GENETIC MONITORING OF THE RIO GRANDE SILVERY MINNOW: GENETIC STATUS OF WILD AND CAPTIVE STOCKS IN 2016

Annual report FY 2016

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EXECUTIVE SUMMARY

We have conducted genetic monitoring of the Middle Rio Grande population of Rio Grande silvery minnow annually from 1999-2012 and resumed monitoring 2014 through 2016. This work has included monitoring stocks that were bred or reared in captivity and were released to the Rio Grande in New Mexico since 2002; when the augmentation program commenced. Monitoring in 2016 was based on genotyping 420 'wild' silvery minnow collected in all three occupied reaches of the Middle Rio Grande, as well as wild-caught hatchery released fish (WCH, n=111), and progeny of captive stocks from Southwestern Native Aquatic Resources and Recovery Center (Southwestern Native ARRC), the Los Lunas silvery minnow refugium, and the Albuquerque Biological Park (n=492).

Major findings for 2016

(1) Microsatellite diversity statistics (N_{ac}, H_{ec} and H_{oc}) were essentially unchanged from 2015 values and exceeded minimum benchmark levels of diversity (established here). This stability is likely the result of the augmentation of the wild population with hatchery produced fish acting to buffer the population against loss of diversity. Average number of alleles (estimated by resampling to account for differences in sample size) has remained relatively stable since 2006.

(2) Mitochondrial gene diversity and haplotype richness increased in 2016 over most previous estimates and was within the range seen in previous years. Across all 2016 samples (including hatchery collections) ten haplotypes were detected including two haplotypes not detected in our sampling since 2012. This may reflect the use of 2012 cohort as broodstock for one of the captive lots and highlights the potential benefit of using these older age classes in production.

(3) Variance genetic effective size using the temporal comparison 2015-2016 was greater (N_{eV}=514-744) than for the previous comparison 2012-2015 (N_{ev}=193-328). Because the rate of loss of genetic diversity (through genetic drift) is inversely proportional to the genetic effective population size, it is of particular importance when effective population size is very small because the rate of loss of genetic diversity is likely to be highest. For Rio Grande silvery minnow, the small 2012-2015 N_{ev} likely resulted from a period of very low density in the wild and near complete replacement with fish reared in captivity. Higher N_{ev} in 2015-2016 suggests more stable allele frequencies.

(4) Mitochondrial DNA (mtDNA) was also used to estimate female variance effective population size using a maximum likelihood approach (MLNE). Female effective population size was less than 100 for the 2015-2016 temporal comparison suggesting relatively few females made a reproductive contribution.

(5) Fewer WCH hatchery fish needed to be collected in 2016 compared to the previous two years (2014 and 2015) because adequate numbers of 'wild' fish were available for collection. Similar levels of diversity were observed between WCH fish collected in 2015 and 2016, regardless of the smaller sample size, indicating that the captive breeding program is maintaining diversity. Furthermore, diversity metrics of the 2015 WCH sample were very similar to the 2016 collected 'wild' fish from the river suggesting that fish stocked in 2015 likely contributed genetic variation to the 2016 'wild' fish.

(6) Approximately 200,000 Rio Grande silvery minnow reared in captivity were released to the Middle Rio Grande in fall 2015 to supplement reproduction with 'wild' 2016 fish to produce the 2017 year-class. We genotyped representatives from five lots (n = 492), including Southwestern Native Aquatic Resources and Recovery Center (n = 1 lot), Albuquerque Biopark (3 lots) and Interstate Stream Commission's Los Lunas Silvery Minnow Refugium (1 lot). Pooled hatchery samples released to the middle Rio Grande had diversity that was comparable to the 'wild' population such that we do not expect to observe large changes in allele frequencies or reduction of diversity of 'wild' fish if the hatchery reared individuals contribute to the next generation.

(7) We evaluated the effect of sample size on estimates of variance genetic effective size (N_{eV}, using microsatellite data). Using the long-term genetic data, we resampled individuals from each year at incrementally larger sample sizes to estimate N_{eV}. Our results showed that the estimates obtained from different sample sizes resulted in comparable estimates to those obtained using the actual sample sizes. This suggests that our sampling strategy does not dramatically impact our effective size estimates.

INTRODUCTION

Genetic monitoring is defined as a collection of two or more temporally spaced genetic samples from the same population (Schwartz et al. 2007). Such studies typically employ neutral genetic markers and occasionally maternally inherited mitochondrial DNA (mtDNA), to track changes in standard genetic diversity metrics (gene diversity [H_e], heterozygosity [H_o], allelic richness [A_R] and genetic effective size [N_e]) over a contemporary time series (see glossary). The reason for tracking these metrics of diversity is because it is widely recognized that erosion of genetic

diversity increases a species' vulnerability through lowered fitness associated with inbreeding depression for example, which can ultimately accelerate a species' path to extinction (e.g. Frankham 2005). The time-scale of genetic monitoring varies considerably among studies from sampling over only a few years to the use of archival samples for a monitoring program that may span decades. In studies that encompass multiple decades, sampling is rarely conducted on an annual basis so linking changes in diversity metrics with specific environmental or management actions may not be plausible. In fish, genetic monitoring to date has been confined largely to marine species and freshwater salmonids. To our knowledge, the data set that we have collected for Rio Grande silvery minnow over the past 18 years represents one of the longest genetic monitoring time series for a non-salmonid freshwater fish.

For genetic monitoring programs, empirical measurements of diversity and genetic effective size are typically obtained from neutrally evolving microsatellite loci. Microsatellites are short tandemly repeating DNA sequences that are found throughout the genome of most species (reviewed in Dowling et al. 1996). They are biparentally inherited and are highly polymorphic among individuals (which is particularly important for endangered species that many have limited genetic diversity) and hence are the most widely used genetic markers in molecular ecology and conservation genetics studies. Mitochondrial DNA (mtDNA) is a haploid marker (i.e., individuals only have one copy as opposed to two copies for microsatellites), so progeny inherit a single mtDNA molecule from the female parent. Due to the differences in how nuclear DNA and mtDNA are inherited, they provide complementary approaches to monitoring genetic diversity.

The Rio Grande silvery minnow population is sampled throughout its current range, using nine microsatellite loci and a mitochondrial DNA gene to measure the trajectory of metrics of genetic diversity including allelic richness, heterozygosity, and genetically effective population size. The temporal component and sampling strategy provides the framework necessary to examine impacts of changes in abundance, management actions and environmental conditions on genetic diversity at these loci.

Negative genetic impacts to a population can occur over relatively short time periods for fishes that are characterized by a short lifespan (the population is dominated by age-1 fish; Horwitz et al. 2011) and in which dramatic changes in abundance occur from year to year (Dudley et al. 2014). Thus, for species such as Rio Grande silvery minnow, genetic monitoring is a crucial component to management. For example, by collecting temporal genetic data for Rio Grande silvery minnow, we have demonstrated that:

i) Genetic effective size is orders of magnitude lower than the census size (Alò and Turner 2005),
ii) The downstream movement of eggs, larvae, and adult fish, and the stocking of the population with captive reared fish, has prevented significant divergence of allele frequencies among fishes collected in each of the three river reaches (Osborne et al. 2005, 2012),
iii) The diversity of the 'wild' component of the population was best represented in captive stocks derived from collections of eggs that were produced by natural spawning events in the wild (Osborne et al. 2012);
iv) When artificial breeding is necessary, a group spawning design with equalized (i.e., 1:1) sex ratio produces fish that have levels of diversity that are statistically equivalent to that achieved through a paired mating design (Osborne et al. 2013).
v) That Rio Grande silvery minnow exhibits substantial spatial and temporal variation at genes of the major histocompatibility complex which is a critical component of the adaptive immune response (Osborne et al. 2016).

These findings have informed the Recovery Plan for the species and have been instrumental in the development of the captive propagation and genetics management plan (USFWS 2009, 2013). Here, we report on the genetic status of the population in 2016 and compare these results to previous years.

MATERIALS AND METHODS

Sampling- Rio Grande population

Throughout this study we use the term 'wild' to refer to unmarked fish sampled directly from the Rio Grande, as opposed to individuals that were marked with a Visible Implant Elastomer (VIE) tag to indicate that they were reared in a hatchery and used to supplement the Rio Grande silvery minnow population (and its reproduction). We use the term 'wild caught hatchery' (WCH) to refer to individuals with a VIE tag. 'Wild' fish may have parents that were wild or bred/reared in captivity, but were hatched in the Rio Grande. In 2016, 420 unmarked (i.e. 'wild') silvery minnow were collected between 5 November 2015 and 15 February 2016. These samples add to the data collected from wild Rio Grande silvery minnow sampled from the Middle Rio Grande annually from 1999 to 2012 and 2014-2016 (between November and April- just prior to reproduction) as well as 43 individuals used in a previous allozyme study of *Hybognathus* and stored in the Museum of Southwestern Biology Division of Genomic Resources (Cook et al. 1992 - referred to as 1987 sample). We also collected genetic samples from 111 VIE tagged fish from the Middle Rio Grande in 2016. These fish represented fish

released (from all breeding facilities) in the fall of 2015 and which are likely breeders in spring 2016. Sampling for WCH fish commenced in 2014 because of inadequate captures of wild 'untagged' fish. The distinction is made between 'wild' and WCH fish for this reason and because population monitoring tracks 'wild' fish separately from hatchery released fish. Collections were made throughout the current distribution (i.e., from Cochiti reservoir to Elephant Butte reservoir in New Mexico) of Rio Grande silvery minnow, with the exception of the Cochiti reach because the species is rare or absent in that area (Bestgen and Platania 1991). Rio Grande silvery minnow were collected by seining. In 2016, wild fish were collected from all three river reaches; Angostura (n=171), Isleta (n=121) and San Acacia (n=128) (Table 1). Fish were anesthetized in river water treated with MS-222 (Tricaine methane sulfonate 200 mg/L river water) at the site of capture. A piece of caudal fin was removed from each individual. Fin clips were preserved in 95% ethanol. Fish were allowed to recover in untreated river water prior to release.

Sampling- Hatchery Lots

In 2016, fin clips from five captive lots provided by Southwestern Native Aquatic Resources and Recovery Center (Southwestern Native ARRC, n = 1 lot), the Interstate Stream Commission (ISC) Los Lunas Refugium (n = 1) and the Albuquerque Biological Park (n = 3) totaling 492 genotyped individuals. The samples from the Albuquerque BioPark were 1) ABP14-003-2011, wild fish collected in 2011 and released in fall 2015 but not spawned, 2) ABP12-003/004, wild fish collected in 2012 from Isleta and San Acacia reaches for broodstock and released in fall 2015, and 3) ABP15-001, produced by spawning 60 males and 60 females from ABP12-003/004 broodstock. Mortality from fin-clipping and VIE-tagging is negligible (Southwestern Native ARRC and New Mexico Fish and Wildlife Conservation Office staff, pers. comm.).

Molecular methods- microsatellites

Total nucleic acids, including genomic DNA and mitochondrial DNA (mtDNA) were extracted from air-dried fin clips using proteinase-K digestion and organic extraction methods (Hillis et al. 1996). Individuals were genotyped at nine microsatellite loci: *Lco1*, *Lco3*, *Lco6*, *Lco7*, *Lco8* (Turner et al. 2004); *Ca6* and *Ca8* (Dimsoski et al. 2000); and *Ppro118* and *Ppro126* (Bessert and Orti 2003). The following pairs of loci were amplified through multiplex PCR: *Lco1*/*Ca6* and *Lco6*/*Lco7* (1X PCR buffer, 3 mM $MgCl_2$, 125 micromol [µM] deoxyribonucleotide triphosphates [dNTPs], 0.40-0.50 µM each primer, 0.375 units *Taq* polymerase); *Lco3* and *Lco8* (1X PCR buffer, 2 mM $MgCl_2$, 125µM dNTPs, 0.40-0.50 µM each primer, 0.375 units *Taq*); and *Ppro 118*/*Ppro126* (1X PCR buffer, 3 mM $MgCl_2$, 125µM dNTPs, 0.40-0.50 µM each primer, 0.375 units *Taq*). *Ca8* was amplified alone (1X PCR buffer, 3 mM $MgCl_2$, 125µM dNTPs, 0.50µM each primer, 0.375 units *Taq* polymerase). PCR cycling conditions for all loci were as follows: one denaturation cycle of 92°C for 2 min followed by 30 cycles of 90 °C for 20s, 50°C for 20 s, 72°C

for 30s. Cycling conditions for *Ppro 118*/*Ppro126* were as follows: one denaturation cycle of 92°C for 2 min followed by 30 cycles of 90 °C for 20s, 60°C for 20 s, 72°C for 30s. Primer concentrations in multiplex reactions were optimized by locus to ensure equal amplification each microsatellite. Fragment size analysis on an ABI 3130 automated capillary sequencer was performed by combining 1 µl of PCR product with 10 µl of formamide + 0.4 µl of HD400 size standard, denatured at 93°C for 5 minutes. Genotype data were scored in GENEMAPPER Version 4.0 (Applied Biosystems).

mtDNA- ND4

A 295 base pair (bp) fragment of the mtDNA ND4 gene from each individual was amplified in a 10 µL reaction containing 1 µL template DNA, 1 µL 10× reaction buffer, 2 mM $MgCl_2$, 125 µM dNTPs, 0.5 µM forward (5'- GAC CGT CTG CAA AAC CTT AA- 3') and reverse primer (5'- GGG GAT GAG AGT GGC TTC AA – 3'), and 0.375 units *Taq*. PCR conditions were 90° C initial denaturation for 2 minutes followed by 30 cycles of 90° C for 30 seconds, 50° C for 30 seconds, and 72◦ C for 30 seconds. Sequence data was obtained for all individuals by direct sanger sequencing (Big Dye vers. 1.1) according to the manufacturer's instructions and using an ABI 3130 DNA Sequencer.

Statistical analysis

MICROSATELLITE TOOLKIT (add-in for Microsoft Excel, written by S. Park, available at http://animalgenomics.ucd.ie/sdepark/ms-toolkit/) was used to estimate microsatellite diversity estimates of observed heterozygosity (H_o), Nei's unbiased gene diversity (H_e), and mean number of alleles (N_a) as well as to check for microsatellite allele scoring errors. GENEPOP (Raymond and Rousett 1995) was used to test for departures from Hardy-Weinberg equilibrium (HWE), using the procedure of Guo and Thompson (1992) and to perform global tests for linkage disequilibrium for all pairs of loci in each collection. Sequential Bonferroni correction (Rice 1989) was applied to account for inflated Type-1 error rates associated with multiple simultaneous tests. For each microsatellite locus and population, inbreeding coefficients (F_{IS}) were obtained using FSTAT vers. 2.9.3.1 (Goudet 1995). Estimates of mtDNA diversity of unbiased haplotype diversity (*h*) were obtained using ARLEQUIN vers. 3.11 (Excoffier et al. 2005).

Mitochondrial diversity was characterized by number of haplotypes (N_h), haplotype diversity (*h*), and haplotype richness (H_R). These metrics are equivalent to the number of alleles, gene diversity (H_{ec}), allelic diversity (N_{ac}) averaged across microsatellite loci. Haplotype richness (H_R) (Petit et al. 1998) was obtained using the program CONTRIB vers.1.02 (available at http://www.pierroton.inra.fr/genetics/labo/Software/Contrib/), which uses a rarefaction approach to correct for unequal sample sizes. The number of haplotypes (N_h) at a locus is also reported for mtDNA (not corrected for difference in sample size). Haplotype diversity (*h*) is a measure of the uniqueness of a haplotype in a population. Values of *h* range from zero (all

individuals have the same haplotype) to one (all individuals have a different haplotype). The calculation of *h* is based on the sample size and the frequency of each haplotype in the population.

In some cases, sample sizes differed between collections, particularly between some samples collected early in the study and those collected more recently. Because number of alleles and heterozygosity are dependent on sample size, we used a resampling approach to correct for sample size effects on diversity measures and make them more comparable across collections. In short, we randomly sampled each collection without replacement using the minimum sample size across all years (n = 43 in 1987). Microsatellite diversity estimates (corrected number of alleles [N_{ac}], gene diversity [H_{ec}] and heterozygosity [observed proportion of heterozygotes] [H_{oc}]) were then calculated for the random sample and the process repeated for 1000 iterations. Corrected diversity estimates are calculated as the mean estimate across all iterations. This analysis was conducted in the R statistical package (www.r-project.org). This resampling technique was also used for comparisons among collections obtained across years and river reaches, we repeated the resampling procedure for microsatellite data with diversity measures based on n=15 (2004, Isleta) and excluding the smallest sample n=6 (2004, San Acacia).

To place levels of diversity across years in context of overall genetic diversity of the species and to develop a biologically relevant benchmark for assessing levels of diversity within samples, we used an additional resampling technique. All 'wild' fish were pooled into one large population (n = 4,958) from which we iteratively took samples (n = 43) to estimate diversity statistics. Because our primary interest is maintaining genetic diversity, we estimated a one-tailed lower 95% confidence interval that corresponds to the upper 95% of the resampled distribution (i.e., 9500 of 10000 iterations). Thus, the distribution contained within this confidence interval corresponds to the null hypothesis of no loss of diversity.

F-statistics

Weir and Cockerham's (1984) *F*-statistics (microsatellites) and Φ-statistics (mtDNA) were calculated in Arlequin ver. 3.11 (Excoffier et al. 2005). Hierarchical analysis of molecular variance (AMOVA) was used to test whether a significant proportion of genetic variance was partitioned into components attributable to differences among 'wild', captive-spawned, and wild-caught hatchery (F_{CT}, Φ_{CT}), among samples within these three groups (F_{SC}, Φ_{SC}) and among all samples (F_{ST}, Φ_{ST}). This analysis was conducted on a subset of the data including 'wild' fish collected in 2012 (prior to the most recent population decline) and 2015-2016, WCH-2014-2016, and hatchery raised fish released in 2015 and 2016. P-values for all statistics were generated using bootstrapping (1000 permutations), as implemented in Arlequin.

Estimation of genetic effective size

Variance genetic effective size (N_{eV}) and 95% confidence intervals (CIs) were estimated from temporal (annual) changes in microsatellite allele frequencies across annual samples, using the temporal method (N_{eV}) (Nei and Tajima 1981; Waples 1989) implemented in NEESTIMATOR (Do et al. 2014). Highly polymorphic loci with many rare alleles, as is typical of microsatellites, can be subject to biased estimates of variance effective size, N_{eV}, (Hedrick 1999; Turner et al. 2001). To account for this potential bias, the unbiased estimator, F_S, (Jorde and Ryman 2007), as implemented in NEESTIMATOR, was also used to estimate N_{eV}. Rio Grande silvery minnow were sampled under Plan I (prior to reproduction, with replacement) for all methods; therefore, calculations of N_{eV} required an estimate of census size (N_c). No reliable, long-term data (i.e., spanning the entire sampling period) were available for N_c, so each pairwise comparison was run under the following two N_c scenarios: a "crashed" (N_c = 10,000) and a "large" (1,000,000 individuals) population. The former value is lower than any census size estimate to date and the latter is within the order of magnitude for which larger N_c have been recorded (Dudley et al. 2011). In all comparisons, differences in mean N_{eV} were negligible between the N_c = 10,000 and N_c = 1,000,000 scenarios, but lower and upper confidence intervals were slightly larger for the latter. Only the most conservative N_{eV} estimates (i.e., based on N_c = 1,000,000) are reported herein. Jackknife estimation over all loci was used to calculate N_{eV} and associated 95% confidence intervals. Multiple temporal methods are used to calculated N_{eV} to ensure consistency across estimators.

For all methods we assumed that migration (except from hatchery stocks) from outside the study area did not affect estimates of N_e. We equated the number of years separating a pair of samples with the number of generations elapsed between samples because Rio Grande silvery minnow have essentially non-overlapping generations (based on unpublished population monitoring data of R. K. Dudley and S. P. Platania). However, to account for small but known deviation from the discrete generation model (G = 1.27), we corrected consecutive estimates of N_e and N_{ef} for overlapping generations (Turner et al. 2006; Osborne et al. 2010), using the analytical method of Jorde and Ryman (1995, 1996). In addition to consecutive pairwise estimates, we also present comparisons between the 1987 and 1999 samples to provide historical context for the contemporary estimates. As these samples (1987-1999) were collected more than 3-5 generations apart, the drift signal should be sufficiently large relative to sampling biases associated with age-structure such that correction for overlapping generations is unnecessary (Waples and Yokota 2007).

To explore the influence of varying sample sizes across years on estimates of N_{eV}, we performed an exploratory resampling exercise. Briefly, random samples that reflected the four smallest sample sizes in our 18-year data set were drawn (with 5 replicates at each sample size [n=45,

n=127, n=143, n=161]). We calculated variance effective size and associated upper and lower 95% confidence intervals for each sample size using the Nei and Tajima method (Nei & Tajima 1981). All consecutive estimates were corrected to account for slightly overlapping generations (Osborne et al. 2012). Mean upper and lower 95% confidence intervals were included for resampling N_{eV} estimates. Infinite estimates of N_{eV} were removed prior to calculating mean effective size across replicates (5 out of 253 resampled estimates were infinite).

In addition to the estimates of N_{eV}, which require samples from different time periods, we used the linkage disequilibrium method (N_{eD}, Hill 1981) that only requires a single temporal sample. Annual N_{eD} was estimated from microsatellite DNA data separately for 'wild', WCH and captive-spawned stocks using the program NEESTIMATOR (Do et al. 2014) and methods described in Osborne et al. (2012). Single sample N_e methods (such as those provided by N_{eD}) yield an estimate of the effective number of parents that produced the progeny from which the sample is drawn, and most closely approximates the inbreeding effective size, N_{el} (Laurie-Ahlberg and Weir 1979; Waples 2005).

Variance effective size was also estimated for the female portion of the population using mtDNA data. To distinguish between variance effective size based on microsatellite data (N_{ev}) we use the designation N_{ef} to represent mtDNA variance effective size. N_{ef} was estimated with temporal (Turner et al. 2001) and pseudo-maximum-likelihood (MLNE) methods. It is useful to estimate genetic effective size from mitochondrial DNA data because it provides information pertaining to the female portion of the population. For example, if very low estimates of N_{ef} were obtained it would suggest that few females are making a genetic contribution to the population.

RESULTS

Microsatellites- genetic diversity

In 2016, we sampled 420 'wild' Rio Grande silvery minnow, and 111 WCH fish (Tables 1 and 2). An additional 492 individuals produced in captivity were analyzed. Within the three reaches of the middle Rio Grande, 171 'wild' fish were collected from the Angostura reach while 121 were collected from the Isleta reach, and 128 were collected from the San Acacia reach (Table 2). Across the entire time series, we have genotyped a total 9553 individuals at nine microsatellite loci representing both fish produced in the wild as well as hatchery stocks.

Characterization of microsatellite genotypes from the 2016 samples revealed two loci (*Ca6* and *Ppro126*) as the least variable, each with 9-10 alleles detected across all samples. Locus *Ppro118* was the most variable with 60 alleles followed by *M1* with 39 alleles detected across

2016 collections. Tests for deviations from Hardy-Weinberg Proportions were significant for 32% of locus-by-site combinations (29/90) following sequential Bonferroni correction for multiple comparisons. Significant departures were confined to four loci (*M7*, *M8*, *Ca8*, *Ppro118*), for which analysis by MICRO-CHECKER (Van Oosterhout et al. 2004) found an excess of homozygous individuals which could be due to the presence of null alleles. The remaining five loci (*Lco1*, *Lco3*, *Lco6*, *Ca6* and *Ppro126*) conformed to Hardy-Weinberg expectations. Significant tests for genotypic disequilibrium occurred in 4 of 360 comparisons following sequential Bonferroni correction and all of these occurred in captive stock samples.

Genetic diversity based on microsatellite data of 'wild' Rio Grande silvery minnow sampled for 2016 were equivalent to observed values in 2015 after accounting for sample size (Table 3, Fig 1.). Corrected diversity statistics observed in 2016 (H_{ec} = 0.813, H_{oc} = 0.731, and N_{ac}= 15.2) were within the range of values observed since monitoring began. The lowest number of alleles and heterozygosity were observed in the 1999 sample (N_{ac} = 12.2 and H_{oc} = 0.65) and lowest gene diversity was recorded for 2002 (H_{ec} = 0.79). Whereas the greatest number of alleles were observed in 2012 (N_{ac} = 14.42) gene diversity was greatest in the 2010 sample (H_{ec} = 0.834) and heterozygosity in 2004 (H_{oc} = 0.737). We used a resampling approach of all 'wild' fish collected between 1987-2016 to determine diversity benchmarks that correspond to annual diversity estimates based on the minimum annual sample size (n = 43). Benchmarks were obtained for microsatellite diversity estimates and were H_e = 0.798, H_o = 0.673, and N_{ac}= 14.7 (Fig 1). Observed levels of diversity for 2016 'wild' fish exceeded these benchmarks. Both wild and WCH fish had similar levels of genetic diversity at microsatellite loci after correcting for differences in sample size (Table 3). Across all stocks, diversity estimates were lowest in the sample taken from the Los Lunas Refugium and from one Albuquerque BioPark collection (ABP15-01). Genetic diversity statistics for pooled captive lots released to the Rio Grande were greater than the 95% CI genetic diversity benchmark (Table 3).

Analysis of microsatellite data by river reach indicated a decline from 2015-2016 in gene diversity in the Angostura and Isleta reaches while this statistic increased marginally between 2015 and 2016 in the San Acacia reach (Figure 2). For heterozygosity and number of alleles, a decline was observed between 2015 and 2016 in the Isleta reach while the opposite trend was seen in the San Acacia reach. In the Angostura reach heterozygosity and number of alleles remained stable between 2015 and 2016.

mtDNA- genetic diversity

A total of 17 mtDNA haplotypes have been identified from assaying 4971 wild (untagged) individuals from the middle Rio Grande from 1987 to 2016 (Table 4; Osborne and Turner 2012). Haplotype A was the most common in almost all samples including the 2016 collections. Three

haplotypes (C, D, F) were present at moderate frequencies (>5%) and 11 haplotypes were uncommon (<5%) or rare (<1%). Across the time series, haplotype diversity was highest in the 1987 sample (*h* = 0.743) and lowest in 2000 (*h* = 0.364) (Table 3, Figure 3). In 2016, ten haplotypes (A, C, D, E, F, K, M and O, Table 3) were observed among all collections with haplotype A the most common, followed by haplotypes C and D. In 2016, haplotype diversity ranged from 0.69 (WCH16) to 0.75 ('wild'), with a value of 0.63 for both wild and WCH; numbers of haplotypes and haplotype diversities observed in 2016 samples were typical of those from previous years (Figure 3; Table 3). In 2016, two haplotypes (I and Q) were detected that had not been observed since 2012. Haplotype Q was detected in a captive stock from the Albuquerque BioPark and haplotype I was detected in three individuals collected from the Angostura reach of the middle Rio Grande. For mtDNA, haplotype diversity and haplotype richness were very similar between reaches (Angostura: *h*= 0.754, H_R= 3.773, Isleta: *h*= 0.741, H_R= 3.715, San Acacia: *h*= 0.735, H_R= 3.656). Mitochondrial diversity statistics increased over 2015 values in all reaches (Figure 4).

Population structure- microsatellites

Total population structure was evaluated using global F_{ST} estimates across all 2016 samples, including hatchery stocks, and although F_{ST} was significantly different from zero it was very small (F_{ST} = 0.005, 95% CI = 0.004 to 0.006). Pairwise F_{ST} was estimated between samples collected in each river reach to assess genetic structure among wild fish. Consistent with analyses in previous years, no significant values were observed indicating no genetic structuring by reach. Pairwise F_{ST} was also estimated between all 2016 wild fish (reaches combined) and captive stocks representing fish released in fall 2014 (CSDX14, ABP14_002, ABP14_001, CSDX14_LL, ABP13_006, ABP14-004, and ABP13_002) to identify the possible source of the wild fish. Comparisons between wild fish were non-significant with the lot from Southwestern Native ARRC (CSDX14) and three Albuquerque BioPark 2014 lots (ABP14_001, ABP14_002, ABP14_004). F_{ST} values between wild fish and Los Lunas (F_{ST} = 0.014) or 2013 BioPark lots (ABP13_006 F_{ST} = 0.013, ABP13_002 F_{ST} = 0.023) were significant but small.

Population structure- mtDNA

Φ-statistics, among river reaches, were calculated across all wild-caught Rio Grande silvery minnow across the time-series (1987, 1999-2016). Genetic differences among the Angostura, Isleta, and San Acacia reaches were not significant (Φ_{CT} = -0.0006, P = 0.734). AMOVA comparing wild (2012, 2015-2016), WCH (2014-2016) and hatchery lots (2015-2016) revealed that these groups were not significantly different from one another (Φ_{CT} = -0.007, P = 0.711), while samples within groups did differ significantly (Φ_{SC} = 0.035, P < 0.00001). Pairwise F_{ST}s were significant between wild 2012 and 2015 samples and the 2016 wild collection (Φ_{ST} = 0.0197, P = 0.0001 and Φ_{ST} = 0.0271, P = 0.0001).

Genetic effective size

Moments and MLNE estimates of variance effective size, N_{eV}, from microsatellites, are shown in Figure 5. For 2015-2016, all estimates of N_{eV} were similar across methods (N_{eV} = 514-744). Both the moments and TempoFs estimates had finite lower and upper 95% CIs. MLNE and moments estimates of female variance effective size, N_{ef}, based on mtDNA are shown in Figure 6. For 2015-2016 temporal comparison, estimates of N_{ef} were less than 100 for both moments and maximum likelihood methods (Figure 6).

Estimates of inbreeding effective size (Figure 7; Table 2) were N_{eD} = 1483 (95% CI 736-infinity) for wild and N_{eD} = 128 (95% CI 99-173) for WCH fish collected in 2016. For captive stocks released in the middle Rio Grande in fall 2015 from Albuquerque BioPark, estimates were N_{eD} were highly variable ranging from 50 to infinity. Effective size of captive stocks from Southwest Native ARRC was N_{eD} = 163 (95% CI 118-235), and Los Lunas Refugium had the smallest effective size N_{eD} = 44 (95% CI 35-57). Pooling the hatchery fish released resulted in N_{eD} estimate of 290 (95% CI 184-520).

To account for the potential effect of sample size on variance genetic effective size estimates (N_{eV}), we used resampling to our four minimum sample sizes and then calculated N_{eV}. At the lowest sample size, occasional aberrant estimates are obtained but in almost all cases, effective size estimates obtained with the actual sample sizes fall well within the 95% CIs obtained from the resampling exercise (Figure 8).

DISCUSSION

Genetic monitoring

Monitoring genetic diversity parameters (H_e, H_o, A_R and N_e) across contemporary time-series can illuminate demographic and evolutionary processes affecting wild and captive populations that are unattainable using standard demographic sampling approaches. To our knowledge, data from Rio Grande silvery minnow is one of the longest genetic monitoring time-series for any non-salmonid freshwater fish. Annual monitoring the genetic status of both 'wild' and WCH Rio Grande silvery minnow in the middle Rio Grande in addition to the captive stocks repatriated to the river allows assessment of whether management actions are maintaining levels of genetic diversity in the species. Maintenance of diversity is critical because genetic diversity allows species to adapt and respond to changing conditions.

Status of the 'wild' (i.e. untagged) Rio Grande silvery minnow population in 2016

The population monitoring program for Rio Grande silvery minnow (1993-2016) shows that the wild population has experienced multiple, order-of-magnitude changes in density over the past

two decades (Dudley et al. 2014, Dudley et al. 2016). In particular, the lowest densities of Rio Grande silvery minnow have been recorded during 2012 – 2014 whilst densities from 2010-2014 were substantially lower than the period 2007-2009 (Dudley et al. 2014). Although population monitoring data shows that Rio Grande silvery minnow has the capacity to rebound rapidly following periods of very low density, declines are expected to gradually erode genetic diversity particularly in the absence of actions to buffer the population (i.e. supportive breeding and augmentation).

From 1987 and 1999-2004, both microsatellites and mtDNA showed considerable inter-annual variability in gene diversity metrics and effective size estimates. Following commencement of population supplementation with fish reared in captivity, inter-annual variability in diversity measures decreased from 2005 to 2012 and during this period there were marginal increases in mtDNA and microsatellite diversity. In 2016, we used resampling of combined temporal samples of 'wild' individuals to establish a benchmark of genetic diversity (lower 95% CI) to facilitate the temporal comparisons of diversity statistics, and as a means for identifying collections that may have unacceptably low genetic diversity. For 2016, the microsatellite diversity statistics from collections representing the middle Rio Grande population had diversity metrics that exceeded the lower 95% CI suggesting that genetic diversity is being maintained at acceptable levels. Hence, genetic monitoring data (1999-2012 and 2015-2016) for Rio Grande silvery minnow has shown that despite repeated declines in density, augmentation with fishes reared in captivity has thus far prevented catastrophic loss of diversity. Others have shown that although genetic erosion can be halted by rapid demographic recovery, the loss of diversity that has occurred is effectively irreversible over short time scales (i.e. tens of generations), hence genetic indicators are not expected to increase substantially (Hoban et al. 2014).

Genetic effective size

Genetic effective population size is a key parameter in genetic monitoring program because this number determines the amount of variation that is transmitted to the next generation. Specifically, the rate at which diversity is lost is inversely proportional to the effective size (i.e., at smaller N_e, diversity is lost more rapidly). Estimates (from microsatellite data) of N_{eV} for the 2015-2016 period calculated using the temporal method increased from values calculated for the previous time period (2012-2015). The genetic effective size estimate for the 2012-2015 time period is the harmonic mean of the effective size over this period which encompassed periods of exceptionally low densities in the wild and heavy supplementation with hatchery produced fish. Changes in allele frequencies caused by the input of hatchery fish can cause lowered estimates of N_{eV} if allele frequencies differ substantially from the recipient population. Estimates of effective size (MLNE) made from mitochondrial DNA haplotype frequency data,

decreased for the 2015-2016 time period to less than 100 suggesting substantial change in haplotype frequencies.

Genetic effective size estimated using the linkage disequilibrium method (N_{eD}=1483) showed more than an order of magnitude decline in 2016 when compared to the 2012 estimate (N_{eD}=10,064) but an increase over the 2015 estimate. This method is a single sample estimator and uses different aspects of the data to estimate the effective size. From a management perspective, there are a number of theoretical and practical distinctions between N_{el} (to which N_{eD} estimates are most closely associated) and N_{eV}. These two measures of effective size should be similar in stable populations but show predictable differences in declining (or growing) populations. For example, in declining populations N_{el} should be larger than N_{eV} because the latter depends on the amount of genetic drift between sampled generations but the former is a measure of inbreeding in the generation prior to sampling, (Allendorf & Luikart 2007); therefore, N_{el} is only reduced once mating between close relatives becomes more common (i.e., homozygosity increases in the population). Values of N_{eD} were uniformly higher than estimates of N_{eV} in previous years and this trend continued in 2016.

We evaluated the relationship between sample size and genetic effective size as it has been noted that there can be a correlation between these. The relationship between sample size and genetic effective size is complex. Specifically, the number of samples that can be collected for genetic analysis is dependent on densities of fish in the wild; low densities result in fewer samples for genetic analysis. Lower wild fish densities also are likely accompanied by a reduction in genetic effective size. To assess the impact of sample size on N_{eV} we used resampling to our four smallest collections. The results of this analysis showed that the estimates obtained using resampling at different sizes resulted in comparable estimates to those obtained using the actual sample size.

Reach Specific Findings

Comparison of diversity metrics between 2015 and 2016, revealed different patterns in each reach. Specifically, in the northern most reach (Angostura) gene diversity declined while heterozygosity, haplotype diversity, allelic and haplotype richness were stable or increased. The greater stability in this reach may be associated with the lack of river drying in this reach and hence less population turnover (associated with augmentation efforts). All diversity metrics declined in the Isleta reach. In contrast, all diversity metrics increased over the 2015 estimates in the San Acacia reach. Higher diversity estimates in the most downstream river reach suggests that this river stretch is likely a sink within the Middle Rio Grande (i.e. receives inputs of diversity from upstream reaches, and that can also be lost due to frequent drying). The extent

of drying in the lower two reaches and the genetic diversity of stocked fish may also impact the diversity in these populations in subsequent generations.

Genetic diversity of captive stocks released to the middle Rio Grande, New Mexico
In fall 2015, 200,549 fish were released in the middle Rio Grande, New Mexico. We sampled five distinct captive lots representing each of the facilities. Microsatellite diversity statistics for wild-caught hatchery fish and the pooled hatchery samples had values that exceeded the lower 95% CI benchmark based on resampling the 'wild' population suggesting that genetic diversity is being maintained at acceptable levels in these stocks. Like 2015, mitochondrial haplotype richness (corrected for differences in sample size) was variable across lots and facilities ranging from 4-6.5. Low haplotype representation in several hatchery lots highlights the importance of spawning large numbers of adults so that rare haplotypes (not just at mitochondrial DNA) are maintained. Interestingly, in 2016 we detected two haplotypes (I and Q) not seen in our sampling since 2012. Haplotype I was detected in 'wild' samples from the Angostura reach and haplotype Q was detected in a broodstock collection made in 2012 from the Isleta and San Acacia reaches that was released in fall 2015. The presence of these haplotypes likely reflects their presence in the captive broodstock used for spawning and releases in fall 2014 (and hence 2015 spawners and detected in 2016 'wild' fish). Detection of these rare haplotypes in 2016 is likely because of the higher number of samples genotyped.

CONCLUSIONS and FUTURE DIRECTIONS

Eighteen years of genetic monitoring of the 'wild' middle Rio Grande population and of released captive reared/bred silvery minnow provides a rare opportunity to track the genetic effects of population fluctuations associated with inter-annual variability in flows and of various management activities. The results of this study indicate that the trajectory of genetic change in the wild Rio Grande silvery minnow population is determined largely by supplementation with captive reared stocks and not by changes in population size (Osborne et al. 2012) and this continues to be true. Levels of genetic diversity including heterozygosity and average number of alleles have so far been maintained over the duration of the study. This highlights the importance of continued monitoring the captive stocks and of the wild population as any detrimental effects (such as losses of diversity) in the captive stocks will ultimately by transferred to the 'wild' population. For this reason, it is also important to begin genotyping the broodstock used to produce the captive stocks as well as a representative sample of their offspring. This would also allow parentage analysis to be conducted and an alternative effective size to be estimated using this data (using the sibship method). This could provide a better way to assess the genetic effective size of the captive stocks.

Recent technological advances now make it feasible (logistically and financially) to potentially screen 100's to 1000's of individuals (typical of monitoring programs) at 100-1000's of single nucleotide polymorphism [SNPs] loci. Once developed, these markers will allow changes in diversity to be monitored at potentially adaptive genes in addition to neutral loci. To date however, very few studies have been published that have employed SNPs in a genetic monitoring context. A recent study evaluated different genetic markers (microsatellites & single nucleotide polymorphisms [SNPs]) and number of loci to assess their resolution in detecting changes in diversity measures on conservation relevant time-scales using computer simulations (Hoban et al. 2014). Because SNPs are typically biallelic (two alleles) many more need to be screened to approach the power of microsatellites (which have 10's of alleles). Hoban et al. (2014) found that although using more markers (as with SNPs) improved power to detect change in diversity metrics, a modest number of microsatellites (n=20) were also sufficient to detect genetic erosion particularly when declines were instantaneous. Another study, compared genetic effective size estimates obtained using SNPs and microsatellites and they found that both methods gave comparable estimates (Linløkken et al. 2016) but this likely need to be evaluated on a species-specific basis. SNP genotyping would permit rapid assessment of diversity of captive lots before they are released to the wild such that any lots that appear to be genetically depauperate could be withheld from release. A SNP panel could be used to genotype individuals through their life in a hatchery (from egg to larvae to the released adult fish or through the life of the refuge broodstock population) to determine whether genetic diversity is being lost or maintained. Additionally, the valuable genetic archive that has been accumulated over the life of the genetic monitoring program will allow us to assess whether variation at adaptively important loci has been maintained and to assess concordance between microsatellite and SNP data.

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GLOSSARY

Allelic richness – The total number of alleles in a population corrected by rarefaction to account for differences in sample size among collections.

Gene diversity – Expected heterozygosity, assuming Hardy-Weinberg equilibrium.

Genetic drift – is the random change in allele frequencies from generation to generation because of sampling error. Specifically, the finite number of genes passed on to progeny will be an imperfect sample of the parental allele frequencies. The effects of genetic drift are (i) allele frequencies will change and (ii) genetic variation will be lost. The smaller the population, the greater the change in allele frequencies due to drift.

Genetic effective size – The effective size of a breeding population under idealized conditions meeting the assumptions of Hardy-Weinberg (i.e., equal sex ratio, random mating).

Hardy-Weinberg equilibrium – The stable frequency distribution of genotypes (AA, Aa, and aa) in the proportions (p^2, 2pq, and q^2) respectively (where p and q are the frequencies of the alleles, A and a). The Hardy-Weinberg principle makes the following assumptions (i) random mating (i.e. there is neither preference or aversion), (ii) no mutation (i.e. genetic information is transmitted from parent to progeny without change), (iii) large or infinite population size, (iv) no natural selection, (v) no immigration.

Heterozygosity – The presence of different alleles at one or more loci on homologous chromosomes. Proportion of heterozygous individuals for a locus in a population.

Inbreeding co-efficient (F) – the probability that two alleles at a locus in an individuals are identical by descent. Used to measure the extent of inbreeding.

Linkage disequilibrium – statistical association of alleles at different loci.

Locus/Loci – A segment of DNA on a chromosome. Loci is the plural form of the noun.

Microsatellite – short tandem repeated DNA sequences e.g. ACACACAC. These loci usually have variable numbers of repeats within/among individuals and high heterozygosity.

Mitochondrial DNA – maternally inherited circular DNA molecule contained within the mitochondria.

Null allele – a mutation that occurs in a PCR primer site that prevents amplification during polymerase chain reaction (PCR).

Primers – short fragments of DNA that flank the DNA region of interest and which are used in PCR.

Polymerase chain reaction – method used to make copies through amplification of a specific segment of DNA (such as a microsatellite locus or mitochondrial DNA gene). DNA is heated in the presence of PCR primers, and the Taq polymerase enzyme, to copy the intervening DNA sequencing using ~30 cycles.

Ryman-Laikre effect – an increase in inbreeding and reduction in the total effective population size that can occur in wild-captive systems that occurs when few individuals contribute large numbers of offspring.

SNP (single nucleotide polymorphism) **–** a variable nucleotide position in a population.

Wahlund effect – is a reduction in heterozygosity compared to Hardy-Weinberg expectations, and occurs in a population divided into partially isolated subpopulations

'Wild' vs. 'captive' – we use the term 'wild' to refer to unmarked fish sampled directly from the Rio Grande. 'Wild' fish may have parents that were wild or bred/reared in captivity, but were hatched in the Rio Grande.

Table 1. Sample sizes and collection localities of wild Rio Grande silvery minnow by river reach for samples collected during 2016 genetic monitoring.

SAMPLING SITE	Angostura	Isleta	San Acacia	Grand Total
Bernalillo	14			14
Rio Rancho	16			16
Calabacillas Arroyo	30			30
Alameda	3			3
Central Ave. Bridge	66			66
Avenida Cesar Chavez bridge	33			33
Rio Bravo Blvd Bridge	9			9
Los Lunas Bridge		17		17
Belen		39		39
Jarales		11		11
Bernardo		26		26
3.5 mi. downstream of Bernardo		25		25
0.6 mi. upstream of SADD		3		3
SADD			14	14
1.5 mi. downstream of SADD			48	48
Lemitar			21	21
Escondida			4	4
Socorro			16	16
4 mi. upstream of US HWY 380 bridge			5	5
Bosque del Apache NWR			3	3
San Marcial RR bridge			2	2
4.5 mi. downstream of San Marcial RR bridge			3	3
8 mi. downstream of San Marcial RR bridge			8	8
10 mi. downstream of San Marcial RR bridge			4	4
Grand Total	**171**	**121**	**128**	**420**

Table 2. All 'wild' samples collected from the Middle Rio Grande by river reach.

Year	Angostura	Isleta	San Acacia	Total
1987	15	-	28	43
1999	-	-	46	46
2000	-	-	194	194
2001	-	65	63	128
2002	67	121	201	389
2003	71	65	33	169
2004	141	15	6	162
2005	190	109	95	394
2006	95	143	145	383
2007	48	128	42	218
2008	165	191	123	479
2009	175	153	150	478
2010	149	146	151	446
2011	71	148	140	359
2012	147	215	154	516
2013	-	-	-	-
2014	5	3	4	12
2015	75	33	35	143
2016	171	121	128	420

Table 3. Diversity statistics for microsatellites and mtDNA. N is sample size, N_{ac} is average number of alleles across loci, H_{ec} is Nei's gene diversity, H_{oc} is observed heterozygosity, F_{IS} is inbreeding co-efficient, N_h is number of haplotypes, *h* is haplotype diversity, and H_R is haplotype richness. Linkage disequilibrium estimates of effective size, N_{eD}, are also given. No genetic monitoring was conducted in 2013. Values from 2016 monitoring year are bolded for emphasis; * indicates samples not included in diversity corrections due to smaller sample sizes. Wild caught hatchery fish (WCH) were included in genetic monitoring beginning 2014.

	Msats								mtDNA			
Wild-MRG	**N**	**N_{ac}**	**H_{ec}**	**H_{oc}**	**F_{IS}**	**N_{eD}**	**-95%**	**95%**	**N**	**N_h**	**h**	**H_R**
1987	43	14.00	0.797	0.710	0.111	∞	139	∞	37	7	0.74	6.00
1999	46	12.23	0.814	0.647	0.210	∞	∞	∞	44	5	0.43	3.82
2000	194	14.33	0.814	0.697	0.145	∞	∞	∞	124	6	0.36	3.36
2001	128	15.01	0.807	0.721	0.107	2008	495	∞	122	10	0.61	6.06
2002	389	14.75	0.793	0.681	0.143	1951	702	∞	387	8	0.63	4.16
2003	169	14.95	0.817	0.709	0.134	2998	564	∞	167	9	0.52	4.89
2004	162	14.85	0.819	0.737	0.100	596	357	1559	161	10	0.62	6.28
2005	394	14.90	0.816	0.724	0.113	2724	1014	∞	396	10	0.61	5.63
2006	383	15.26	0.826	0.727	0.122	2562	1291	34064	378	10	0.62	5.67
2007	218	15.08	0.828	0.726	0.123	∞	1211	∞	218	10	0.58	5.36
2008	474	15.16	0.823	0.713	0.135	4459	1479	∞	466	11	0.57	5.30
2009	476	15.11	0.830	0.689	0.172	3608	1677	∞	472	12	0.59	5.65
2010	440	15.19	0.834	0.692	0.172	∞	2023	∞	433	9	0.65	6.09
2011	362	15.32	0.830	0.724	0.130	∞	3117	∞	359	11	0.63	5.74
2012	517	15.42	0.827	0.728	0.123	10064	1782	∞	522	11	0.66	5.68
2013	-	-	-	-	-	-	-	-	-	-	-	-
2014	12	-	-	-	-	-	-	-	-	-	-	-
2015	144	15.33	0.815	0.731	0.103	468	281	1189	143	8	0.63	5.42
2016	**420**	**15.23**	**0.813**	**0.73**	**0.114**	**1483**	**736**	**∞**	**420**	**9**	**678.35**	**6.01**
Wild caught hatchery	**N**	**N_{ac}**	**H_{ec}**	**H_{oc}**	**F_{IS}**	**N_{eD}**	**-95%**	**95%**	**N**	**N_h**	**h**	**H_R**
2014	184	14.80	0.831	0.774	0.069	133*	101	184	182	6	0.61	3.87
2015	300	15.43	0.825	0.731	0.115	289	206	443	297	8	0.63	5.25
2016	**111**	**14.23**	**0.813**	**0.706**	**0.144**	**128**	**99**	**173**	**107**	**7**	**0.69**	**4.61**

Table 3 (cont.). Diversity statistics for microsatellites and mtDNA. N is sample size, N_{ac} is average number of alleles across loci, H_{ec} is Nei's gene diversity, H_{oc} is observed heterozygosity, F_{IS} is inbreeding co-efficient, N_h is number of haplotypes, *h* is haplotype diversity, and H_R is haplotype richness. Linkage disequilibrium estimates of effective size, N_{eD}, are also given. No genetic monitoring was conducted in 2013. Values from 2016 monitoring year are bolded for emphasis; * indicates samples not included in diversity corrections due to smaller sample sizes. Wild caught hatchery fish (WCH) were included in genetic monitoring beginning 2014.

	Msats								mtDNA			
Wild caught eggs	**N**	**N_{ac}**	**H_{ec}**	**H_{oc}**	**F_{IS}**	**N_{eD}**	**-95%**	**95%**	**N**	**N_h**	**h**	**H_R**
WCE_01*	178	14.76	0.819	0.651	0.206	1380	656	∞	157	8	0.63	7.00
WCE_SA_01	50	13.95	0.830	0.727	0.070	86	54	173	51	6	0.62	6.00
WCE_AN_02	50	12.12	0.784	0.731	0.126	∞	238	∞	49	3	0.48	2.95
WCE_SA_02	81	14.95	0.818	0.680	0.171	∞	462	∞	80	8	0.70	7.38
WCE_SA_03	51	14.99	0.830	0.696	0.164	5009	308	∞	51	8	0.71	7.85
MJO_07_005	54	15.31	0.827	0.738	0.091	60	48	79	53	7	0.60	6.73
MJO_07_006	49	15.64	0.814	0.723	0.108	1065	196	∞	48	6	0.58	5.96
MJO_07_015	49	15.42	0.818	0.694	0.154	871	270	∞	49	7	0.63	5.40
MJO_07_016	50	15.29	0.837	0.756	0.097	2425	359	∞	50	7	0.60	5.79
MJO_07_017	50	14.49	0.813	0.720	0.115	277	143	2070	46	8	0.76	6.57
2013	-	-	-	-	-	-	-	-	-	-	-	-
2014_WCE_RG	144	14.23	0.818	0.721	0.118	173	123	269	143	5	0.64	3.84
2014_WCE_RGNC	144	13.54	0.817	0.721	0.118	46	39	54	139	5	0.58	3.32
2014_WCE_ALL	288	14.25	0.821	0.722	0.122	117	88	162	281	5	0.61	3.68
ABP14_001	50	14.40	0.814	0.706	0.135	194	115	535	50	5	0.41	3.67
ABP14_002	49	15.28	0.838	0.722	0.140	189	114	485	48	5	0.62	3.72
Captive spawned	**N**	**N_{ac}**	**H_{ec}**	**H_{oc}**	**F_{IS}**	**N_{eD}**	**-95%**	**95%**	**N**	**N_h**	**h**	**H_R**
MJO_06_29	50	11.37	0.804	0.745	0.074	42	29	69	50	5	0.52	5.00
CS_01	64	12.81	0.794	0.658	0.172	44	36	55	58	5	0.46	4.98
CS_AN_02	51	8.48	0.685	0.675	0.015	22	15	33	51	1	0.00	1.00
CS_SA_02	53	13.15	0.802	0.673	0.163	73	53	111	53	6	0.75	5.92
TFT_03_09	51	12.77	0.806	0.7	0.133	106	56	434	52	4	0.56	4.00
CS_04	50	14.09	0.823	0.69	0.163	66	46	106	47	6	0.59	5.91
TFT_04_23	50	11.65	0.779	0.683	0.124	20	17	25	47	5	0.59	5.00
TFT_04_24	48	11.76	0.828	0.717	0.135	40	30	58	48	5	0.61	4.95
TFT_04_25	50	11.66	0.81	0.768	0.053	25	20	32	53	6	0.70	5.93
TFT_04_29	54	14.01	0.839	0.762	0.092	-424	532	∞	53	5	0.61	4.90
TFT_04_30	56	14.70	0.825	0.727	0.121	323	134	∞	45	5	0.66	4.79
TFT_04_31	50	12.80	0.805	0.701	0.13	83	55	155	50	7	0.71	6.87
TFT_05_06	50	10.31	0.792	0.649	0.183	49	39	66	50	6	0.63	5.80
TFT_05_07	49	12.15	0.797	0.704	0.117	87	53	191	48	5	0.55	4.88
TFT_05_08	50	11.15	0.804	0.663	0.178	32	27	40	49	5	0.61	4.93

Table 3 (cont.). Diversity statistics for microsatellites and mtDNA. N is sample size, N_{ac} is average number of alleles across loci, H_{ec} is Nei's gene diversity, H_{oc} is observed heterozygosity, F_{IS} is inbreeding co-efficient, N_h is number of haplotypes, *h* is haplotype diversity, and H_R is haplotype richness. Linkage disequilibrium estimates of effective size, N_{eD}, are also given. No genetic monitoring was conducted in 2013. Values from 2016 monitoring year are bolded for emphasis; * indicates samples not included in diversity corrections due to smaller sample sizes. Wild caught hatchery fish (WCH) were included in genetic monitoring beginning 2014.

	Msats								mtDNA			
Captive spawned	**N**	**N_{ac}**	**H_{ec}**	**H_{oc}**	**F_{IS}**	**N_{eD}**	**-95%**	**95%**	**N**	**N_h**	**h**	**H_R**
TFT_05_09	50	12.90	0.804	0.717	0.109	220	99	∞	50	4	0.51	4.00
TFT_05_11	51	12.56	0.808	0.693	0.144	137	81	354	53	6	0.57	5.85
MJO_06_25	50	14.85	0.813	0.721	0.115	185	110	488	49	5	0.64	4.93
MJO_06_28	50	12.41	0.805	0.705	0.125	88	57	164	50	5	0.74	5.00
MJO_07_07	50	13.16	0.813	0.739	0.114	∞	521	∞	50	5	0.61	4.87
LL_11	50	14.18	0.829	0.738	0.11	302	123	∞	49	5	0.68	0.37
MJO_10_05	49	14.04	0.839	0.7	0.167	260	87	∞	44	6	0.71	3.00
MJO_10_06	49	12.36	0.782	0.698	0.108	59	32	163	49	6	0.66	4.88
MJO_10_07	48	14.06	0.825	0.742	0.101	106	60	312	48	7	0.52	5.48
MJO_11_05	48	13.97	0.81	0.73	0.1	118	82	201	47	4	0.59	3.00
MJO_11_11	50	11.87	0.769	0.693	0.101	37	30	45	51	8	0.69	6.73
MJO_11_12	50	11.61	0.785	0.712	0.094	27	21	35	50	5	0.56	3.92
MJO_11_13	48	13.35	0.806	0.715	0.115	46	34	68	48	5	0.34	3.70
MJO_11_14	50	13.77	0.829	0.754	0.092	68	52	97	50	6	0.47	4.60
LL_12	49	12.48	0.794	0.684	0.141	41	33	52	48	6	0.63	4.49
MJO_12_09	50	14.03	0.829	0.721	0.133	62	46	88	49	4	0.60	3.00
MJO_12_10	50	14.16	0.81	0.719	0.113	121	69	371	50	7	0.64	5.71
2013_LLR	100	14.51	0.825	0.765	0.075	74	62	90	100	6	0.63	4.56
2013_DEX	100	14.70	0.818	0.765	0.066	112	87	152	99	6	0.53	4.23
ABP13_006	36	12.22*	0.792*	0.716*	0.097	36	28	49	36	4	0.67	7.00
ABP13_002	50	12.90	0.799	0.703	0.122	27	22	33	50	3	0.50	2.00
ABP14_004	49	13.90	0.807	0.683	0.155	133	86	262	49	8	0.56	6.01
CSDX14_SNARRC	150	14.65	0.827	0.728	0.120	179	127	279	147	7	0.6	4.49
CSDX14_Los_lunas	55	11.66	0.789	0.744	0.058	21	18	25	52	3	0.67	2.00
ABP14-003-2011	**50**	**14.84**	**0.808**	**0.721**	**0.12**	**771**	**196**	**∞**	**50**	**7**	**0.66**	**5.40**
ABP15-001	**49**	**12.24***	**0.8**	**0.703**	**0.133**	**50**	**41**	**61**	**48**	**6**	**0.79**	**4.94**
ABP12-003/004	**49**	**14.49**	**0.806**	**0.741**	**0.093**	**infinity**	**271**	**∞**	**48**	**8**	**0.73**	**6.48**
15CSDX_LLSMR	**50**	**12.13***	**0.817**	**0.697**	**0.16**	**44**	**35**	**57**	**50**	**5**	**0.76**	**4.00**
15CSDX	**294**	**14.38**	**0.822**	**0.735**	**0.118**	**163**	**118**	**235**	**298**	**7**	**0.74**	**4.56**
Global 2016 Hatchery	492	14.73	0.821	0.726	0.125	290	184	520	494	9	0.748	5.233

Table 4. MtDNA haplotype frequencies (%) for the wild middle Rio Grande population, wild caught hatchery fish, fish reared from wild-caught eggs, and fish reared from captive spawning. Values from 2016 monitoring year are bolded for emphasis.

Wild-MRG		A	C	D	E	F	I	J	K	M	N	O	P	Q	S	T	W	V
	1987	45.9	16.2	16.2	5.4	8.1	-	-	2.7	5.4	-	-	-	-	-	-	-	-
	1999	75.0	-	11.4	6.8	4.5	-	-	2.3	-	-	-	-	-	-	-	-	-
	2000	79.0	0.8	4.8	4.8	9.7	-	-	0.8	-	-	-	-	-	-	-	-	-
	2001	60.7	9.0	5.7	3.3	9.8	0.8	1.6	7.4	0.8	-	0.8	-	-	-	-	-	-
	2002	55.6	19.9	13.7	1.0	5.9	-	0.3	3.4	-	-	0.3	-	-	-	-	-	-
	2003	67.1	5.4	15.0	3.0	5.4	-	0.6	1.2	0.6	-	1.8	-	-	-	-	-	-
	2004	59.6	8.7	10.6	1.9	7.5	1.2	-	5.0	1.9	-	3.1	0.6	-	-	-	-	-
	2005	59.8	12.6	8.8	2.8	8.6	1.5	0.3	1.8	2.8	-	1.0	-	-	-	-	-	-
	2006	58.7	13.5	9.3	4.8	4.8	0.3	-	4.8	2.9	-	0.8	-	-	-	0.3	-	-
	2007	62.8	11.0	8.3	2.3	8.7	0.5	-	3.7	0.5	-	1.8	-	0.5	-	-	-	-
	2008	63.5	12.0	7.9	2.6	6.7	0.4	-	4.5	0.9	-	0.6	0.2	-	0.6	-	-	-
	2009	61.4	14.0	7.6	2.8	6.4	0.6	0.4	3.4	1.9	-	1.1	0.2	-	0.2	-	-	-
	2010	56.2	12.4	9.7	3.2	6.9	1.4	-	5.3	1.6	-	3.2	-	-	-	-	-	-
	2011	57.4	14.2	10.9	2.8	6.4	0.6	-	3.1	3.1	-	1.1	-	0.3	0.3	-	-	-
	2012	53.8	16.5	11.6	3.4	7.2	0.4	0.4	3.0	1.7	-	1.7	-	0.2	-	-	-	-
	2013	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2014	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2015	57.3	12.6	13.3	1.4	6.3	-	-	2.8	1.4	-	4.9	-	-	-	-	-	-
	2016	**40.9**	**25.4**	**9.5**	**1.7**	**6.9**	**0.7**	**-**	**2.9**	**3.3**	**-**	**8.6**	**-**	**-**	**-**	**-**	**-**	**-**

WCH		A	C	D	E	F	I	J	K	M	N	O	P	Q	S	T	W	V
	2014	57.7	20.9	6.0	-	11.5	-	-	0.1	-	-	3.3	-	-	-	-	-	-
	2015	57.1	13.3	15.0	2.3	2.7	-	-	2.0	2.0	-	5.6	-	-	-	-	-	-
	2016	**45.5**	**25.0**	**9.8**	**-**	**7.1**	**-**	**-**	**-**	**0.9**	**-**	**6.3**	**-**	**0.9**	**-**	**-**	**-**	**-**

Table 4 (cont.). MtDNA haplotype frequencies (%) for the wild middle Rio Grande population, wild caught hatchery fish, fish reared from wild-caught eggs, and fish reared from captive spawning. Values from 2016 monitoring year are bolded for emphasis.

Wild caught eggs	A	C	D	E	F	I	J	K	M	N	O	P	Q	S	T	W	V
WCE_01*	57.3	19.7	5.1	6.4	6.4	-	-	3.2	1.3	0.6	-	-	-	-	-	-	-
WCE_SA_01	56.9	13.7	5.9	5.9	9.8	-	-	7.8	-	-	-	-	-	-	-	-	-
WCE_AN_02	65.3	2.0	32.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-
WCE_SA_02	48.8	22.5	5.0	1.3	13.8	-	-	5.0	3.8	-	-	-	-	-	-	-	-
WCE_SA_03	49.0	7.8	19.6	5.9	9.8	-	-	3.9	2.0	-	2.0	-	-	-	-	-	-
MJO_07_005	60.4	9.4	1.9	1.9	17.0	-	1.9	7.5	-	-	-	-	-	-	-	-	-
MJO_07_006	60.4	8.3	12.5	2.1	8.3	-	-	4.2	-	-	4.2	-	-	-	-	-	-
MJO_07_015	57.1	22.4	4.1	2.0	4.1	-	-	8.2	2.0	-	-	-	-	-	-	-	-
MJO_07_016	62.0	12.0	6.0	-	8.0	-	-	4.0	4.0	-	4.0	-	-	-	-	-	-
MJO_07_017	43.5	19.6	6.5	4.3	13.0	-	-	8.7	2.2	-	-	-	2.2	-	-	-	-
2013	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2014_WCE_RG	54.2	7.0	22.5	-	4.9	-	-	-	-	-	11.3	-	-	-	-	-	-
2014_WCE_RGNC	58.3	7.2	28.1	-	1.4	-	-	-	-	-	5.0	-	-	-	-	-	-
2014_WCE_ALL	56.2	7.1	25.3	-	3.2	-	-	-	-	-	8.2	-	-	-	-	-	-
ABP14-001	76.0	8.0	2.0	4.0	10.0	-	-	-	-	-	-	-	-	-	-	-	-
ABP14-002	56.0	4.0	19.0	-	2.0	-	-	-	-	-	19.0	-	-	-	-	-	-
Captive spawned	**A**	**C**	**D**	**E**	**F**	**I**	**J**	**K**	**M**	**N**	**O**	**P**	**Q**	**S**	**T**	**W**	**V**
MJO_06_29	68.0	14.0	8.0	-	6.0	-	-	-	4.0	-	-	-	-	-	-	-	-
CS_01	72.4	5.2	-	3.4	6.9	-	-	12.1	-	-	-	-	-	-	-	-	-
CS_AN_02	-	-	100.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CS_SA_02	43.4	7.5	17.0	13.2	17.0	-	-	-	-	-	-	1.9	-	-	-	-	-
TFT_03_09	59.6	26.9	3.8	-	-	-	-	9.6	-	-	-	-	-	-	-	-	-
CS_04	59.6	25.5	2.1	-	4.3	-	-	6.4	-	-	2.1	-	-	-	-	-	-
TFT_04_23	61.7	4.3	19.1	-	-	-	-	4.3	-	-	10.6	-	-	-	-	-	-
TFT_04_24	58.3	12.5	20.8	-	2.1	-	-	6.3	-	-	-	-	-	-	-	-	-
TFT_04_25	43.4	5.7	11.3	5.7	28.3	-	-	5.7	-	-	-	-	-	-	-	-	-
TFT_04_29	56.6	24.5	-	7.5	-	-	-	9.4	1.9	-	-	-	-	-	-	-	-
TFT_04_30	40.0	33.3	-	-	-	-	-	24.4	-	-	-	2.2	-	-	-	-	-

Table 4 (cont.). MtDNA haplotype frequencies (%) for the wild middle Rio Grande population, wild caught hatchery fish, fish reared from wild-caught eggs, and fish reared from captive spawning. Values from 2016 monitoring year are bolded for emphasis.

Captive spawned	A	C	D	E	F	I	J	K	M	N	O	P	Q	S	T	W	V
TFT_04_31	42.0	34.0	2.0	-	6.0	-	-	4.0	10.0	-	2.0	-	-	-	-	-	-
TFT_05_06	50.0	36.0	2.0	-	2.0	-	-	8.0	2.0	-	-	-	-	-	-	-	-
TFT_05_07	62.5	29.2	2.1	6.3	-	-	-	0.0	-	-	-	-	-	-	-	-	-
TFT_05_08	59.2	8.2	-	10.2	-	-	-	22.4	-	-	-	-	-	-	-	-	-
TFT_05_09	68.0	16.0	-	-	-	-	-	12.0	4.0	-	-	-	-	-	-	-	-
TFT_05_11	62.3	5.7	11.3	1.9	17.0	-	-	-	1.9	-	-	-	-	-	-	-	-
MJO_06_25	55.1	24.5	6.1	-	6.1	-	-	8.2	-	-	-	-	-	-	-	-	-
MJO_06_28	40.0	14.0	22.0	-	22.0	-	-	2.0	-	-	-	-	-	-	-	-	-
MJO_07_07	56.0	2.0	12.0	28.0	2.0	-	-	-	-	-	-	-	-	-	-	-	-
LL_11	46.9	22.4	24.5	-	4.1	-	-	-	2.0	-	-	-	-	-	-	-	-
MJO_10_05	47.7	18.2	15.9	-	13.6	-	-	2.3	-	-	2.3	-	-	-	-	-	-
MJO_10_06	53.1	22.4	4.1	6.1	-	10.2	-	-	4.1	-	-	-	-	-	-	-	-
MJO_10_07	68.8	6.3	4.2	2.1	8.3	-	-	8.3	-	-	-	-	2.1	-	-	-	-
MJO_11_05	59.6	21.3	10.6	-	8.5	-	-	-	-	-	-	-	-	-	-	-	-
MJO_11_11	52.9	5.9	3.9	3.9	3.9	-	-	17.6	5.9	-	-	-	-	-	-	-	-
MJO_11_12	64.0	12.0	4.0	-	-	-	-	-	14.0	-	6.0	-	-	-	-	-	-
MJO_11_13	81.3	6.3	6.3	4.2	-	-	-	-	2.1	-	-	-	-	-	-	-	-
MJO_11_14	72.0	4.0	6.0	4.0	-	-	-	12.0	-	-	2.0	-	-	-	-	-	-
LL_12	56.3	4.2	12.5	-	22.9	-	-	2.1	-	-	-	-	2.1	-	-	-	-
MJO_12_09	59.2	18.4	8.2	-	14.3	-	-	-	-	-	-	-	-	-	-	-	-
MJO_12_10	58.0	8.0	10.0	-	10.0	-	-	6.0	-	-	2.0	-	6.0	-	-	-	-
2013_LLR	57.0	20.0	4.0	9.0	3.0	-	-	-	-	-	7.0	-	-	-	-	-	-
2013_DEX	66.7	11.1	9.1	-	5.1	-	-	-	-	-	7.1	-	-	-	-	-	1.0
ABP13_002	66.0	8.0	26.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ABP13_006	42.0	14.0	39.0	-	-	-	-	-	-	-	6.0	-	-	-	-	-	-
ABP14_004	65.0	6.0	2.0	2.0	12.0	-	-	8.0	2.0	-	2.0	-	-	-	-	-	-
CSDX14_SNARRC	61.0	13.0	0.14	-	1.0	-	-	3.0	2.0	-	7.0	-	-	-	-	-	-
CSDX14_LL	37.0	-	38.0	-	-	-	-	25.0	-	-	-	-	-	-	-	-	-

Table 4 (cont.). MtDNA haplotype frequencies (%) for the wild middle Rio Grande population, wild caught hatchery fish, fish reared from wild-caught eggs, and fish reared from captive spawning. Values from 2016 monitoring year are bolded for emphasis.

Captive spawned	A	C	D	E	F	I	J	K	M	N	O	P	Q	S	T	W	V
15CSDX	**41.6**	**18.1**	**7.4**	**-**	**12.4**	**-**	**-**	**1.7**	**0.3**	**-**	**18.5**	**-**	**-**	**-**	**-**	**-**	**-**
ABP12_03_04	**46.9**	**16.3**	**4.1**	**2.0**	**8.2**	**2.0**	**-**	**-**	**6.1**	**-**	**12.2**	**-**	**2.0**	**-**	**-**	**-**	**-**
ABP14_03_2011	**54.0**	**18.0**	**14.0**	**4.0**	**6.0**	**-**	**-**	**2.0**	**-**	**-**	**2.0**	**-**	**-**	**-**	**-**	**-**	**-**
ABP15_01	**22.4**	**30.6**	**6.1**	**-**	**12.2**	**-**	**-**	**-**	**-**	**-**	**22.4**	**-**	**-**	**-**	**-**	**-**	**-**
LLSMR	**40.0**	**18.0**	**20.0**	**-**	**-**	**-**	**-**	**12.0**	**-**	**-**	**10.0**	**-**	**-**	**-**	**-**	**-**	**-**

Figure 1. Diversity metrics (annual) from microsatellite loci. Estimates of gene diversity and heterozygosity obtained from resampling of microsatellites (H_{ec} and H_{oc}) are shown in the upper panel, and number of alleles (N_{ac}) are shown in the lower panel. Dashed (H_{ec} and N_{ac}) and dotted (H_{oc}) lines indicate diversity benchmarks obtained using a resampling procedure and correspond to a minimum sample size of n=43.

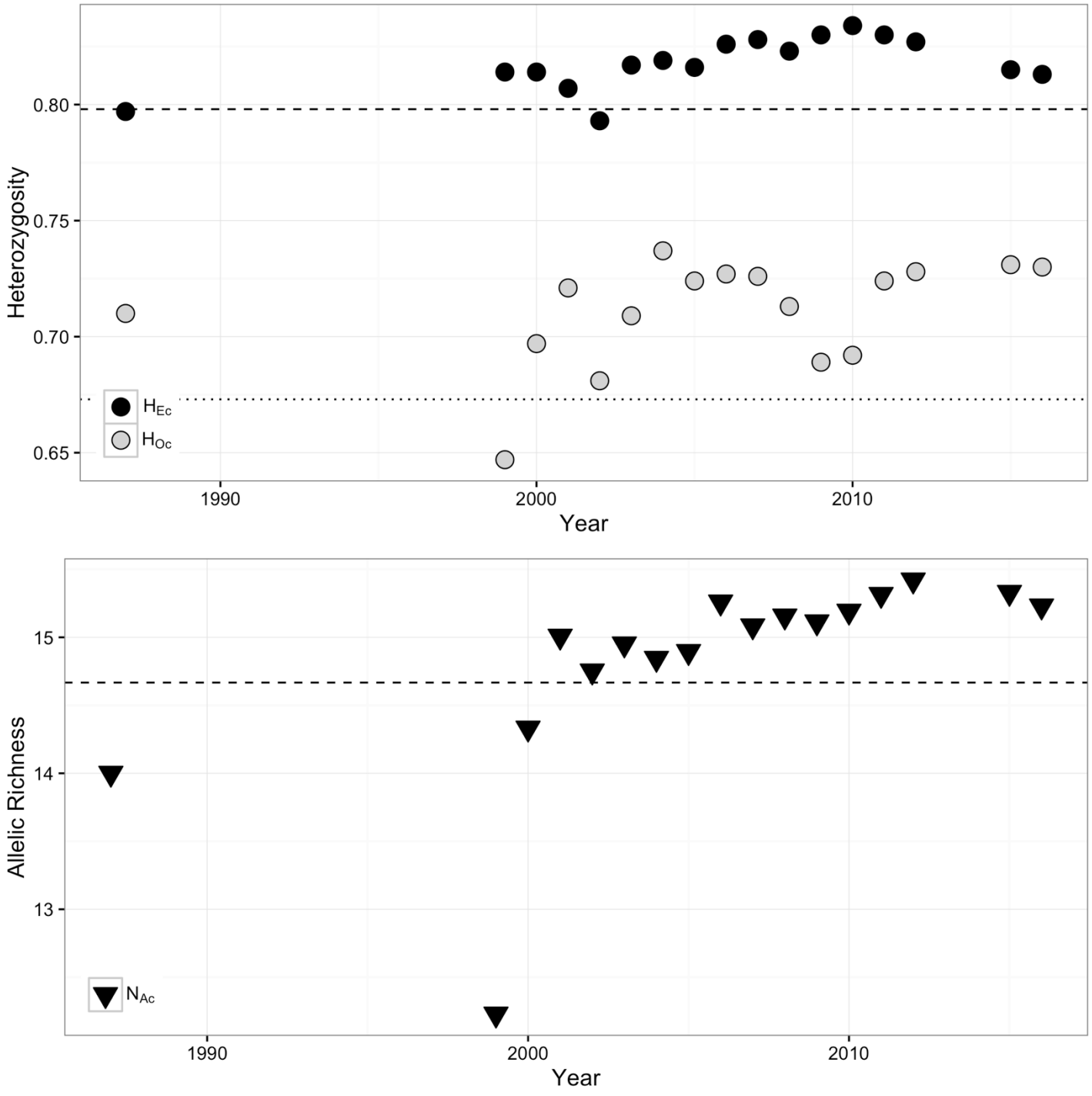


Figure 2. Annual diversity metrics of wild Rio Grande silvery minnow across the 18-year study by reach. Microsatellites diversity estimates, H_{ec} (top), H_{oc} (middle), N_{ac} (bottom), were corrected for differences in sample sizes across years by resampling.

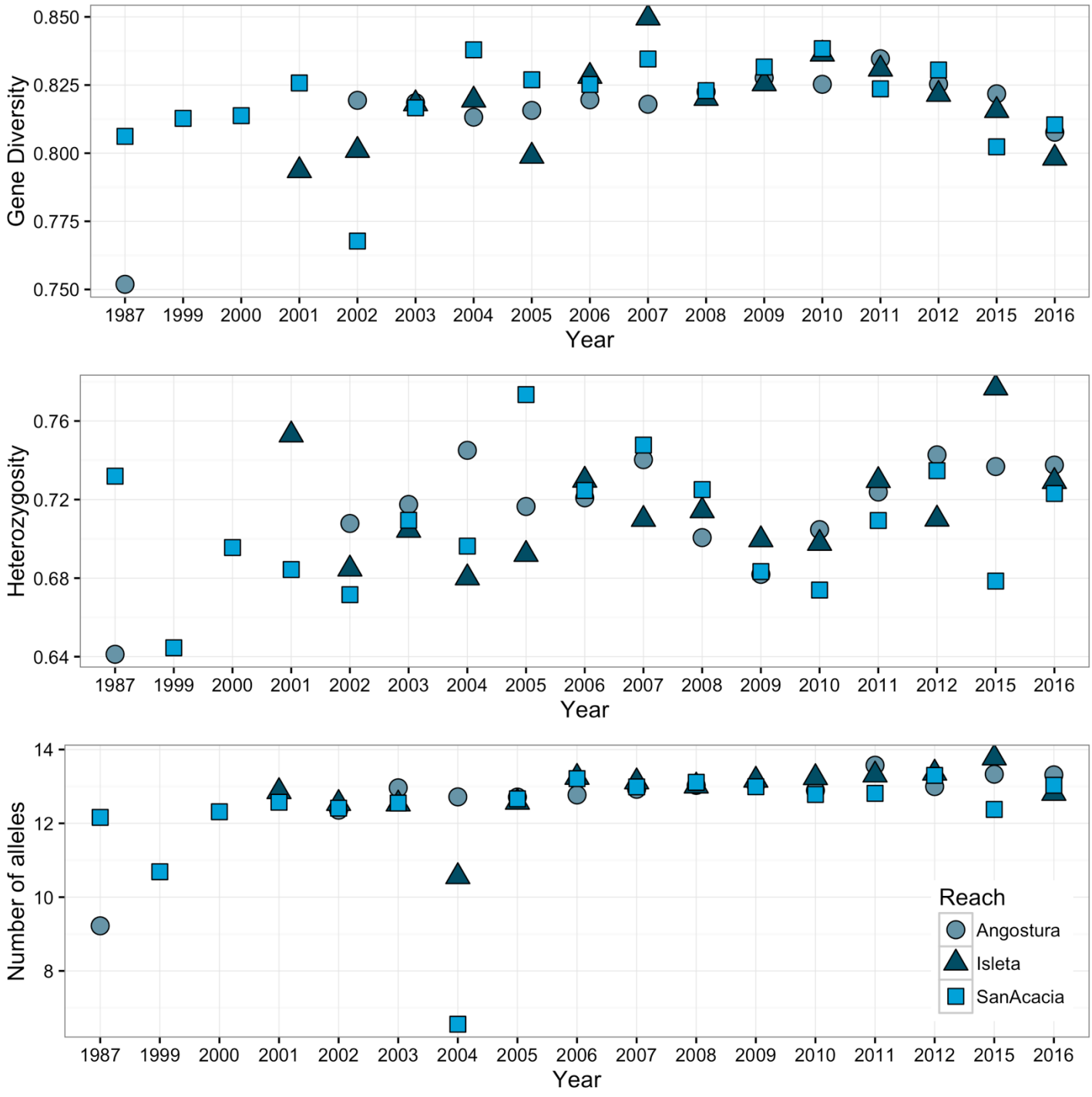
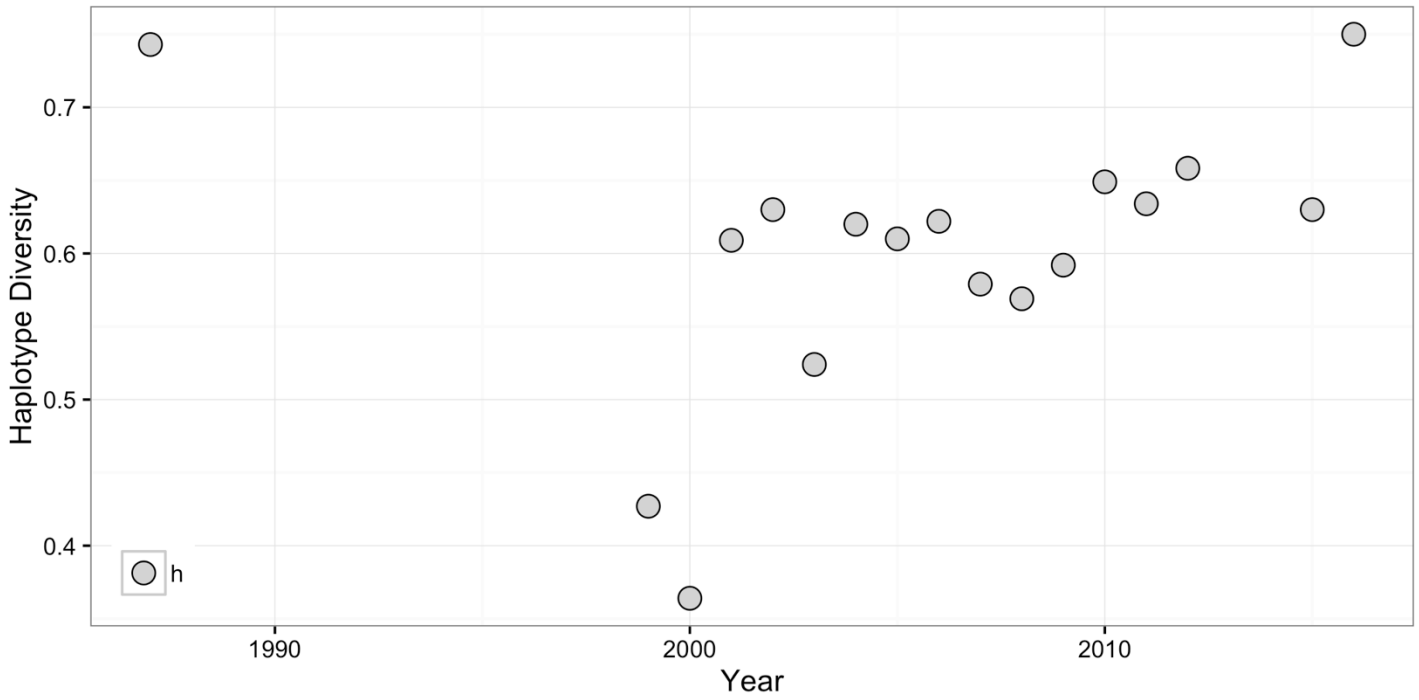


Figure 3. Diversity metrics (annual) from the mtDNA locus. Estimates mtDNA haplotype diversity (*h*) are shown in the upper panel and haplotype richness (H_R) are shown in the lower panel.



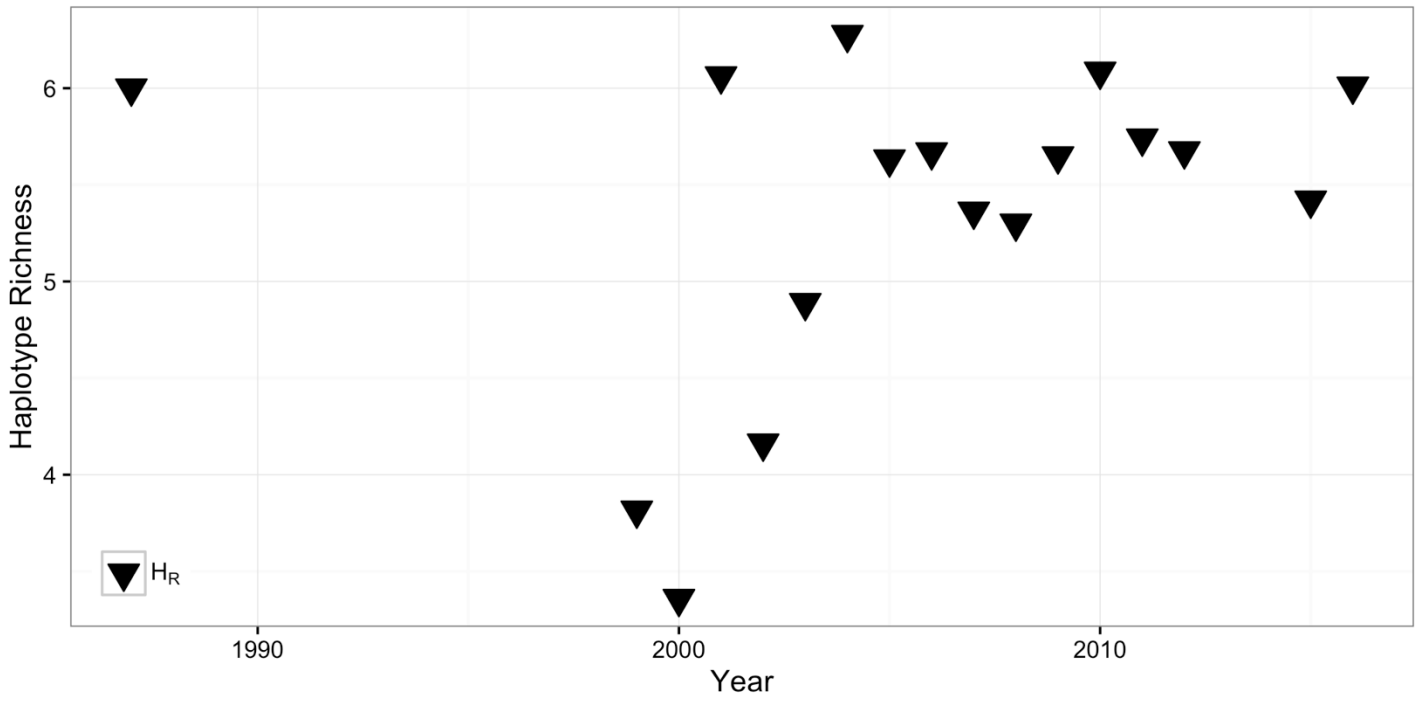


Figure 4. Annual mtDNA diversity metrics of wild Rio Grande silvery minnow across the 18-year study by reach. Estimates mtDNA haplotype diversity (*h*) are shown in the upper panel and haplotype richness (H_R) are shown in the lower panel.

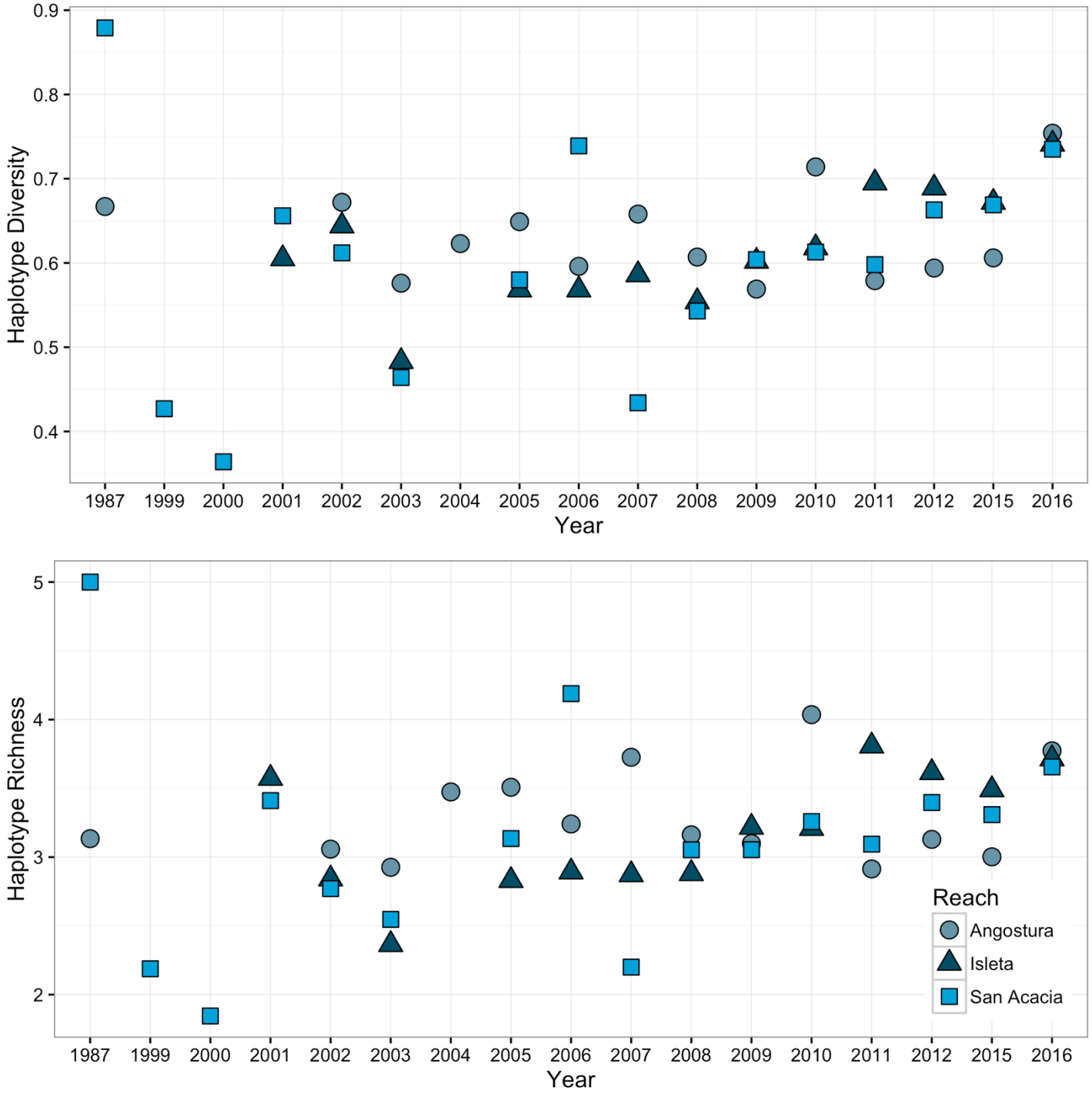


Figure 5. Variance effective size (N_{eV}) calculated from microsatellite data, as based on MLNE (upper), moments (middle), and TEMPOFS (lower), estimates and their associates 95% CIs. Mean TEMPOFS estimate from 2011-2012 (value not shown) was infinite, and upper error bars extending to y-maxima indicate infinite upper bounded 95% CI.

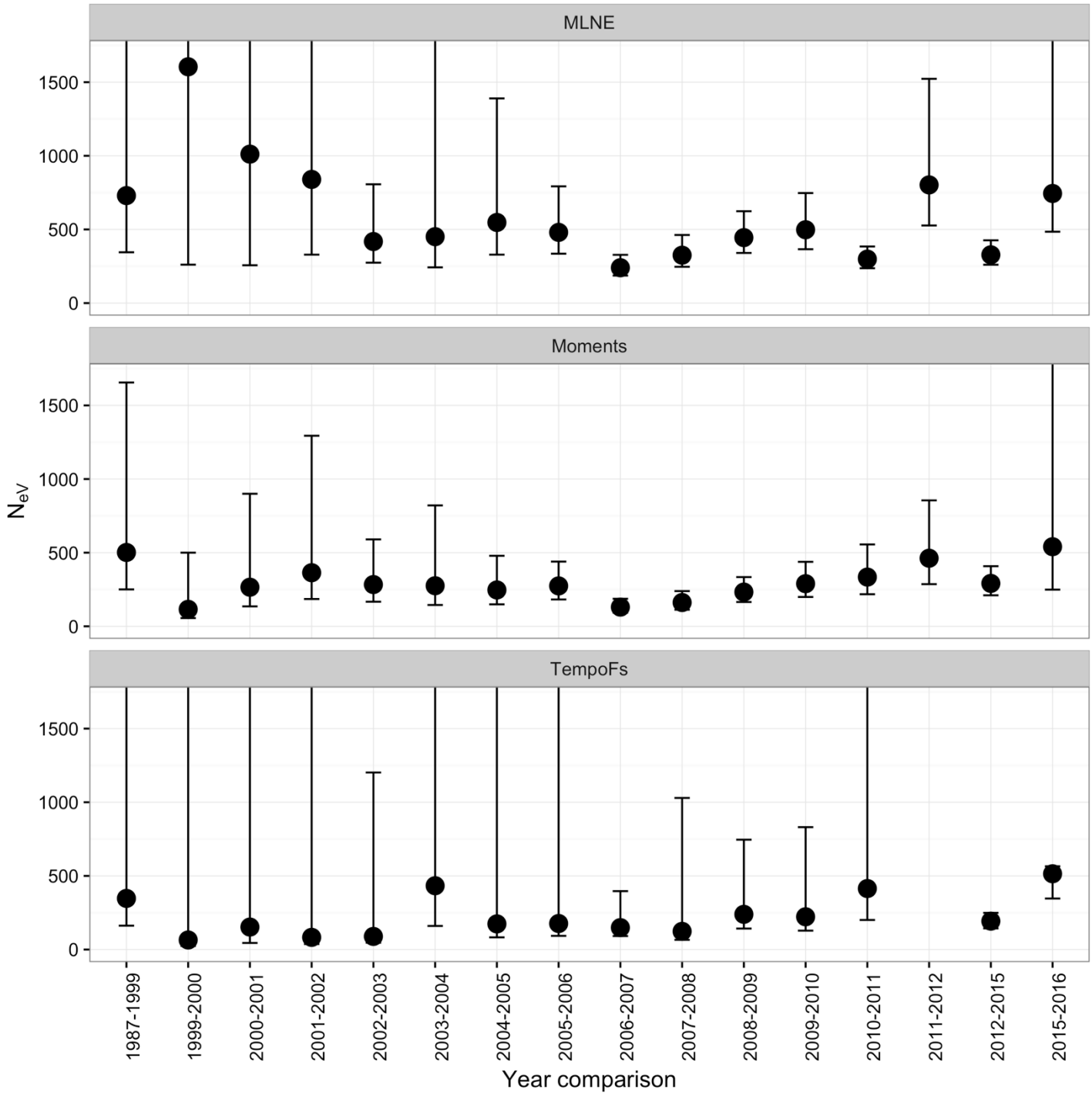


Figure 6. Female variance effective size estimates (N_{ef}) and their associated 95% CIs, based on mtDNA data and calculated using MLNE (upper) and moments (lower) methods. Infinite mean estimates are indicated by points falling outside of the plot area and upper error bars extending to y-maxima indicate infinite upper bounded 95% CI.

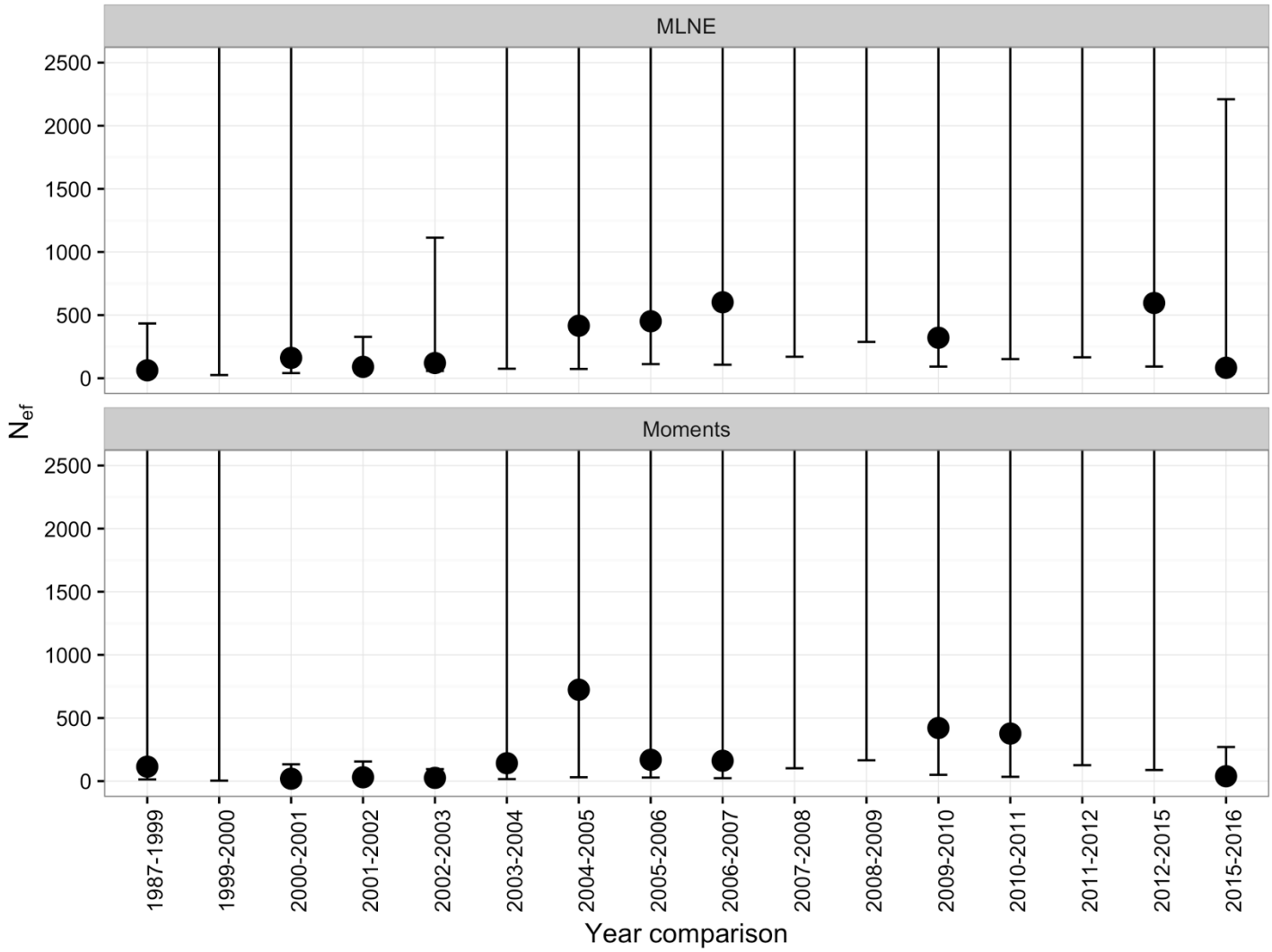


Figure 7. Estimates of inbreeding effective size (N_{eD}) and their associated 95% confidence intervals. Note the logarithmic scale on y-axis. Infinite mean estimates are indicated by points lying at y-maximum, and upper error bars extending to y-maximum indicate infinite upper bounded 95% CI.

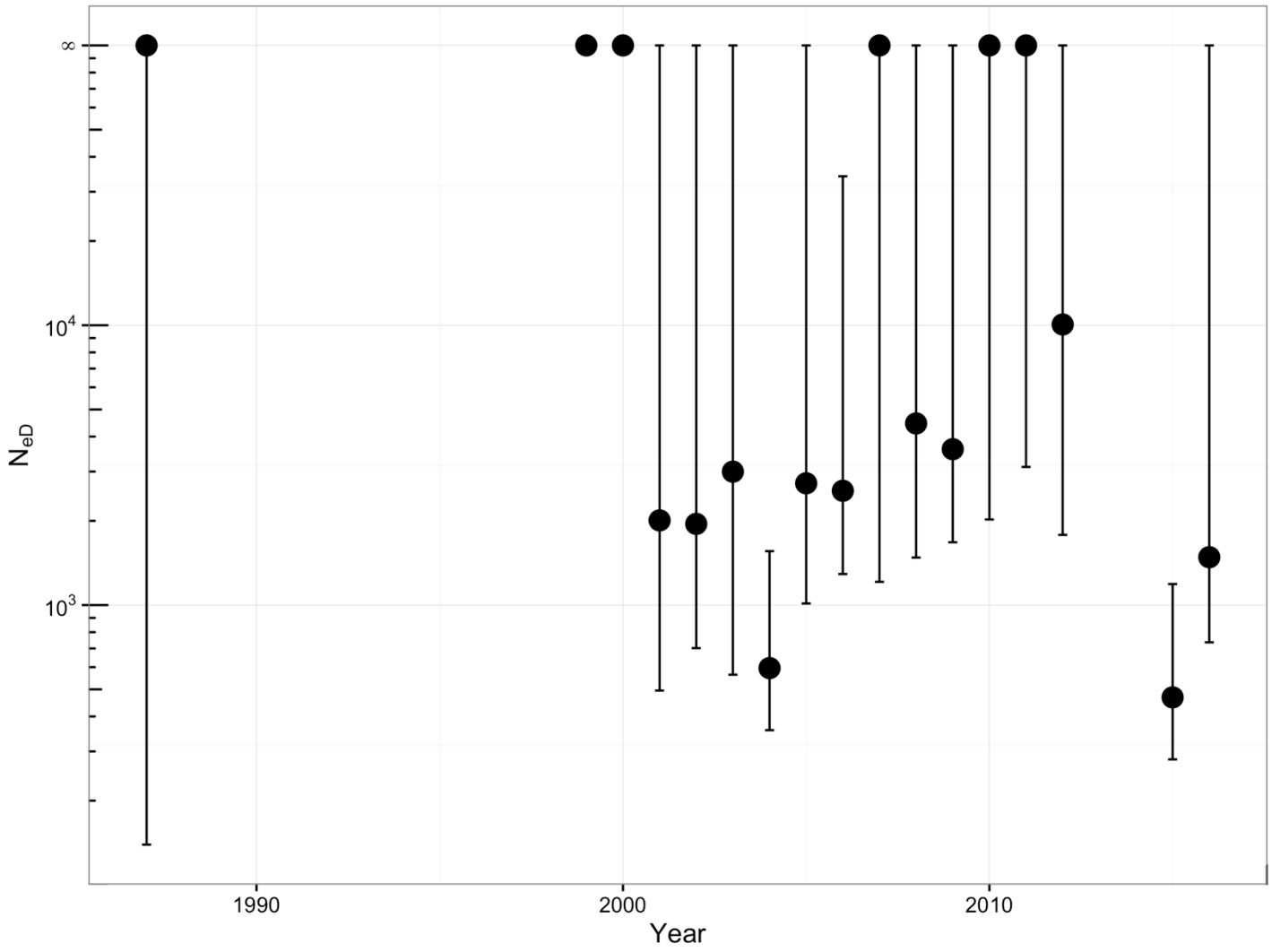


Figure 8. Genetic effective size (N_{eV})obtained using resampling to sample sizes of n=45, n=127, n=143 and n=161 and averaged across 5 replicates. Error bars represent 95% confidence intervals for the resampled estimate. Also plotted are the values of N_{eV} obtained using the actual sample size. Note the log scale on the y-axis.