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NEWS AND VIEWS

Comment

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

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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, Saglam 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 lock (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

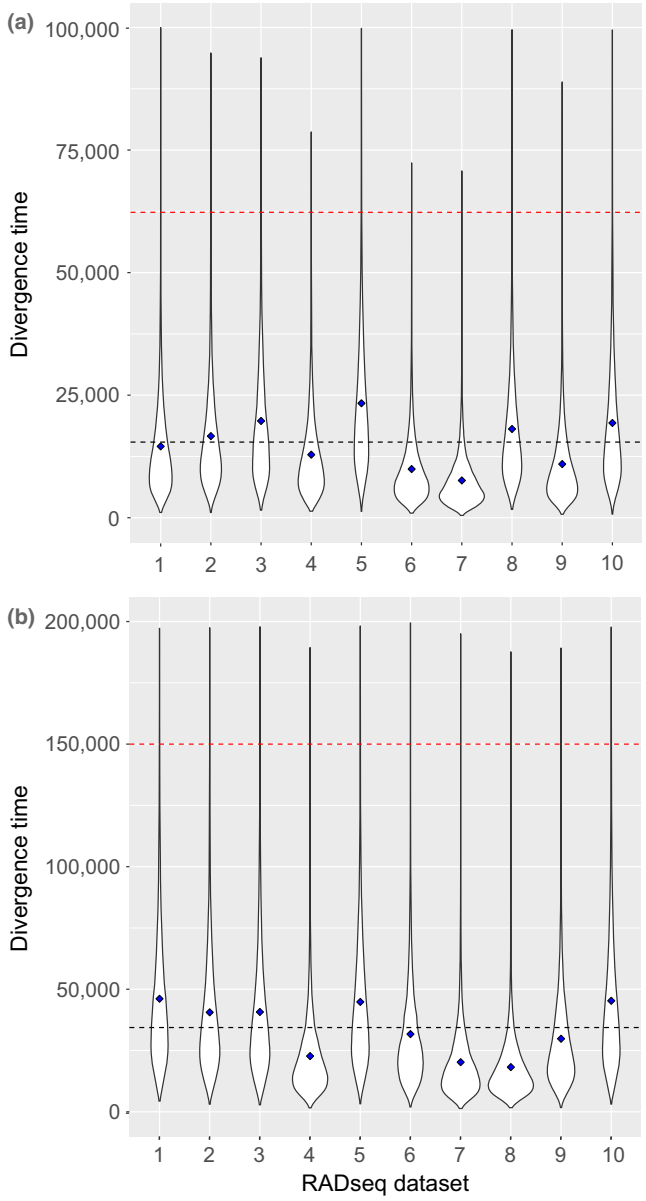

FIGURE 1 (a) Estimated divergence time (years) between Devil's Hole pupfish and the neighbouring Amargosa pupfish population across ten independent RADseq data sets of twenty loci each. (b) Estimated divergence time between Owen's pupfish (OWP) and Death Valley pupfishes (DHP + AMP) across the same ten 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

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 also important to note that an older age for DHP of 60 kya would imply divergence between sister *C. salinus salinus* and *C. salinus milleri* 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.

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REFERENCES

Arnold, B., Corbett-Detig, R. B., Hartl, D., & Bomblies, K. (2013). RADseq underestimates diversity and introduces genealogical biases due to nonrandom haplotype sampling. *Molecular Ecology*, *22*, 3179–3190. https://doi.org/10.1111/mec.12276

Beissinger, S. R. (2014). Digging the pupfish out of its hole: Risk analyses to guide harvest of Devils Hole pupfish for captive breeding. *PeerJ*, *2*, e549. https://doi.org/10.7717/peerj.549

Bjedov, I., Tenaillon, O., Gerard, B., & Souza, V. (2003). Stress-induced mutagenesis in bacteria. *Science*, *300*, 1404–1409. https://doi.org/10.1126/science.1082240

Bleiweiss, R. (1998). Slow rate of molecular evolution in high-elevation hummingbirds. *Proceedings of the National Academy of Sciences*, *95*, 612–616. https://doi.org/10.1073/pnas.95.2.612

Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T., Wu, C.-H., Xie, D., … Drummond, A. J. (2014). BEAST 2: A software platform for Bayesian evolutionary analysis. *PLoS Computational Biology*, *10*(4), e1003537. https://doi.org/10.1371/journal.pcbi.1003537

Bromham, L. (2009). Why do species vary in their rate of molecular evolution? *Biology Letters*, *5*, 401–404. https://doi.org/10.1098/rsbl.2009.0136

Bromham, L., Rambaut, A., & Harvey, P. H. (1996). Determinants of rate variation in mammalian DNA sequence evolution. *Journal of Molecular Evolution*, *43*, 610–621. https://doi.org/10.1007/BF02202109

Burridge, C. P., Craw, D., Fletcher, D., & Waters, J. M. (2008). Geological dates and molecular rates: Fish DNA sheds light on time dependency. *Molecular Biology and Evolution*, *25*, 624–633. https://doi.org/10.1093/molbev/msm271

Clarke, A., & Johnston, N. (1999). Scaling of metabolic rate with body mass and temperature in teleost fish. *Journal of Animal Ecology*, *68*, 893–905. https://doi.org/10.1046/j.1365-2656.1999.00337.x

Coyne, J. A., & Orr, H. A. (2004). *Speciation*. Sunderland, MA: Sinauer Associates.

Drummond, A. J., & Rambaut, A. (2007). BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology*, *7*, 214. https://doi.org/10.1186/1471-2148-7-214

Echelle, A. A., Carson, E. W., Echelle, A. F., Van Den Bussche, R. A., Dowling, T. E., & Meyer, A. (2005). Historical biogeography of the New-World pupfish genus *Cyprinodon* (Teleostei: Cyprinodontidae) (RM Wood, Ed.). *Copeia*, *2005*, 320–339. https://doi.org/10.1643/CG-03-093R3

Echelle, A. A., & Connor, P. J. (1989). Rapid, geographically extensive genetic introgression after secondary contact between two pupfish species (*Cyprinodon*, Cyprinodontidae). *Evolution*, *43*, 717–727. https://doi.org/10.1111/evo.1989.43.issue-4

Edwards, S. V. (2009). Is a new and general theory of molecular systematics emerging? *Evolution*, *63*, 1–19. https://doi.org/10.1111/evo.2009.63.issue-1

Eldridge, M. D. B., King, J. M., Loupis, A. K., Spencer, P., Taylor, A. C., Pope, L. C., & Hall, G. P. (1999). Unprecedented low levels of genetic variation and inbreeding depression in an island population of the black-footed rock wallaby. *Conservation Biology*, *13*, 531–541. https://doi.org/10.1046/j.1523-1739.1999.98115.x

Erwin, T. L. (1991). An evolutionary basis for conservation strategies. *Science (New York, N.Y.)*, *253*, 750–752. https://doi.org/10.1126/science.253.5021.750

Faith, D., Reid, C., & Hunter, J. (2004). Integrating phylogenetic diversity, complementarity, and endemism for conservation assessment. *Conservation Biology*, *18*, 255–261. https://doi.org/10.1111/cbi.2004.18.issue-1

Gillooly, J., Brown, J., West, G., & Savage, V. (2001). Effects of size and temperature on metabolic rate. *Science*, *293*, 2248–2251. https://doi.org/10.1126/science.1061967

Gutenkunst, R., & Hernandez, R. (2009). Inferring the joint demographic history of multiple populations from multidimensional SNP frequency data. *PLoS Genetics*, *5*, e1000695.

Harris, K. (2015). Evidence for recent, population-specific evolution of the human mutation rate. *Proceedings of the National Academy of Sciences of the United States of America*, *112*, 3439–3444. https://doi.org/10.1073/pnas.1418652112

Harris, K., & Pritchard, J. (2016). Rapid evolution of the human mutation spectrum. *eLife*, *6*, e24284. https://doi.org/10.1534/g3.112.003897

Heled, J., & Drummond, A. J. (2010). Bayesian inference of species trees from multilocus data. *Molecular Biology and Evolution*, *27*, 570–580. https://doi.org/10.1093/molbev/msp274

Hendry, A. (2017). *Eco-evolutionary dynamics*. Princeton and Oxford: Princeton University Press. https://doi.org/10.1515/9781400883080

Hendry, A., & Kinnison, M. (1999). Perspective: The pace of modern life: Measuring rates of contemporary microevolution. *Evolution*, *53*, 1637–1653. https://doi.org/10.1111/evo.1999.53.issue-6

Heuton, M., Ayala, L., Burg, C., & Dayton, K. (2015). Paradoxical anaerobism in desert pupfish. *Journal of Experimental Biology*, *218*, 3739–3745. https://doi.org/10.1242/jeb.130633

Hillyard, S., Burg, G., McKenna, K., & Urbina, N. (2014). Oxygen consumption in a hot hypoxic world, the Devils Hole pupfish (879.24). *The FASEB Journal*, *28*, 879–924.

Ho, S. Y. W., Lanfear, R., Bromham, L., Phillips, M. J., Soubrier, J., Rodrigo, A. G., & Cooper, A. (2011). Time-dependent rates of molecular evolution. *Molecular Ecology*, *20*, 3087–3101. https://doi.org/10.1111/j.1365-294X.2011.05178.x

Ho, S. Y. W., & Phillips, M. J. (2009). Accounting for calibration uncertainty in phylogenetic estimation of evolutionary divergence times. *Systematic Biology*, *58*, 367–380. https://doi.org/10.1093/sysbio/syp035

Ho, S. Y. W., Phillips, M. J., Cooper, A., & Drummond, A. J. (2005). Time dependency of molecular rate estimates and systematic overestimation of recent divergence times. *Molecular Biology and Evolution*, *22*, 1561–1568. https://doi.org/10.1093/molbev/msi145

Hulbert, A., Pamplona, R., & Buffenstein, R. (2007). Life and death: Metabolic rate, membrane composition, and life span of animals. *Physiological Reviews*, *87*, 1175–1213. https://doi.org/10.1152/physrev.00047.2006

Jaillon, O., Aury, J.-M., Brunet, F., Petit, J. L., Stange-Thomann, N., Mauceli, E., … Nicaud, S. (2004). Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. *Nature*, *431*, 946–957. https://doi.org/10.1038/nature03025

Ji, J., Ng, S., Sharma, V., Neculai, D., & Hussein, S. (2012). Elevated coding mutation rate during the reprogramming of human somatic cells into induced pluripotent stem cells. *Stem Cells*, *30*, 435–440. https://doi.org/10.1002/stem.1011

Kasahara, M., Naruse, K., Sasaki, S., Nakatani, Y., Qu, W., Ahsan, B., … Jindo, T. (2007). The medaka draft genome and insights into vertebrate genome evolution. *Nature*, *447*, 714–719. https://doi.org/10.1038/nature05846

Kautt, A. F., Machado-Schiaffino, G., & Meyer, A. (2016). Multispecies outcomes of sympatric speciation after admixture with the source population in two radiations of Nicaraguan crater lake cichlids. *PLOS Genetics*, *12*, e1006157. https://doi.org/10.1371/journal.pgen.1006157

Kennedy, E. S., Grueber, C. E., Duncan, R. P., & Jamieson, I. G. (2014). Severe inbreeding depression and no evidence of purging in an extremely inbred wild species–the Chatham Island black robin. *Evolution*, *68*, 987–995. https://doi.org/10.1111/evo.2014.68.issue-4

Kinnison, M. T., Hendry, A. P., & Stockwell, C. A. (2007). Contemporary evolution meets conservation biology II: Impediments to integration and application. *Ecological Research*, *22*, 947–954. https://doi.org/10.1007/s11284-007-0416-6

Lema, S. C., & Nevitt, G. A. (2006). Testing an ecophysiological mechanism of morphological plasticity in pupfish and its relevance to conservation efforts for endangered Devils Hole pupfish. *The Journal of Experimental Biology*, *209*, 3499–3509. https://doi.org/10.1242/jeb.02417

Li, W., & Tanimura, M. (1987). The molecular clock runs more slowly in man than in apes and monkeys. *Nature*, *326*, 93–96. https://doi.org/10.1038/326093a0

Liu, S., Lorenzen, E. D., Fumagalli, M., Li, B., Harris, K., Xiong, Z., … Wray, G. (2014). Population genomics reveal recent speciation and rapid evolutionary adaptation in polar bears. *Cell*, *157*, 785–794. https://doi.org/10.1016/j.cell.2014.03.054

Lowenstein, T., Li, J., Brown, C., & Roberts, S. (1999). 200 ky paleoclimate record from Death Valley salt core. *Geology*, *27*, 3–6. https://doi.org/10.1130/0091-7613(1999)027<0003:KYPRFD>2.3.CO;2

Lyle, M., Heusser, L., Ravelo, C., Yamamoto, M., Barron, J., Diffenbaugh, N. S., … Andreasen, D. (2012). Out of the tropics: The Pacific, Great Basin lakes, and late Pleistocene water cycle in the western United States. *Science*, *337*(6102), 1629–1633. https://doi.org/10.1126/science.1218390

Lynch, M. (2010). Evolution of the mutation rate. *Trends in Genetics*, *26*, 345–352. https://doi.org/10.1016/j.tig.2010.05.003

Martin, C. H. (2012). Weak disruptive selection and incomplete phenotypic divergence in two classic examples of sympatric speciation: Cameroon Crater Lake cichlids. *The American Naturalist*, *180*, E90–E109. https://doi.org/10.1086/667586

Martin, C. H. (2013). Strong assortative mating by diet, color, size, and morphology but limited progress toward sympatric speciation in a classic example: Cameroon Crater Lake cichlids. *Evolution*, *67*, 2114–2123. https://doi.org/10.1111/evo.12090

Martin, C. H. (2016a). The cryptic origins of evolutionary novelty: 1000-fold faster trophic diversification rates without increased ecological opportunity or hybrid swarm. *Evolution*, *70*, 2504–2519. https://doi.org/10.1111/evo.13046

Martin, C. H. (2016b). Context-dependence in complex adaptive landscapes: Frequency and trait-dependent selection surfaces within an adaptive radiation of Caribbean pupfishes. *Evolution*, *70*, 1265–1282. https://doi.org/10.1111/evo.12932

Martin, C. H., Crawford, J. E., Turner, B. J., & Simons, L. H. (2016). Diabolical survival in Death Valley: Recent pupfish colonization, gene flow, and genetic assimilation in the smallest species range on earth. *Proceedings of the Royal Society B: Biological Sciences*, *283*, 23–34.

Martin, C. H., Cutler, J. S., Friel, J. P., Dening Touokong, C., Coop, G., & Wainwright, P. C. (2015). Complex histories of repeated colonization and hybridization cast doubt on the clearest examples of sympatric speciation in the wild. *Evolution*, *69*, 1406–1422. https://doi.org/10.1111/evo.12674

Martin, A. P., Echelle, A. A., Zegers, G., Baker, S., & Keeler-Foster, C. L. (2011). Dramatic shifts in the gene pool of a managed population of an endangered species may be exacerbated by high genetic load. *Conservation Genetics*, *13*, 349–358.

Martin, C. H., & Feinstein, L. C. (2014). Novel trophic niches drive variable progress towards ecological speciation within an adaptive radiation of pupfishes. *Molecular Ecology*, *23*, 1846–1862. https://doi.org/10.1111/mec.12658

Martin, A. P., & Palumbi, S. R. (1993). Body size, metabolic rate, generation time, and the molecular clock. *Proceedings of the National Academy of Sciences of the United States of America*, *90*, 4087–4091. https://doi.org/10.1073/pnas.90.9.4087

Martin, C. H., & Wainwright, P. C. (2013a). A remarkable species flock of *Cyprinodon* pupfishes endemic to San Salvador Island, Bahamas. *Bulletin of the Peabody Museum of Natural History*, *54*, 231–240. https://doi.org/10.3374/014.054.0201

Martin, C. H., & Wainwright, P. C. (2013b). Multiple fitness peaks on the adaptive landscape drive adaptive radiation in the wild. *Science (New York, N.Y.)*, *339*, 208–211. https://doi.org/10.1126/science.1227710

Martin, C. H., Höhna, S., Crawford, J. E., Turner, B. J., Richards, E. J., & Simons, L. H. (2017). The complex effects of demographic history on the estimation of substitution rate: Concatenated gene analysis results in no more than twofold overestimation. *Proceedings Biological Sciences*, *284*(1860), 20170537.

McGirr, J., & Martin, C. H. (2016). Novel candidate genes underlying extreme trophic specialization in Caribbean pupfishes. *Molecular Biology and Evolution*, *34*, 873–888.

Millar, C. D., Dodd, A., Anderson, J., Gibb, G. C., Ritchie, P. A., Baroni, C., … Lambert, D. M. (2008). Mutation and evolutionary rates in Adélie penguins from the Antarctic. *PLoS Genetics*, *4*, e1000209.

Moorjani, P., Gao, Z., & Przeworski, M. (2016). Human germline mutation and the erratic evolutionary clock. *PLoS Biology*, *14*, e2000744.

Nachman, M. W., & Crowell, S. L. (2000). Estimate of the mutation rate per nucleotide in humans. *Genetics*, *156*, 297–304.

Nadachowska-Brzyska, K., Burri, R., Olason, P. I., Kawakami, T., Smeds, L., & Ellegren, H. (2013). Demographic divergence history of pied flycatcher and collared flycatcher inferred from whole-genome re-sequencing data. *PLoS Genetics*, *9*, e1003942. https://doi.org/10.1371/journal.pgen.1003942

Nosil, P. (2012). *Ecological speciation*. Oxford: Oxford University Press. https://doi.org/10.1093/acprof:osobl/9780199587100.001.0001

Puritz, J. B., Matz, M. V., Toonen, R. J., Weber, J. N., Bolnick, D. I., & Bird, C. E. (2014). Demystifying the RAD fad. *Molecular Ecology*, *23*, 5937–5942. https://doi.org/10.1111/mec.12965

Ravi, V., & Venkatesh, B. (2008). Rapidly evolving fish genomes and teleost diversity. *Current Opinion in Genetics and Development*, *18*, 544–550. https://doi.org/10.1016/j.gde.2008.11.001

Recknagel, H., Elmer, K. R., & Meyer, A. (2013). A hybrid genetic linkage map of two ecologically and morphologically divergent Midas cichlid fishes (*Amphilophus* spp.) obtained by massively parallel DNA sequencing (ddRADSeq). *G3*, *3*, 65–74. https://doi.org/10.1534/g3.112.003897

Reed, J., & Stockwell, C. (2014). Evaluating an icon of population persistence: The Devil's Hole pupfish. *Proceedings of the Royal Society B: Biological Sciences*, *281*, 1648–1658.

Richards, E. J., & Martin, C. H. (2017). Adaptive introgression from distant Caribbean islands contributed to the diversification of a microendemic radiation of trophic specialist pupfishes. *PLoS Genetics*, *13*(8), e1006919. https://doi.org/10.1371/journal.pgen.1006919

Riggs, A., & Deacon, J. (2002). Connectivity in desert aquatic ecosystems: The Devils Hole story. *Spring-Fed Wetlands: Important Scientific and Cultural Resources of the Intermountain Region*, *11*, 1–38.

Roach, J. C., Glusman, G., Smit, A. F. A., Huff, C. D., Hubley, R., Shannon, P. T., … Galas, D. J. (2010). Analysis of genetic inheritance in a family quartet by whole-genome sequencing. *Science*, *328*(5978), 636–639.

Robinson, J. A., Vecchyo, D. O., Fan, Z., et al. (2016). Genomic flatlining in the endangered island fox. *Current Biology*, *26*, 1183–1189. https://doi.org/10.1016/j.cub.2016.02.062

Romiguier, J., Gayral, P., Ballenghien, M., Bernard, A., Cahais, V., Chenuil, A., … Loire, E. (2014). Comparative population genomics in animals uncovers the determinants of genetic diversity. *Nature*, *515*, 261–263. https://doi.org/10.1038/nature13685

Rosenfield, J. A., & Kodric-Brown, A. (2003). Sexual selection promotes hybridization between Pecos pupfish, *Cyprinodon pecosensis* and sheepshead minnow, *C. variegatus*. *Journal of Evolutionary Biology*, *16*, 595–606. https://doi.org/10.1046/j.1420-9101.2003.00557.x

Sağlam, İ. K., Baumsteiger, J., Smith, M. J., Linares-Casenave, J., Nichols, A. L., O'Rourke, S. M., & Miller, M. R. (2016a). Phylogenetics support an ancient common origin of two scientific icons: Devils Hole and Devils Hole pupfish. *Molecular Ecology*, *25*, 3962–3973.

Sağlam, İ. K., Baumsteiger, J., Smith, M. J., Linares-Casenave, J., Nichols, A. L., O'Rourke, S. M., & Miller, M. R. (2016b). Data from: Phylogenetics support an ancient common origin of two scientific icons: Devils Hole and Devils Hole pupfish. Dryad Digital Repository. https://doi.org/10.5061/dryad.2bm21

Santos, C., Montiel, R., Sierra, B., Bettencourt, C., Fernandez, E., Alvarez, L., … Aluja, M. P. (2005). Understanding differences between phylogenetic and pedigree-derived mtDNA mutation rate: A model using families from the Azores Islands (Portugal). *Molecular Biology and Evolution*, *22*, 1490–1505. https://doi.org/10.1093/molbev/msi141

Scally, A., & Durbin, R. (2012). Revising the human mutation rate: Implications for understanding human evolution. *Nature Reviews Genetics*, *13*, 745–753. https://doi.org/10.1038/nrg3353

Schluter, D. (2000). *Ecology of adaptive radiation*. Oxford: Oxford University Press.

Seehausen, O., Butlin, R. K., Keller, I., Wagner, C. E., Boughman, J. W., Hohenlohe, P. A., … Brelsford, A. (2014). Genomics and the origin of species. *Nature Reviews: Genetics*, *15*, 176–192. https://doi.org/10.1038/nrg3644

Smith, G., Dowling, T., Gobalet, K., Lugaski, T., Shiozawa, D. K., & Evans, R. P. (2002). Biogeography and rates of evolution of Great Basin fishes. In R. Hershler & D. Curry (Eds.), *The Great Basin: Cenozoic geology and biogeography* (pp. 175–234). Washington, DC: Smithsonian Institution.

Stockwell, C. A., Heilveil, J. S., & Purcell, K. (2013). Estimating divergence time for two evolutionarily significant units of a protected fish species. *Conservation Genetics*, *14*, 215–222. https://doi.org/10.1007/s10592-013-0447-1

Stoike, S. L., & Pister, E. P. (2010). Threatened fishes of the world: *Cyprinodon diabolis* (Wales, 1930). *Environmental Biology of Fishes*, *88*, 399–400. https://doi.org/10.1007/s10641-010-9651-8

Tech, C. (2006). Postzygotic incompatibilities between the pupfishes, *Cyprinodon elegans* and *Cyprinodon variegatus*: Hybrid male sterility and sex ratio bias. *Journal of Evolutionary Biology*, *19*, 1830–1837. https://doi.org/10.1111/jeb.2006.19.issue-6

Tobler, M., & Carson, E. W. (2010). Environmental variation, hybridization, and phenotypic diversification in Cuatro Ciénegas pupfishes. *Journal of Evolutionary Biology*, *23*, 1475–1489. https://doi.org/10.1111/j.1420-9101.2010.02014.x

Turelli, M., Barton, N. H., & Coyne, J. A. (2001). Theory and speciation. *Trends in Ecology and Evolution*, *16*, 330–343. https://doi.org/10.1016/S0169-5347(01)02177-2

Vane-Wright, R. I., Humphries, C. J., & Williams, P. H. (1991). What to protect? Systematics and the agony of choice. *Biological Conservation*, *55*, 235–254. https://doi.org/10.1016/0006-3207(91)90030-D

Wainwright, P. C., Smith, W. L., Price, S. A., Tang, K. L., Sparks, J. S., Ferry, L. A., … Near, T. J. (2012). The evolution of pharyngognathy: A phylogenetic and functional appraisal of the pharyngeal jaw key innovation in Labroid fishes and beyond. *Systematic Biology*, *61*, 1001–1027. https://doi.org/10.1093/sysbio/sys060

Warnock, R. C. M., Parham, J. F., Joyce, W. G., Lyson, T. R., & Donoghue, P. C. J. (2014). Calibration uncertainty in molecular dating analyses: There is no substitute for the prior evaluation of time priors. *Proceedings of the Royal Society B*, *282*, 1013–1023.

Wayne, R., & Shaffer, H. (2016). Hybridization and endangered species protection in the molecular era. *Molecular Ecology*, *25*, 2680–2689. https://doi.org/10.1111/mec.13642

Welch, J., & Bininda-Emonds, O. (2008). Correlates of substitution rate variation in mammalian protein-coding sequences. *BMC Evolutionary Biology*, *8*, 53. https://doi.org/10.1186/1471-2148-8-53

Winograd, I. J., Coplen, T. B., Landwehr, J. M., Riggs, A. C., Ludwig, K., Szabo, B., … Revesz, K. (1992). Continuous 500,000-year climate record from vein calcite in devils hole, Nevada. *Science (New York, N.Y.)*, *258*, 255–260. https://doi.org/10.1126/science.258.5080.255

Woodhams, M. (2006). Can deleterious mutations explain the time dependency of molecular rate estimates? *Molecular Biology and Evolution*, *23*, 2271–2273. https://doi.org/10.1093/molbev/msl107

SUPPORTING INFORMATION

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