

# Species designation of the Bruneau Dune tiger beetle (*Cicindela waynei*) is supported by phylogenetic analysis of mitochondrial DNA sequence data

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**Abstract** Beetles comprise nearly one quarter of described species and show high levels of morphological and ecological diversification. Because of their wide distribution, ease of detection, and correlation of species richness patterns with other taxa, tiger beetles have been recommended for use as a global indicator of regional biodiversity, requiring accurate taxonomic designations. The Bruneau Dune tiger beetle (*Cicindela waynei*), whose habitat consists of an isolated dune field in southern Idaho, was recently described as a distinct species from the St. Anthony Dunes tiger beetle (*C. arenicola*) based on morphological characteristics. While these characteristics include distinct differences in genital morphology that could indicate intrinsic reproductive isolation, morphological characteristics have sometimes been misleading in tiger beetle taxonomy. To evaluate genetic support for this species designation, we analyzed 1,751 base pairs of mitochondrial DNA sequence from 147 tiger beetles collected throughout the range of both *C. arenicola* and *C. waynei*. Maximum-likelihood and Bayesian phylogenetic analyses indicated monophyly for

*C. waynei* on a well-supported, short branch nested within *C. arenicola*. Bayesian species delimitation analyses strongly supported *C. waynei* as a distinct species (speciation probability = 1.0) with the estimated time of divergence ca. 14,500–67,000 years ago. This lack of reciprocal monophyly and recency of speciation is consistent with *C. waynei* as a member of an evolutionary front where speciation has occurred at a rapid rate. Mitochondrial sequence data supports the species designation of *C. waynei*, emphasizing the need to determine appropriate management for this species and its restricted habitat.

**Keywords** Bayesian species delimitation ·  
*Cicindela arenicola* · *Cicindela waynei* ·  
Mitochondrial DNA · Tiger beetle

## Introduction

Beetles represent unparalleled diversity among known organisms, making up nearly one quarter of described species (Liebherr and McHugh 2003). Phylogenetic analysis of this group shows high survival of lineages through evolutionary time and diversification into a large variety of niches (Hunt et al. 2007). One of the most well-known groups of beetles is the tiger beetles (Coleoptera: Carabidae: Cicindelinae), with over 2300 species distributed throughout the world (Cassola and Pearson 2000). Because of their wide distribution, relatively stable taxonomy, specialized habitat requirements, ease of detection, and correlation of species richness patterns with other taxa, tiger beetles have been recommended for use as a global indicator of regional biodiversity (Pearson and Cassola 1992).

Prior to widespread availability of molecular tools, the taxonomy of tiger beetles in North America was based

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primarily on the analysis of male genital morphology (Rivalier 1954). Since that time, ecological and morphological characteristics of tiger beetles have been found to be evolutionarily convergent, requiring genetic methods to clarify species' relationships (Barraclough et al. 1999; Vogler et al. 1998). Recently, analysis of mitochondrial DNA sequence data has led to an increased understanding of tiger beetle systematics and evolution (e.g., Vogler et al. 2005), as well as the elevation of several taxa from the subspecific level to full species (Morgan et al. 2000; Woodcock et al. 2007).

In North America, the western clade of the *C. maritima* group of tiger beetles is estimated to have diverged from a more widespread lineage ca. 1 mya and represents an evolutionary front where speciation occurs at a rapid rate (Barraclough and Vogler 2002; Erwin 1991; Vogler et al. 1998). Within this clade, the Bruneau Dune tiger beetle (*Cicindela waynei*) was recently described as a species distinct from St. Anthony Dunes tiger beetle (*C. arenicola*), based on several non-intergrading morphological features, including mandible characteristics and male and female genitalia (Leffler 2001). Additionally, *C. waynei* is unique among the *C. maritima* group for having a laterally displaced penultimate mandibular tooth, likely used to maintain grip during mating (Leffler 2001). *C. waynei* and *C. arenicola* are allopatric inland dune specialists and habitat for these species is localized and irregularly distributed. The Bruneau Dunes occupied by *C. waynei* is an isolated patch in an otherwise continuous geology of loess and eolian (wind-blown) deposits that contain geographically distinct habitat patches for *C. arenicola* populations (Busacca et al. 2004). Habitat for both species is impacted by human recreation, livestock, and invasive plants. Additionally, these species have been extensively targeted by collectors for commercial trade (Shook and Clark 1988). Currently, the only extant population of *C. waynei* persists in a single dune field in southern Idaho.

Appropriate resource management decisions depend on accurate and robust taxonomic designation. To investigate whether genetic data support the species designation of *C. waynei*, we collected 147 samples from throughout the range of *C. waynei* and *C. arenicola* and sequenced 1,751 base pairs of mitochondrial DNA from three gene regions for each individual. Species delimitation using genealogical data often relies on establishing a threshold for genetic distances between species and/or requiring gene tree monophyly to establish species boundaries (Sites and Marshall 2004). Because speciation is an ongoing process (e.g., de Queiroz 2007), species delimitation methods that use genetic data must incorporate the stochastic nature of the coalescent process, especially when evaluating recent speciation events because gene tree uncertainties due to coalescent stochasticity are more pronounced. Here we

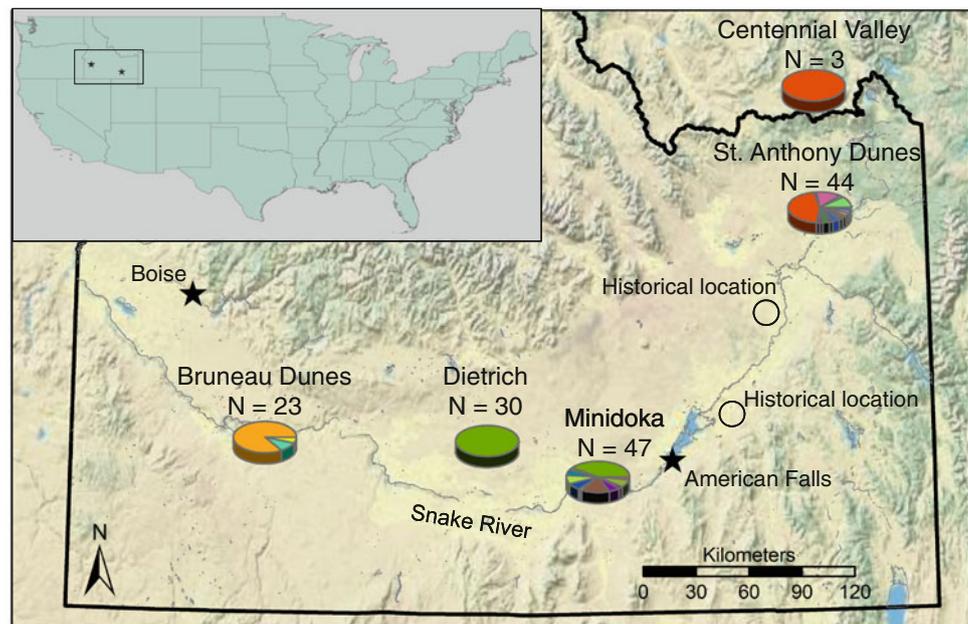
estimated phylogenetic relationships between *C. waynei* and *C. arenicola* using both maximum likelihood (ML) and Bayesian phylogenetic methods. In addition, we used a Bayesian species delimitation method (Yang and Rannala 2010) that uses a reversible-jump Markov chain Monte Carlo (rjMCMC) in a coalescent framework to accommodate gene tree uncertainty and estimate the posterior distribution for competing species delimitation models that are contained within a user-specified guide tree. The guide tree is a fully resolved species tree and the rjMCMC algorithm evaluates nested species delimitation models (i.e., subtrees generated by collapsing or splitting nodes on the guide tree), and thus provides speciation probabilities for each divergence event, in addition to estimates of  $\theta$  (the product of effective population size and mutation rate),  $\tau_a$  (origination time of a species) and  $\tau$  (the time at which a species diverges into two descendent lineages).

## Methods

### Taxon sampling and molecular methods

We collected 147 whole tiger beetles from all known populations in the range of *C. waynei* and *C. arenicola* in Spring 2009 and immediately preserved them in 95% ethanol (we were not able to obtain samples from two additional locations; one site with most recent record from 1989 (Ft. Hall) could not be accessed, and no beetles were found at a site open to public sand removal (Idaho Falls); Fig. 1). Individuals collected from Centennial Valley were reported in Winton et al. (2010). We extracted DNA from one middle and one rear leg of these samples using a DNeasy Tissue Kit (Qiagen, Inc., Valencia, CA), substituting PBS (pH 7.2) for ATL and macerating tissue with a mortar and pestle in the presence of PBS, Proteinase K, and AL. We amplified three regions of the mitochondrial genome that have been used previously to analyze the phylogenetic relationships in this genus by Vogler and Welsh (1997): cytochrome oxidase III and adjacent tRNA<sup>gly</sup> and ND3 (COIII) using region I primers from Volger et al. (1993); cytochrome b (CytB) using primers CB1 and CB2 (Crozier et al. 1991); and the 16S rRNA gene, adjacent tRNA<sup>leu</sup> and ND1 (16S) using the 16F primer from Volger and Welsh (1997) and 16sar from Simon et al. (1991). PCR conditions were as described in Vogler and Welsh (1997), except for the 16S region where we raised the annealing temperature to 46°C and included 0.5× Q-solution (Qiagen, Inc.) to increase stringency. Amplicons were sequenced with the amplifying primers (COIIIF, CB2, and both primers for 16S) using a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). Resulting chromatograms were edited and contigs were

**Fig. 1** Sampling locations, sample sizes, and haplotype frequencies for *C. waynei* (Bruneau Dunes) and *C. arenicola*. Haplotypes were derived from 1,751 base pairs of the COIII (634 bp), CytB (407 bp) and 16S rRNA (720 bp) mtDNA gene regions



assembled using Sequencher v.4.7 (Gene Codes Corp., Ann Arbor, MI). Previously published sequences of *C. columbica* were used as an outgroup (Vogler et al. 1998; GenBank Accession numbers: AF438838.2, AF438856.1, AF438847.1).

#### Phylogenetic analyses

For each of the three gene regions, sequence alignment was straightforward, i.e., introducing gaps in the alignment was not necessary, and was done manually using the program Se-AL v.2.0a11 (Rambaut 2002).

The program jModelTest v.0.1.1 (Posada 2008) was used to determine the model of sequence evolution best fit to the data according to the Akaike Information Criterion (AIC). ML analyses were conducted using RAxML v.7.2.4 (Stamatakis 2006; Stamatakis et al. 2008) and consisted of 1,000 rapid bootstrap replicates. To assess incongruence between gene regions, each region was analyzed separately; however, because the mitochondrion is inherited as a single unit, there is no a priori reason to not treat the separate gene regions as a single locus. Therefore, we also conducted a combined, 3-gene analysis with each region treated as a separate partition, to account for the inherent differences that exist between regions in base composition and among site rate variation. Every fifth bootstrap tree generated by the rapid bootstrap analyses was used as a starting tree for full ML searches and the trees with the highest ML scores were chosen. Bayesian phylogenetic analyses were conducted using MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003), again with each gene region treated as a separate partition. Each analysis consisted of

two runs of 10,000,000 generations from a random starting tree using a variable rate prior and four Markov chains (using the default heating values) sampled every 1,000 generations. Convergence of the chains was determined by examining the plot of all parameter values and the  $-\ln L$  against generation time using the program Tracer v.1.4 (Rambaut and Drummond 2004). Stationarity was assumed when all parameter values and the  $-\ln L$  had stabilized. Burn-in trees were then discarded and the remaining trees, and their associated parameter values, were saved. To explore more tree space and to decrease the chance of obtaining stationarity on local optima, two independent analyses were performed for each data set.

To test the hypothesis that *C. waynei* and *C. arenicola* are reciprocally monophyletic, we used the approximately unbiased (AU) test (Shimodaira 2002) as implemented in CONSEL v.1.19 (Shimodaira and Hasegawa). Per site likelihoods were calculated using RAxML v.7.2.4 (Stamatakis 2006) on both the ML tree from our partitioned 3-gene analysis and a ML tree from a constrained analysis where monophyly was enforced on both *C. waynei* and *C. arenicola*.

#### Bayesian species delimitation

The program BPP v.2.1 (Bayesian Phylogenetics and Phylogeography; Rannala and Yang 2003; Yang and Rannala 2010) was used to test alternative species delimitation models. BPP utilizes a completely resolved guide tree depicting the relationships of the putative species to estimate the posterior probabilities of competing species delimitation models (Yang and Rannala 2010). It is

important to note that because BPP does not intrinsically infer phylogenetic relationships, (i.e., the guide tree is assumed to be the true species tree) posterior probabilities on nodes are not intended to be evidence for relationships but rather as probabilities of speciation. High posterior probabilities indicate that the rjMCMC algorithm visited more species delimitation models where those two lineages descended from that node (Leaché and Fujita 2010).

Because the guide tree influences the nested species delimitation models that are tested, we investigated two guide trees corresponding to a two-species model with *C. arenicola* populations treated as one species and a three-species model with *C. arenicola* populations grouped into two putative species based on shared haplotypes (Fig. 1; Dietrich + Minidoka and St. Anthony Dunes + Centennial Valley).

Prior distributions were assigned to both ancestral population size ( $\theta$ ) and the age of the root in the species tree ( $\tau_0$ ). Following Leaché and Fujita (2010), the gamma prior  $G(1, 10)$  was used for  $\theta$  and the gamma prior  $G(2, 2000)$  was used for  $\tau_0$ . This configuration of priors favors models that do not over-split populations and is most appropriate for biological systems that include putatively large ancestral population sizes and recent lineage divergences (Leaché and Fujita 2010; Yang and Rannala 2010). All other divergence time parameters were assigned the Dirichlet prior (Yang and Rannala 2010).

Finally, to assess consistency of the results and proper mixing of the rjMCMC algorithm, analyses consisted of two runs of  $10^6$  generations each (sampling every 5th generation) under algorithm 0 and fine-tuning parameter  $\varepsilon = 2$ , and a burn-in period of 250,000 generations.

## Results

### Taxon sampling and molecular methods

Complete sequences were obtained for all 147 samples for each of the three gene regions sequenced, COIII, CytB, and 16S. The COIII alignment was 624 bp in length, the CytB alignment was 407 bp in length, and the 16S alignment was 720 bp in length. Each of these regions aligned unambiguously, requiring no gaps to be introduced into the alignment. The combined, 3-gene alignment had a total length of 1,751 bp. Thirty-five variable sites were observed, and these sites defined 29 unique haplotypes. Newly generated sequences used in this study were deposited in GenBank (Accession numbers JN016622 – JN016708).

### Phylogenetic analyses

Model selection using the AIC, as implemented in jModel-Test v.0.1.1 (Posada 2008), resulted in the TPM1uf + I,

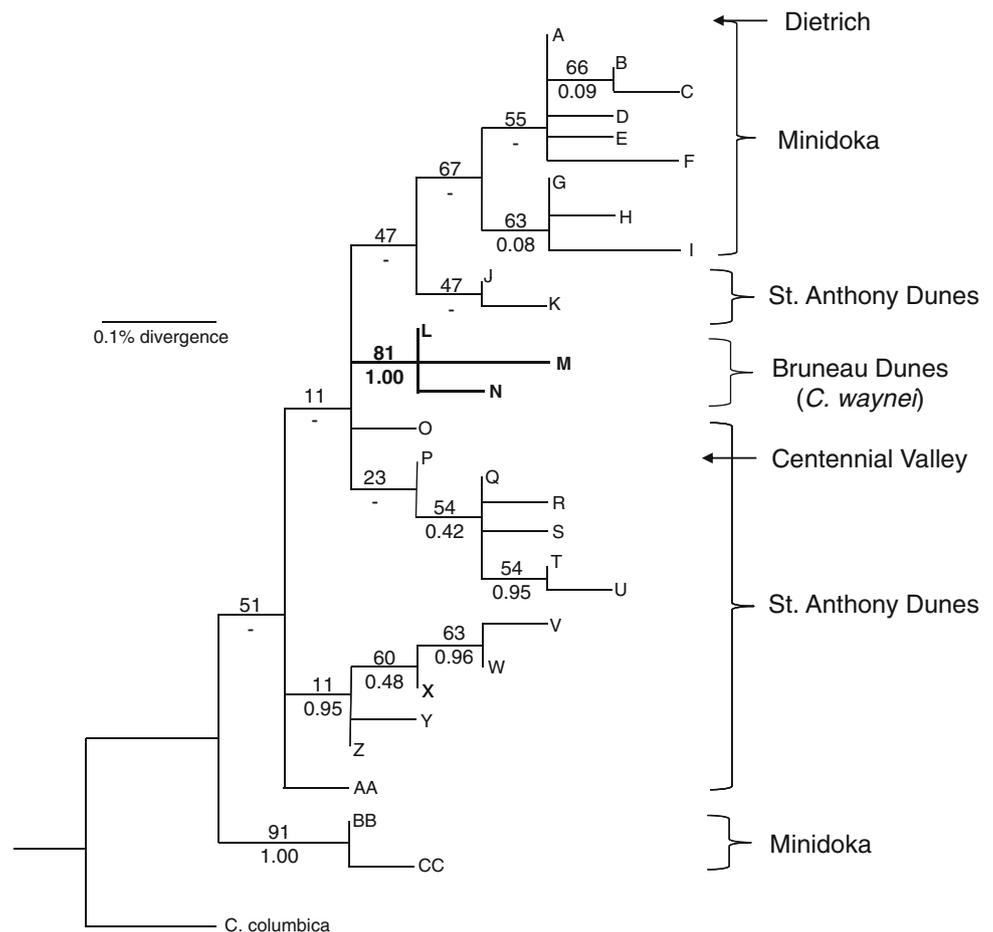
TPM2uf + I, and TPM3uf + I (TPM = three parameter model, uf = unequal base frequencies, I = proportion of invariable sites) models of sequence evolution for the COIII, CytB, and 16S regions, respectively. The TPMuf model (Kimura 1981) is a submodel of the GTR (GTR = general time reversible) model of sequence evolution. Because Stamatakis (2006) has only implemented the proportion of invariable sites parameter (I) along with gamma distributed variable sites (G) in RAxML, our ML analyses were conducted using the GTRGAMMAI model of sequence evolution (GTR + I + G) as implemented by the rapid bootstrap algorithm in RAxML (Stamatakis et al. 2008). Likewise, for the Bayesian analyses using MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003), we used a six substitution model including a parameter for the proportion of invariable sites (GTR + I) for each of the three gene regions.

Trees from ML and Bayesian analyses of the individual gene regions (trees not shown) were all nearly identical in topology, only differing in regions that received little to no statistical support as measured by ML bootstrap percentages and Bayesian posterior probability values. Therefore, we have chosen to only show results from the partitioned 3-gene ML and Bayesian analyses (Fig. 2). None of the populations of *C. arenicola* were found to be monophyletic (except those with only one haplotype), and *C. arenicola* and *C. waynei* were not reciprocally monophyletic (Fig. 2). However, *C. waynei* was monophyletic in both the ML and Bayesian analyses, forming a strongly supported clade (ML bootstrap value = 81%, Bayesian posterior probability = 1.0) nested within *C. arenicola* (Fig. 2). The best ML gene trees also recovered the *C. waynei* clade for CytB and COIII, but with low bootstrap support. Results of the AU test (Shimodaira 2002) as implemented in CONSEL v. 1.19 (Shimodaira and Hasegawa 2001) based on sequence data from these three gene regions indicated that the best ML tree from the partitioned 3-gene analysis was not significantly different from a topology where *C. waynei* and *C. arenicola* were constrained to be reciprocally monophyletic ( $P = 0.205$ ). Uncorrected sequence divergence between individuals of *C. waynei* and *C. arenicola* ranged from 0.11 to 0.57%, divergence within *C. waynei* ranged from 0.06 to 0.17%, and divergence within *C. arenicola* ranged from 0.06 to 0.57%.

### Bayesian species delimitation

Speciation between *C. waynei* and *C. arenicola* was strongly supported by all BPP analyses. Speciation probabilities of 1.0 were recovered for the split between *C. waynei* and *C. arenicola* using both of the guide trees (Figs. 3 and S1). Under the three-species guide tree, where populations of *C. arenicola* were split into two putative species based on shared haplotypes and geography, the

**Fig. 2** Best ML tree (GTR + I + G model) for *C. waynei* and *C. arenicola* haplotypes derived from 1,751 base pairs from the COIII (634 bp), CytB (407 bp) and 16S rRNA (720 bp) mtDNA gene regions with branch lengths drawn to scale. Bootstrap values (1,000 replicates) are indicated *above branches* and posterior probabilities from Bayesian analysis are indicated *below branches*



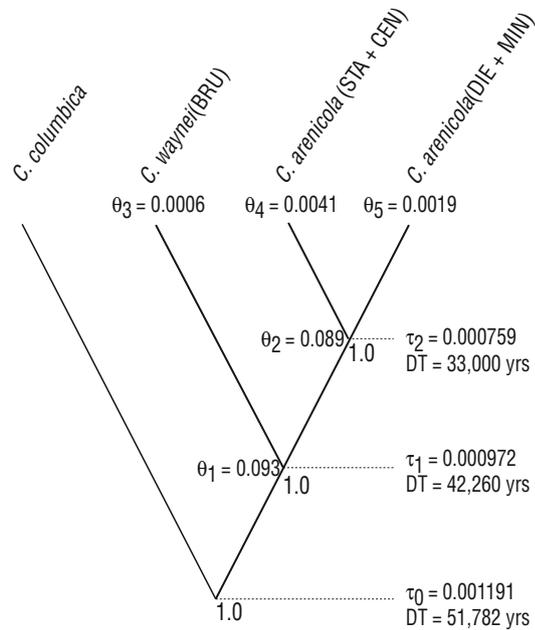
additional split within *C. arenicola* received a speciation probability of 1.0 (Fig. 3). To illustrate the presence of further population structure within *C. arenicola*, we have chosen to show BPP results from the three-species guide tree, including both speciation probabilities (Fig. 3), as well as estimates of  $\tau$  (Table 1A) and  $\theta$  (Table 1B). However, because the guide tree can have a large effect on the outcomes of the analyses (Leaché and Fujita 2010), the most conservative interpretation of parameter estimates should include the 95% confidence intervals from the three species and five species guide trees as well (Table S1). In this light, values for  $\tau_1$  marking the divergence between *C. waynei* and *C. arenicola* ranged from 0.00034 to 0.00155 mutations/site. Using the widely cited estimate of mtDNA mutation rate in arthropods of 2.3% sequence divergence per million years (Brower 1994), this corresponds to a divergence time of approximately 14,500–67,000 years (Tables 1 and S1).

**Discussion**

Species delimitation - especially during the early stages of population divergence – can be extremely challenging.

Early in the process of speciation, both morphological and molecular characters are likely to show very little differentiation, and incomplete lineage sorting at genetic loci is likely to confound the discovery of reciprocally monophyletic evolutionary lineages (de Queiroz 2007). Despite this, molecular data can provide a wealth of demographic and phylogenetic information when analyzed in a coalescent framework. We did not find reciprocal monophyly between *C. waynei* and *C. arenicola*, which is not surprising, given the recency of divergence among species in the *C. maritima* evolutionary front, where sequence divergence between *C. arenicola* and the closely related *C. columbica* is only 0.6% using the same mtDNA gene regions employed here (Morgan et al. 2000). Results of our Bayesian species delimitation analyses strongly support *C. waynei* as a distinct lineage that is separate from *C. arenicola* (Figs. 3 and S1). Following de Queiroz’s (2007) unified species concept where a species is recognized as an independently evolving metapopulation lineage and not solely by the presence of a specific criterion (e.g., reciprocal monophyly, reproductive isolation, apomorphies), the combination of (1) putative reproductive isolation due to distinct genital morphology and geographic isolation, (2) the strongly supported, unique phylogenetic

lineage (Fig. 2) that includes the molecular synapomorphy of a non-homoplastic base pair change found only in *C. waynei*, and (3) a speciation probability of 1.0 based on



**Fig. 3** Bayesian species delimitation results for *C. waynei* and *C. arenicola* for the three species model with *C. columbica* used as the outgroup. Values below nodes are speciation probabilities. Mean estimates of  $\theta$  for current and ancestral populations are indicated, and mean divergence times are shown in substitutions/site ( $\tau$ ) and years before present (DT) calculated using the molecular evolutionary rate of  $2.3 \times 10^{-8}$  substitutions/site/my for arthropod mtDNA (Brower 1994). BRU Bruneau Dunes, DIE Dietrich, MIN Minidoka, STA St. Anthony Dunes, CEN Centennial Valley

the coalescent-based Bayesian species delimitation method, all provide lines of evidence to support the species designation of *C. waynei* that had formerly been established using morphological characters (Leffler 2001).

Given the short branch lengths and lack of reciprocal monophyly between the two taxa, *C. waynei* may have arisen from the isolation of an ancestral *Cicindela* lineage following late Pleistocene flood events in the Snake River. Although the underlying geological formations of the western Snake River Plain that contain Bruneau Dunes are older than those in the eastern Snake River Plain (Malde 1991), the dunes themselves are thought to have been created by eolian processes after the Bonneville Flood ca. 14,500 ya (Murphy 1973; Oviatt et al. 1992), while at least some of the dunes in the eastern part of the state may be older (Malde 1991). These dates correspond well with the divergence times estimated in our BPP analyses (Figs. 3 and S1).

Populations of *C. arenicola* in the center and eastern part of the range do not share haplotypes and may also represent separate species. Our Bayesian species delimitation analysis with the three species guide tree indicated significant population structure between the eastern (St. Anthony Dunes and Centennial Valley) and western (Minidoka and Dietrich) populations of *C. arenicola* (Figs 3 and S1). However, additional DNA sequence data and evidence for morphological and/or ecological differentiation would be necessary to clarify species designations, as the data from this study are not sufficient for answering this question. In addition, discovery and analysis of *C. arenicola* populations from intervening localities

**Table 1** Estimates of  $\tau$  (A) and  $\theta$  (B) from the Bayesian species delimitation analyses using the three species guide tree shown in Fig. 3

	$\tau_0$	DT <sub>0</sub>	$\tau_1$	DT <sub>1</sub>	$\tau_2$	DT <sub>2</sub>
A)						
Mean	0.001191	51782.6	0.000972	42260.9	0.000759	33000
S.D.	0.000308	13391.3	0.000261	11347.8	0.000219	9521.7
2.5%	0.000687	29869.6	0.000533	23173.9	0.000396	17217.4
97.5%	0.001887	82043.5	0.001551	67434.8	0.001248	54260.9
ESS	11397.8	11397.8	22749.8	22749.8	27003.8	27003.8
	$\theta_1$	$\theta_2$	$\theta_3$	$\theta_4$	$\theta_5$	
B)						
Mean	0.0934	0.0891	0.0006	0.0041	0.0019	
S.D.	0.0992	0.0938	0.0004	0.0014	0.0007	
2.5%	0.002	0.0032	0.0001	0.002	0.0009	
97.5%	0.3643	0.344	0.0017	0.0075	0.0034	
ESS	13006	14229.4	6350.3	40067.9	9447.4	

Divergence times are shown in substitutions/site ( $\tau$ ) and years before present (DT) calculated using the molecular evolutionary rate of  $2.3 \times 10^{-8}$  substitutions/site/my for arthropod mtDNA (Brower 1994), and population size ( $\theta$ ) estimates for current and ancestral populations are given as the product of effective population size and per site mutation rate

would help determine whether species designation is appropriate for these populations. We detected only one haplotype in the population of *C. arenicola* at the Dietrich site, where we sampled 30 individuals (Fig. 1). This is a strikingly low amount of genetic diversity compared to the other locations for this species, indicating a putative founder event and/or fixation of a single haplotype via genetic drift. These dunes formerly consisted of a large complex, but stabilization projects and the spread of invasive plants in the latter part of the 20th century greatly reduced habitat in this area (Logan 1995). Additionally, this population is isolated from the nearest known population of *C. arenicola* by >55 km of intensive agriculture. Given the recent landscape changes and geographic isolation, this population is likely to be small and experiencing a loss of diversity through genetic drift, which could lead to a variety of issues, including lower adaptability to new stressors (Reed et al. 2003) and increased vulnerability to disease (Spielman et al. 2004). Our analyses also revealed only a single haplotype from the Centennial Valley population, but the sample size was small ( $N = 3$ ; Fig. 1), so additional sampling would be required to determine if this population is also experiencing a genetic bottleneck. The addition of more informative mtDNA regions and multiple independent nuclear loci would allow for a more comprehensive analysis of population demography including the detection of putative population bottlenecks, population expansions, and contemporary and ancestral levels of gene flow between populations.

The *C. waynei* distribution is currently restricted to a single dune field, where invasive weeds and human recreation are threats to population persistence. Additionally, extensive collecting of these tiger beetles may be detrimental to these already small, isolated populations. Molecular phylogenetic support for the species designation of *C. waynei* emphasizes the need to determine appropriate management strategies for this species and protect this unique lineage from further threats to its persistence.

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