Temporal patterns of genetic diversity in *Baetis tricaudatus* (Ephemeroptera:Baetidae) from the Russian River, northern California

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Abstract: The mayfly *Baetis tricaudatus* is an abundant, widespread, and ecologically important multivoltine benthic macroinvertebrate that is found throughout most of North America. *Baetis tricaudatus* belongs to the *Baetis rhodani* species group, which is known to have cryptic species. Some investigators have found that *B. tricaudatus* morphospecies have cytochrome oxidase I (COI) diversity >20%. However, no investigators have examined whether this diversity is structured temporally, with some haplotypes being more common in certain years or seasons than others. We sequenced COI from 371 *B. rhodani* specimens. The 371 *rhodani* species group sequences generated fell into 2 well-supported clades, one with 38 *Baetis adonis* specimens and another with 333 *B. tricaudatus* specimens, which were the focus of our study. We examined the temporal and spatial dynamics of genetic diversity in *B. tricaudatus* populations from northern California using COI haplotype networks. The maximum genetic diversity among *B. tricaudatus* specimens was 1.7% and was found at a single site (Austin Creek). The same 2 dominant haplotypes of *B. tricaudatus* were consistent through years, sites, and seasons, and Φ_{ST} values were correspondingly low. In 2 intensive sampling events, each with >40 individuals examined, intrapopulational divergence was 1.2 to 1.4%. This result suggests that most of the genetic diversity for this species in this system could be captured in 1 high-effort sampling event rather than in smaller, long-term monitoring events. Our results suggest that, based on the sites examined, Russian River populations of *B. tricaudatus* constitute a single species with no evidence of cryptic diversity.

Key words: Ephemeroptera, *Baetis tricaudatus*, COI gene region, genetic diversity, temporal population genetic structuring, biodiversity, California

Biodiversity is an important aspect of the natural world and a cornerstone of resilience in ecosystems (Folke et al. 2004). Despite its importance, some investigators examining the genetic diversity of organisms have found that species concepts based on morphology have underestimated the actual biodiversity of systems (Witt et al. 2006, Saunders 2008, Gebiola et al. 2012, Kieneke et al. 2012). Underestimation of biodiversity has occurred in many taxa, including the mayfly genus *Baetis* (Williams et al. 2006, Ståhls and Savolainen 2008, Lucentini et al. 2011, Jackson et al. 2014). Studies on the genetic diversity of organisms have contributed to a variety of scientific discussions, including those on species concepts and species delimitation (e.g., Agapow et al. 2004, Sites and Marshall 2004, DeSalle et al. 2005, Pons et al. 2006, De Queiroz 2007, White et al. 2014), concerns about the accurate estimation of biodiversity (e.g., Isaac et al. 2004, Zachos et al. 2013), and the relationships between diversity and geography (e.g., Hughes et al. 2003b, Szpiech et al. 2008, Spitzer 2014). Nevertheless, much remains to be learned about the patterns and dynamics of genetic diversity.

An understanding of the dynamics of genetic diversity in freshwater ecosystems is especially important because of recent biodiversity losses in these environments (Jenkins 2003, Dudgeon et al. 2006, Moyle et al. 2011). Increased genetic structure, which is one aspect of population diversity, can contribute to a stabilizing portfolio effect (Carlson and Satterthwaite 2011). A portfolio effect describes the increased production and resiliency of a population that comprises spatially or temporally segregated subpopulations with diverse adaptations (Carlson and Satterthwaite 2011). The portfolio effect can be an important component in the stability and survival of freshwater species, such as Sockeye Salmon (Schindler et al. 2010). Other investigators

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examining the genetic diversity of freshwater organisms have found evidence for cryptic species, which are morphologically similar but, genetically, appear to be separately evolving lineages when described by methods such as an arbitrary threshold of genetic divergence (De Queiroz 2007). Investigators have found evidence for cryptic species in freshwater taxa, such as Ephemeroptera (e.g., Sweeney and Funk 1991, Ståhls and Savolainen 2008, Zhou et al. 2010, Lucentini et al. 2011, Webb et al. 2012, Jackson et al. 2014), Trichoptera (e.g., Jackson and Resh 1992, 1998, Pauls et al. 2010, Zhou et al. 2011, Harvey et al. 2012), Plecoptera (e.g., Mynott et al. 2011), Diptera (e.g., Smith et al. 2006a, Kim et al. 2012, Renaud et al. 2012), and other groups (e.g., Monaghan et al. 2005, Larson et al. 2012).

Genetic methods are particularly useful for examining biodiversity of freshwater invertebrates for many reasons (Mynott et al. 2011, Webb et al. 2012, Stein et al. 2014). For example, species-level identifications generally require adult specimens rather than the aquatic larval forms that are the life stage collected in many bioassessment and biomonitoring programs to assess the quality of freshwater habitats (Carter and Resh 2013). Furthermore, morphological identification of freshwater invertebrates, particularly the immature stages, is exceptionally difficult because many are small or have numerous instars, dynamic life histories, and systematic uncertainties (McCafferty et al. 2008). Many *Baetis* species, including *B. tricaudatus*, cannot be accurately and consistently identified using traditional morphological methods. Separating *B. tricaudatus* larvae from other congeneric taxa living in the same stream is currently impossible, so Jacobus et al. (2014) recommended identifying these taxa to the *rhodani* group. These taxonomic challenges can be mitigated and potentially solved by integrating molecular and morphological methods (Webb et al. 2012).

Many investigators using molecular methods to study genetic diversity in mayflies, including in the genus *Baetis*, have focused on spatial patterns, such as isolation by distance, and have found mixed results. Some investigators have found increases in genetic diversity or population structure with increasing geographic distance between populations (e.g., Smith et al. 2006b, Alexander 2007, Watanabe et al. 2010, Baggiano et al. 2011). Others have not found increasing genetic diversity or population structure with increasing distance between populations (e.g., Schmidt et al. 1995, Bunn and Hughes 1997, Hughes et al. 2003a, Peckarsky et al. 2005, Rebora et al. 2005, Zickovich and Bohonak 2007, Múrria et al. 2014). Population structure also can vary between closely related mayfly species (Peckarsky et al. 2005) and species that occupy the same geographic region (Monaghan et al. 2001, Baggiano et al. 2011).

Temporal variation in the genetic diversity of mayfly populations is an aspect of biodiversity that has received less study than spatial issues (Lucentini et al. 2011). Genetic diversity, whether spatially or temporally structured, is part of the population diversity that gives rise to the portfolio effect found in salmon populations (Carlson and Satterthwaite 2011, Schindler et al. 2010). Like salmon, mayflies respond to unpredictable and dynamic environmental conditions, which might select for unique adaptations in certain cohorts or populations. Over time, these adaptations might accumulate to create a diverse portfolio of co-occurring populations with substantial genetic structure or even cryptic species, such as Lucentini et al. (2011) found in *B. rhodani*. Lucentini et al. (2011) studied populations from Italy and the UK and found evidence for 3 sympatric cryptic species that were temporally segregated. Adults of each putative species emerged at different times of year and took advantage of unique resources (Lucentini et al. 2011). Temporally structured portfolio effects might be especially important in mayfly species given the increase in disturbance of natural systems (Lucentini et al. 2011). Despite its importance, temporal population structuring of mayfly populations has received little study (Lucentini et al. 2011).

Baetis tricaudatus is thought to be the most widespread *Baetis* species in North America and occurs throughout the continent, except in the extreme southeast (Morihara and McCafferty 1979, McCafferty et al. 2010). Larvae of this species are collected frequently in bioassessment and biomonitoring programs, and *B. tricaudatus* is one of the most abundant mayfly species in some systems, including a brown-water stream in Alberta, Canada (Clifford 1978) and the San Bernardino mountains in southern California (Spitzer 2014). Larvae play key roles in freshwater ecosystems as consumers of periphyton and particulate matter (Culp and Scrimgeour 1993) and as components in stream drift (Ciborowski 1983). *Baetis tricaudatus* populations also have dynamic life histories. For example, bivoltine *B. tricaudatus* populations have been observed in the northern part of its range in Saskatchewan (Webb 2002), whereas multivoltine *B. tricaudatus* populations have been observed in more central and southern parts of its range, including Idaho (Robinson et al. 1992).

We examined the genetic diversity of the COI gene region in *B. tricaudatus* collected from several sites in the Russian River drainage in northern California. Our objectives were to: 1) assess whether population structuring was present among years, seasons, or presumed cohorts; 2) assess whether population structuring was present among sites, streams, and watersheds; and 3) search for evidence to support cryptic diversity of *B. tricaudatus* at sites and times examined.

METHODS

Study area

The Russian River watershed in northern California drains ~3800 km^2 of Sonoma and Mendocino Counties and flows into the Pacific Ocean. The region has a Medi-

terranean climate, with 93% of precipitation occurring as rain during winter (NOAA 2009). The watershed is mostly rural–residential and agricultural, and grapes are the most notable and common crop (NOAA 2009).

The Russian River-mainstem collection site 1 (Fig. 1) was in a wide, sunny channel with thick riparian vegetation and mostly silt and gravel sediment. The mainstem is part of a managed system in which water flow is regulated by upstream dams, but at least one scouring event occurred each winter during the study period and probably affected the benthic fauna (Resh et al. 1988). Collection site 2 is ~11 km from site 1 (Table 1, Fig. 1) and is on Austin Creek, an unregulated tributary of the Russian River. Sediment at this site ranges from mostly large gravel in riffles to silt in pools. We chose sites 1 and 2 because high density of *B. tricaudatus* was expected year-round. We sampled 3 more sites in the Russian River watershed and 3 sites in the Sacramento River watershed (Table 1, Fig. 1). We selected these sites on the basis of the presence of *B. tricaudatus* and to provide ecological contrast (e.g., land use and elevation) to the Russian River sites.

Taxon sampling and identification

We made monthly collections at sites 1 and 2, and we sampled the other 6 sites at least once (Table 2). We made monthly collections at sites 1 and 2 from September 2012 to September 2015 (total = 37 events). Sampling events were ~1 mo apart to ensure collection of *B. tricaudatus* cohorts

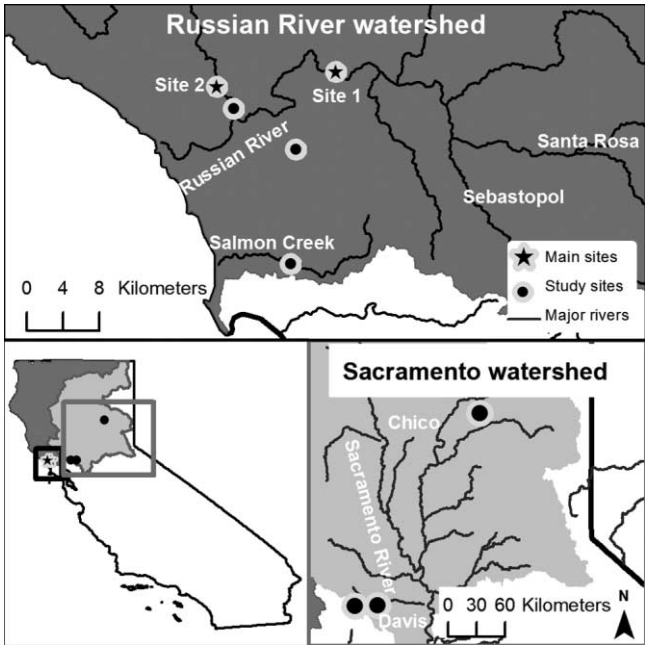

Figure 1. Map of collection sites. In the map of California (lower left), watersheds are delineated by hydrologic unit code (HUC) 6. Sites 1 and 2 are indicated with stars and the other 6 collecting sites are marked with circles.

Table 1. Global positioning system coordinates for all sites where *Baetis tricaudatus* samples were collected and successfully sequenced.

Site	Latitude (°N)	Longitude (°W)	Elevation (m)
Russian River watershed			
Russian River mainstem	38.504	−122.93	9
Austin Creek	38.511	−123.075	46
Salmon Creek	38.356	−123.004	30
Austin Creek Site 2	38.506	−123.07	20
Dutch Bill Creek	38.453	−122.984	53
Sacramento River watershed			
Capell Creek	38.495	−122.243	156
Putah Creek	38.492	−122.027	45
Schneider Creek	39.917	−121.065	1253

and to capture the natural variation of populations of *B. tricaudatus* within and between years. Two monthly collections at site 1 were more intensive than the rest to capture the genetic diversity within populations. One intensive collection was made before the rains in autumn 2014 (26 November 2014) and the other followed the rains in spring 2015 (15 May 2015). These 2 collection events are referred to as autumn and spring collections, respectively.

We collected larvae by disturbing the sediment in a variety of habitats, including riffles and pools, and catching suspended material with a D-frame net. We also used a D-net with a smaller mesh bag (500-μm) in an effort to capture early instars. To ensure consistent sampling effort among sites and dates, we collected for 20 min/sampling event, except for the 2 intensive sampling events, which were 40 min long.

We preserved specimens in 95% ethanol immediately after collection and transported them to the laboratory at University of California (UC) Berkeley for identification. All specimens were identified to the *rhodani* species group based on diagnostic characters outlined by Jacobus et al. (2014). Specimens were identified to species group because diagnostic characters for *B. tricaudatus* are sometimes indistinguishable and inconsistent (Jacobus et al. 2014). The *rhodani* group includes 4 species known from northern California: *B. tricaudatus, B. adonis*, *Baetis palisade*, and *Baetis piscatoris* (Meyer and McCafferty 2008). For consistency, all identifications were made by a single taxonomist (NSO) and more difficult identifications were confirmed by other experienced taxonomists (e.g., L. Jacobus from Indiana University–Purdue University of Columbus and J. Webb from Rhithron Associates Inc.).

DNA extraction, amplification, and sequencing

Following identification, we chose *rhodani* species group specimens for DNA extraction. The number of spec-

Table 2. Number of sequences, number of haplotypes (H_N), number of unique haplotypes (H_U), and % genetic diversity present in each temporal and spatial grouping based on all data and rarefied data. For temporal groups, numbers are from collections made within the Russian River watershed. Numbers in parentheses are calculated based on specimens collected from all sites.

	All data				Rarefied data			
Site/time	Sequences	H_N	H_U	% diversity	Sequences	H_N	H_U	% diversity
2012	4	3	0	0.9	–	–	–	–
2013	82 (89)	12 (14)	3 (5)	1.4 (1.5)	82	12	4	1.4
2014	113	20	10	1.5	82	14	5	1.1
2015	126 (127)	18 (19)	9 (10)	1.5 (1.5)	82	14	7	1.3
Spring cohort	113	13	7	1.2	65	9	4	1.3
Summer cohort	147 (148)	19 (20)	10 (10)	1.7 (1.7)	65	11	3	1.4
Autumn cohort	65 (72)	16 (18)	5 (7)	1.6 (1.7)	65	16	8	1.6
Spring	178	24	15	1.4	36	8	3	1.1
Summer	49 (50)	8 (9)	1 (1)	1.6 (1.6)	36	8	1	1.2
Autumn	62 (69)	16 (18)	5 (7)	1.6 (1.7)	36	11	5	1.6
Winter	36	7	2	1.1	36	7	2	1.1
Site 1	178	26	16	1.6	8	4	2	0.7
Site 2	128	18	9	1.7				
Russian River watershed	19	6	0	1.7	8	3	0	1.0
Sacramento River watershed	8	5	2	1.2	8	5	2	1.2
Autumn intensive	45	14	–	1.4	44	14	–	1.4
Spring intensive	44	10	–	1.2	44	10	–	1.2

imens selected from each monthly collection at sites 1 and 2 ranged from 0 to 14 individuals, based on the number of specimens collected. We sequenced 45 and 44 specimens, respectively, from the November 2014 and May 2015 intensive sampling events (Table 2) and extracted DNA from a total of 1 to 178 specimens from each of the 8 sites.

We used reagents from a Qiagen DNeasy DNA extraction kit (Qiagen, Alameda, California) to extract DNA with slight modifications to the manufacturer's protocol. We removed specimens from 95% ethanol and dried them on a clean tissue before putting them into microcentrifuge tubes, to which we added proteinase K and AL buffer. We lysed samples for ≥5 h at 56°C. After lysing, we placed samples in 95% ethanol and stored them as vouchers at the UC Berkeley Essig Museum of Entomology (EMEC numbers 1173400–1173692). We followed manufacturer's instructions for the rest of the protocol.

We amplified and sequenced the mitochondrial gene region cytochrome oxidase I (COI) because it is variable at the intraspecific level (Hebert et al. 2003a, b, Hajibabaei et al. 2006, Webb et al. 2012). Sequence divergence <2% is regarded as the amount of variation expected within populations or among individuals of the same species (Hebert et al. 2003b, Zhou et al. 2009, White et al. 2014), but the cutoff to differentiate species varies with taxon and study (DeWalt 2011). The COI gene region has been used effectively to identify *Baetis* species in previous studies (Webb et al. 2012, White et al. 2014). We used universal primers LCO 1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO 2198 (5′-TAAACTTCAGGGTGACCAAA-AAATCA-3′) (Folmer et al. 1994) to amplify a 658 base pair (bp) fragment of COI. Polymerase chain reaction (PCR) for COI was done with the following ratio of reagents: 17.5 μL sterile H_2O, 2.5μL iTaq (BioRad) buffer, 2.5 μL $MgCl_2$ (25 μM), 0.5 μL deoxynucleotide (dNTPs) (10 μM), 2.5 μL of each primer (10 μM), 0.25 μL iTaq polymerase, and 1 to 2 μL of the extracted template DNA. For amplification, the following protocol was used: 5 min initial denaturing at 94°C, 15 cycles of 30 s at 94°C, 30 s at 45°C, and 45 s at 72°C, 20 cycles of 30 s at 94°C, 30 s at 55°C, and 45 s at 72°C, and a final extension step of 72° C for 5 min.

We cleaned PCR products with Exonuclease I - Shrimp Alkaline Phosphatase (ExoSAP) following the manufacturer's protocol (Thermo Fisher Scientific, Waltham, Massachusetts). We incubated samples at 37°C for 15 min, then 80°C for another 15 min. Once cleaned, we sent PCR products to the UC Berkeley DNA Sequencing Facility.

Sequence editing and phylogenetics

We used the program Geneious Pro (version 6.1.4; Kearse et al. 2012) to create and edit contigs of individual sequence reads, build an alignment, generate basic sequence statistics,

and conduct phylogenetic analyses. Contigs were edited individually and most had 99 to 100% high-quality (HQ) bases. We discarded contigs with <80% HQ bases because base-pair calls were not reliable. We also discarded sequences with <80% overlap between forward and reverse reads to ensure that sequences used in analyses were effectively proofread by overlapping strands. Overall, 37 of 408 contigs were removed because of low-quality reads or insufficient overlap. Contigs were translated into amino acids to check for stop codons and shifts in reading frame that could indicate the presence of nuclear-mitochondrial copies (numts), but none were detected.

After editing, we made an alignment with the MAFFT Geneious Pro plugin (version 7.017; Katoh and Standley 2013) and uploaded it to Figshare (available from: https://figshare.com/s/c7f7b1c33f4600256586). We uploaded all sequences to the Barcode of Life Database (BOLD; samples: NAT1–NAT827) and GenBank (accession numbers: KY580901–KY581193). We used the Basic Local Alignment Search Tool (BLAST) to compare generated sequences against GenBank sequences from laboratories with well-established Ephemeroptera taxonomists.

We created phylogenetic trees to explore species identifications and to evaluate relationships among taxa sampled from northern California. jModelTest 2 (version 01.10; Darriba et al. 2012) indicated that the HKY85 substitution model best fit our data, so we used it to infer maximum likelihood trees with the Geneious Pro plugin PhyML (version 2.2.0; Guindon and Gascuel 2003). We assessed support for relationships by running 100 bootstrap replicates (Felsenstein 1985) and on the basis of selected GenBank sequences for the *B. tricaudatus* and *B. adonis* clades generated.

Population genetics analyses

For temporal analyses, we grouped genetic sequences from sites within the Russian River watershed and from all sites according to year, presumed cohort (January–April, May–August, September–December), and season (winter: December–February; spring: March–May; summer: June–August; and autumn: September–November) (Table 2). The presumed cohort grouping used may not represent actual cohort and emergence timing, which were not measured. However, cohorts were identified based on our field observations in the study area and provided an approximate estimate that is useful for comparison. We chose multiple temporal groupings to reflect a variety of life-history and temporal categories. For spatial analyses, we grouped sequences from sites 1 and 2 and compared them with: 1) sequences grouped from other sites within the watershed, and 2) sequences grouped from sites outside the watershed (Table 2) to reflect different scales of geographic distance.

Sample sizes in the temporal and spatial groups were unequal, so we performed analyses on: 1) all sequences and 2) standardized sample sizes (Table 2) based on rarefication (Szpiech et al. 2008). We removed sequences from 2012 from the rarefied comparison because only 4 sequences from 2012 existed. For the spatial analysis, we randomly chose 8 sequences from the 2 main sites and compared them to 8 sequences from other sites within the Russian River watershed and 8 sequences collected from the Sacramento River watershed. We compared analyses based on all sequences to analyses based on standardized sample sizes to understand the effect of sample size on observed genetic diversity and population genetic statistics.

Table 3. Population genetics statistics for each temporal and spatial grouping based on all data and rarefied data. ns = not significant, $p > 0.05$.

	All data		Rarefied data	
Grouping	Φ_{ST}	p	Φ_{ST}	p
Year	ns	<0.001	ns	<0.001
Presumed cohort	0.003	0.707	0.007	0.177
Season	0.003	0.263	0.019	0.071
Russian River watershed	ns	<0.001	ns	<0.001
All sites	0.008	0.05	0.098	0.049

We exported sequence alignments from Geneious Pro (version 6.1.4; Kearse et al. 2012) for further analyses. We imported nexus files to POPART (Population Analysis with Reticulate Trees) (Leigh and Bryant 2015) for analysis and FASTA files were converted to Arlequin project files (.arp) in PGDSpider (Lischer and Excoffier 2012) for analysis in Arlequin (version 3.5.1.2; Excoffier and Lischer 2010). We used these programs to describe intraspecific genetic variation and to create haplotype networks. Results were largely congruent, so our subsequent focus was on population genetic statistics generated in POPART, which were: Φ_{ST} (Table 3), number of haplotypes (H_N), and number of unique haplotypes (H_U; haplotypes that did not occur at any other site/time). We calculated Φ_{ST} with analysis of molecular variance (AMOVA; 1000 permutations) (Leigh and Bryant 2015). We grouped sequences based on collection times or locations as described above and calculated population genetic statistics and haplotype networks based on these groups. We used POPART to create median-joining haplotype networks to reconstruct intraspecific relationships and to identify temporal relationships among haplotypes.

RESULTS

Phylogenetic trees and clades

A total of 371 sequences identified as belonging to the *rhodani* species group were collected. Phylogenetic analyses indicated 2 well-supported clades: a *B. adonis* clade with 38 sequences (93% support) and a *B. tricaudatus* clade with 333 sequences (100% support). Based on the well-supported

B. adonis and *B. tricaudatus* clades and the much larger number of *B. tricaudatus* sequences, we focused further analyses on sequences within the *B. tricaudatus* clade.

Of the 333 sequences that fell into the *B. tricaudatus* clade, 178 were from site 1, 128 were from site 2, 19 were from other sites within the Russian River watershed, and 8 were from sites outside the watershed (Table 2). The *B. tricaudatus* sequences were generated from 29 of the monthly collections from 2012–2015 (Table 2). Of the 38 sequences that fell into the *B. adonis* clade, 24 were from site 1, 6 were from site 2, 6 were from other sites within the Russian River watershed, and 2 were from sites outside of the watershed. *Baetis adonis* sequences were collected on 17 sampling dates. Genetic divergence between the *B. tricaudatus* and *B. adonis* clades was ≥8.2%. Sequences within the *B. adonis* clade had 2% within-group diversity and sequences within the *B. tricaudatus* clade had 1.7% within-group diversity (Table 2).

Species identifications based on BLAST results were generally consistent with our own identifications. For both *B. tricaudatus* and *B. adonis* clades, many of our sequences were most similar to those from Southern California Coastal Water Research Project (SCCWRP) and Webb et al. (2012). For example, sequences in our *B. tricaudatus* clade were similar to *B. tricaudatus* sequences with accession numbers HQ938581 (from SCCWRP) and JQ663270 (Webb et al. 2012). Sequences within our *B. adonis* clade were similar to *B. adonis* sequences with accession numbers HQ941363 (from SCCWRP) and JQ661573 (from Webb et al. 2012).

Temporal dynamics of genetic diversity

We analyzed data in several ways to assess whether genetic diversity or population structure varied from year to year, between presumed cohorts, or from season to season. Analyses were done with all sequences generated for a given treatment (Appendix 1) and with rarefied sampling to control for unequal sampling between events (see below). Overall, genetic diversity in temporal samples varied by <0.5% (1.1–1.6%) across the rarefied annual, cohort, and seasonal comparisons within the Russian River watershed (Table 2). Population structuring was nonsignificant or very weak in the 3 temporal groupings, as indicated by the low Φ_{ST} values (Table 3). However, for presumed cohorts and season, *p*-values were >0.05, so accurate comparisons could not be made among the temporal groupings. Regardless, population structuring by time appeared very weak at best.

Annual comparisons. Annual variation in genetic diversity in rarefied comparisons varied by only 0.3%, from a low of 1.1% in 2014 to a high of 1.4% in 2013 (Table 2). Sample year 2013 had the lowest number of haplotypes (H_N = 12) and unique haplotypes (H_U = 4) in the rarefied samples (Table 2). The years 2014 and 2015 shared the highest H_N (14) while 2015 had the highest H_U (7) (Table 2). Two major haplotypes, A (N = 91 individuals, 37%) and B (N = 98 individuals, 40%), were present across the 3 years included in rarefied analyses (Fig. 2). Other minor haplotypes, most of which differed by only a single base-pair change from either Haplotype A or B also were present (Fig. 2). The total number of base-pair changes separating haplotypes ranged from 1 to 9 (Figs 2–7), with Haplotypes A and B differentiated by 2 base-pair changes (Figs 2–7).

Cohort comparisons. The presumed spring cohort had the lowest genetic diversity (1.3%) and the lowest number of haplotypes (9), but not the lowest number of unique haplotypes (the summer cohort had only 3; Table 2). The presumed autumn cohort had the highest genetic diversity (1.6%) and correspondingly high H_N (16) and H_U (8) (Table 2). Figure 3 shows the haplotype network for the presumed cohort comparisons. Haplotype A (N = 62 individuals, 32%) and Haplotype B (N = 85 individuals, 44%) were represented in similar proportions in each presumed cohort, revealing temporally well-mixed populations with no cryptic diversity.

Seasonal comparisons. Seasonal comparisons of genetic diversity showed the greatest variation (0.5%) among the temporal comparisons examined, ranging from a low of 1.1% in winter and spring samples to a high of 1.6% in autumn (Table 2). Absolute and unique haplotype numbers

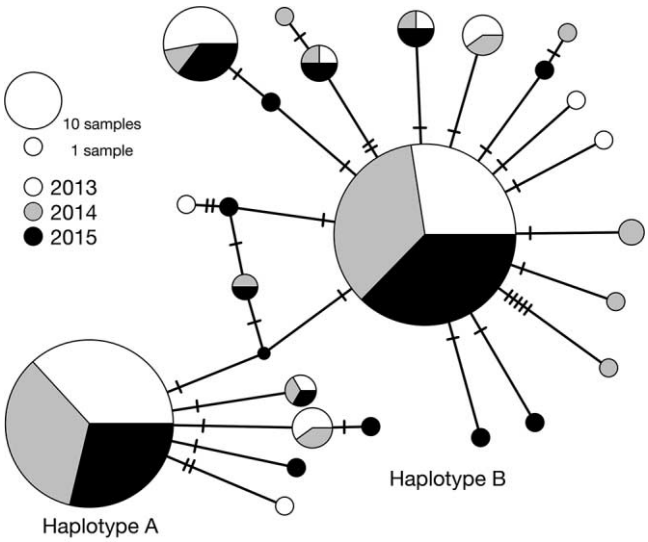

Figure 2. Median-joining haplotype network with rarefied data that indicates years that haplotypes were present. Circles represent haplotypes, and circle sizes represent the number of individuals with that haplotype. Shading corresponds to collection year, so solid circles represent haplotypes that were present in only 1 year. The small hash marks on lines connecting the different haplotypes represent base-pair changes, which are additive on either side of a circle.

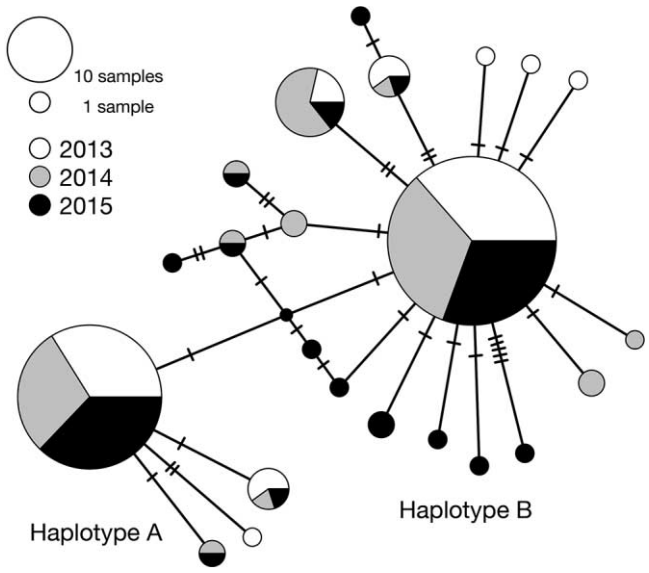

Figure 3. Median-joining haplotype network with rarefied data that indicates presumed cohorts in which haplotypes were present (spring: January–April, summer: May–August, autumn: September–December). See Fig. 2 for explanation of figure.

were correlated, with winter having the lowest H_N (7) and H_U (2), and autumn having the highest H_N (11) and H_U (5) (Table 2). As with the previous 2 comparisons, Haplotype A ($N = 49$ individuals, 34%) and Haplotype B ($N = 55$ individuals, 38%) were again represented in similar proportions among seasons. The haplotype network for seasonal comparisons showed no structuring or cryptic diversity (Fig. 4).

Intensive sampling events. The spring (2015) intensive sampling event at the Russian River mainstem site yielded 1.2% genetic diversity, or 71% of the genetic diversity collected within the Russian River watershed over the entire study period. The autumn (2014) intensive sampling event captured 1.4% genetic diversity, or 82% of the genetic diversity collected within the Russian River watershed over the entire study period. The autumn intensive sampling event also revealed a higher H_N (14) than did the spring intensive sampling event (10) (Table 2). Haplotype networks for the 2 intensive sampling events in November 2014 (Fig. 5) and May 2015 (Fig. 6) showed the higher diversity in autumn than in spring samples. Haplotypes A and B were captured in both events.

Spatial distribution of sampled haplotypes

Spatial analyses were done with all sequences generated for a given treatment (Appendix 2) and with rarefied sampling to control for unequal sampling among events (see below). Rarefied analyses of sequences grouped spatially indicated that diversity at sites 1 and 2 was lower (0.7%) than diversity captured at sites within the Russian River watershed (1.0%), which was lower than diversity captured at sites outside the watershed (1.2%) (Table 2). The low Φ_{ST} value indicated a well-mixed population with minor structuring spatially and no cryptic diversity (Table 3). The *p*-value associated with the rarefied spatial Φ_{ST} was <0.05. The small amount of population structuring spatially probably was the result of the presence of 2 unique haplotypes at Putah Creek site that were not collected at other locations. The haplotype network confirmed a population with little structuring (Fig. 7).

DISCUSSION

The COI gene region of sampled *B. tricaudatus* populations showed genetic diversity indicative of a single species with little or no temporal or spatial population structuring. The *rhodani* group species collected in our study, *B. tricaudatus* and *B. adonis*, formed distinct clades that had genetic differences expected between 2 congeneric species (Ball et al. 2005). Morphological differentiation between these species can be difficult or impossible, but our genetic approach supports the existence of 2 discrete species.

We found little evidence for cryptic diversity within *B. tricaudatus* from the Russian River, a result that differs from those of previous studies done on various ranges of this taxon (Webb et al. 2012, Spitzer 2014). A number of factors, including sample sizes, differing geographic coverage, and environmental factors may explain the conflicting results observed between our and earlier studies. For example, Webb et al. (2012) examined a large number of sites across most of North America, whereas populations were

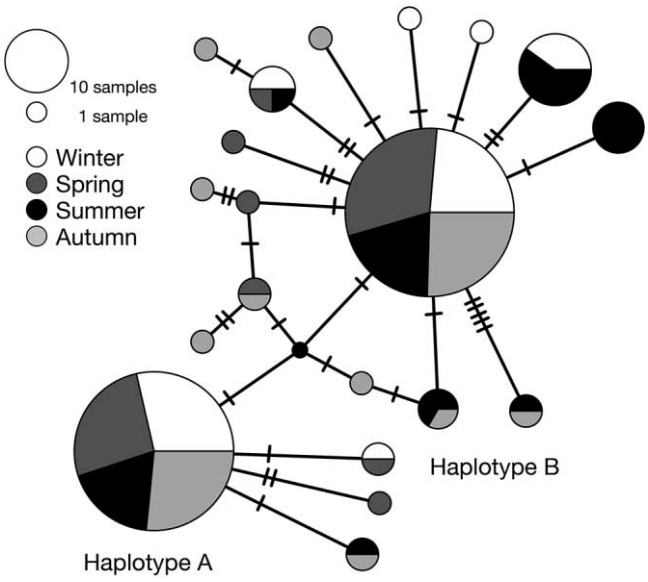

Figure 4. Median-joining haplotype network with rarefied data that indicates seasons when haplotypes were present (winter: December–February, spring: March–May, summer: June–August, autumn: September–November). See Fig. 2 for explanation of figure.

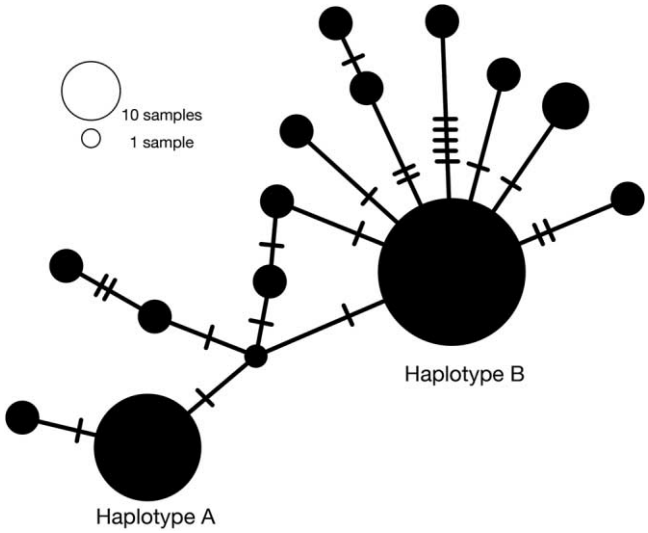

Figure 5. Median-joining haplotype network for intensive autumn (November 2014) sampling. See Fig. 2 for explanation of figure.

sampled from 2 neighboring watersheds in our study. Cryptic diversity is more likely to be detected over a broad than over a narrow geographic range. Spitzer (2014) sampled populations throughout the mountains of southern California across a geographic range similar in size to our study, but the drier intervening habitats in southern California may have limited dispersal among streams, increasing population structuring. Populations subject to the more extreme droughts in southern California may have increased genetic diversity as a result of genetic drift. For example, if Haplotypes A and B occurred at sites 1 and 2 but drought resulted in occurrence of Haplotype A only at site 1 and Haplotype B only at site 2, populations might appear more diverse than they really are.

Authors of studies based on DNA barcoding approaches often interpret cryptic diversity at different levels of sequence divergence. Maximum % divergence in our study was 1.7%. Some authors have recommended a conservative cutoff of ≥3% for cryptic species (Hebert et al. 2003a, Sweeney et al. 2011). Others consider populations diverging by only 1–2% as distinct species (Hebert et al. 2003b, Zhou et al. 2009, White et al. 2014). Based on the less conservative cutoff of 1–2%, cryptic species might have been suspected in our study, but the patterns of population structure based on haplotype networks provided no evidence of cryptic diversity. In comparison, authors of studies of *B. tricaudatus* populations (Jackson et al. 2014, Stein et al. 2014) have suggested that a 1% divergence in the COI gene region is sufficient diversity to support cryptic species. However, these authors did not report how the diversity they found was structured, e.g., via haplotype networks.

The lack of temporal population structuring of *B. tricaudatus* in our study differs from results found by Lucentini et al. (2011) in *B. rhodani*. Their molecular analyses of western European populations of *B. rhodani* supported the existence of 3 co-occurring, temporally segregated cryptic species (Lucentini et al. 2011). However, we found no evidence for temporal structuring in northern California populations of *B. tricaudatus*, in spite of the similar life-history characteristics between these 2 taxa; e.g., both species can be multivoltine and have dynamic life histories with differing voltinism in different habitats (Humpesch 1979, Brittain 1982, Robinson et al. 1992, Webb 2002).

Lucentini et al. (2011) hypothesized that temporal segregation of cryptic species within the *B. rhodani* species group arose from different emergence times of adult cohorts. We have limited information on emergence times, but we have no genetic evidence of temporally segregated cohorts in this system. Our data indicate that interbreeding does occur among cohorts, and *B. tricaudatus* emergence probably is less synchronous in the Russian River watershed than emergence of *B. rhodani* cohorts sampled by Lucentini et al. (2011) in western Europe. Our analyses support previous reports that larval *B. tricaudatus* populations often consist of multiple cohorts without discrete cohort emergence (Robinson et al. 1992, Webb 2002, Spitzer 2014).

Lucentini et al. (2011) suggested that cryptic diversity in *B. rhodani* was advantageous for resource partitioning and conferred a type of portfolio effect. A portfolio effect, or temporal structuring, did not appear to be the case for *B. tricaudatus* in the Russian River watershed because we did not find evidence for either cryptic species or temporal structuring of genetic diversity. Resource partitioning could

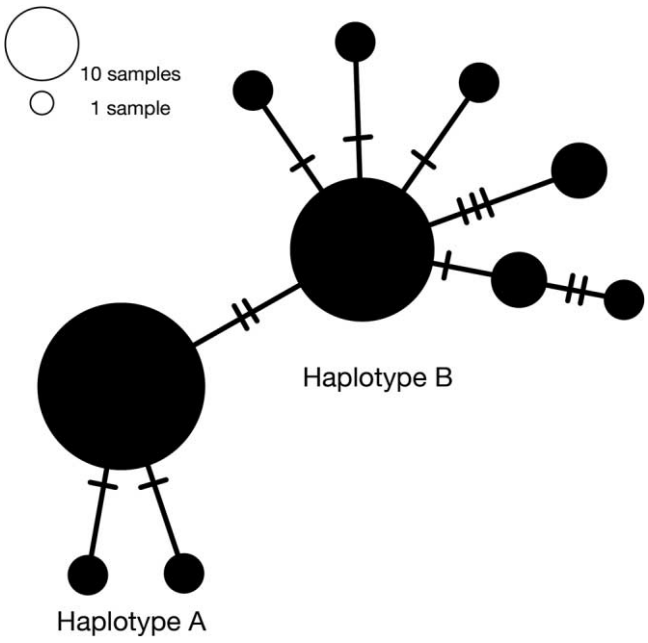

Figure 6. Median-joining haplotype network for intensive spring (May 2015) sampling. See Fig. 2 for explanation of figure.

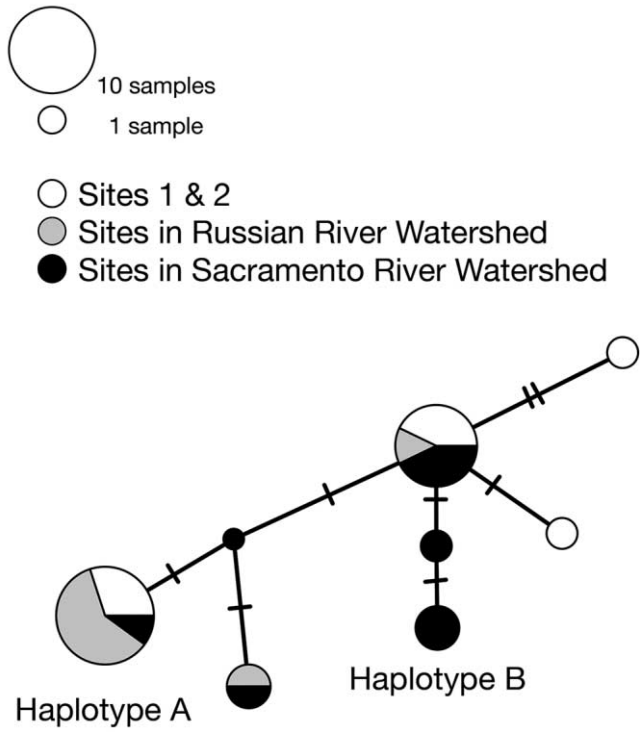

Figure 7. Median-joining haplotype network with rarefied data that indicates sites where haplotypes were collected. See Fig. 2 for explanation of figure.

be advantageous if sites were variable temporally, which is not the case for the Russian River mainstem site, which is in a managed river with relatively consistent flow throughout spring and summer. The lack of temporal genetic structuring in our study might be explained in part by phenotypic plasticity within the sampled populations. For example, Peckarsky et al. (2005) found that traits associated with local adaptations in streams with and without fish were plastic and not captured by analysis of COI diversity.

Our results also indicate that populations of *B. tricaudatus* are well-mixed within and between watersheds, a finding that has been reported for other species within *Baetis* (Monaghan et al. 2001, Hughes et al. 2003b, Peckarsky et al. 2005). Highly mobile larvae, such as those of *B. bicaudatus* (Peckarsky 1996), probably maintain mixing within watersheds, whereas dispersal by adults probably maintains mixing among watersheds, as has been reported for other *Baetis* species (Peckarsky et al. 2005).

Flight ability affects population structure. Jackson and Resh (1989) examined dispersal of adult aquatic insects in the Russian River watershed and found that species richness, number of individuals, and biomass decreased as distance from the stream increased. Petersen et al. (2004) caught >90% of mayflies within 60 m of their natal stream. Limited adult dispersal could partially explain the relationship between genetic diversity and geographic distance and the unique haplotypes found outside the watershed. The slight increase in genetic diversity (0.7% at sites 1 and 2; 1.0% at other sites within the watershed; and 1.2% at sites outside the watershed) that corresponded with increased distances between sites might indicate the amount of gene flow that occurs at varying spatial scales. Nevertheless, gene flow certainly occurs between sampled *B. tricaudatus* populations, as indicated by the similar proportions of the same 2 dominant haplotypes across sampling sites and watersheds and the consistently low Φ_{ST} values.

Patterns in genetic structure can be the result of historical events and may not necessarily indicate current dispersal patterns. A population bottleneck in the mayfly *Ephemerella inconstans* was hypothesized as the cause of low levels of polymorphism across a large (200 km) and diverse (forested, agricultural, and residential) study area (Alexander and Lamp 2008). Our study had a similar range, so an historical event, such as a population bottleneck, could explain the low genetic diversity in our study.

The higher % diversity at Austin Creek (site 2) than over the entire study could indicate patchy recruitment by a small number of females. Patchy recruitment has been suggested in other studies of mayflies (Schmidt et al. 1995, Rebora et al. 2005). However, the 1.7% diversity in our study was spread out over time, as indicated in the haplotype networks, so we think patchy recruitment from only a few egg masses is unlikely. The higher diversity at site 2 could be a result of larger numbers of individuals, but additional research is needed to test this hypothesis. Our results indicate that gene flow between streams and watersheds in the study area is sufficient to keep populations temporally and spatially well-mixed.

Our autumn and summer intensive sampling results suggest that, for *B. tricaudatus* in the Russian River, most diversity within a watershed can be captured in a single intensive collection event. Bergsten et al. (2012), who studied *Agabini* diving beetles in the family Dytiscidae, found that a sample size of 70 individuals was necessary to capture 95% of intraspecific diversity, a finding in agreement with ours. However, results from studies in which evidence of cryptic diversity was found within a single watershed or neighboring watersheds (e.g., White et al. 2014, Jackson et al. 2014) suggest the need for more robust sampling schemes to fully capture the genetic diversity of a taxon.

Extensive cryptic diversity has been found in freshwater organisms (e.g., Funk et al. 1988, Lucentini et al. 2011, Webb et al. 2012) and has contributed to the ideas that freshwater biodiversity is underestimated and that molecular methods can help identify the true biodiversity of freshwater systems (Sweeney et al. 2011, Jackson et al. 2014). Our data support separation of *B. adonis* and *B. tricaudatus* as 2 species, despite the morphological similarity of their larvae. Interpretation of results, such as the value for % genetic divergence used to differentiate putative species, can change conclusions. The *B. tricaudatus* populations we sampled do not differ genetically more

than expected for a single species and have little or no genetic structure at the COI gene region. We acknowledge limitations associated with use of a single mitochondrial gene region and suggest continued research on temporal and spatial patterns of genetic diversity. An improved understanding of genetic diversity will be needed to conserve biodiversity and manage natural systems.

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APPENDIX 1

Nonrarefied temporal dynamics of genetic diversity

When all sequences were compared, % genetic diversity varied ≤0.8% (0.9–1.7%) among years, approximate generations, and seasons in the Russian River watershed. Diversity captured in 2012 (which had only 4 sequences) was the least among the years (0.9%) and corresponded with the lowest H_N (3) and H_U (0) among years. 2014 and 2015 both contained 1.5% genetic diversity, but 2014 had the highest H_N (20) and H_U (10) (Table 2). The presumed spring cohort contained the lowest diversity (1.2%), H_N (13), and H_U (7) of the presumed cohorts. The presumed summer cohort had the highest % diversity (1.7%), H_N (19), and H_U (10) of the presumed cohorts. Winter samples had the lowest % diversity (1.1%) and H_N (7). Summer and autumn had the highest % diversity (1.6%) of the seasons. However, summer also had the lowest H_U (1) among seasons, whereas spring had the highest H_N (24) and H_U (15) (Table 2).

APPENDIX 2

Non-rarefied spatial distribution of sampled haplotypes

When all sequences were considered, the maximum diversity captured within the Russian River watershed (1.7%) was present at site 2 (Table 2). In comparison, diversity among sequences collected from outside of the watershed was 1.2%. Spatial population structuring was very weak, as indicated by the low Φ_{ST} values (Table 3). When all sequences were compared, sites outside the Russian River watershed had the lowest H_N (5) and sites within the Russian River watershed (excluding sites 1 and 2) had the lowest H_U (0). Site 1 had the highest H_N (26) and H_U (16) (Table 2).