

# Estimating divergence time for two evolutionarily significant units of a protected fish species

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**Abstract** The evolutionarily significant unit concept provides a powerful tool for conserving biodiversity below the species level, but temporal criteria are often used explicitly or implicitly in the operational definitions of evolutionarily significant units (ESUs). Such temporal considerations have important implications for recently diverged taxa, as is the case with the White sands pupfish (*Cyprinodon tularosa*). This species consists of two native populations previously designated as the Malpais Spring and Salt Creek ESUs based on allele frequency differences at nuclear markers and their ecologically divergent habitats; despite a lack of reciprocal monophyly. Isolation of these two ESUs *presumably* occurred during the mid-Holocene, but an alternative hypothesis is that the populations were isolated due to changes in surface hydrology associated with overgrazing in the late 19th and early 20th centuries. We assayed 13 microsatellite loci and applied an Approximate Bayesian Computation analysis to estimate time of divergence between the two populations. Our reference table consisted of 1,000,000 simulated data sets, and we used three different models, each having different

combinations of summary statistics. Estimates of median divergence time varied from approximately 6,500–11,000 generations (3,250–11,000 years). These findings support the hypothesis that Malpais Spring and Salt Creek having been isolated for a least a few millennia, and together with previously documented adaptive divergence, argues for continued management as separate conservation units. We consider the temporal constraints for defining evolutionary significance as it relates to recently diverged populations occupying ecologically divergent habitats.

**Keywords** *Cyprinodon tularosa* · White Sands pupfish · ESU · Molecular divergence · Conservation units

## Introduction

The temporal scale of diversification is of great interest to evolutionary and conservation biologists (Darwin 1859; Reznick et al. 1997; Reznick and Ghalambor 2001; Stockwell et al. 2003). Our fascination with the temporal aspects of diversification is reflected by the long-standing interest in the tempo of evolution (Darwin 1859; Simpson 1944), as well as the emerging interest in contemporary diversification (Hendry and Kinnison 1999; Reznick and Ghalambor 2001; Stockwell et al. 2003). In fact, assigning conservation value to a lineage often involves an assessment of its age. Temporal criteria are often used either explicitly or implicitly in the operational definitions of evolutionarily significant units (ESUs). Moritz (1994) defined ESUs as unique genetic lineages that are reciprocally monophyletic to one another based on mitochondrial DNA (mtDNA) markers, a condition that theoretically takes 4 N generations of isolation (Neigel and Avise 1986). Similarly, Crandall et al. (2000) argued that conservation

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status should be based on whether ecological and/or genetic divergence had occurred over “recent” or “historic” time periods.

A temporal component to defining ESUs is also related to the type of traits/markers used. Many studies have relied on molecular markers for designating ESUs (Guia and Saitoh 2007), but some workers have argued that evaluations should include a broad array of markers, ecological data, and phenotypic traits (Crandall et al. 2000; Rader et al. 2005; Guia and Saitoh 2007). Different traits/markers evolve at different rates; therefore, their sensitivity for detecting divergence will vary across different time scales. While reciprocal monophyly at mtDNA markers typically takes 100,000 generations or more, divergence at microsatellites can occur over much shorter time scales (Neigel and Avise 1986; Hedrick et al. 2006). Rader et al. (2005) suggested that adaptive divergence for phenotypic traits, such as life history traits, can be used as a criterion for designating conservation units. Phenotypic divergence can, however, occur over contemporary time scales (years to decades; Hendry and Kinnison 1999; Stockwell et al. 2003; Collyer et al. 2011). Thus, assessing conservation significance may depend on the markers/traits used, and this is of particular concern for recently diverged taxa.

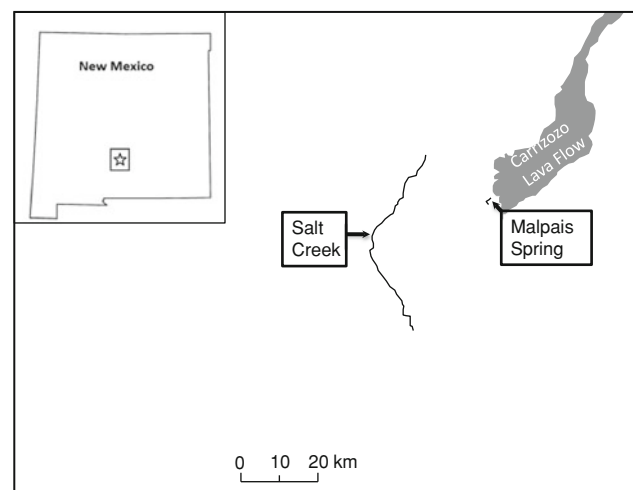
Freshwater and anadromous fishes of the western United States provide useful model systems for evaluating conservation significance as it relates to time of isolation. The rapid diversification of many of these fishes coincided with their isolation into ecologically distinct habitats following the desiccation of Pleistocene lakes (Miller 1948, 1981), while other fishes, such as the salmonids, colonized novel ecologically-divergent habitats (Waples et al. 2008). Rapid and substantial diversification followed, as evidenced by substantial morphological divergence (Miller 1948, 1981; Meyer et al. 1990). Many species have been described based on such differences (Miller 1948, 1981; Meyer et al. 1990), yet many of these taxa have not been isolated long enough for reciprocal monophyly to develop (Duvernell and Turner 1998; Stockwell et al. 1998; Hedrick et al. 2006) and thus would not qualify as separate ESUs under the most commonly used genetic criteria (Moritz 1994; Guia and Saitoh 2007).

These studies suggest that evaluations relying solely on neutral genetic markers may under-identify ESUs of concern for conservation, which is particularly problematic for species where populations occupy a variety of ecologically distinct habitats and may be locally adapted. Crandall et al. (2000) acknowledged the importance of local adaptation by suggesting that conservation status should be linked to whether populations are genetically and ecologically divergent (non-exchangeable), and whether such divergence occurred historically or recently. This would suggest that estimating time of divergence is a critical step for

evaluating the conservation status of locally adapted populations.

Here, we consider the conservation status of two populations of the White Sands pupfish (*Cyprinodon tularosa*) that occupy ecologically divergent habitats at Malpais Spring and Salt Creek in the Tularosa Basin of southern New Mexico (Fig. 1). Malpais Spring and Salt Creek differ markedly in salinity, flow regime, physicochemical stability, and parasite communities (Miller and Echelle 1975; Stockwell and Mulvey 1998; Collyer and Stockwell 2004; Collyer et al. 2005; Rogowski and Stockwell 2006; Stockwell et al. 2011), and the two respective populations have fixed and nearly fixed differences at microsatellite and allozyme loci, respectively (Stockwell et al. 1998; Heilveil and Stockwell, unpublished manuscript). The different ecological conditions taken together with the observed genetic divergence led Stockwell et al. (1998) to recognize the Malpais Spring and Salt Creek populations as separate ESUs; despite a lack of reciprocal monophyly for mtDNA or any notable difference in haplotype frequency.

Evolutionary divergence in body shape was subsequently shown for the two ESUs (Collyer et al. 2005, 2011). Specifically, fish occupying Salt Creek were streamlined, whereas fish occupying Malpais Spring were deep bodied, and a common garden experiment showed these traits to be heritable (Collyer et al. 2011). These findings supported the ESU status of the two populations; however, adaptive divergence was also observed for the Mound Spring pupfish population of *C. tularosa* (Collyer et al. 2011) which was genetically descended from Salt Creek fish introduced approximately 30 years earlier (Collyer et al. 2011). Interestingly, the Mound Spring body shape divergence (not just the divergence rate) exceeded the divergence between the native populations at Salt Creek and Malpais Spring



**Fig. 1** Map of habitats hosting the two native populations of White Sands pupfish

(Collyer et al. 2011). These findings suggest the possibility that the Malpais Spring and Salt Creek populations may also have rapidly diverged soon after isolation, thus adaptive phenotypic divergence in and of itself offers little insight regarding time of divergence.

The Malpais Spring and Salt Creek populations are the only native populations of *C. tularosa* and were first observed as early as 1900 and 1911, respectively (Herrick 1900; Pittenger and Springer 1999; Fig. 1). These two populations were presumably isolated following the eruption of the Carrizozo Volcano (Pittenger and Springer 1999), which occurred approximately 5,000 years BP (Salyards 1991; Dunbar 1999). The resulting Carrizozo lava flow in-filled the upper reaches of the proto-Salt Creek drainage (Dunbar 1999; Pittenger and Springer 1999; Fig. 1). Malpais Spring formed at the foot of the lava flow and historically flowed to the west, feeding a wetland that sloped toward a tributary of Salt Creek (John Pittenger, personal communication). The Salt Creek and Malpais Spring populations could have been isolated about 5,000 years ago concordant with the formation of Malpais Spring; however, an alternative hypothesis is that Malpais Spring and Salt Creek might have had more connectivity in the relatively recent past (John Pittenger, personal communication). This hypothesis is supported by the observation that surface hydrology was apparently altered in the late 19th century due to grazing pressure, as evidenced by a head-cut waterfall in Salt Creek (Pittenger and Springer 1999).

When the two ESUs were first designated, it was assumed that they shared ancestry in the mid-Holocene (Stockwell et al. 1998). Based on these findings, the conservation plan for *C. tularosa* calls for increasing security for each ESU by creating “refuge” populations for both ESUs (Stockwell et al. 1998; Pittenger and Springer 1999). The Malpais Spring ESU has not yet been replicated, but populations historically established at Lost River (1970) and Mound Spring (between 1967 and 1973) are both genetically descended from the Salt Creek population (Stockwell et al. 1998; Pittenger and Springer 1999). The conservation status and associated plans are more difficult to define, if the Malpais Spring and Salt Creek populations shared ancestry in the 1800s. Without reciprocal monophyly to elucidate the issue, verifying time of isolation has critical implications for managing this New Mexico state Threatened species.

Here, we evaluate the time of isolation for the Malpais Spring and Salt Creek populations. The emergence of approximate Bayesian computational (ABC) methodology provides an avenue for using genetic data to evaluate population history as well as time of divergence (Cornuet et al. 2008; Csilléry et al. 2010; Bertorelle et al. 2010; Guillemaud et al. 2010). We performed an ABC analysis to

estimate long-term effective population size ( $N_E$ ) and time of divergence for the Malpais Spring and Salt Creek populations of the White Sands pupfish. We contrast our findings with the two competing hypotheses concerning time of isolation; 5,000 years ago versus late 1800s.

## Methods

White Sands pupfish were caught by minnow-trapping and seining during March, 2003, at Malpais Spring below the USGS flow gauge ( $n = 40$ ), and Salt Creek, below Range Road 316 ( $n = 40$ ; Table 1). Fish were subsequently sacrificed, frozen, and stored at  $-80\text{ }^\circ\text{C}$  upon return to the Laboratory.

We extracted whole genomic DNA, and amplified 13 polymorphic microsatellite loci as per Collyer et al. (2011). After automated fragment analysis for pool-plexed groups of PCR products, alleles were called by hand and verified at least thrice for each sample. We used GENEPOP 4.0 (Raymond and Rousset 1995; Rousset 2008) to test for Hardy–Weinberg Equilibrium (*HWE*) and linkage disequilibrium (*LD*). Sequential Bonferroni correction (Rice 1989) was applied to tests of significance involving simultaneous comparisons.

We used GENALEX 6.4 (Peakall and Smouse 2006) to calculate the number of alleles per locus ( $A$ ), the observed ( $H_O$ ) and expected ( $H_E$ ) heterozygosity and the mean number of private alleles ( $P_A$ ) for each population. We tested for divergence between the two populations with  $F_{ST}$  using FSTAT 2.9.3 (Goudet 1995), and tests of allelic and genotypic differentiation using GENEPOP 4.0 (Raymond and Rousset 1995; Rousset 2008).

We used Approximate Bayesian Computation (DI-YABC v. 1.0.4.38; Cornuet et al. 2008) to estimate time of divergence and long-term effective size of both populations. The *reference table*, which forms the basis of the parameter estimation, consisted of 1,000,000 simulated data sets. Each record in the reference table was based on a set of summary statistics (Table 2). We followed Guillemaud et al. (2010) and used various combinations of the following summary statistics: one-sample statistics for mean number of alleles ( $A$ ), heterozygosity ( $H$ ; Nei 1987), allele size variance ( $V$ ; Estoup et al. 2004), and Garza–Williamson’s  $M$  (Garza and Williamson 2001;  $M$ ), two-sample statistics for the mean number of alleles ( $A-2P$ ), genic diversity ( $H-2P$ ), allele size variance ( $V-2P$ ), pairwise  $F_{ST}$  (Weir and Cockerham 1984), and mean of individual likelihood assignments collected from population ( $i$ ) but assigned to population ( $j$ ) ( $L_{i-j}$ ; Pascual et al. 2007; Table 2). We ran three different simulations, each with a different combination of summary statistics; *Beaumont*, *Cornuet-Miller*, and *Guillemaud* models (Miller et al.

**Table 1** Genetic characteristics summary table for both populations

Population	Spatial coordinates		<i>N</i>	<i>A</i>	<i>H<sub>E</sub></i>	<i>H<sub>O</sub></i>	<i>P<sub>A</sub></i>
	Lat.	Lon.					
Malpais Spring	33°17'16"	106°18'36"	40	3.23	0.337	0.354	1.85
Salt Creek	33°16'33"	106°23'50"	40	3.46	0.356	0.340	1.62

Symbols indicate number of samples (*N*), allelic diversity (alleles/locus) (*A*), expected heterozygosity (*H<sub>E</sub>*), observed heterozygosity (*H<sub>O</sub>*), and number of private alleles (*P<sub>A</sub>*)

**Table 2** Summary statistics used for each of the 3 ABC models

	Cornuet–Miller		Beaumont		Guillemaud	
	Malpais	Salt Creek	Malpais	Salt Creek	Malpais	Salt Creek
One population summary statistics						
Mean number of alleles ( <i>A</i> )	✓	✓	✓	✓		
Heterozygosity ( <i>H</i> )	✓	✓	✓	✓		
Allele Size Variance ( <i>V</i> )			✓ <sup>a</sup>	✓		
Garza's <i>M</i> ( <i>M</i> )	✓ <sup>a</sup>	✓				
Two population summary statistics						
Mean number of alleles ( <i>A-2P</i> )				✓		✓
Heterozygosity ( <i>H-2P</i> )				✓		✓
Allele size variance ( <i>V-2P</i> )				✓ <sup>a</sup>		✓
<i>F<sub>ST</sub></i>		✓				✓
<sup>+</sup> <i>L<sub>i-j</sub></i>	✓	✓			✓	✓

<sup>+</sup> *L<sub>i-j</sub>* = mean of individual likelihood assignments from population *i* but assigned to population *j*

<sup>a</sup> Summary statistics where observed and simulated were significantly different

2005; Beaumont 2008; Cornuet et al. 2008; Guillemaud et al. 2010, respectively) (Table 2). We used the default generalized stepwise mutation model (Estoup and Jarne 2002; Guillemaud et al. 2010) with a mean mutation rate across loci set at  $10^{-3}$ – $10^{-4}$ . We used uniform priors and allowed for a range of values for both parameters under evaluation (Malpais Spring *N*: 10–10,000; Salt Creek *N*: 10–10,000; time of divergence: 1–20,000 generations).

## Results

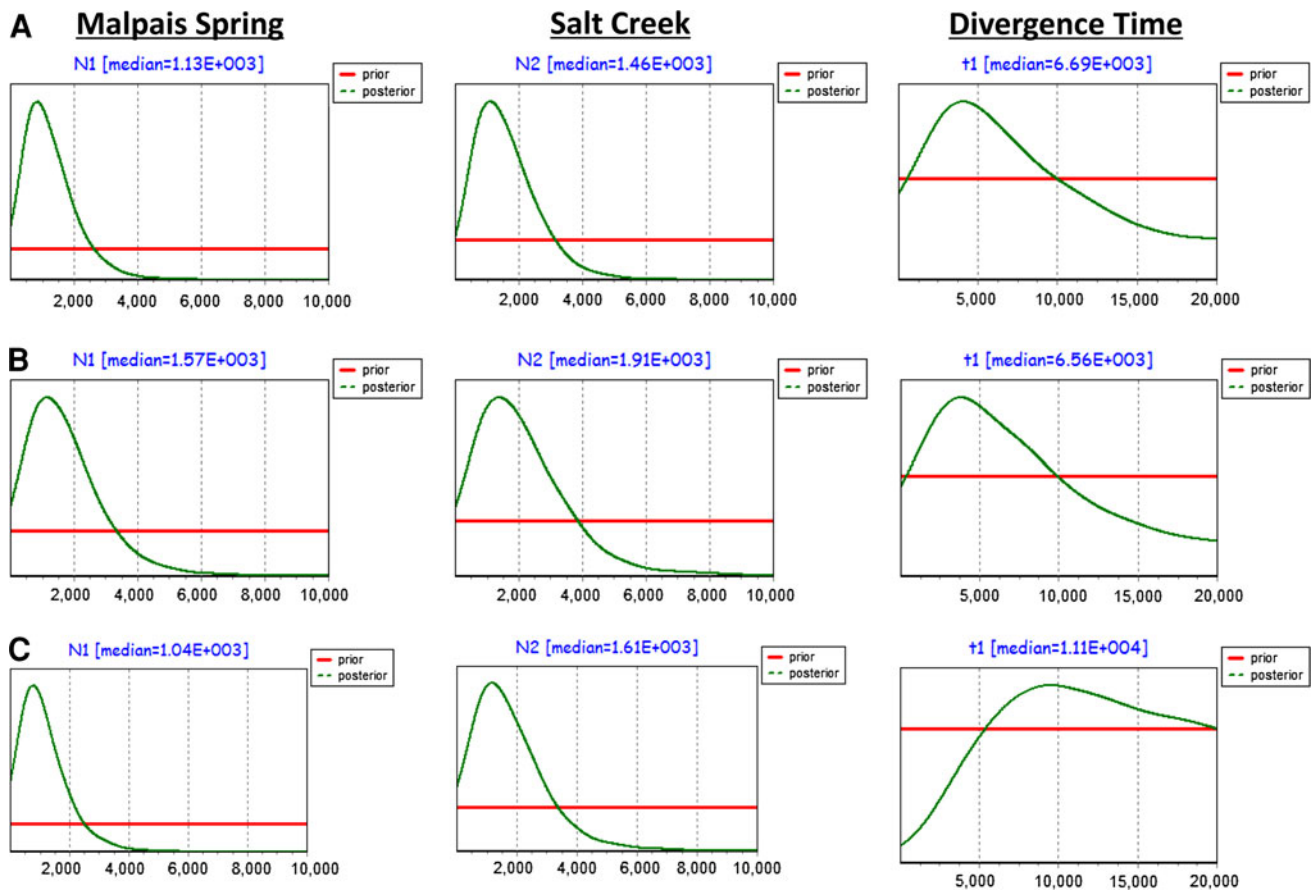
Multilocus genotypes were successfully obtained for all fish sampled, eliminating the need for rarefaction or other sub-sampling methods. We found no indications of statistical violations of *HWE* or *LD* within the sampled dataset. Heterozygosity values were relatively similar for the two populations (Malpais: *H<sub>E</sub>* = 0.337, *H<sub>O</sub>* = 0.354; Salt Creek *H<sub>E</sub>* = 0.356; *H<sub>O</sub>* = 0.340; Table 1). Likewise, both populations had similar levels of allelic diversity (*A* = 3.23 and 3.46, at Malpais Spring and Salt Creek, respectively) and average number of private alleles per locus (Malpais Spring: *P<sub>A</sub>* = 1.85; Salt Creek *P<sub>A</sub>* = 1.62; Table 1).

Genetic differentiation between the two populations was revealed by a large *F<sub>ST</sub>* value (0.386). Further, private alleles accounted for 48 and 53 % of the total number of alleles, with 12 and 15 of these at frequencies  $\geq 0.10$  in Malpais Spring and Salt Creek, respectively (Heilveil and Stockwell, unpublished manuscript). Finally, all 13 loci showed highly significant differences in both genotypic and allele frequency distributions between the two populations.

The three different simulations, using unique combinations of summary statistics, provided relatively consistent estimates for long-term effective population sizes. The median varied from 1,040 to 1,570 for Malpais Spring and 1,460 to 1,910 for Salt Creek (Fig. 2; Table 3). Median times of divergence estimates were relatively consistent for the Beaumont model (6,690 generations) and Cornuet-Miller model (6560 generations), but notably higher for the Guillemaud model (11,100 generations; Fig. 2; Table 3).

## Discussion

Our simulations indicate that the Malpais Spring and Salt Creek populations of White Sands Pupfish have been



**Fig. 2** DIYABC posterior probability distributions (green lines) for the three parameters of interest: Malpais Spring  $N_E$ ; Salt Creek  $N_E$ ; and Time of Divergence, under three different models with each using

a different combination of summary statistics (Table 2); **a** Beaumont model, **b** Cornuet-Miller model and **c** Guillemaud model (see Table 2). (Color figure online)

**Table 3** Median effective population size ( $N_E$ ) estimates (and 95 % credible intervals) and time of divergence from each of three ABC models

Population	Beaumont	Cornuet-Miller	Guillemaud
Malpais Spring $N_E$	1,130 (287–3,300)	1,570 (352–4,980)	1,040 (226–3,270)
Salt Creek $N_E$	1,460 (337–4,060)	1,910 (424–5,970)	1,610 (365–5,240)
Divergence Time	6,690 (1,150–18,800)	6,560 (1,160–18,500)	11,100 (2,730–19,400)

diverging for approximately 6,500–11,000 generations (3,250–11,000 years; with 1–2 generations/year). These results are consistent with the hypothesis that isolation of the two populations was concurrent with the Carrizozo Lava flow (~5,000 years BP; Salyards 1991; Dunbar 1999). The disparity in time of divergence between the Guillemaud model and the other two models might reflect differences in model parameters, with the Guillemaud model using only two-sample (population) summary statistics, while the other two models use a combination of

both two-sample and one-sample summary statistics. In all cases, however, time of divergence was a number of millennia ago, well before anthropogenic hydrological changes in the late 19th century (Pittenger and Springer 1999).

Molecular assessment of time of divergence would provide valuable tests for many recently evolved species with times of divergence inferred from geological data. For example, the isolation of many desert fish species is assumed to have occurred following the desiccation of Pleistocene lakes, and thus time of isolation is inferred to

be coincident (Miller 1948; Hedrick et al. 2006). In a particularly problematic example, earlier workers suggested that the Devils Hole pupfish (*C. diabolis*) was isolated within Devils Hole 10,000–20,000 years ago (Miller 1948, 1981); however, Riggs and Deacon (2004) pointed out that there is no evidence for a surface hydrological connection to Devils Hole, suggesting that this population of fish was introduced by either some unusual hydrological event or introduced in some other un-documented fashion, meaning that time of isolation is not known. The case of the White Sands pupfish demonstrates how molecular data can be used to examine geologically-derived hypotheses of divergence time.

Documenting time of divergence is relevant, as conservation biologists have traditionally attached added value to taxa with more ancient evolutionary histories (Bruton and Stobbs 1991; Wilson 1992; Crandall et al. 2000). Had we found evidence for recent isolation, the conservation status would be complicated. For instance, establishing a Malpais Spring refuge population would be less pressing, given that two refuge populations have been established; Salt Creek derived populations at Mound Spring and Lost River. On the other hand, observed adaptive divergence between the two native populations would place high value on managing them as separate conservation units, especially since gene flow can constrain local adaptation (Tufto 2001; Lenormand 2002).

Our work, however, provides evidence that the two native populations have been isolated since the mid-Holocene and we therefore echo Stockwell et al. (1998) in recommending that these two populations be managed as separate ESUs. The case study of the White Sands pupfish (Stockwell et al. 1998) is an example of using both genetic and ecological criteria to define ESUs, an approach advocated by many other workers (Waples 1991, 1995; Crandall et al. 2000; Fraser and Bernatchez 2001; Rader et al. 2005). Guia and Saitoh (2007), however, recently reported that ESUs have largely been delineated solely using molecular makers and the criterion of reciprocal monophyly (Moritz 1994).

The ESU criterion of reciprocal monophyly proposed by Moritz (1994) is appealing because of its crisp dichotomous nature; ESU or not. Further, this approach is quite useful for systems that have not been well studied. While ESU delineation is relatively straightforward for long-separated populations, the rate at which reciprocal monophyly forms limits the designation of recently diverged forms. Moritz (1994) also created a designation of MUs, for those populations which are distinct, but not yet monophyletic. Moritz et al. (1995) suggested that conservation protection would be warranted for MUs as well as ESUs; however, while there is precedent for defining ESUs

as *distinct population segments* of endangered species under the Endangered Species Act (Waples 1991, 1995; see Federal Register 70:37,160–37,204), we are unaware of any similar protection being offered to specific Management Units.

Thus, the common practice of evaluating species by looking solely for reciprocal monophyly (Guia and Saitoh 2007) has real-world consequences for the recognition and conservation of biodiversity. We argue that widespread dependence on reciprocal monophyly (Guia and Saitoh 2007) undervalues more recently derived biodiversity and may blind us to important ESUs. Similar to the case of *C. tularosa*, the desiccation of Pleistocene lakes also led to rapid speciation of pupfishes in the Death Valley region (Miller 1948, 1981); however, even recognized species from this system, such as the Devils Hole pupfish, do not exhibit reciprocal monophyly (Duvernell and Turner 1998) and would therefore be undervalued by studies focused on reciprocal monophyly.

Studies that include a broad array of neutral and functional markers/traits should provide the richest data set for populations occupying ecological divergent habitats. This approach should allow workers to evaluate not only the degree of divergence, but also estimate time of divergence. Without information on time of divergence, ESU status could be assigned to populations that adaptively diverged in contemporary time. While some would argue that such populations have lower conservation value (Crandall et al. 2000), we conclude by suggesting that such temporal constraints ignore the tempo and mode of evolution. In fact, adaptive divergence may well occur in starts and fits (Kinnison and Hendry 2001), where rapid initial population divergence is followed by long periods of stasis. Following traditional views that link evolutionary significance to lineage longevity, such populations only accrue “evolutionary significance” over time.

A provocative approach may be to remove all temporal constraints for defining evolutionary and conservation value which, at the extreme, would provide protection for populations diverging in contemporary time. Protecting recently diverged populations may be justified based on both the scientific insights they offer as well as the diversity generated. In the case of the White Sands pupfish, identifying the most unique population depends on the markers/traits used. The Mound Spring population has the most divergent body shape, while the Malpais Spring population has the most molecular divergence at neutral markers. In our view, both are evolutionarily significant and thus worthy of protection. In fact, protecting anthropogenically driven diversification may provide unique opportunities for limiting the net loss of biodiversity during the Anthropocene.

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