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Inferring past demographic changes in a critically endangered marine fish after fishery collapse

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Several worldwide marine fish stocks need to recover from collapse or overexploitation. However, the effects of a fishery collapse at the genetic level are still largely unknown, as is the extent of reduction in genetic diversity caused by fisheries and the consequences for extinction risk. Here we present a case study of totoaba, the first marine fish considered as critically endangered. We assessed 16 microsatellite loci to determine whether the demographic collapse of the species resulted in a loss of genetic diversity. Our data indicate that genetic diversity of totoaba is in the range of values observed for fish with similar biological traits without a documented fishery collapse. Contemporary demographic analysis indicated no loss of genetic diversity. Long-term genealogical analysis showed a substantial reduction in effective population size. However, the time and causal effects for population decline cannot be inferred because of the large uncertainty in estimates. Our results indicate that the totoaba in the Gulf of California has not suffered a measurable contemporary reduction in genetic diversity, and that genetic diversity is driven by long-term climatic events. Estimates of current effective size indicate that it is large enough that genetic factors may not be a major problem for conservation. We conclude that the recent fishery collapse of totoaba did not have sufficient consequences at the genetic level to increase the risk of extinction from genetic drift. However, selective effects of fishing on the adaptive potential in totoaba remain unclear.

Keywords: critically endangered, demographic history, effective population size, evolutionary potential, fishery collapse, microsatellites, *Totoaba macdonaldi*.

Introduction

Marine fish populations exhibit a broad range of responses to fishing pressure, which can involve several levels of biological organization, from biomass reduction to genetic consequences (Hilborn and Walters, 1992; Enberg *et al.*, 2009), and many marine fish stocks have collapsed, presumably as a result of overexploitation (Worm *et al.*, 2009; Costello *et al.*, 2012). However, the effects of fishery collapse on genetic diversity have been poorly documented, and it is not clear whether fisheries can reduce genetic variability so much so that they lead to extinction. Many collapsed stocks have failed to recover, and the mechanisms for limited recovery (genetic or ecological factors) are unclear (Hutchings, 2000; Enberg *et al.*, 2009). Several studies have reported loss of genetic diversity for collapsed and overexploited stocks of marine fish (Smith *et al.*, 1991; Hauser *et al.*, 2002; Hutchinson *et al.*, 2003; Hoarau *et al.*, 2005; Ruggeri *et al.*, 2012), whereas other studies did not detect a loss of diversity (Ruzzante *et al.*, 2001; Poulsen *et al.*, 2006; Therkildsen *et al.*, 2010; Chapman *et al.*, 2011; Cuveliers *et al.*, 2011). These results imply that a fishery collapse does not necessarily reduce genetic variability measurably at neutral markers, and underscore the importance of understanding the evolutionary history of marine species to ensure long-term conservation. However, fishery management regulations are usually formulated for short periods and ignore evolutionary principles (Hauser and Carvalho, 2008; Reiss *et al.*, 2009; Lankau *et al.*, 2011), even though the effects of reductions of genetic diversity after fishery collapse are unclear (Therkildsen *et al.*, 2010).

A practical way to integrate genetic information into fishery management is to monitor effective population size (N_e). This is one of the most important parameters in evolutionary biology because it determines the level of genetic variation that can be maintained and provides insight into the risk of extinction and long-term evolutionary potential (Frankham, 2005; Charlesworth, 2009).

As such, genetic diversity and N_e can be used as a proxy for the level of threat in fish with limited data available for a full stock assessment (Spielman *et al.*, 2004; Palstra and Ruzzante, 2008; Hare *et al.*, 2011).

Long-term genetic monitoring programs can provide information on population status, as well as insights into how population genetic diversity responds to fishery pressure, if tissue samples are taken and archived (Ruzzante *et al.*, 2001; Hoarau *et al.*, 2005; Therkildsen *et al.*, 2010, 2013a); however, archived samples are unavailable for most marine species. Fortunately, there are several methods available for using molecular genetic data from contemporary samples to infer past fluctuations in N_e (Luikart *et al.*, 1998; Beaumont, 1999; Garza and Williamson, 2001; Storz and Beaumont, 2002; Cornuet *et al.*, 2008). Many of these methods provide inference at different temporal scales, which can potentially be used to distinguish between contemporary population reduction and natural long-term cycles (Wirth and Bernatchez, 2003; Karlsson *et al.*, 2009). This information about past and current levels of N_e can then be used in management actions (Peter *et al.*, 2010; Hare *et al.*, 2011; Lankau *et al.*, 2011).

Totoaba (*Totoaba macdonaldi*) is the largest fish in the family Sciaenidae and is endemic to the Gulf of California (Chute, 1928). It is distributed from the mouth of the Colorado River to the mouth of the Río Fuerte along the eastern coastline of the Gulf, and from the mouth of the Colorado River to Bahía Concepción on the west coast of the Gulf (Figure 1) (Arvizu and Chávez, 1972). Despite this distribution, totoaba is more common during the breeding season in the Upper Gulf and is only occasionally observed in the rest of its distribution range. Totoaba is considered an estuarine spawner and historically spawned primarily in the estuary of the Colorado River, which was desiccated in the middle of the last century, after it was dammed. However, the totoaba appears to not be completely dependent upon estuarine conditions, as the species still spawns in the same general vicinity, even though non-estuarine conditions prevail (Cisneros-Mata *et al.*, 1995; Bobadilla *et al.*, 2011; Valenzuela-Quiñonez *et al.*, 2011).

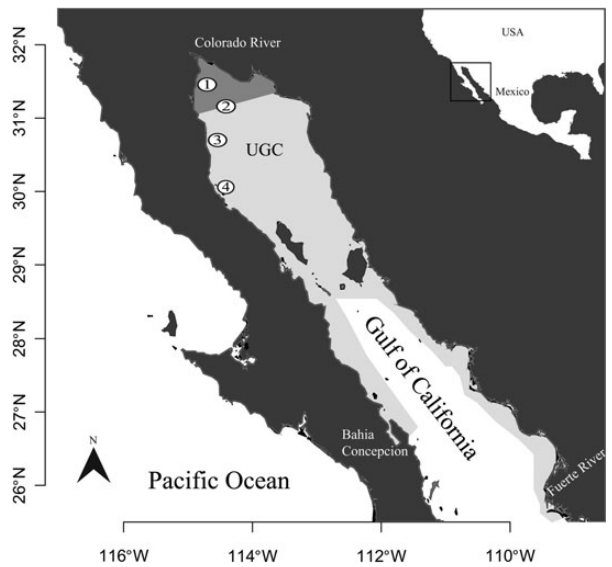

Figure 1. Distribution of *Totoaba macdonaldi* in the Gulf of California (light gray). The Biosphere Reserve of the Upper Gulf of California and Colorado Delta River is indicated in dark gray. Numbers in circles represent sampling locations: (1) Core Zone ($n = 39$), (2) Roca Consag ($n = 66$), (3) South of San Felipe ($n = 12$), (4) San Luis Gonzaga 2010 ($n = 37$), 2005 ($n = 26$).

Totoaba was also the target of an important fishery, which collapsed shortly after the damming of the Colorado River. Fishery records indicate that the catch of totoaba reached 2000 t in 1940 but decreased to 52 t by 1975. This prompted the Mexican Government to completely close the fishery (Cisneros-Mata *et al.*, 1995). A year earlier (1974), a reserve zone, in which all fishing activities were prohibited, was established at the mouth of the Colorado River (Flanagan and Hendrickson, 1976; Rosales-Juárez and Ramírez-González, 1987). Illegal and unreported catches continued, however, and the Mexican Government designated the totoaba estuarine habitat as a Biosphere Reserve in 1993. Since the totoaba fishery was closed in 1975, no formal demographic studies or catch records are available (Cisneros-Mata *et al.*, 1995; Lercari and Chavez, 2007; Valenzuela-Quiñonez *et al.*, 2011). Some combination of loss of habitat and overfishing caused a steep demographic decline (Cisneros-Mata *et al.*, 1995; Flanagan and Hendrickson, 1976), and totoaba was the first marine fish listed as critically endangered under CITES Appendix I in 1976 and was also listed as endangered under the US Endangered Species Act in 1979 (Barrera-Guevara, 1990; CITES–UNEP, 2011). More recently, totoaba was listed as critically endangered by the IUCN in 1996 (Cisneros-Mata *et al.*, 1995; Findley, 2010).

While overfishing and loss of habitat are the primary hypotheses for the fishery collapse (Flanagan and Hendrickson, 1976; Cisneros-Mata *et al.*, 1995; Cisneros-Mata *et al.*, 1997; Lercari and Chavez, 2007), the loss of genetic variation from demographic decline may be an ongoing threat that is contributing to the lack of totoaba recovery (García-de-León *et al.*, 2010; Valenzuela-Quiñonez *et al.*, 2011). Population decline could have resulted in the loss of genetic diversity that compromised the evolutionary potential of the species. This raises three important questions. (i) Did the decline in the population of totoaba affect genetic diversity? (ii) Can we distinguish between population decline caused by contemporary anthropogenic pressure and prehistoric population oscillations? (iii) Is the current effective population size sufficiently large to conserve long-term evolutionary potential?

Here, we evaluate whether the population collapse of totoaba was accompanied by a measurable loss of genetic diversity that may be an obstacle to stock recovery. Genetic data from microsatellite markers were used to estimate the amount of genetic diversity in the species and to reconstruct its demographic history. Current and past effective population sizes were estimated to determine whether current anthropogenic pressure has affected genetic diversity and evolutionary potential of the critically endangered totoaba.

Material and methods

Sampling and DNA extraction

Totoaba were caught at sea with hook and line and gillnet fishing surveys in April and November 2010 and February and March 2011 in the Upper Gulf of California in four sampling areas where totoaba are frequently observed (Figure 1): Core Zone, Roca Consag, south of San Felipe, and San Luis Gonzaga. Fish that were collected in San Luis Gonzaga in 2005 were also included. Approximately 1 cm^2 of pectoral fin tissue was excised and preserved in 96% ethanol. Genomic DNA was then extracted using the chloroform/isoamyl alcohol DNA extraction method, as modified by Correa-Ramírez *et al.* (2010).

Microsatellite markers

DNA samples were analysed at 19 microsatellite loci. Of these, 13 were developed for totoaba (García-de-León *et al.*, 2010) and six (*Soc418, Soc423, Soc428, Soc430, Soc442* and *Soc443*) were developed for red drum (*Sciaenops ocellatus*) but were successfully amplified and variable in totoaba (O'Malley *et al.*, 2003). PCR was performed in 15 μl total reaction volumes containing 1.5 μl PCR buffer ($\times$10), 0.97 μl $MgCl_2$ (25 mM), 0.6 μl dNTPs (10 mM total), 1 μl primers (5 μM ea, pooled), and 0.2 U Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). Thermal cycling conditions were 94°C for 4 min, followed by 34 cycles at 94°C for 45 s, at a locus-specific annealing temperature (Table S1) for 45 s, and at 74°C for 45 s, with a final extension at 74°C for 4 min. PCR products were electrophoresed on an ABI Prism 377 automated sequencer (Applied Biosystems, Foster City, CA, USA). Allele sizes were determined with the Genotyper software program (Applied Biosystems). Two independent persons scored the genotypes, and discrepancies were resolved by a third independent person.

Microsatellite data

Microsatellite data were checked for evidence of null alleles, using FreeNa (Chapuis and Estoup, 2007). Deviations from linkage and Hardy–Weinberg equilibria were tested with Markov chain Monte Carlo approximations of an exact test implemented in GenePop 4.0 (Rousset, 2008). The observed number of alleles (k), effective number of alleles (n_e), observed heterozygosity (H_o), and expected heterozygosity (H_e) were calculated using GenAlEx 6.2 (Peakall and Smouse, 2006). Allelic richness (Ar) was calculated in FSTAT 2.9.3.2 using a sample size of 159 individuals (Goudet, 2001).

Test of demographic changes

Prior to evaluating demographic changes, population structure analysis was performed to detect any cryptic population structure in totoaba (highest F_{st} value between sampling locations $\leq$0.00608; $p \geq 0.27$). To infer past demographic changes, single sample tests for recent reductions in N_e (i.e. bottlenecks) were performed. The heterozygosity excess test developed by Cornuet and Luikart (1996), implemented in the program Bottleneck 1.2.02 (Piry *et al.*, 1999), was used with variable proportions of single-step mutations (95, 90 and 85%). This method assumes that following a severe reduction in N_e, heterozygosity is higher than expected in a population at mutation-drift equilibrium with the same number of alleles. To determine the significance of heterozygosity excesses, Wilcoxon signed rank tests were used. The allele frequency distribution mode-shift method (Luikart *et al.*, 1998) was also used. This method examines the distribution of allele frequencies in the population with the idea that bottlenecked populations can potentially be discriminated from stable ones by the "shape" of the distribution. An L-shaped distribution is expected under mutation-drift equilibrium and a distribution with more intermediate frequency alleles (i.e. a mode-shift) is expected in bottlenecked ones, as a consequence of a higher rate of loss of rare alleles. This bottleneck signature is detectable over a relatively short period, about two to four N_e generations for heterozygosity excess and a few dozen generations for the mode-shift test (Cornuet and Luikart, 1996). The M ratio test (Garza and Williamson, 2001) was also performed. This method exploits the same differential loss of rare alleles following a reduction in N_e but examines the ratio of the number of alleles, k, to the range of allele size, r. The M ratio is expected to be smaller in recently reduced populations than in populations in equilibrium (Garza and Williamson, 2001). Estimates of the M ratio for totoaba were calculated with Arlequin 3.0 (Excoffier *et al.*, 2005).

Long-term changes in N_e were assessed with the Bayesian method implemented in the program MsVar 1.3 (Beaumont, 1999). This method uses the genealogical history of microsatellite loci to estimate rates of population expansion or decline with Markov chain Monte Carlo simulations of mutation-coalescent history (Beaumont, 1999; Storz and Beaumont, 2002). This model estimates four parameters: N_0 (current effective size), N_1 (ancestral effective size), μ (average of mutation rate for all loci), and T_a (time in years since change in population size). Three independent chains were run with different sets of *a priori* log values for the mean (M) and standard deviation (V) of N_0 and N_1 (Prior log values: Run 1: $MVN_0 = 2,1$, $MVN_1 = 4,2$; Run 2: $MVN_0 = 4,2$, $MVN_1 = 4,2$; Run 3: $MVN_0 = 4,2$, $MVN_1 = 2,1$) to test stability of estimates. Prior values of time since the population change ($MVT_a = 5,2$) and mutation rate ($MV\mu = -3.5,1$) were the same for all runs. The mutation rate was based on published estimates (Schlötterer, 2000; Storz and Beaumont, 2002; Selkoe and Toonen, 2006). The default values were used for the hyperpriors (Storz and Beaumont, 2002). Each chain was run for 1.25×10^9 steps, with parameter estimates recorded each 50 000 steps. Convergence was assessed using Gelman–Rubin diagnostics (GRD) with the Coda package 0.14–4 (Plummer *et al.*, 2006) implemented in the R programming language (R Development Core Team, 2011). GRD values from 1 to 1.1 indicate reasonable convergence; values >1.1 indicate poor convergence (Girod *et al.*, 2011). To support either population growth or decline, we used Bayes factors (BFs) (Beaumont, 1999; Storz and Beaumont, 2002). BFs for two models can be defined as the ratio where the numerator represents the posterior probability divided by its prior probability of model 1, and the denominator represents the posterior probability divided by its prior probability of model 2 (Girod *et al.*, 2011). BF for population decline can be estimated from simulated chains using posterior probability of population contraction: $BF = (N_0/N_1 \leq 1)/(N_0/N_1 \geq 1)$, where $(N_0/N_1 \leq 1)$ is the posterior probability of population contraction and $(N_0/N_1 \geq 1)$ is the posterior probability of population expansion (Storz and Beaumont, 2002). Posterior probabilities are the number of states in the chain in which the population has contracted or expanded (Girod *et al.*, 2011). The magnitude of BF in favour of population contraction indicates strong support when BF$\geq$10, substantial support when BF = 3–10, no support when BF = 0.33–3 and false detection when BF < 0.33 (Girod *et al.*, 2011). No information about generation time for totoaba is available; thus, we used the age at first maturity of seven years (Cisneros-Mata *et al.*, 1995) as a proxy for generation time, as in other studies (e.g. Allen *et al.*, 2012).

Effective population size

Several methods were used to estimate effective population size. As a first approximation, long-term effective population size was estimated following Nei (1987), based on microsatellite heterozygosity and assuming mutation-drift equilibrium: $N_e = (1/[1 - H_e]^2 - 1)/8\mu$, where H_e is expected heterozygosity and μ is the mutation rate. Mean H_e was calculated without loci *Soc442, Soc430* and *Tmac74* because of the departures from the Hardy–Weinberg equilibrium at these loci. Two different values of μ were used. The first ($\mu = 0.00054$) was estimated from the data with DIYABC 0.7 (Cornuet *et al.*, 2008), and the second ($\mu = 0.0005$) was estimated from the literature (Ellegren, 2000; Garza and Williamson, 2001; Selkoe and Toonen, 2006).

Another approach used to estimate N_e was with linkage disequilibrium (LD). The principle behind LD methods is that, as N_e decreases, genetic drift generates non-random associations among alleles at different loci or gametic disequilibrium (Hill, 1981). The level of LD should directly reflect N_e in small and moderate sized populations (Waples, 2006; Waples and Do, 2010). N_e was estimated using the LD bias-corrected method (Waples, 2006; Waples and Do, 2010), as implemented in NeEstimator 2.0 (Do *et al.*, 2013). A *pcritic* value of 0.05 was chosen to reduce the potential bias for low frequency alleles (Waples and Do, 2010).

Approximate Bayesian computation (ABC) was also used to estimate N_e from the microsatellite data using the program OneSamp (http://genomics.jun.alaska.edu/asp/Default.aspx (last accessed 15 September 2013); Tallmon *et al.*, 2008). ABC uses multiple summary statistics, and thus more information from the data than single summary statistic methods, which is expected to improve accuracy and precision of estimates. OneSamp created 50 000 populations with the same number of individuals and loci as contained in the genetic dataset and with N_e drawn uniformly from *a priori* values ranging from 100 to 5000. Values of N_e from simulated populations with summary statistic values close to the values from the focal population were accepted and used in a weighted local regression to estimate N_e of the focal population (Tallmon *et al.*, 2008).

Finally, a different ABC estimation for N_e was performed with the program DIYABC (Cornuet *et al.*, 2008) using all of the genetic diversity measures as summary statistics. This demographic model to estimate N_e for a population requires temporal sampling. To approximate temporal sampling in the observed data, three age groups were created, based on body length, and translated to age (<5 years, 970 mm; ~5 years, 970–1160 mm; >7 years, 1160 mm; unpublished data). The summary statistics from the original dataset were compared with summary statistics from 500 000 datasets sampled temporally from simulated populations with N_e drawn from a uniform distribution with *a priori* values ranging from 100–5000. Then 1% (5000) of the simulated datasets with the closest summary statistic values to those observed in the data were selected to estimate the posterior distribution of N_e through a local linear regression procedure (Cornuet *et al.*, 2008).

Results

Multilocus microsatellite genotypes were obtained for 180 totoaba. Null alleles were evident in *Soc442*. Seven of the 19 loci deviated from Hardy–Weinberg equilibrium ($p < 0.05$), but only three loci (*Tmac74, Soc442, Soc430*) were not in Hardy–Weinberg equilibrium after Bonferroni correction ($p \leq 0.0026$). These loci were omitted from further analysis. Four comparisons showed significant linkage disequilibrium ($p < 0.05$), but none were significant after Bonferroni correction ($p > 0.00014$).

The number of alleles per locus (k) varied from 3 to 31 (mean = 11.6), the effective number of alleles (n_e) varied from 1.3 to 19.2 (mean = 5), and allelic richness (Ar) ranged from 3 to 31 (mean = 11.5). Mean H_o and H_e were 0.62 ± 0.22 and 0.67 ± 0.20, respectively, and the mean F_{is} over all loci was 0.08 ± 0.19 (Table 1).

No evidence of significant heterozygosity excess was found with any of the mutational model parameters. Similarly, mode-shift analysis revealed no noticeable departure from the L-shaped allele frequency distribution expected for populations at equilibrium. The M ratio test also failed to find evidence of a recent reduction in genetic effective size, and the mean value for all loci was $M = 0.77$ (Table 1); thus, all summary statistic methods failed to find evidence of a significant recent reduction in N_e in totoaba.

Table 1. Genetic diversity values of *Totoaba macdonaldi*.

Locus	N	k	n_e	Ar	H_o	H_e	F_{is}	H–W	M
Tmac74	160	31	19.2	31.0	0.70	0.95	0.26	*	–
Tmac56	174	12	4.3	11.6	0.71	0.77	0.07	NS	0.60
Tmac55	177	14	5.9	13.8	0.80	0.83	0.04	NS	0.70
Tmac51	176	26	12.0	25.8	0.95	0.92	−0.04	NS	0.90
Tmac44	159	9	4.0	9.0	0.70	0.75	0.06	NS	0.75
Tmac43	179	6	1.3	5.9	0.21	0.21	0.01	NS	0.46
Tmac25	172	17	9.4	17.0	0.89	0.89	0.00	NS	0.71
Tmac10	172	9	3.4	8.8	0.74	0.70	−0.05	NS	0.60
Tmac08	167	8	3.2	8.0	0.68	0.69	0.01	NS	1.00
Tmac07a	173	4	1.8	4.0	0.42	0.44	0.03	NS	1.00
Tmac06	166	18	6.5	17.9	0.75	0.85	0.11	NS	0.72
Tmac05	177	5	2.0	4.9	0.44	0.50	0.11	NS	1.00
Tmac03	179	5	1.8	4.9	0.48	0.45	−0.07	NS	0.83
Soc443	172	4	1.8	4.0	0.44	0.45	0.02	NS	0.57
Soc442	178	3	2.0	3.0	0.10	0.50	0.80	*	–
Soc430	173	10	2.1	9.7	0.46	0.53	0.13	*	–
Soc428	179	8	4.3	8.0	0.80	0.77	−0.05	NS	0.80
Soc423	178	11	2.9	10.7	0.66	0.66	−0.01	NS	0.83
Soc418	169	21	7.0	20.9	0.81	0.86	0.05	NS	0.88
Mean		11.6	5.0	11.5	0.62	0.67	0.08	NS	0.77

N = Sample size, k = number of alleles, n_e = effective number of alleles, Ar = allelic richness, based on a sample size of 159 individuals, H_o = observed heterozygosity, H_e = expected heterozygosity, F_{is} = fixation index, $H–W$ = Hardy–Weinberg disequilibrium, M = M ratio, NS = No significant departures from $H–W$ equilibrium. *Significant departures from $H–W$ equilibrium.

Table 2. Estimates of effective population size (N_e) and confidence interval (CI) for totoaba using different methods.

Software	Estimator	N_e	CI
MsVar	*Genealogy* (N_0)	2 669	603–11 625
MsVar	*Genealogy* (N_1)	10 849	2 303–51 701
	Nei*	1 894	
	Nei**	2 046	
NeEstimator	*LD*	2 759	697–∞
OneSamp	*Bayesian*	1 803	1 094–4 932
DIYABC	*Bayesian*	2 680	1 540–3 900

Long-term demographic history estimates of current N_e (N_0) and ancestral N_e (N_1). Long-term *Nei*, linkage disequilibrium (LD) and Bayesian N_e estimates. *($\mu = 0.00054$; DIYABC), **($\mu = 0.0005$; Ellegren, 2000; Garza and Williamson, 2001; Selkoe and Toonen, 2006 from literature reports).

GRD indicated reasonable convergence among three independent MsVar runs for N_0 (GRD = 1.08), N_1 (GRD = 1.02), and μ (GRD = 1.01), and poor convergence for T_a (GRD = 1.37). The analysis found a population decline in totoaba with ancestral effective size (N_1) of 10 849 (90% HDP: 2303–51701) and current effective size (N_0) of 2669 (90% HDP: 603–11 625), approximately a fourfold reduction (Table 2). This reduction was strongly supported (BF > 10), but the 90% HDP of N_0 and N_1 overlap, so the hypothesis of a constant population cannot be excluded (Table 2). The mean time elapsed since the population reduction started (T_a) was estimated at 7413 years ago (90% HDP: 926–72 144; Figure 2).

Estimates of N_e obtained for totoaba were consistent among different methodologies and are summarized in Table 2. Nei's long-term N_e was estimated at 1894–2046 with two different mutation rates for microsatellite loci. The LD method implemented in NeEstimator yielded an estimate of 2759 (C.I.: 697–∞). ABC estimates were likewise similar, with OneSamp yielding an estimate of 1803 (C.I.: 1094–4932) and DIYABC of 2680 (95% HDP: 1540–3900) (Table 2).

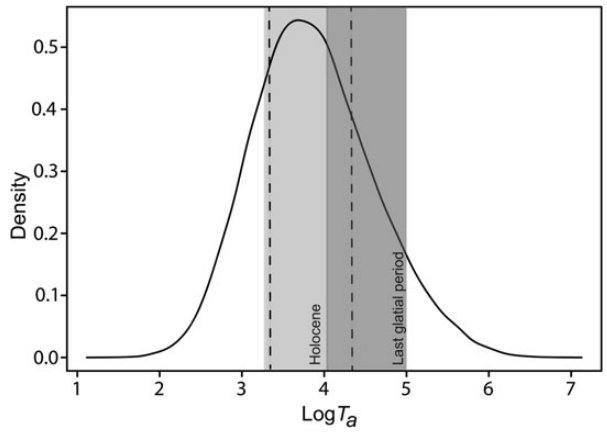

Figure 2. Posterior distribution (solid line) of time since the population started to decline (T_a), from MsVar. Time is in $\log_{10}$ scale and represents years before present. Vertical dashed lines represent the 50% of data around the mean T_a estimate. Dark and light gray shades represent the last glacial period and Holocene, respectively.

Discussion

Two different hypotheses to distinguish between contemporary and long-term effects of demographic changes on totoaba genetic diversity were evaluated with methods that provide inference on different time-scales.

First, contemporary overfishing and habitat loss were hypothesized as factors that could reduce genetic diversity of totoaba. To ascertain if this is the case, several summary statistic tests for recent reductions in N_e were performed. Both the heterozygosity excess test and allele frequencies distribution (mode shift) analysis provided results consistent with those expected in populations that have not experienced a bottleneck (Cornuet and Luikart, 1996; Luikart *et al.*, 1998; Luikart and Cornuet, 1998). The *M* ratio test also failed to find significant support for a recent reduction in effective size; the *M* ratio for totoaba was 0.77, which was higher than a commonly used threshold value ($M = 0.68$) for populations that have suffered such reductions (Garza and Williamson, 2001). Genetic diversity was also compared with that of species in the same family of fish. The optimal way to compare genetic diversity between closely related species is to assay variation at the same set of molecular markers, ideally markers that were initially ascertained in other species, in samples that provide similar representation in the species being compared (Pastor *et al.*, 2004). When this is not feasible, a general comparison of patterns may still provide insight into genetic diversity. Totoaba exhibit genetic diversity that is similar to other, less depleted, sciaenid species that are not threatened (for comparisons see Table S2). Levels of genetic diversity in fish have been related to habitat and life history, with a general trend of increasing genetic diversity from freshwater to anadromous to marine fish (DeWoody and Avise, 2000). Genetic diversity of totoaba was similar to anadromous fish and slightly less than the mean for marine fish, but within the range of observed values, in spite of the severe population decline in the last century (Cisneros-Mata *et al.*, 1995).

Second, the hypothesis that long-term fluctuations in N_e are the primary determinants of genetic diversity was evaluated by Bayesian coalescent analysis and indicated that totoaba have experienced a fourfold historical reduction in effective population size. Bayes factors also indicate strong support for a population decline scenario. Even so, the broad range of T_a estimates (7413 years ago; 90%HPD: 926–72 144) makes it difficult to determine the exact time when the population started to decline (Figure 2). Consequently, these results should be treated with caution. This idea is supported by the overlap in the 90%HDP of N_0 and N_1, as well as the Nei's long-term estimate of N_e that assumes mutation drift equilibrium, which was similar to contemporary N_e estimates. Other scenarios could be considered. For example, 50% of the data around mean T_a are in the range of 2200–22 000 years ago, which corresponds to the last glaciation maximum and the Holocene (Figure 2). In these epochs, several potential climatic events related to large scale oceanographic and ecological changes (Keigwin and Jones, 1990; Barron *et al.*, 2004; Yasuhara *et al.*, 2008) could have caused declines in the totoaba population. Although the proximate factors that caused this reduction in effective size cannot be inferred, it is likely to have been driven by these large-scale climatic events. Such patterns of long-term fluctuations in effective size have also been inferred in other marine species, including North Atlantic eels (Wirth and Bernatchez, 2003) and lane snapper (Karlsson *et al.*, 2009).

Mean N_e estimated for totoaba varied from 1894 to 2759, depending on the method employed. These values fall within the N_e range recommended for long-term conservation (N_e: 1000–5000), when considering mutation, drift and selection (Franklin and Frankham, 1998; Lynch and Lande, 1998). This suggests that totoaba still maintain sufficient genetic variation to cope with potential environmental changes that may affect its life history, which is contrary to some views (Flanagan and Hendrickson, 1976; Valenzuela-Quiñonez *et al.*, 2011).

Effective population size estimates for totoaba were also consistent with those of several commercial fishery stocks that have collapsed and some that have not (Turner *et al.*, 2002; Chapman *et al.*, 2002; Hauser *et al.*, 2002; Hutchinson *et al.*, 2003; Hoarau *et al.*, 2005; Poulsen *et al.*, 2006; Saillant and Gold, 2006; Riccioni *et al.*, 2010; Chapman *et al.*, 2011; Ruggeri *et al.*, 2012) (see Table S3 for summary comparisons). However, these comparisons should be viewed cautiously because of the different methods of estimation and the number and variability of the selected markers. However, our study was based on 16 microsatellite loci, while most N_e estimates in marine fish have been based on less than 11 microsatellite loci (Table S3), and the estimates for totoaba should therefore be relatively robust.

Taken together, these results suggest that the contemporary reduction in population size due to overfishing, habitat degradation, and poaching have not affected neutral genetic diversity, that the totoaba population is currently large enough that biological extinction caused by genetic effects is not likely to be an immediate threat, and that totoaba are likely to maintain the evolutionary potential necessary to cope with environmental changes.

Methodological issues

Methods of detecting reductions in N_e assume a single panmictic population, but it is known that population structure can lead to false positive signals of reduction (Chikhi *et al.*, 2010). No evidence of population structure was detected in totoaba (highest F_{st} value between sampling locations $\leq 0.006\,08$; $p \geq 0.27$). Although the presence of loci that are out of Hardy–Weinberg equilibrium could indicate cryptic population structure, only three (<20%) of the loci used here were out of Hardy–Weinberg equilibrium after Bonferroni correction. Methods for inferring recent reductions in N_e from contemporary genetic variation should be treated with

caution, as statistical power depends on sample size, number of loci employed, and the magnitude and timing of demographic changes (Williamson-Natesan, 2005; Girod *et al.*, 2011; Peery *et al.*, 2012). However, the number of microsatellite loci used here (16) and the population sample size (180 individuals) should provide sufficient statistical power to detect large reductions in effective population size (Peery *et al.*, 2012). That all methods provided concordant results lends additional support to the conclusion that there has not been a recent measurable loss of genetic variation in totoaba.

The Bayesian method in MsVar performs better than summary statistic methods for detecting changes in effective population size (Girod *et al.*, 2011). The MsVar method has higher precision and less biased estimates with severe ($N_0/N_1<0.1$) and ancient population size reductions (Girod *et al.*, 2011), and the 90%HDP of parameter estimates decreases with severe population reductions. In this study, a less severe ($N_0/N_1 = 0.25$) population size reduction was inferred, and the wide 90%HPDs in parameter estimates was thus expected (Girod *et al.*, 2011). The overlap in the 90%HDP of N_0 and N_1 estimates implies that the hypothesis of constant population cannot be ruled out. Assumptions about the mutation model could also bias the results (Storz and Beaumont, 2002), as the MsVar method assumes single-step mutations, but most microsatellite loci more closely follow a two-phase mutation model (Di Rienzo *et al.*, 1994). Inaccurate mutation rate estimates could produce biased estimates of some parameters (N_0,N_1,T_a) (Storz and Beaumont, 2002) and hinder precise dating of past demographic changes. However, the mutation rate estimates used here are based on a large number of estimates from other species and have been used previously (Ellegren, 2000; Garza and Williamson, 2001; Storz and Beaumont, 2002; Selkoe and Toonen, 2006).

Estimates of N_e can also be biased due to assumption of the underlying assumptions. The methods employed here assume a single panmictic population and discrete generations. The assumption of discrete generations was clearly violated here, although overlapping generations are common in N_e estimation. In this situation, estimates from the LD method can be interpreted as an estimate of the number of breeders (Nb) if only one cohort was sampled. If the number of cohorts sampled is approximately equal to generation length, estimates can be interpreted as N_e for the generation, but this relationship is still unclear for this method (Waples, 2006; Waples and Yokota, 2007; Waples and Do, 2010). The LD method has low precision at larger N_e (>1000) because the drift signal is too weak (Waples and Do, 2010). Marine fish populations, in general, have large N_e, and LD estimates usually include infinity (Palstra and Ruzzante, 2008; Hare *et al.*, 2011), but the lower boundary is still informative (Waples and Do, 2010; Hare *et al.*, 2011).

Similarly, Bayesian methods to estimate N_e are biased by overlapping generations, but the effect of this bias has not been evaluated (Waples and Yokota, 2007). Bayesian methods yielded finite interval boundaries, despite low genetic drift. This difference can be explained because Bayesian methods use more information from the data to get better approximations for large N_e (Tallmon *et al.*, 2008; Therkildsen *et al.*, 2010). Potential biases associated with the use of reconstructed cohorts as a proxy to represent temporal sampling have not been evaluated in DIYABC, but both this and the Bayesian method in OneSamp use a similar approach (simulated data, summary statistics comparison, and posterior parameter estimation) (Cornuet *et al.*, 2008; Tallmon *et al.*, 2008). DIYABC uses coalescence theory in different complex scenarios; OneSamp does not and only uses the three general steps of the ABC approach. Despite the potential bias induced by cohort reconstruction and methodological approach, they produced very similar results. Even so, one method does not validate the other, and results from the Bayesian methods should be viewed with caution, given the possible biases associated with their N_e estimates.

Conservation implications

Understanding the evolutionary history of marine species can help to distinguish between contemporary and long-term population fluctuations and help to identify the level of conservation concern. Many commercially exploited marine fish have been considered as threatened, based mainly on demographic criteria (Musick *et al.*, 2000; Powles *et al.*, 2000; Dulvy *et al.*, 2003; Reynolds *et al.*, 2005) without consideration of genetic factors that underlie the species evolutionary potential and, therefore, its long-term conservation (Allendorf and Luikart, 2007).

Although some empirical work has found evidence of reductions in genetic diversity of marine fish caused by overexploitation (Hauser *et al.*, 2002; Hutchinson *et al.*, 2003), many other studies, including the present one, have failed to find reductions in genetic diversity in heavily exploited species and have concluded that N_e is large enough to alleviate long-term conservation concerns from loss of evolutionary potential (Ruzzante *et al.*, 2001; Hoarau *et al.*, 2005; Poulsen *et al.*, 2006; Therkildsen *et al.*, 2010; Chapman *et al.*, 2011; Cuveliers *et al.*, 2011; Pujolar *et al.*, 2011). In a meta-analysis of marine fish, Palstra and Ruzzante (2008) found that populations of conservation concern had significantly smaller N_e estimates (7–1160) than stable populations without conservation concern (19–8935). In addition, commercially exploited marine fish had significantly larger N_e estimates (N_e: in the range of 560–19 535) than conservation concern and without conservation concern categories of populations.

Under the prevailing hypothesis of estuarine dependence of totoaba for spawning, a reduction in genetic diversity was expected. After the Colorado River was dammed and diverted, the estuary was almost entirely desiccated. However, our results do not support this hypothesis as the cause for the population decline. Totoaba continue to spawn in the upper Gulf in non-estuarine conditions (Lavín *et al.*, 1998; Lavín and Sánchez, 1999; Valdez-Muñoz *et al.*, 2010; Valenzuela-Quiñonez *et al.*, 2011), and it is now known that the totoaba is able to tolerate a wide range of salinity conditions and can complete its life cycle entirely in marine conditions (Ortíz-Viveros, 1999; Valenzuela-Quiñonez *et al.*, 2011). This supports non-stringent dependence of totoaba on the estuary (Cisneros-Mata *et al.*, 1995; Bobadilla *et al.*, 2011; Valenzuela-Quiñonez *et al.*, 2011).

This study showed that the totoaba population has not suffered a measurable contemporary reduction in neutral genetic diversity, but may have experienced a long-term, fourfold reduction in effective population size, which could be related to large-scale oceanographic and climatic events. Although totoaba has experienced very large declines in abundance in the last century, the remaining population is still large enough that genetic factors may not be a major problem for conservation. This represents a change in perception of the threats to the species after the fishery collapse in the 20th century (Flanagan and Hendrickson, 1976; Hendrickson, 1979; Cisneros-Mata *et al.*, 1995; CITES, 2010).

While evidence of substantial loss of neutral genetic diversity was not found, fishery and habitat loss may have caused loss or altered the frequency of selectively important variation, which could still have negative consequences. Changes in genetic diversity that are not easily measured by surveying neutral genetic markers may have significant effects on fitness and the genetic architecture of quantitative

traits (Russello *et al.*, 2012; Therkildsen *et al.*, 2013a). In the future, next-generation DNA sequencing technologies will be a powerful approach to examining the consequences of fisheries-induced, or other, selection on populations of marine fish with historically large effective sizes by providing genotypes at thousands of markers and assaying genomic levels of variation and signals of selection (Hemmer-Hansen *et al.*, 2013; Therkildsen *et al.*, 2013a, 2013b). These approaches will help to define new management units, based on the adaptive uniqueness of populations, and assess microevolutionary changes induced by harvest and other selective forces (Funk *et al.*, 2012; Therkildsen *et al.*, 2013a).

In spite of the apparent lack of genetic consequences from the population decline of totoaba over the last half century, the current demographic abundance of the species is still unknown. It is necessary to assess demographic parameters, such as biomass, mortality rates, and recruitment, to understand its population status. There is a request to reopen a totoaba sport fishery in the Gulf. The absence of a formal monitoring program or other approach to estimate abundance since the fishery was closed means that there is no contemporary information on population status. Coupled with a lack of information about other aspects of the species' biology, such as potential population structure and population dynamics, this makes any decision to reopen a fishery a risky venture (Valenzuela-Quiñonez *et al.*, 2011). We recommend a long-term monitoring program be implemented to provide insight into demographic and evolutionary processes of this species and a formal population assessment then be performed to inform any future changes in management.

Supplementary data

The following supplementary data is available at *ICES Journal of Marine Science* online.

Table S1. Annealing temperatures (*Tm*) for loci used in this study.

Table S2. Genetic diversity estimates for fish in the family Sciaenidae. Mean estimates for all loci in all populations. Number of loci, number of alleles (k), observed heterozygosity (H_o) and expected heterozygosity (H_e).

Table S3. Estimated effective population sizes for fish with microsatellite loci. Number of loci used (No. Loci), effective population size (N_e), and confidence interval (CI). Modified from Hauser and Carvalho (2008).

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