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ABSTRACT

I used meristic, mitochondrial DNA, and nuclear DNA methods to infer the most likely ancestry of a putative hybrid specimen of *Percina* captured in the Roanoke River of Virginia. Potential parental species included *Percina rex*, *P. nevisense*, and *P. roanoka*. All nine of the meristic characters that I counted for the putative hybrid were within the published ranges of counts for *P. nevisense*, whereas counts for five characters were outside of published ranges for either *P. roanoka* or *P. rex*. These results were consistent with pure *P. nevisense* ancestry as well as various hybridization scenarios. Based on analysis of 1037 bp of the *ND2* mitochondrial gene, the haplotype of the putative hybrid was identical to a known *P. rex* haplotype, but <86% similar to the closest-matching haplotypes for either of the other two species. Bayesian admixture analysis using seven nuclear microsatellite markers indicated a high probability of *P. rex* or *P. nevisense* ancestry and a low probability of *P. roanoka* ancestry. Taking all evidence together, the most parsimonious explanation is that the specimen was a hybrid between *P. rex* and *P. nevisense*.

INTRODUCTION

Hybridization is a relatively common phenomenon among freshwater fishes (Scribner et al., 2001; Keck and Near, 2009). Although many hybrid offspring are not viable, those that are may contribute to subsequent introgression. Introgression can increase phylogenetic diversity via the creation of novel evolutionary trajectories (e.g., Dowling and Secor, 1997), but also can negatively impact native genomes and species (e.g., Echelle and Echelle, 1997; Seehausen et al., 1997). Documentation of hybridization events in nature therefore is important from both scientific and conservation standpoints.

On 16 July 2004, while using a backpack electrofisher to sample fishes in the Roanoke River (Roanoke County, Virginia), a montane warmwater stream in the Ridge and Valley physiographic province, I captured a putative hybrid of the genus *Percina*. The specimen exhibited pigmentation patterns unlike any other *Percina* species known from the Roanoke River drainage (Fig. 1; Jenkins and Burkhead, 1994). The right pectoral fin was removed for genetic analysis and both specimen and fin were preserved in 95% ethanol.

The only *Percina* species known to occur in the Roanoke drainage (Jenkins and Burkhead, 1994) are Roanoke Logperch, *Percina rex* (Jordan and Evermann); Chainback Darter, *P. nevisense* (Cope); and Roanoke Darter, *P. roanoka* (Jordan and Jenkins). All three species are syntopic in riffle-run habitats in the montane section of the Roanoke River, but the species vary greatly in abundance; *P. roanoka* is by far the most abundant, *P. rex* is intermediately abundant, and *P. nevisense* is by far the least abundant *Percina* species (J. Roberts, pers. obs.). Numerous darter hybrids involving *Percina* species have been observed previously in nature (Hocutt and Hambrick, 1973; Keck and Near, 2009). Of the three Roanoke River *Percina* species, hybridization has been observed only in *P. roanoka*, but there have been reported hybrids involving species closely related to the other two Roanoke River *Percina* species (e.g., *P. caprodes*, *P. peltata*). Furthermore, prezygotic reproductive isolating barriers (RIBs) for these species may be weak because of their preferences for similar spawning habitats and times, similar egg-burying strategies and modest sexual dimorphism. In the present study, I used meristic counts and molecular genetic markers to infer the most likely parental species of the putative hybrid individual.

METHODS

Meristic counts were made under a dissecting microscope on both sides of the specimen. Results were compared to published ranges of meristic counts from potential parental species, as follows: *P. rex* ranges were based on Jenkins and Burkhead's (1994) analysis of 112 Virginia collections, *P. roanoka* ranges were based on Jenkins and Burkhead's (1994) analysis of 80 Virginia collections combined with their synthesis of Mayden and Page's (1979) data, and *P. nevisense* ranges were based on Goodin et al. (1998) analysis of 115 collections from throughout the species' range.

To infer the matrilineal ancestry of the putative hybrid, I sampled 1037 bp of the *ND2* mitochondrial DNA gene. Whole genomic DNA was extracted from the fin clip using a Pure Gene DNA Extraction Core Kit A (Gentra Systems,

Minneapolis, Minnesota, USA). Forward and reverse primers for polymerase chain reactions were *ND2* 562L and *ND2* 449H, respectively, from George et al. (2006). PCR employed 25-µl reactions with the following reagent mix: 2 µL of 2.5-mM each dNTPs (premixed); 2.5 µL of 10X NH_4 Ex*Taq* buffer ($MgCl_2$ included); 1 µL each of 20-µM ND2 562L and ND2 449H primers; 0.15 µL of 5 Units μL^{-1} Ex*Taq* polymerase (TaKaRa Bio, Inc., Otsu, Shiga, Japan); 3 µL of 20 ng μL^{-1} template DNA; and 15.35 µL of dH_2O. I conducted PCR in a MyCycler Thermal Cycler (BioRad, Hercules, California, USA) using an initial denaturation step (94 °C, 3 min), followed by 35 cycles of denaturation (94 °C, 40 s), annealing (60 °C, 40 s), and extension (72 °C, 60 s), and a final extension step (72 °C, 2 min). Non-specific amplification products were removed using ExoSAP-IT (USB Corp., Cleveland, Ohio, USA) and the cleaned DNA was subjected to forward and reverse sequencing in an ABI 3130 automated sequencer at the Virginia Bioinformatics Institute at Virginia Tech. Forward and reverse sequence fragments then were aligned and edited in SEQUENCHER version 3.0 (Gene Codes Corp., Ann Arbor, Michigan, USA). The resulting sequence was accessioned in the GenBank public database (Benson et al., 1999; accession number JF944898) and compared to published *ND2* sequences from potential parent species using a BLAST search of the database, conducted 23 May 2011.

I further examined the ancestry of the putative hybrid using nuclear DNA markers, which are biparentally inherited and recombinant, thus providing information on both parents. I analyzed the putative hybrid plus a suite of known-identity individuals from the three possible parent species (*i.e.*, 15 *P. rex*, 4 *P. nevisense*, and 5 *P. roanoka*) using 12 microsatellite markers (*Prex33*, *Prex34*, *Prex36*, *Prex37*, *Prex38*, *Prex41*, *Prex42*, *Prex43*, *Prex44*, *Prex45*, *Prex46* and *Prex47*) and conditions described by Dutton et al. (2008). Forward primers were labeled using one of the following four fluorescent dyes: NED, VIC, PET or FAM (Applied Biosystems, Inc., Foster City, California, USA). PCR was conducted in a MyCycler Thermal Cycler (BioRad, Hercules, California, USA). Amplification products were separated in an ABI 3130 automated sequencer at the Virginia Bioinformatics Institute at Virginia Tech and sized using GeneMapper version 3.5 and a LIZ500HD size standard (Applied Biosystems, Inc., Foster City, California, USA).

Only seven of the microsatellite markers (*Prex37*, *Prex38*, *Prex41*, *Prex43*, *Prex44*, *Prex46*, and *Prex47*) amplified reliably across all three potential parental species, presumably because of inter-specific mutations in microsatellite-flanking regions that prevented annealing of primers. I used data from these seven markers to infer the ancestry of the putative hybrid using two types of admixture analyses. First, NewHybrids 1.1 (Anderson, 2003) was used to estimate the Bayesian posterior probabilities that the putative hybrid belonged in each of six discrete hybrid categories (Anderson and Thompson, 2002). NewHybrids could accommodate only two parental species at a time, so two separate models were run. The first model estimated the probabilities that the putative hybrid was: 1) a pure *P. rex*, 2) a pure *P. nevisense*, 3) an F1 cross of these two species, 4) an F2 cross of two F1s, 5) an F1 x *P. rex* backcross, or 6) an F1 x *P. nevisense* backcross. In the second model, categories were similar except that *P. roanoka* was substituted for *P. nevisense*. I did not attempt to model the possibility of a *P. roanoka* x *P. nevisense* hybrid, given that mtDNA analysis indicated that *P. rex* was one of the ancestral species (see Results and Discussion). In both models, I used a Jeffreys-type prior distribution for the parental species' allele frequencies, as recommended by Anderson and Thompson (2002), and made an exhaustive set of 2.5 x 10^6 "sweeps" through the Markov-Chain-Monte-Carlo (MCMC) simulation algorithm.

The second admixture analysis employed STRUCTURE 2.1 (Pritchard et al., 2000) to estimate the probabilities of the putative hybrid's genome originating from each of the three possible parental species. The STRUCTURE model assumed three *a priori* genetic populations (i.e., K = 3) with independent allele frequencies, but allowed for potential background admixture and did not incorporate prior knowledge of individuals' species-identities. Parameter space was searched using 10^6 recorded MCMC chains, following a burn-in of 10^5 chains. To estimate the potential accuracy of admixture analyses for detecting hybrids, estimates of genetic differentiation (F_{ST}) were calculated in ARLEQUIN 3.11 (Excoffier et al., 2005) and compared to the simulation results of Vähä and Primmer (2006).

RESULTS AND DISCUSSION

All meristic counts were within meristic ranges previously observed for *P. nevisense*, but counts were not consistently within known ranges for the other two species (Fig. 2). Meristic counts on the putative hybrid for pectoral fin rays (13), anal fin spines (2) and anal fin rays (10) overlapped with the known meristic ranges of all three potential parent species. In contrast, the dorsal spine count (12) overlapped only with *P. roanoka* and *P. nevisense* and the count of scales above the lateral line (8) overlapped only with *P. rex* and *P. nevisense*. Remaining meristic counts for the putative hybrid, including dorsal fin rays (13), circum-caudal-peduncle scales (22), scales below the lateral line (11) and lateral line scales (63), overlapped with the range for *P. nevisense* only. Thus, meristic results were inconsistent with the hypotheses of pure *P. rex* or pure *P. roanoka* ancestry. However, because meristic characteristics of hybrid individuals may or may not be intermediate to parental species (e.g., Ross and Cavender, 1981), alternative hypotheses regarding meristics are possible, including: (1) pure *P. nevisense* ancestry, (2) meristic intermediacy in a *P. rex* x *P. roanoka* hybrid, or (3) meristic non-intermediacy in a hybrid cross involving *P. nevisense*.

Based on a BLAST search of the GenBank database, the haplotype of the putative hybrid was identical (1037 bp matching) to a published *P. rex ND2* mitochondrial DNA haplotype (accession number JF929012). The *P. rex* indi-

vidual bearing this haplotype was captured in the upper Roanoke River (J. Roberts, unpublished data). In contrast, the closest-matching *P. roanoka ND2* haplotype (AY225722) was only 85% similar (883 of 1037 bp) to the hybrid haplotype. No *P. nevisense ND2* haplotypes were contained in the GenBank database, but the closest-matching *ND2* haplotype from *P. peltata* (AY770845), a close relative of *P. nevisense*, was only 84% similar (869 of 1033 bp) to the hybrid haplotype. Thus, there was strong evidence that one of the ancestral species was *P. rex*, though I could not conclude from this analysis, how far back in time the ancestry occurred (i.e., the hybrid individual could have been an F1, F2, backcross, etc.).

Analyses of nuclear DNA microsatellite data suggest that the most likely parental species of the putative hybrid were *P. rex* and *P. nevisense*. Estimates of F_{ST} were 0.13, 0.20, and 0.21 in pairwise comparisons of known-identity *P. rex* versus *P. roanoka*, *P. roanoka* versus *P. nevisense*, and *P. rex* versus *P. nevisense* specimens, respectively. Given this level of differentiation and the use of seven loci, STRUCTURE could detect hybrid ancestry ≥ 20% with estimated 60-80% accuracy, whereas NewHybrids could assign hybrid status with estimated 50-80% accuracy (Vähä and Primmer, 2006). In the NewHybrids model hypothesizing *P. rex* and/or *P. nevisense* as parental species, there was an essentially equal Bayesian posterior probability that the putative hybrid was a pure *P. rex* or a pure *P. nevisense* ($P = 0.35$) and the highest-probability hybrid category was F1 ($P = 0.14$) (Table 1). In the model hypothesizing *P. rex* and/or *P. roanoka* as parental species, there was a low probability for any category involving full or partial *P. roanoka* ancestry ($P < 0.02$), so the model assigned most of the probability to the pure *P. rex* category ($P = 0.96$). Both models performed well at classifying individuals of known identity to the correct species, with probabilities > 0.97 in all cases. NewHybrids thus indicated strong support for *P. rex* and *P. nevisense* ancestry, but weak support for *P. roanoka* ancestry. Difficulties teasing apart pure from hybrid ancestry may have stemmed from the somewhat low statistical power of the analysis, given only 7 loci (Vähä and Primmer, 2006).

The STRUCTURE analysis also performed well at assigning known-identity individuals to the correct species, but assignment of the putative hybrid was more ambiguous (Fig. 3). Assuming that the markers were not linked, we would expect an F1 hybrid to exhibit an approximately 0.5 probability of originating from each of two parental species. However, the Bayesian posterior probabilities of the putative hybrid being a *P. nevisense*, *P. roanoka*, or *P. rex* were 0.73, 0.17, and 0.09, respectively. Thus, STRUCTURE indicated strong support for *P. nevisense* ancestry, but weaker support for either other parent. Lack of strong support for *P. rex* ancestry suggests that the hybrid may have been a backcross with *P. nevisense*, though this hypothesis was not supported by the results of NewHybrids. Previous studies have revealed hybrid backcrosses of various other darter taxa that lacked strong nuclear introgression despite complete mitochondrial introgression (e.g., Bossu and Near, 2009; Keck and Near, 2009).

Given the preponderance of meristic and genetic evidence, the most parsimonious explanation is that the specimen was a hybrid between *P. rex* and *P. nevisense*. Analysis of mitochondrial DNA clearly indicated *P. rex* matrilineal ancestry, whereas nuclear DNA results were most consistent with admixture between *P. rex* and *P. nevisense*. Meristic data indicated either pure *P. nevisense* ancestry or hybridization, but could not be used to proffer one hybrid pairing over another. Although I cannot conclusively rule out the possibility of *P. roanoka* ancestry, this species was the least supported parental species across the analyses performed herein. Furthermore, data were inconclusive as to how many generations ago the hybridization event occurred, given that neither of the two admixture analyses clearly indicated that the hybrid was an F1.

No hybrids previously have been reported involving either *P. rex* or *P. nevisense*, yet this hybridization event is not especially surprising: both species are relatively large-bodied darters with similar ecological requirements and modest sexual dimorphism. Thus, prezygotic RIBs may be weak for these species. RIBs are known to break down following disturbances (Hubbs, 1955; Seehausen et al., 1997), though I am unaware of any novel environmental pressures that would increase hybridization rates in the Roanoke River. The prevalence and significance of such hybridization events are unknown. However, I presume that hybridization between *P. rex* and *P. nevisense* has been rare in the Roanoke River over the past 40 years, given that this was its first observation despite frequent surveys over this time period by workers from Virginia Tech and Roanoke College (Jenkins and Burkhead, 1994; R. Jenkins, Roanoke College, pers. comm.). Biologists working in this area in the future should be particularly observant for additional *Percina* hybrids. Further analyses of this and future suspected hybrids should seek to determine the direction of hybridization and whether crossings are one-time events or if introgression is ongoing. A targeted search for mitochondrial introgression between *P. rex* and *P. nevisense* in the Roanoke River also may be useful.

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TABLE 1. Results of Bayesian admixture analysis of the putative hybrid *Percina* specimen and 24 known-identity specimens at seven microsatellite markers.

Model 1		**Bayesian posterior probabilities**					
		Pure	Pure	F1	F2	F1 x *P. rex*	F1 x *P. nevisense*
Species	*n*	*P. rex*	*P. nevisense*	hybrid	hybrid	backcross	backcross
Hybrid	1	0.350	0.347	0.136	0.023	0.041	0.104
P. rex	15	>0.976	0.000	0.000	<0.001	<0.020	0.000
P. nevisense	4	0.000	>0.989	0.000	<0.001	0.000	<0.010
Model 2		**Bayesian posterior probabilities**					
		Pure	Pure	F1	F2	F1 x *P. rex*	F1 x *P. roanoka*
Species	*n*	*P. rex*	*P. roanoka*	hybrid	hybrid	backcross	backcross
Hybrid	1	0.962	0.009	0.002	0.016	0.014	0.004
P. rex	15	>0.996	0.000	0.000	<0.001	<0.004	0.000
P. roanoka	5	0.000	>0.990	0.000	<0.002	0.000	<0.008

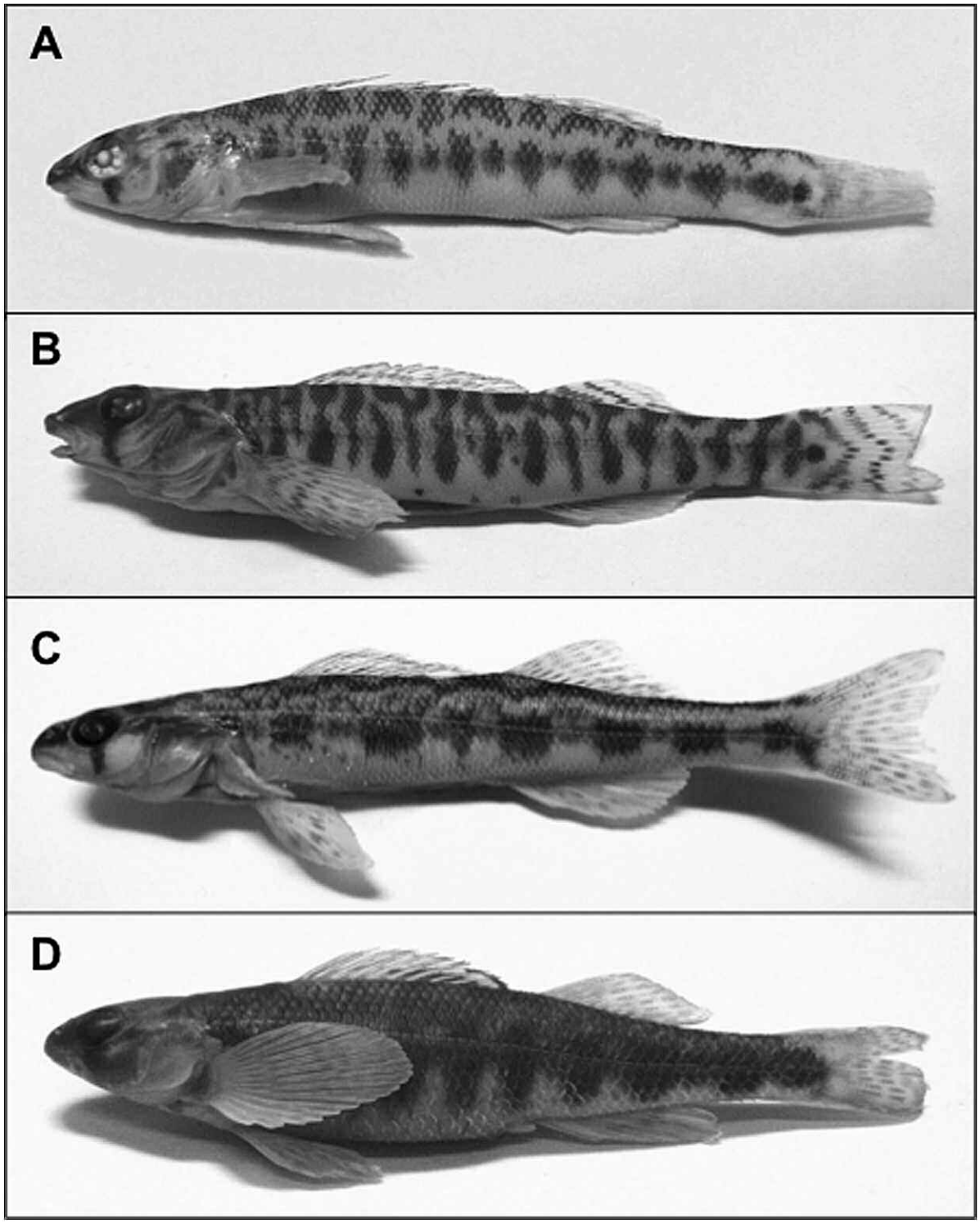

FIGURE 1. Photographs of all *Percina* species known to occur in the upper Roanoke River, including **A**) the putative hybrid specimen, **B**) *P. rex*, **C**) *P. nevisense*, **D**) *P. roanoka*. Pictured specimens were collected in the Roanoke River, Roanoke County, Virginia, and measured 58, 62, 76, and 58 mm total length respectively.

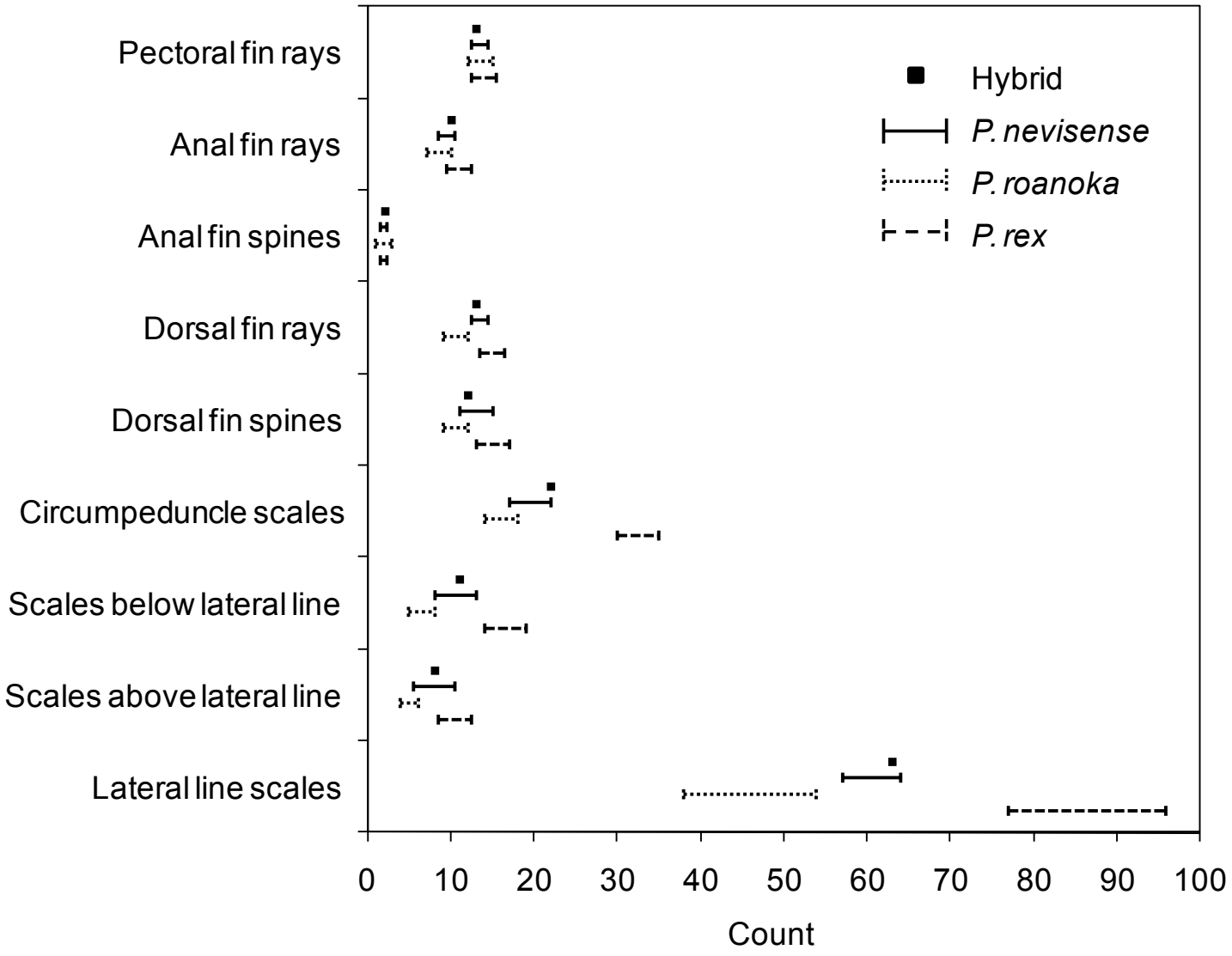

FIGURE 2. Measured values from the putative hybrid specimen and published ranges for *P. nevisense*, *P. roanoka*, and *P. rex* of counts for nine meristic characters.

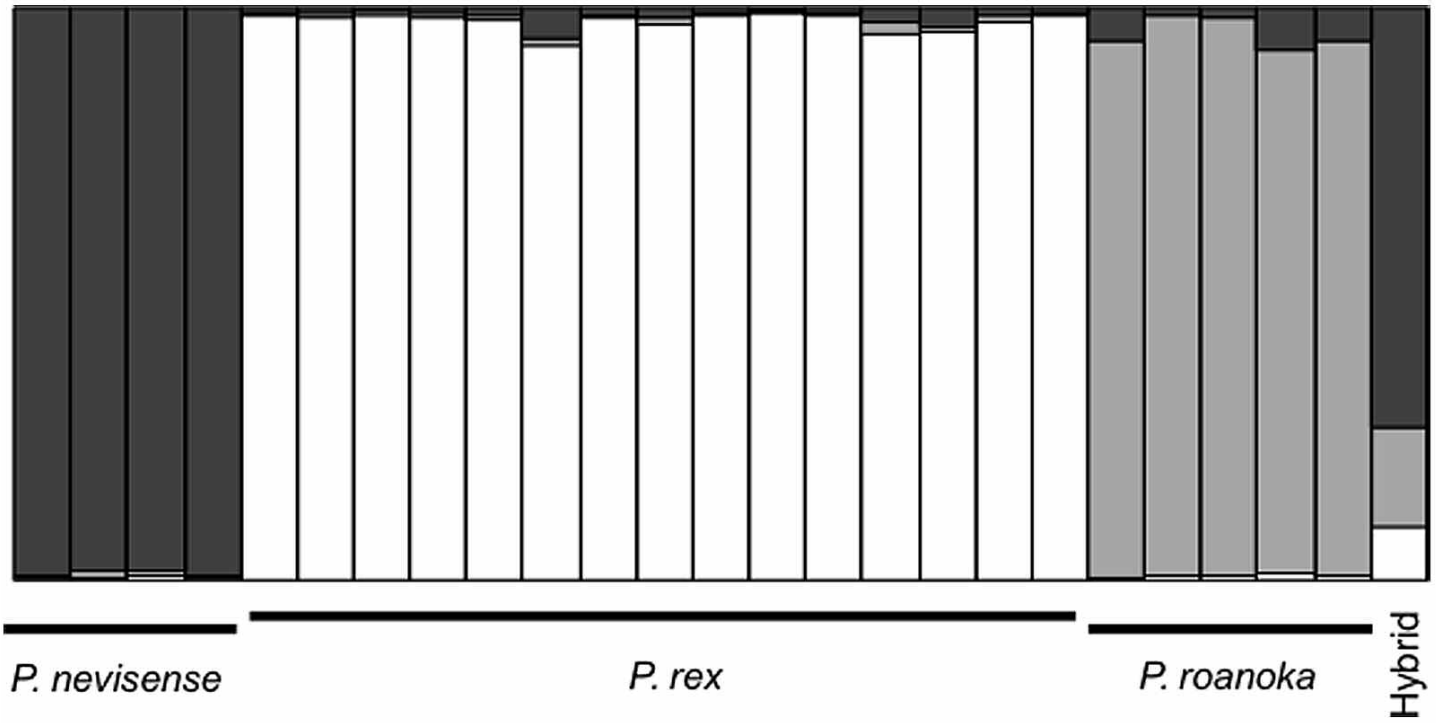

FIGURE 3. Results from STRUCTURE admixture analysis, showing Bayesian posterior probabilities of 25 individuals' origination from each of three inferred ancestral populations (represented by shading). Each individual is represented by a single horizontal bar.