection [Ph.D. thesis]. Dublin (Republic of Ireland): University of Dublin.

Pinheiro HT, Joyeux J, Martins AS. 2010. Reef fisheries and underwater surveys indicate overfishing of a Brazilian coastal island. Nat Conserv. 8(2):151-159.

Pollock DD, Bergman A, Feldman MW, Goldstein DB. 1998. Microsatellite behavior with range constraints: parameter estimation and improved distances for use in phylogenetic reconstruction. Theor Popul Biol. 53:256-271.

Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. Genetics. 155:945-959.

Pritchard JK, Wen X, Falush D. 2009. Documentation for structure software [version 2.2]. University of Chicago. Available from pritch.bsd.uchicago.edu/software.

Rambaut A. 2014. FigTree [version 1.4.1] Tree figure drawing tool. Edinburgh, UK, University of Edinburgh, Institute of Evolutionary Biology. http://tree.bio.ed.ac.uk/software/figtree/

Ranalla B, Mountain J. 1997. Detecting immigration by using multilocus genotypes. Proc Natl Acad Sci U S A, 94: 9197-9201.

Randall JE. 1983. Caribbean reef fishes, Rev. ed. Neptune City (NJ), TFH Publications Inc.

Raymond M, Rousset F. 1995. An exact test for population differentiation. Evolution. 49:1280-1283.

Reed DL, Carpenter KE, Gravelle MJ. 2002. Molecular systematics of the jacks (Perciformes: Carangidae) based on mitochondrial cytochrome b sequences using parsimony, likelihood, and Bayesian approaches. Mol Phylogenet Evol. 23:513-524. DOI: 10.1016/S1055-7903(02)00036-2

Rice WR. 1989. Analyzing tables of statistical tests. Evolution. 43:223-225.

Richard M., Thorpe RS. 2001. Can microsatellites be used to infer phylogenies? Evidence from population affinities of the Western Canary Island Lizard (*Gallotia galloti*). Mol Phylogenet Evol. 20(3):351-360.

Robins CR, Ray GC. 1986. A field guide to Atlantic coast fishes of North America. Boston (MA), Houghton Mifflin Company.

Rosenberg NA. 2004. DISTRUCT (version 1.1): a program for the graphical display of sample structure. Mol Ecol Notes. 4:137-138.

Ross SW, Lancaster JE. 2002. Movements and site fidelity of two juvenile fish species using surf zone nursery habitats along the southeastern North Carolina coast. Environ Biol Fishes. 63:161-172.

Rousset F. 2008. Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. Mol Ecol Resour. 8:103-106.

Rozen S, Skaletsky HJ. 2000. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (editors) Bioinformatics methods and protocols: Methods in Molecular Bology 132-:365-86

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol, 4:406-425.

Saloman CH, Naughton SP. 1978. Benthic macroinvertebrates inhabiting the swash zone of Panama City Beach, Florida. Northeast Gulf Sci. 2(1):65-72.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor [NY]: Cold Spring Harbor Laboratory Press.

Seyoum S, Denison SH, Tringali MD. 2006. Isolation and characterization of 13 polymorphic microsatellite loci for the Florida Pompano, *Trachinotus carolinus*. Mol Ecol Notes. 7:141-143.

Solomon JJ, Tremain DM. 2009. Recruitment timing and spatial patterns of estuarine use by young-of-the-year Florida Pompano, *Trachinotus carolinus,* in northeastern Florida. Bull Mar Sci. 85(2):133-148.

Springer VG, Woodburn KD. 1960. An ecological study of the fishes of the Tampa Bay area. St. Petersburg,-Florida Biodiversity Conservation Marine Laboratory. Professional Paper Series No.1: 1-104.

St. John J, Quinn TW. 2008. Rapid capture of DNA targets. BioTechniques. 44:259-264.

Szöllosi GJ, Tannier E, Daubin V, Boussau B. 2013. The inference of gene trees with species trees. arxiv:1311.0651v1 [q-bio.PE]

Takezaki N, Nei M. 1996. Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. Genetics. 144:389-399.

Takezaki N, Nei M. 2008. Empirical tests of the reliability of phylogenetic trees constructed with microsatellite DNA. Genetics. 178(1):385-392.

Takezaki N, Nei M, Tamura K. 2010. POPTREE2: software for constructing population trees from allele frequency data and computing other population statistics with Windows-interface. Mol Biol Evol. 27:747-752.

Thouard E, Soletchnik P, Marion J-P. 1989. Selection of finfish species for aquaculture development in Martinique (F.W.I.). In: Advances in tropical aquaculture. AQUACOP IFREMER Actes de Colloque 9. p. 499-510.

Tringali MD, Denison SH, Seyoum S, Mcvey J, Rivera JA. 2006. Preliminary investigation of genetic stock structure in Pompano (*Trachinotus carolinus*) from Puerto Rico and southwest Florida. St. Petersburg: Florida Fish and Wildlife Conservation Commission, Fish and Wildlife Research Institute. Report No. IHR2006-007.

Weir BS, Cockerham CC. 1984. Estimating F-statistics for the analysis of population structure. Evolution. 38:43-44.

Zhou M-Y, Gomez-Sanchez CE. 2000. Universal TA cloning. Curr Issues Mol Biol. 2(1):1-7.