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Martin, Christopher H
Höhna, Sebastian

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NEWS AND VIEWS**Comment**

New evidence for the recent divergence of Devil's Hole pupfish and the plausibility of elevated mutation rates in endangered taxa

Christopher H. Martin¹  | Sebastian Höhna^{2,3,4}

¹Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

²Department of Integrative Biology, University of California, Berkeley, CA, USA

³Department of Statistics, University of California, Berkeley, CA, USA

⁴Division of Evolutionary Biology, Ludwig-Maximilians-Universität, München, Germany

Correspondence

Christopher H. Martin, Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA.
Email: chmartin@unc.edu

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Sağlam et al. recently argued that the Devil's Hole pupfish (*Cyprinodon diabolis*), a conservation icon with the smallest known species range, was isolated 60 kya based on a new genomic data set. If true, this would be a radically long timescale for any species to persist at population sizes <500 individuals, in contrast to conservation genetics theory. However, here we argue that their analyses and interpretation are inappropriate. They placed highly restrictive prior distributions on divergence times, which do not appropriately model the large uncertainty and result in removing nearly all uncertainty from their analyses, and chose among models by assuming that pupfishes exhibit human mutation rates. We reanalysed their data with their same methods, only using an informative prior for the plausible range of mutation rates observed across vertebrates, including an estimate of the genomewide mutation rate from a pedigree analysis of cichlid fishes. In fact, Sağlam et al.'s phylogenetic data support much younger median divergence times for *C. diabolis*, ranging from 6.2 to 19.9 kya, overlapping with our previous phylogenetic divergence time estimates of 2.5–6.5 kya. There are many reasons to suspect an even younger age and higher mutation rate in *C. diabolis*, as we previously estimated, due to their high metabolism, small adult size, small population size and severe environmental stressors. In conclusion, our results highlight the need for measuring mutation rate in this fascinating species and suggest that the ages of endangered taxa present in small, isolated populations may frequently be overestimated.

KEYWORDS

divergence time estimation, phylogeny, speciation, species tree, time calibration

1 | INTRODUCTION

Estimation of species divergence time is critical not only for understanding the evolutionary history of a group (Drummond & Rambaut, 2007; Edwards, 2009) and testing fundamental concepts in evolutionary ecology (Coyne & Orr, 2004; Hendry & Kinnison, 1999; Schluter, 2000) but also for the management of small populations and their future conservation (Kinnison, Hendry, & Stockwell, 2007; Martin, Crawford, Turner, & Simons, 2016; Reed & Stockwell, 2014;

Stockwell, Heilveil, & Purcell, 2013). Older lineages—particularly relict lineages such as the coelacanth and tuatara—are widely regarded as higher conservation value due to their greater reservoir of evolutionary history and unique traits (Faith, Reid, & Hunter, 2004; Vane-Wright, Humphries, & Williams, 1991). However, radiations of young taxa are also valuable and provide the most direct insights into the speciation process (Erwin, 1991; Hendry, 2017; Martin, 2012, 2013; Martin & Wainwright, 2013a,b; Nosil, 2012; Seehausen et al., 2014; Turelli, Barton, & Coyne, 2001). Searching

for complex histories of gene flow and introgression within endangered taxa can also highlight the need for alternative management strategies to preserve this dynamic: Are endangered species relics that must be isolated or complex interconnected communities that would not persist without periodic influxes of secondary gene flow (Martin et al., 2016; Wayne & Shaffer, 2016)? Answering this question is of critical importance for proper management (e.g., Eldridge et al., 1999; Kennedy, Grueber, Duncan, & Jamieson, 2014; Martin et al., 2015; Robinson et al., 2016); however, scientists should not fall into the trap of claiming that a species' age "is vital for determining its status as a critically endangered species" (Sağlam et al., 2016a).

The age of the Devil's Hole pupfish (DHP) is highly controversial. Published estimates range from 500,000 to 200 years, largely reflecting the choice of outgroup fossil or recent calibration priors and phylogenetic or demographic analyses (note that Devil's Hole itself has been physiochemically dated to 60 kya based on calcite deposition rates: Winograd et al., 1992; Riggs & Deacon, 2002; Echelle et al., 2005; Martin et al., 2016; Smith et al., 2002). The age of this species is also critically important for setting the timescale for the survival of such a small population, historically fluctuating between 35 and 500 individuals (Beissinger, 2014; Reed & Stockwell, 2014; Stoike & Pister, 2010), the evolution of postzygotic intrinsic incompatibilities in Cyprinodontidae (e.g., Tech, 2006) and determining the frequency of periodic renewal of genetic diversity in isolated desert populations through gene flow (Martin et al., 2016). The historical frequency of gene flow is also vital for informing management actions (e.g., Wayne & Shaffer, 2016).

Sağlam et al. (2016a); hereafter SEA) recently estimated the age of DHP. They compared divergence times with the neighbouring Ash Meadows Amargosa pupfish population (*C. nevadensis mionectes*) and one outgroup (Owens pupfish, *C. radiosus*) by placing restrictive prior distributions on divergence times corresponding to four different scenarios, which has the effect of eliminating nearly all uncertainty in their divergence time estimates. These restrictive priors do not appropriately capture the large amount of uncertainty in these estimates. They also used the simple Jukes-Cantor model for nucleotide substitution rates without rate variation among sites or loci, which is probably inappropriate for genomic data (however, divergence times seemed robust when analysed under the more complex GTR+ Γ model (data not shown)). SEA then compared the four divergence time hypotheses based on the inferred mutation rate under each scenario and chose the model predicting the slowest mutation rate (1.08×10^{-8} mutations/site/generation) as correct based on its similarity to what they claim is the average vertebrate mutation rate based on citations to (Lynch, 2010; Roach et al., 2010). However, both these publications only refer to 1×10^{-8} mutations/site/generation as the human mutation rate; indeed, Lynch (2010) further demonstrates that mutation rate scales negatively with effective population size over more than an order of magnitude across taxa, suggesting a much greater rate in DHP. The true mutation rate of

DHP is unknown; simply using the human mutation rate is not appropriate (Martin & Palumbi, 1993).

There are many reasons to suspect in general that DHP may have a genomewide mutation rate that differs from other vertebrates. First, despite intense scrutiny, even the human mutation rate is controversial (Harris, 2015; Harris & Pritchard, 2016; Moorjani, Gao, & Przeworski, 2016; Nachman & Crowell, 2000; Scally & Durbin, 2012). Indeed, there appears to be a "hominoid-slowdown" in mutation rate due to increased generation times, violating assumptions of a strict molecular clock (Li & Tanimura, 1987). Second, the correct mutation rate to use for calibration is particularly controversial in exactly the scenario discussed here: very recent timescales (BurrIDGE, Craw, Fletcher, & Waters, 2008; Ho & Phillips, 2009; Ho, Phillips, Cooper, & Drummond, 2005; Ho et al., 2011). Pedigree analysis and mutation accumulation lines generally detect much faster mutation rates than fossil and ancient geological calibrations used to estimate substitution rates on phylogenies, likely due to purifying selection over the extended period from spontaneous mutations appearing in a population to fixation (substitution) over million-year evolutionary timescales (Liu et al., 2014; Millar et al., 2008; Santos et al., 2005). Third, teleost fish mutation rates are known to be higher than other vertebrates, possibly due to their whole-genome duplication (Jaillon et al., 2004; Kasahara et al., 2007; Ravi & Venkatesh, 2008; Recknagel, Elmer, & Meyer, 2013). Fourth, variation in mutation rates among vertebrates has long been known, leading Martin et al. in 1992 to directly advise against the strategy used by SEA "it is inappropriate to use a [molecular substitution rate] calibration for one group to estimate divergence times or demographic parameters for another group."

In addition, there are many reasons to suspect that the Devil's Hole pupfish in particular may exhibit much higher mutation rates than other teleost fishes (which would result in a younger age estimate for this species). First, higher mutation rates are correlated with higher metabolism in vertebrates (Bleiweiss, 1998; Martin & Palumbi, 1993). Higher metabolism is strongly correlated with environmental temperature in fishes (Clarke & Johnston, 1999) and DHP persist in one of the hottest environments of any vertebrate species at 32°C year-round in Devil's Hole (Stoike & Pister, 2010). Furthermore, DHP periodically goes into states of anaerobic metabolism for up to 2 hr, paradoxically reducing its metabolic efficiency even in the presence of oxygen and driving up its metabolic rate (Heuton, Ayala, Burg, & Dayton, 2015; Hillyard, Burg, McKenna, & Urbina, 2014). Second, higher metabolism is also correlated with smaller size (Gillooly, Brown, West, & Savage, 2001) and Devil's Hole pupfish are the smallest species of *Cyprinodon* (Stoike & Pister, 2010). Third, higher metabolism is correlated with lifespan (Bromham, 2009; Hulbert, Pamplona, & Buffenstein, 2007) and Devil's Hole pupfish have the shortest lifespan of any Cyprinodontidae at approximately one year (Stoike & Pister, 2010). Although these life history traits are all correlated, combined analyses of large data sets have generally been able to disentangle separate contributions from each factor (e.g., Bromham, Rambaut, & Harvey, 1996; Romiguier et al., 2014; Welch & Bininda-Emonds,

2008). Fourth, laboratory experiments demonstrate that populations experiencing severe environmental stress increased their mutation rate (Bjedov, Tenaillon, Gerard, & Souza, 2003; Ji, Ng, Sharma, Neculai, & Hussein, 2012), which may also be expected in the low-resource, high temperature environment of Devil's Hole; for example, this extreme environment may stunt DHP growth rates and prevent normal pelvic fin development (Lema & Nevitt, 2006). Fifth, purifying selection is weaker in smaller populations, leading to a more rapid accumulation of deleterious mutations, including within DNA repair machinery. This effect is the leading explanation for increased substitution rates observed at more recent timescales (Ho et al., 2011; Woodhams, 2006) and is likely to be amplified in one of the smallest vertebrate populations known (Lynch, 2010; Martin et al., 2016). For example, a previous small refuge population of DHP may have exhibited a severe genetic load (Martin, Echelle, Zegers, Baker, & Keeler-Foster, 2011). Thus, the extreme life history, environment and population size of the Devil's Hole pupfish suggest that it may exhibit much higher mutation rates than other teleost fishes. This list includes many of the same reasons that they are fascinating to evolutionary and conservation biologists, suggesting that elevated mutation rates may be common within small populations of endangered species.

Here we used estimates of vertebrate mutation rates from the literature while not taking into account the many exceptional circumstances surrounding Devil's Hole pupfish, thus likely overestimating the age of this species in our analyses. For the purposes of this study and to compare to SEA, we performed divergence time estimation using biologically informed prior distributions on the substitution rate. We specified a 0.025 accumulated prior density bound of 2.09×10^{-8} mutations per site per year (i.e., we put only 0.025 prior probability on rates being smaller than 2.09×10^{-8}) based on estimates of the human mutation rate (Scally & Durbin, 2012) and a 0.975 accumulated prior density bound of 2.09×10^{-7} mutations per site per year from comparisons of isolated riverine fishes (BurrIDGE et al., 2008). The latter estimate comes from mtDNA substitution rates, but the authors note that it is a lower bound based on river basin divergence and we use it only as an extreme upper bound. Note that our prior bounds cannot precisely match the estimates in these studies to accommodate a lognormal distribution. We centred our mutation rate prior on our best guess from the nonpupfish literature, 6.6×10^{-8} mutations per site per year, from a pedigree analysis of spontaneous mutation rates in a cichlid F2 intercross (Recknagel et al., 2013). These cichlids are relatively closely related to Cyprinodontiform fishes (Wainwright et al., 2012), but do not exhibit any of the life history traits that might predispose them to higher mutation rates as discussed above. Furthermore, to remain independent from our previous analysis in Martin et al. (2016), we avoided using the mutation rate estimated specifically for *Cyprinodon*, 5.23×10^{-7} mutations per site per year from a recent calibration on the age of a geographic basin as recommended for estimation of recent divergence events (Ho & Phillips, 2009; Ho et al., 2005, 2011).

2 | METHODS

We obtained the 10 twenty-locus RADseq data sets used by SEA from the Dryad Digital Repository (Sağlam et al., 2016b: <https://doi.org/10.5061/dryad.2bm21>). We ran *BEAST (v. 2.4.4; Heled & Drummond, 2010; Bouckaert et al. 2014) following the methods of SEA with a strict molecular clock, Yule birth process, a linear population size change model and a Jukes-Cantor model of nucleotide substitution. Hence, we kept all modelling choices as in SEA except replacing the narrow lognormal prior distribution on divergence times with a wide, uninformative uniform (0, 10^6 years) prior distribution. Additionally, we used a lognormal distribution for the mutation rate prior with a mean parameter of 6.6×10^{-8} per site per year and standard deviation of 0.587, thus placing 95% prior probability between 2.087×10^{-8} and 2.087×10^{-7} per site per year. We examined 100 MCMC runs for each data set to assess convergence and ran each chain for 100 million steps. The large number of replicate runs was necessary because of severe convergence problems with the MCMC algorithm in *BEAST. We discarded the first 10 million steps as burn-in. We then corrected the estimated mutation rate by multiplying by 0.75 years per generation, reflecting 1.33 generations per year for the Devil's Hole pupfish from Martin et al., 2016; similar to the 1.5 generations per year used in SEA. Laboratory pupfish generation times of 4 months (Martin, 2016a, 2016b; Martin & Wainwright 2013b) suggest that our generation time choice is conservative, erring on the side of overestimating the age of DHP.

We also performed a second analysis based only on the pedigree estimate of the cichlid spontaneous mutation rate, our best guess for the most appropriate pupfish mutation rate (without relying on our earlier pupfish mutation rate estimate in Martin et al., 2016). Recknagel et al. (2013) made two estimates of the mutation rate in F1 and F2 progeny with a mean \pm 1 standard deviation of $6.6 \times 10^{-8} \pm 1.13 \times 10^{-8}$ mutations per site per generation. We used a lognormal prior with mean = 6.6×10^{-8} and standard deviation = 0.168 to capture this distribution for our analysis following similar procedures as described above.

3 | RESULTS

Using a conservative prior for the range of mutation rates observed in vertebrates, we used SEA's data to estimate the median age of the Devil's Hole pupfish between 6.2 and 19.9 kya across all ten data sets, with a mean of 12.6 kya (range of 95% highest posterior density intervals (HPD): 1.7–63.8 kya; Figure 1, Table 1). These estimates partially overlap with the phylogenetic divergence times estimated by Martin et al. (2.5–6.5 kya; Fig. 2; Martin et al., 2016). Our analysis also illustrates the large amount of uncertainty still present in this estimate due to the limited data set used and substantial range of plausible vertebrate mutation rates.

We estimated the median divergence time between the Owens pupfish and Death Valley pupfishes between 15.1 and 39.5 kya

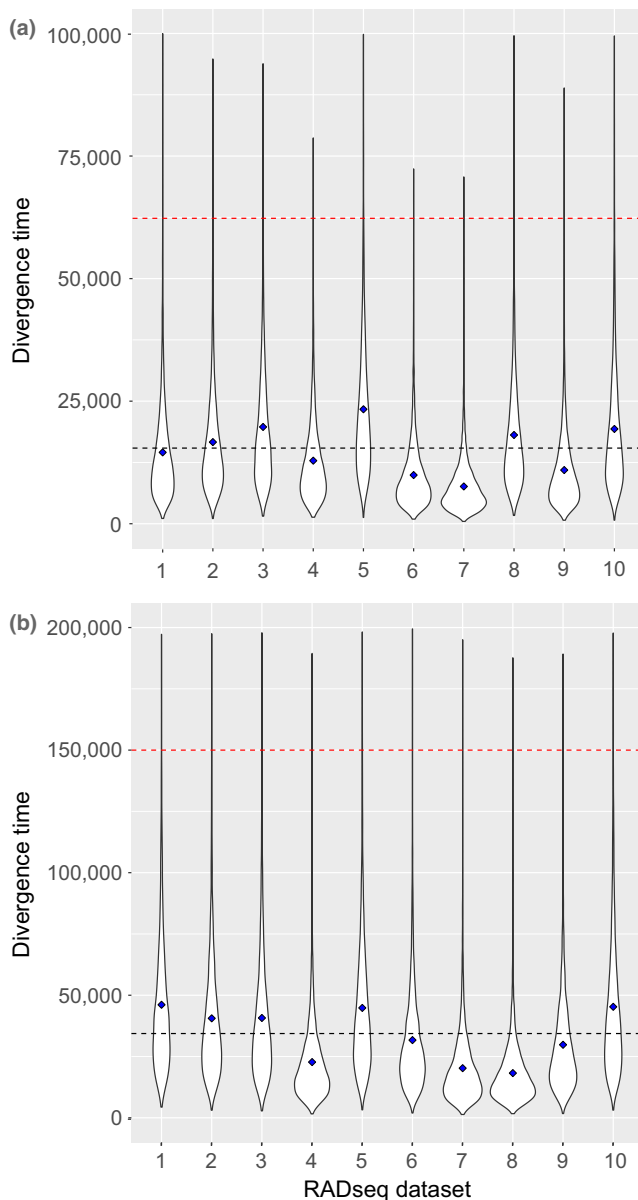


FIGURE 1 (a) Estimated divergence time (years) between Devil's Hole pupfish and the neighbouring Amargosa pupfish population across ten independent RADseq data sets of twenty loci each. (b) Estimated divergence time between Owen's pupfish (OWP) and Death Valley pupfishes (DHP + AMP) across the same ten RADseq data sets. Violin plots indicate the density of the marginal posterior distribution for divergence time. The dashed red line indicates the estimate from Sağlam et al. (2016a); the dashed black line indicates the mean estimate in this study [Colour figure can be viewed at wileyonlinelibrary.com]

across all ten data sets, with a mean of 28.4 kya (95% HPD: 4.6–128.96; Figure 1, Table 1). This divergence across the entire Death Valley region and Owens River Valley is still younger than the age of Devil's Hole (60 kya) and overlaps with the wettest period during the last glacial maximum 15 kya in which Lake Manly within Death Valley was flooded and connected to the neighbouring Owens Lake and Amargosa River within Ash Meadows National Wildlife Refuge (Lowenstein, Li, Brown, & Roberts, 1999; Lyle et al. 2012). Our

TABLE 1 Median divergence times (in thousands of years; 95% HPD in parentheses) estimated using *BEAST and a conservative prior on mutation rate variation for ten independent data sets of 20 RADseq loci from SEA. DHP, AMP: divergence between Devil's Hole pupfish and Ash Meadows Amargosa pupfish (*C. nevadensis mionectes*). OWP (DHP + AMP): divergence between Owen's pupfish (*C. radiosus*) and two Ash Meadows/Death Valley species. Bold values represent minimum and maximum median divergence time estimates for DHP

Data set	Divergence time: DHP, AMP	Divergence time: OWP (DHP + AMP)
1	11.8 (3.3–41.9)	39.5 (11.6–126.3)
2	13.7 (4.2–47.2)	34.1 (10.3–114.0)
3	16.3 (4.8–58.7)	33.8 (9.5–115.4)
4	10.6 (3.0–36.6)	18.7 (5.4–63.7)
5	19.9 (5.9– 63.8)	38.1 (11.5–124.7)
6	8.0 (2.4–28.5)	26.1 (7.8–90.4)
7	6.2 (1.7 –21.7)	16.5 (4.6 –58.5)
8	15.1 (4.7–49.7)	15.1 (4.7–49.7)
9	8.8 (2.4–32.0)	24.5 (7.3–83.8)
10	15.8 (4.5–55.5)	37.7 (11.1– 128.6)

analyses based only on the cichlid pedigree estimate of spontaneous mutation rate (Recknagel et al., 2013) produced similar results, with substantially less uncertainty around these estimates (Fig. S1).

4 | DISCUSSION

Here we reanalysed a new RADseq data set provided by SEA and estimated the age of the Devil's Hole pupfish by setting a conservative prior on the plausible range of mutation rates across vertebrates. We estimate that pupfish first colonized Devil's Hole between 6.2 and 19.9 kya (range of median estimates across ten data sets). This age is approximately six times younger than the age of Devil's Hole and SEA's previous estimate (SEA: mean 62.3 kya, 95% HPD: 29.6–99.5 kya), consistent with the sixfold higher mutation rate observed in closely related cichlid fishes (Recknagel et al., 2013) that we used to centre our prior. Our phylogenetic estimate in this study also overlaps with our previous phylogenetic estimate of the age of DHP (2.5–6.5 kya; Fig. 2: Martin et al., 2016). Furthermore, our estimate for the divergence between all Death Valley pupfishes and Owens Valley pupfish from 15.1 to 39.5 kya overlaps with a prolonged period of pluvial lakes in the region from 10 to 20 kya.

We did not conduct demographic analyses such as *dadi* (Gutenkunst & Hernandez, 2009) which incorporate secondary gene flow among populations and variation in ancestral and derived population sizes; this approach previously suggested a much younger age for DHP (105–830 years: Martin et al., 2016) than our phylogenetic analysis using the same mutation rate. Thus, our reanalysis of SEA's data most likely overestimated the age of DHP by a substantial factor. Such overestimation of divergence times in phylogenetic

analyses is expected because these phylogenetic methods do not account for variation in ancestral population sizes (Gutenkunst & Hernandez, 2009; Nadachowska-Brzyska et al., 2013; Liu et al., 2014).

Although SEA did not include additional species in their analysis, it is also important to note that an older age for DHP of 60 kya would imply divergence between sister *C. salinus salinus* and *C. salinus milneri* populations residing within Badwater basin in Death Valley at around 30 kya (see Martin et al., 2016; : Fig. 2d). However, 15 kya this basin was the inland sea, Lake Manly, with a depth of 10 m; Badwater basin still periodically floods (Lowenstein et al., 1999; Lyle et al. 2012). It is unlikely that these two basin populations would remain isolated within a single lake as no reproductive isolating barriers are known (and pupfishes are known for rampant hybridization upon secondary contact: Echelle & Connor, 1989; Rosenfield & Kodric-Brown, 2003; Tobler & Carson, 2010; Martin & Wainwright 2013a; Martin & Feinstein, 2014; McGirr & Martin, 2016; Richards & Martin, 2017).

Our phylogenetic age estimate remains a conservative upper bound on the age of the Devil's Hole pupfish because we did not account for the many life history and environmental factors associated with DHP, including high temperatures, high metabolic rate, small body size, short generation times, severe environmental stressors and small population size which likely predispose the species to exhibit much higher mutation rates than contained within our prior. In line with this expectation, our previous concatenated phylogenetic analysis of *Cyprinodon* pupfishes estimated a mutation rate about 10-fold higher than the recent high-quality estimate of the mutation rate in cichlids over two generations in the laboratory (Recknagel et al., 2013) used as a conservative prior in this study. We also demonstrated that concatenation of RADseq loci in our previous phylogenetic analysis, as opposed to multispecies coalescent analysis of these loci, would only overestimate mutation rates by a maximum of twofold (Martin et al. 2017).

Furthermore, SEA's *BEAST analysis and our own follow-up *BEAST analyses here do not take into account complex demographic histories, such as secondary gene flow, and result in an overly simplistic model of Death Valley populations, given evidence of substantial gene flow in our previous study. SEA used a simple linear model of changes in population size through time, but new demographic models relax this assumption and allow for estimation of independent effective population sizes within the ancestor and each descendant lineage (Gutenkunst & Hernandez, 2009; Nadachowska-Brzyska et al., 2013; Kautt, Machado-Schiaffino, & Meyer, 2016; Martin et al., 2016). Failing to account for ancestral changes in population size led to a several million year overestimate of polar bear divergence times, indicating that phylogenetic models which do not account for variable population size changes through time, such as a population bottleneck in only one lineage after colonizing a new isolated environment (e.g., Devil's Hole), are biased and lead to an overestimate of divergence times (Liu et al., 2014; Nadachowska-Brzyska et al., 2013).

An additional factor that may result in elevated mutation rates in our study and SEA is the method of down-sampling from the

genome. Reduced-representation restriction site-associated sampling (RADseq) is not unbiased nor random (Puritz et al., 2014); indeed, both studies used restriction enzymes (SbfI and PstI), which target GC-rich regions. Theoretical studies point out that RADseq will systematically underestimate heterozygosity in general due to allele dropout at polymorphic restriction sites (Arnold, Corbett-Detig, Hartl, & Bomblies, 2013). Indeed, when we compared genetic diversity (π) among San Salvador, Bahamas pupfish species sampled using our ddRADseq protocol (Martin & Feinstein, 2014; : Fig. 5c: π : generalist = 0.002, snail-eater = 0.0018, scale-eater = 0.0016) and in our more recent whole-genome resequencing study (McGirr & Martin, 2016: generalist = 0.00402, snail-eater = 0.00321, scale-eater = 0.00324), we found that genetic diversity was generally about 50% lower using the RADseq method. Nonetheless, as we originally discussed in Martin et al. (2016), "although our data set may be biased, Bayesian posterior estimates of divergence time are extremely sensitive to calibration priors, rather than the observed heterozygosity within a data set (Warnock, Parham, Joyce, Lyson, & Donoghue, 2014)." Thus, our estimate of the age of DHP depends mainly on the accuracy of our calibration choice, not the underlying bias in our data set, because any mutational bias present is rescaled to an external timescale and we used this same data set for later demographic analysis.

Finally, SEA claim that their analysis of secondary gene flow "refutes the claim that the evolutionary history of Death Valley pupfish was influenced by frequent overland dispersals and mixing on a scale of hundreds to thousands of years (Martin et al., 2016)." This is highly misleading because they did not sample either Death Valley pupfish species showing significant evidence of secondary gene flow with DHP that was originally analysed in Martin et al. (2016) (Table S4 and discussed in main text): *C. nevadensis amargosae* and *C. nevadensis pectoralis* were not included in SEA's analyses of gene flow. Only *C. nevadensis mionectes* was included by SEA; similarly, Martin et al. (2016) found no significant evidence of gene flow between *C. nevadensis mionectes* and DHP.

In conclusion, our new analyses of SEA's data set continue to suggest a young age for this iconic endangered species. We further discuss life history features shared across many endangered taxa that may lead to an underestimation of their age if their mutation rate is assumed to be similar to even closely related nonendangered taxa. We also did not conduct demographic analyses of the age of DHP incorporating gene flow which previously indicated a much younger age (105–830 years) than our phylogenetic estimates (2,500–6,500 years; Fig. 2: Martin et al., 2016). We conclude that Saglam et al.'s conclusions and analyses are based on incorrect assumptions and omission of taxa showing evidence of secondary gene flow. Their assertion of "overwhelming support for an older divergence time and isolation of DHP" is unwarranted.

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DATA ACCESSIBILITY

Data sets analysed in this study are deposited on the Dryad Digital Depository: <https://doi.org/10.5061/dryad.2bm21>.

AUTHOR CONTRIBUTION

C.H.M. and S.H. conceived of the study design together. C.H.M. wrote the manuscript and S.H. ran all *BEAST analyses.

ORCID

Christopher H. Martin  <http://orcid.org/0000-0001-7989-9124>

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