Phylogeographic comparison of five large-bodied aquatic insect species across the western USA

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Abstract: Glacial legacy, barriers to migration, and dispersal abilities are important determinants of intraspecific genetic diversity. Genetic comparisons can elucidate the distribution of genetic variants among populations, but for many groups of organisms the concordance of population genetic structure and historical refugia among co-occurring species remains unclear. We compared phylogeographic histories of 4 stoneflies (*Calineuria californica*, *Hesperoperla pacifica*, *Pteronarcys californica*, and *Pteronarcys princeps*) and 1 caddisfly (*Dicosmoecus gilvipes*) across their species ranges. Study species had large body and wing sizes that suggest strong flying ability and dispersal potential. Nevertheless, riverine habitat restrictions and mating behaviors can inhibit dispersal. We used mitochondrial and nuclear gene sequences to examine population genetic structure relative to potential past and present barriers to dispersal in the western USA. North–south population genetic structure was present for each species but was more pronounced for 2 stoneflies (*C. californica* and *P. californica*) and the caddisfly. For these 3 species, phylogenies indicated concordant clades north and south of San Francisco Bay, a large, saltwater estuary in California. Basal phylogenetic nodes and regional centers of haplotype diversity suggested common historical refugia in northern California or southern Oregon, similar to that found in previous studies of salamanders. For 1 stonefly (*C. californica*) and the caddisfly, distinct populations in the Sierra Nevada Mountains suggested potential barriers to gene flow. The presence of population genetic structure suggests vulnerability to loss of intraspecific diversity under climate change scenarios, particularly for populations at high elevations.

Key words: Plecoptera, Trichoptera, glacial refugia, life-history traits, biodiversity, North America, dispersal, population genetic structure

Physical, genetic, and historical factors govern the geographic distribution of genetic diversity within a species. Geographic barriers, whether glacial or landscape features, may affect dispersal among populations (Avise et al. 1987, Green et al. 1996, Avise 2000), as can a species' dispersal ability (Hughes 2007, Lehrian et al. 2010). Even when species distributions appear continuous, the genetic legacy of past events can result in cryptic hotspots of intraspecific diversity within the species range (Bálint et al. 2011). Whether because of historical barriers or biological behaviors, strong population genetic structure may isolate certain populations and make some species more susceptible than others to loss of cryptic biodiversity resulting from range contractions associated with global climate change (Pauls et al. 2013).

Physical barriers, such as mountain ranges and inhospitable habitat, can restrict dispersal with the result that populations that are geographically close have reduced gene flow (Wake 1997). Glacial oscillations in temperate climates can act as barriers, reinforcing differences between populations and perhaps leading to speciation (Brunsfeld et al. 2001). Periods of glacial advancement fragment populations as species retreat to 1 or more isolated refugia, which can increase interpopulation diversity through drift, mutation, or natural selection operating on local populations (Hughes et al. 2009, Kuchta et al. 2009). As glaciers retreat and refugial populations become reconnected, genetic diversity may increase relative to preglaciation levels (Avise 2000).

Biological behaviors, physiological tolerances, and life-history traits also can either enhance or restrict dispersal potential (Bunn and Hughes 2007). For example, salamanders (Campbell Grant et al. 2010) and the aquatic stages of some insects (Finn et al. 2007) can physiologically tolerate the terrestrial environment for certain time intervals, and

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therefore, can enhance dispersal by crawling between small headwater streams. In contrast, behaviors related to mating, such as territoriality, or energetic tradeoffs, such as energy expended for wing growth vs egg production, can restrict gene flow (Zera and Harshman 2001, Hanski et al. 2006). Some marine invertebrate larvae are physiologically able to survive longer in the pelagic zone than others and can drift farther and increase gene flow among populations (Doherty et al. 1995, Dawson et al. 2014), whereas aquatic insects that emerge in autumn or winter can be constrained by cool temperatures during their window of dispersal (Briers et al. 2003, Lehrian et al. 2010). Regardless of cause, increased dispersal ability enables colonization of new habitats and maintenance of gene flow among populations, whereas limitations to dispersal can increase genetic differences among isolated populations through genetic drift or natural selection (Slatkin 1985, Bilton et al. 2001).

Gene flow among populations of aquatic insects from different river drainages requires individuals to disperse across terrestrial landscapes and, potentially, past physical barriers. The primary opportunity for dispersal of aquatic insects between rivers is during the winged, adult life-history stage. However, even with wings to promote dispersal, some insects have common behaviors (e.g., territoriality of dragonflies; Kormondy 1961) or short adult lifespans (e.g., mayflies; Merritt et al. 2008) that may restrict actualized dispersal. Long-distance dispersal is extremely difficult to study by using marked individuals (e.g., McCauley 2010), so genetic markers have been widely used to understand evolutionary history and infer species dispersal (Pauls et al. 2006, Hughes et al. 2009). Furthermore, genetic markers can be used to identify intraspecific diversity relative to geography and to infer the history of dispersal for a species (Theissinger et al. 2011, Pessino et al. 2014).

We used 5 common aquatic insect species, representing the orders Plecoptera and Trichoptera, that co-occur in the western USA to examine population genetic structure across their species ranges. We used phylogenetic and population genetic methods to assess the distribution of genetic diversity in association with current geographic barriers, historical glaciation patterns, and species behaviors and traits. Our goals were to: 1) assess geographic concordance in population genetic structure among species, 2) examine the degree of cryptic genetic diversity within each species, and 3) infer the phylogeographic history of each species.

METHODS

Study organisms

The stoneflies *Hesperoperla pacifica* (Perlidae), *Calineuria californica* (Perlidae), *Pteronarcys californica* (Pteronarcyidae), and *Pteronarcys princeps* (Pteronarcyidae), and the caddisfly *Dicosmoecus gilvipes* (Limnephilidae) are common species occurring west of the Rocky Mountains, USA. These species are large (>20 mm), have similar ranges, and perform a range of ecological functions within the stream ecosystem (Merritt et al. 2008). *Hesperoperla pacifica* and *C. californica* are predatory stoneflies that co-occur in western North America as far north as British Columbia (Fig. 1), but the southern limits of their ranges vary. Only *C. californica* is found in southern California, and *H. pacifica* is more common in Arizona, Rocky Mountain streams, and Montana than is *C. californica* (Sheldon 1980, Stewart and Stark 2008).

Pteronarcys californica and *P. princeps* are shredders that feed on the microbial communities associated with submerged leaf litter (Merritt et al. 2008). *Pteronarcys californica* is found primarily in large, lower-elevation rivers along the Pacific coast and in the Great Basin, whereas *P. princeps* is found primarily in small streams in California, particularly at higher elevations (Baumann 1979). In contrast, *D. gilvipes* is a grazer of the periphyton on submerged rocks and is found at both low and high elevations in California, Oregon, and Washington (Resh et al. 2011).

All study species have anatomical traits that would be likely to promote dispersal. Compared to other aquatic insects, they are relatively large bodied (>20 mm) and large winged (20–30 mm), which suggests strong flying ability and, consequently, high dispersal potential (Stark and Gaufin 1974). However, stoneflies in general are thought to have relatively short lifespans (2–4 wk) and high incidence of brachyptery (Stewart and Stark 2008), both of which may limit long-distance travel. Brachyptery is relatively rare in caddisflies (Holzenthal et al. 2007), and emergence windows for *D. gilvipes* are relatively longer (~8 wk) than for stoneflies, suggesting greater potential for dispersal.

Each of these species has behaviors related to mating systems that may limit their dispersal. For example, mating systems for many stoneflies, including the species in our study, rely on auditory communication between males and females (Maketon and Stewart 1984, Sandberg 2011). Adult males 'drum' on logs and other riverside structures, create cadences of rhythmic sounds, and females receiving this sound can respond with different cadences. This short-distance communication may tend to discourage widespread dispersal.

In contrast, *D. gilvipes*, like many limnephilid caddisflies, relies on pheromone communication between females and males (Resh and Wood 1985). From observational studies, caddisflies as a group are thought to be better fliers than stoneflies (Jackson and Resh 1989, Wiggins 2002), and limnephilids fly farthest relative to other caddisflies from a Swedish stream (Svensson 1974). However, like drumming, pheromones are short-distance strategies for finding mates and, when combined with short adult lifespans, these factors may cause adults to remain close to the stream from which they emerged and consequently, may limit their dispersal ability.

We chose closely related co-occurring species as outgroups for each study species. The caddisfly *Allocosmoecus partitus* ($n = 10$ specimens from 2 study sites) served as out-

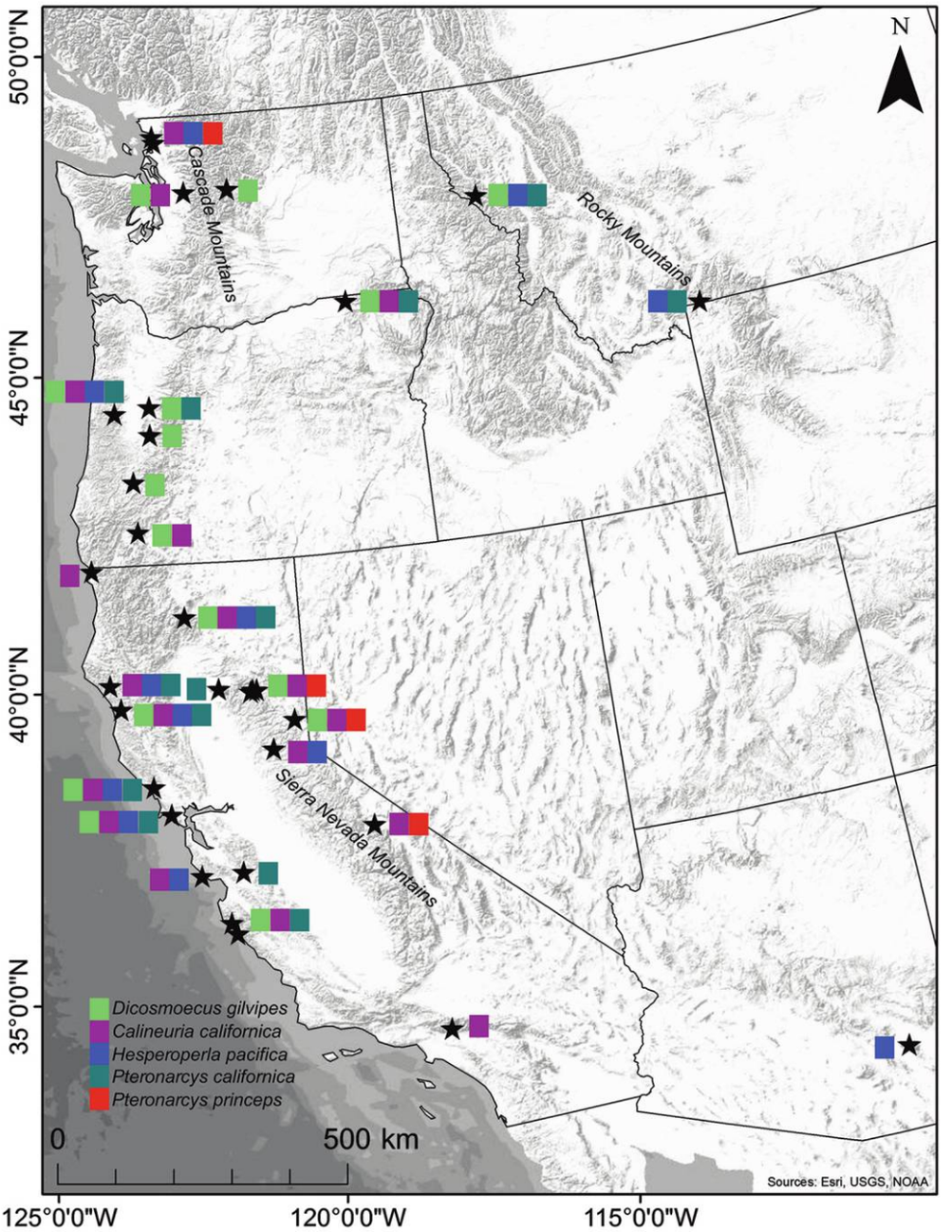

Figure 1. Map of stream sampling locations in western North America. Individual species (*Dicosmoecus gilvipes, Calineuria californica, Hesperoperla pacifica, Pteronarcys californica, Pteronarcys princeps*) collected at each site are coded by color.

group for *D. gilvipes* because these species co-occur in the western USA and are both limnephilids. The stonefly *Doroneuria baumanni* (n = 10 specimens from 3 study sites) is in the family Perlidae and commonly occurs with *C. californica* throughout the study area. The stonefly *P. princeps* is sister species to and has overlapping geographic distribution with *P. californica* so they served as outgroups for each other. The stonefly *Hesperoperla hoguei* is a sister species of *H. pacifica*, but it is rare, so *H. hoguei* (n = 3 specimens from 2 study sites) and *D. baumanni* were used as outgroups for *H. pacifica*.

Study locations

We collected late-instar larvae/nymphs of each species from streams along the west coast of the USA (Fig. 1, Appendix S1), which included locations representing most of

the elevational range for each species. The streams sampled encompassed most of the north–south range of these species, a span of nearly 2000 km, and for some species, as far east as the Rocky Mountains of Montana, a span of 1200 km. We collected >100 specimens from >12 watersheds/species (Table 1). *Pteronarcys californica* and *P. princeps* were treated as 1 group for phylogenetic analyses because both species co-occur with *D. gilvipes*, *C. californica*, and *H. pacifica*. This approach enabled broad-level comparison of genetic diversity across the entire study area. In particular, the combination of *P. californica* and *P. princeps* furthered assessment of phylogeographic concordance among species for lowland (<300 m) vs montane (>700 m) study sites.

Prior to DNA extraction, we identified all specimens to species level with the aid of keys provided for stoneflies by Stewart and Stark (2008) and for caddisflies by Wiggins (2004). A subset of *P. princeps* and *P. californica* specimens were confirmed by R. Baumann (Department of Biology and Monte L. Bean Life Science Museum, Brigham Young University, Utah). We stored all specimens in 95% ethyl alcohol immediately after collection and deposited voucher specimens at the University of California, Berkeley Essig Museum of Entomology.

Molecular laboratory methods

We removed several legs (large specimens) or the head (smaller specimens) from the insect body with forceps and dried them. We extracted DNA with the Qiagen DNeasy Extraction kit (Qiagen, Alameda, California). We amplified 2 mitochondrial genes, cytochrome *c* oxidase subunit I (COI) and subunit II (COII), and 1 nuclear gene, *wingless*, with primers described in previous insect phylogenetic studies (Table 2). We chose COI and COII based on studies (e.g., Beckenbach et al. 1993, Simon et al. 1994, Lessios 2008, Yassin et al. 2010) indicating that their rate of evolution is rapid enough to differentiate among populations and closely related species. Furthermore, COI is commonly used in species-level barcoding for aquatic insects (Sweeney et al. 2011). We chose *wingless* as a more conservative marker of intraspecific diversity (Brower and DeSalle 1998) and used the primers demonstrated in European limnephilid caddisflies (Pauls et al. 2008) and the European stonefly *Dinocras cephalotes* (Elbrecht et al. 2014). We sequenced all specimens for COI and COII and ≥33% of specimens for each species for *wingless*, with multiple representatives from all geographic areas.

Table 1. Total specimens collected and number of sampling locations for *Calineuria californica*, *Dicosmoecus gilvipes*, *Pteronarcys californica*, *Pteronarcys princeps*, and *Hesperoperla pacifica*.

Family	Species	Sampling locations	Specimens sequenced
Perlidae	*C. californica*	22	165
Perlidae	*H. pacifica*	15	120
Pteronarcyidae	*P. californica*	13	110
Pteronarcyidae	*P. princeps*	5	30
Limnephilidae	*D. gilvipes*	17	145

We performed polymerase chain reaction (PCR) for COI based on the following protocol: 5 min initial denaturing step 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. For each 2 μL of extracted template DNA, the reaction consisted of 17.5 μL nano-pure H_2O, 2.5 μL iTaq (Bio-Rad, Hercules, California) buffer, 2.5 μL $MgCl_2$ (25 μM), 0.5 μL dNTPs (10 μM), 1.5 μL of each primer (10 μM), and 0.15 μL iTaq polymerase. We performed PCR for COII based on the following protocol: 5 min initial denaturing step at 94°C; 35 cycles of 30 s at 94°C, 30 s at 53°C, and 45 s at 72°C; and a final extension step of 72°C for 5 min. For each 1 μL of extracted template DNA, the reaction consisted of 17.5 μL nano-pure H_2O, 2.5 μL iTaq buffer, 3.0 μL $MgCl_2$ (25 μM), 0.5 μL dNTPs (10 μM), 1.5 μL of each primer (10 μM), and 0.15 μL iTaq polymerase. We performed PCR for *wingless* based on the following protocol: 5 min initial denaturing step at 94°C; 30 cycles of 30 s at 94°C, 30 s at 59°C, and 45 s at 72°C; and a final extension step of 72°C for 5 min. For each 2 μL of extracted template DNA, the reaction consisted of 13.5 μL nano-pure H_2O, 2.5 μL iTaq buffer, 3.0 μL $MgCl_2$ (25 μM), 0.5 μL dNTPs (10 μM), 1.5 μL of each primer (10 μM), and 0.15 μL iTaq polymerase.

PCR amplification was confirmed based on agarose gel electrophoresis. We cleaned successfully amplified PCR products with Exonuclease I - Shrimp Alkaline Phosphatase (ExoSAP; Thermo Fisher Scientific, Waltham, Massachusetts) with the following ratio of reagents: 5 μL PCR product, 0.5 μL Exonuclease I, 0.5 μL Exonuclease I Buffer, 0.5 μL FastAP, and 1.0 μL nano-pure H_2O. ExoSAP conditions were 15 min at 37°C, followed by 15 min at 80°C. Amplicons were sequenced in both directions at the University of California, Berkeley DNA Sequencing Facility.

Sequence alignment and phylogenetic analyses

We assembled and edited sequences in Geneious Pro (version 8; Kearse et al. 2012) and created alignments with the MAFFT plugin (Katoh and Standley 2013). Sequence lengths were edited, and the number of base pairs analyzed was consistently 655 for COI, 680 for COII, and 400 for *wingless*. Sequences can be accessed at GenBank, National Center for Biotechnology Information (accession no. KX218458–KX219574).

We used individual gene sequences and concatenated sequences for each sample to create phylogenetic trees based on maximum likelihood (ML) and Bayesian inference. We used the program PhyML (version 2.2.0; Guindon and Gascuel 2003) plugin for Geneious Pro to perform maximum

Table 2. Primers and references for mitochondrial and nuclear genes sequenced. COI = cytochrome *c* oxidase subunit I, COII = cytochrome *c* oxidase subunit II.

Gene	Name	Primer sequence	Reference
COI	2198 (HCO)	5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′	Folmer et al. 1994
	1490 (LCO)	5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′	
COII	3037	5′-ATG GCA GAT TAG TGC AAT GG-3′	Liu and Beckenbach 1992
	3791	5′-GTT TAA GAG ACC AGT ACT TG-3′	
wingless	Wingnut1	5′-GAA ATG CGN CAR GAR TGY AA-3′	Pauls et al. 2008
	Wingnut3	5′-ACY TCR CAR CAC CAR TGR AA-3′	

likelihood analyses with the substitution model HKY85, selected based on jModelTest (Posada 2008). We assessed support for relationships on each phylogeny by performing 500 bootstrap replicates (Felsenstein 1985). For Bayesian trees, we used the program MrBayes (version 3.2.5; Ronquist and Huelsenbeck 2003) in Geneious Pro with substitution model HKY85, 1,100,000 Monte Carlo–Markov Chain length, and 100,000 iteration burn-in length. We considered clades with >70% of bootstraps in ML analyses and 90 to 100% Bayesian posterior probability well supported. To address unequal sample sizes, we created additional phylogenetic trees for each species based only on watersheds with ≥7 specimens to corroborate population genetic structure found with phylogenetic trees based on all watersheds.

Population genetic analyses

We calculated nucleotide diversity and haplotype diversity to describe intrapopulation genetic variation in Arlequin (version 3.5.1.2; Excoffier and Lischer 2010). In addition, we used Arlequin to estimate population genetic structure using analysis of molecular variance (AMOVA) and global φST (AMOVA, Kimura-2-parameter distance, 10,000 bootstrap replicates). Further, to identify relationships among haplotypes across sampling sites, we reconstructed relationships within each species based on a median-joining network in PopART (Leigh and Bryant 2015). We inferred ancestral populations of species from the concordance of basal branches of Bayesian and ML phylogenetic trees and historical refugia based on the centers of highest haplotype diversity in median-joining haplotype networks. For haplotype richness, we included rarefaction in analyses with the software ADZE (version 1.0; Szpiech et al. 2008) based on the smallest sample size in each species. We excluded sites with ≤2 specimens. Therefore, rarefied richness was calculated at $N = 6$ for *P. californica*, $N = 5$ for *D. gilvipes*, and $N = 4$ for *H. pacifica*, *C. californica*, and *P. princeps*.

RESULTS

Geographic distribution of genetic diversity

The study species have similar distribution, but they do show some differences in occurrence across their reported ranges. Among the *Pteronarcys* stoneflies, *P. californica* was found only in lowland streams, and *P. princeps* was found primarily in mountain streams of the Sierra Nevada Mountains (Fig. 1). *Calineuria californica*, *D. gilvipes*, and *H. pacifica* were collected from both lowland and montane streams. *Calineuria californica* and *H. pacifica* were found in southern California and Arizona, respectively, which was further south than the other species and occurred in streams that were geographically remote (>500 km) from other collection sites. *Dicosmoecus gilvipes*, *P. californica*, and *H. pacifica* were found in Montana streams, whereas *C. californica* was found only as far east as eastern Washington (Fig. 1).

Population genetic structuring was strong for *D. gilvipes* with distinct north–south clades (Figs 2, 3A). Both Bayesian and ML trees of concatenated genes supported a separate population in 2 coastal streams near Big Sur, which is south of San Francisco and Monterey Bays in California (Fig. 2). Within the northern clade, Bayesian trees suggested further north–south distinctions between a Montana/Washington/Oregon clade and a northern California/Oregon clade (Fig. 2), but ML trees did not show this relationship. Both Bayesian and ML trees of concatenated genes indicated a well-supported population in the Sierra Nevada Mountains. Bayesian and ML trees of only *wingless* gene sequences also showed strong support for a Sierra Nevada Mountain population of *D. gilvipes*, but no support for different populations among all remaining streams.

Calineuria californica had similar overall population genetic structure to *D. gilvipes* (Fig. 2), but with additional populations in Washington State and the San Gabriel Mountains of southern California, a site where no other study species were found. Concatenated trees indicated distinct populations along a north–south gradient, including well-supported clades in northern Washington, Oregon/northern California, California central coast, and southern California (Fig. 2). Both approaches indicated a well-supported Sierra Nevada Mountain clade. Similar to *D. gilvipes*, *wingless* gene trees showed strong support for a Sierra Nevada Mountain population of *C. californica*, and no support for different populations among the remaining streams.

Pteronarcys californica had the most pronounced population structure among study species (Fig. 2). We found

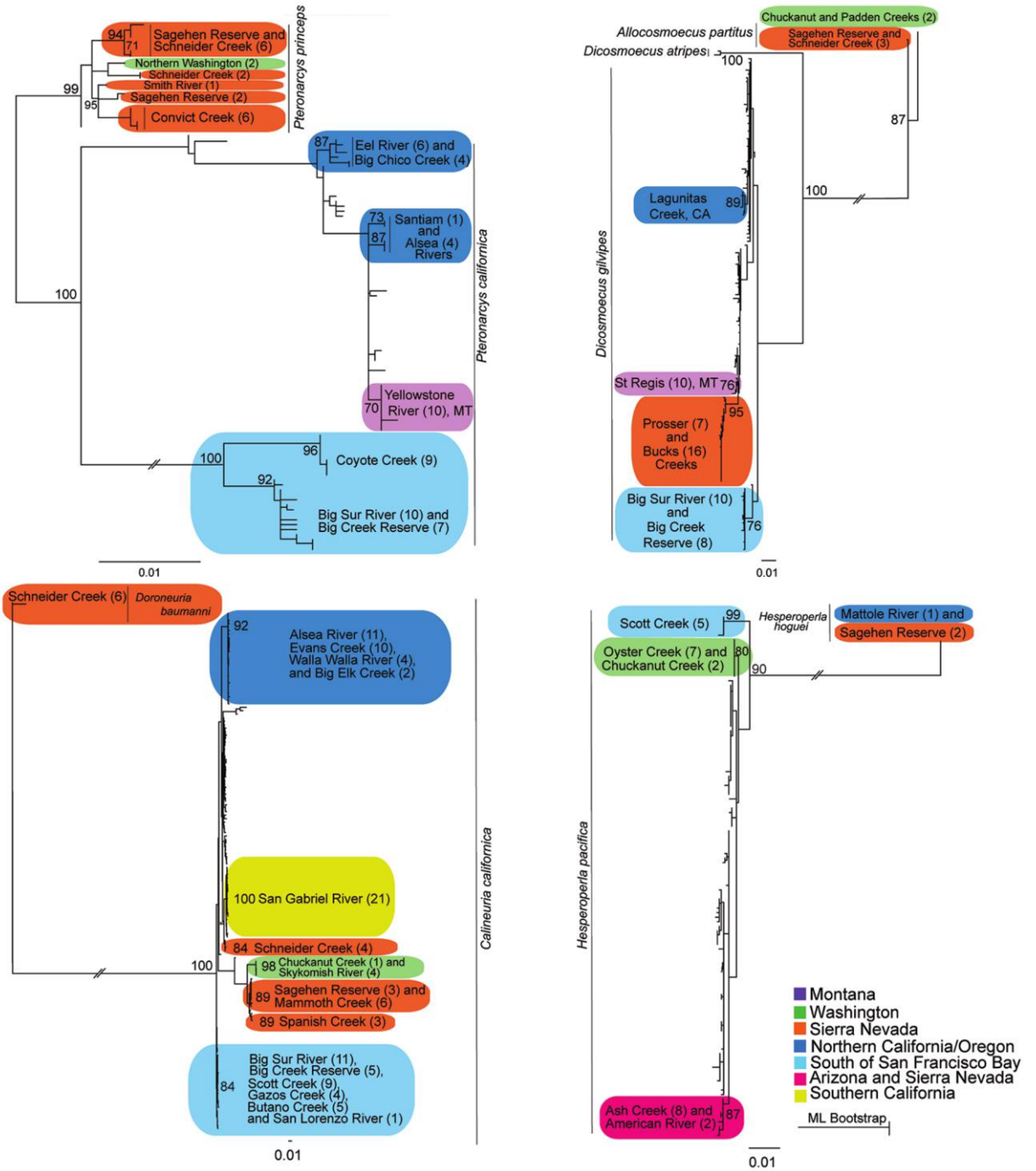

Figure 2. Comparison of concatenated (cytochrome *c* oxidase subunit I and II and *wingless* genes) maximum likelihood (ML) phylogenetic trees for 5 study taxa: *Dicosmoecus gilvipes, Calineuria californica, Hesperoperla pacifica, Pteronarcys californica, Pteronarcys princeps*, and outgroups. *Pteronarcys californica* and *P. princeps* were combined for phylogenetic analysis. ML bootstrap percentages are noted for clades ≥70%, and these clades all were supported by Bayesian posterior probability values of 90 to 100%. Color codes represent geographic regions and identify populations where ML bootstrap percentages were ≥70% and Bayesian posterior probability values were 90 to 100%.

broad north–south structure similar to that of *D. gilvipes* and *C. californica*, with San Francisco Bay as the break point. Among specimens collected south of San Francisco Bay, concatenated trees indicated 2 populations, 1 at coastal sites near Big Sur, California, and 1 composed of specimens from Coyote Creek, California, which occurs 150 km inland (Fig. 1). In addition, concatenated trees indicated a distinct population from the Yellowstone River in southern Montana

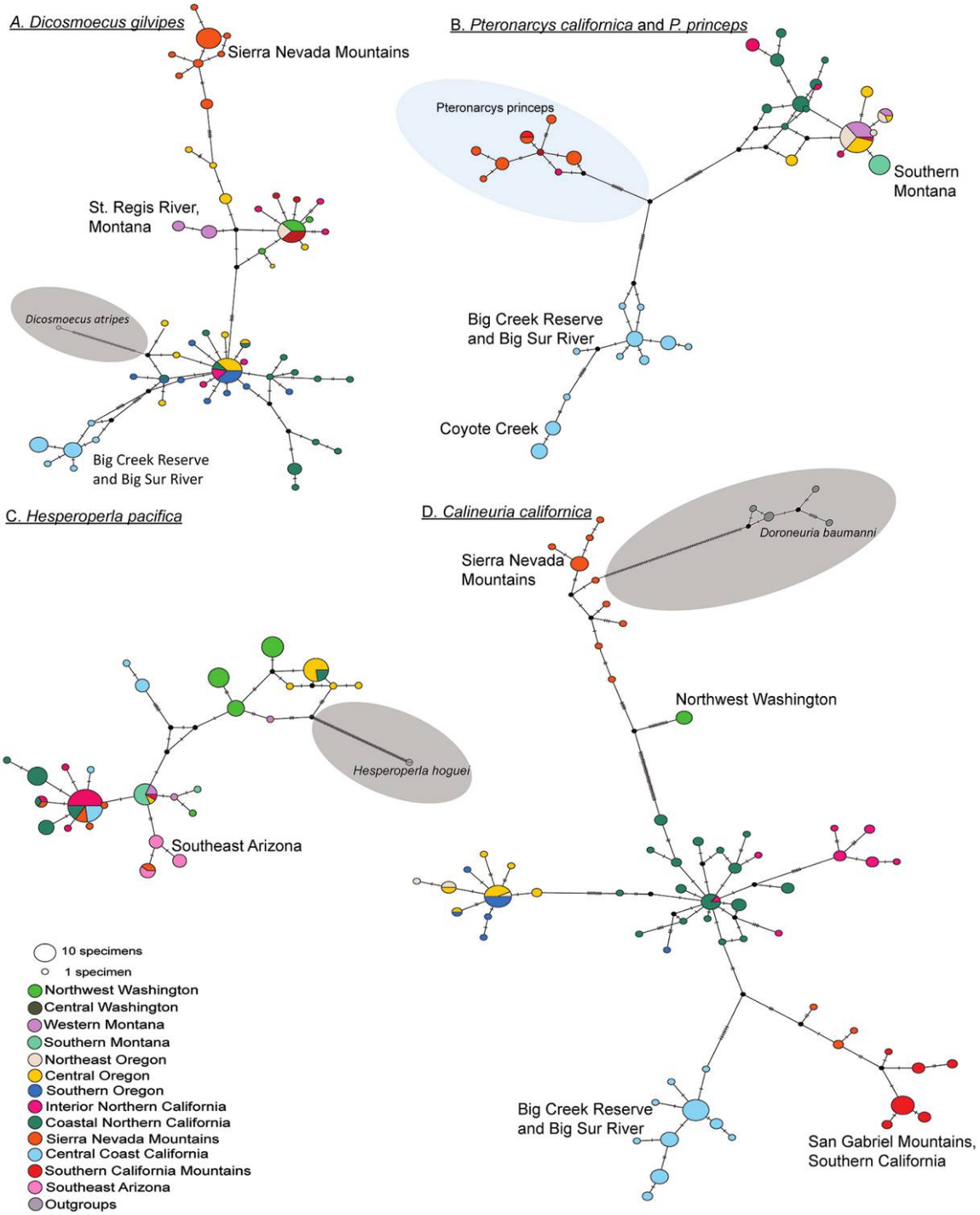

Figure 3. Comparison of median-joining haplotype networks for the 5 study taxa: *Dicosmoecus gilvipes* (A), *Pteronarcys californica* and *Pteronarcys princeps* (B), *Hesperoperla pacifica* (C), and *Calineuria californica* (D). *Pteronarcys californica* and *P. princeps* (shaded light blue) are combined. Haplotype networks were generated from concatenated sequences of cytochrome *c* oxidase subunit I and II and *wingless* genes. Circles represent individual haplotypes and geographic regions are coded by colors, which are consistent across taxa. The size of the circle represents the number of individuals sharing that haplotype. Short hash-marks perpendicular to haplotype branches indicate the number of basepair differences between haplotypes. Certain study sites are labeled for increased clarity, and outgroups are shaded in gray.

(Fig. 2). *Wingless* gene trees indicated northern (Washington and Montana) and southern (northern California and Oregon) populations of *P. californica*. In addition, the difference between *P. californica* and *P. princeps* specimens was 4 to 6.25% for mitochondrial sequences (COI + COII) and 1.5 to 3.5% for *wingless*.

Hesperoperla pacifica had less population structure than the other species (Fig. 2). Concatenated gene trees showed strong support for a distinct population composed of some specimens from 2 streams in northern Washington and a distinct population composed of some, but not all, specimens of Scott Creek, California, which occurs south of San Francisco Bay. Phylogenetic trees also supported a clade that included all specimens from Ash Creek, a montane stream in Arizona that was the most remote site (600 km from the nearest site and 3500 km from the farthest), and 2 specimens from the Sierra Nevada Mountains in California. *Wingless* gene trees showed no support for population structure in *H. pacifica*.

In summary, north–south population genetic structure was present in each species, but was more subtle in *H. pacifica*. In addition, concatenated gene trees revealed genetic diversity between Sierra Nevada Mountain and lowland populations for *D. gilvipes*, *C. californica*, and the 2 species of *Pteronarcys*. *Calineuria californica* and *D. gilvipes* each had distinct populations composed of specimens from Sierra Nevada Mountain streams. For *Pteronarcys*, gene trees indicated strong support for genetic separation between lowland *P. californica* specimens and *P. princeps* of Sierra Nevada Mountain streams.

Historical refugia

For *C. californica*, *D. gilvipes*, and *P. californica*, Bayesian inference and ML trees demonstrated basal branching of California clades relative to clades representing Oregon, Washington, and Montana (Fig. 2). Haplotype networks indicated northern California and in some cases southern Oregon streams as the centers of high haplotype diversity (Fig. 3A–D). Mean haplotype richness for *C. californica*, *D. gilvipes*, and *P. californica* was higher at sites in northern California/Oregon than in Washington or Montana (Fig. 3A, B, D, Table 3). *Hesperoperla pacifica* showed basal branching for some California specimens, but with less resolution. Haplotype networks for *H. pacifica* also showed less clarity of haplotype richness patterns, but the centers of highest diversity occurred in both northern California and southern Oregon streams (Fig. 3C).

DISCUSSION

Glacial legacy

Broad concordance in population genetic structure suggests that the co-occurring species *C. californica*, *P. californica*, and *D. gilvipes* probably have had similar historical and current geographic barriers, despite representing 3 families in 2 orders. In particular, genetic structure along the north–south axis of the range of each of the study species suggests the genetic legacy of glacial refugia and population expansion. For example, phylogenetic trees of *D. gilvipes*, *C. californica*, and *P. californica* show north–south population breakpoints and, in the case of *C. californica*, several population breakpoints from southern California to Washington. In addition, haplotype distribution in these 3 species indicates fewer haplotypes in Washington, and for *P. californica* and *D. gilvipes* in Montana, the northernmost sites, results that suggest post-glacial expansion into these regions. *Hesperoperla pacifica* had distinct populations in the northern and southern portions of their range, but with only subtle indications of refugia and expansion.

Other taxa (e.g., salamanders) studied in western North America (Steele et al. 2005) and aquatic insects in central Europe (Pauls et al. 2006, Engelhardt et al. 2011) show similar genetic differences attributable to isolation of ancestral populations in multiple southern refugia during periods of glacial maxima. During the last glacial maximum in Europe, multiple aquatic species, including vertebrates and invertebrates, persisted in ≥1 refugia in southern Europe and expanded to new habitats as glaciers receded northward. Similar patterns may have resulted from the last period of glaciation (2.5 million to 11,000 y ago) in northwestern North America, where glaciers may have isolated populations of many northern species in ≥1 southern refugia (Wake 1997, Steele et al. 2005).

Phylogenies and haplotype networks suggest ancestry in northern California or southern Oregon for *D. gilvipes*, *C. californica*, and *P. californica*. Phylogenetically basal populations for *D. gilvipes*, *C. californica*, and *P. californica* indicate a Californian ancestral population. Moreover, the comparatively high haplotype richness found in northern California and southern Oregon relative to in Washington or Montana for *D. gilvipes*, *C. californica*, *P. californica*, and to a lesser degree *H. pacifica*, also suggest that ancestral populations persisted in this geographic region. Regions of highest haplotype diversity often are regarded as past refugia and represent the full array of genetic diversity, which is a source of more recently established populations within the current range. In contrast, populations that were founded more recently often have signatures of founder effects or bottlenecks (Theissinger et al. 2011, Pessino et al. 2014). Previous studies suggest the Klamath–Siskiyou mountains in northern California as a potential Pleistocene glacial refugia for aquatic-associated rough-skinned newts, Pacific giant salamanders, and black salamanders (Kuchta and Tan 2005, Steele and Storfer 2006, Reilly et al. 2013). Some studies of salamanders have suggested multiple refugia, including the Columbia River in northern Oregon/Washington (Steele and Storfer 2006) or the Sacramento River in California (Reilly et al. 2013). Our sampling sites did not allow for fine resolution of refugia. All 3 species probably used refugia

in the northern California region, which suggests the potential for concordant refugial geography with co-occurring amphibians.

Geographic barriers

Population structure of *D. gilvipes, C. californica*, and *P. californica* align with several geographic barriers that may restrict insect dispersal on the Pacific coast of the USA and inland to east of the Sierra Nevada Mountains. At present, the arid environment of the California Central Valley provides a probable barrier between coastal and inland populations. The California Central Valley was a freshwater lake until 600,000 y ago (Sarna-Wojcicki et al. 1985, Dupre 1990), which would have acted as a barrier to east–west migration between mountain and coastal streams in California (Kuchta et al. 2009). Previous studies of *Ensatina* salamanders, whose inferred biogeography as a 'ring species' demonstrates the concept of dispersal around a central barrier, show evidence of the potential of the California Central Valley to affect the distribution of genetic diversity (Wake 1997, Kuchta et al. 2009). For the aquatic species in our study, the distinct Sierra Nevada Mountain clades vs lowland clades for *C. californica* and *D. gilvipes* may represent restricted dispersal across or around the California Central Valley (Fig. 1), but the locations of populations in our study do not provide the resolution needed to investigate the presence of a ring pattern.

The historical freshwater lake of the California Central Valley originally fed a large river with an outlet at Monterey Bay, so the Monterey region is an historical north–south break point for many coastal taxa, including reptiles (Feldman and Spicer 2006) and amphibians (Kuchta and Tan 2006, Rissler et al. 2006). Our results for *C. californica*, *P. californica*, and *D. gilvipes* differ from those of these herpetological studies because they did not demonstrate a break point at Monterey Bay, but rather at San Francisco Bay, a much younger geographic feature (Sarna-Wojcicki et al. 1985, Dupre 1990). San Francisco Bay is a saline estuary that is inhospitable to many taxa, including riverine insects, and presents a potential barrier to current dispersal north–south along the Pacific coast of California. For example, *H. pacifica* had a distinct population in Scott Creek, California, which lies north of Monterey Bay and south of San Francisco Bay, but the lack of a coastal location south of Monterey Bay where this species was found limits interpretation of a broader break point.

Multiple well-supported Sierra Nevada Mountain clades for *C. californica* and *P. princeps* and a single well-supported clade for *D. gilvipes* in the Sierra Nevada Mountains align with results from European studies that indicate montane habitats are important for intraspecific biodiversity (Pauls et al. 2006, Engelhardt et al. 2011, Taubmann et al. 2011). Other montane locations also harbored distinct populations of *C. californica* and *H. pacifica*. *Calineuria californica* had a distinct population in the San Gabriel Mountains, and *H. pacifica* had a distinct population composed of individuals from montane sites in Arizona and the Sierra Nevada Mountains.

Dispersal traits and behaviors

Despite being co-distributed, differences in population genetic structure exist among our study species, results suggesting differences in biological traits or dispersal potential. For these species, winged flight, large body size, and large range size suggest strong dispersal potential. However, behaviors may constrain realized dispersal. Little is known about the adult lifespan of individual stonefly species, but the stoneflies in our study are estimated to live 2 wk as adults (Stewart and Stark 2008), which leaves narrow time windows for mating (~2–4 wk), and their acoustic mating systems keep females near the stream environment, which provides strong tradeoffs with dispersal. Results of previous observational studies of post-emergence behavior of adult stoneflies suggest that adults remain near their emergence location (Briers et al. 2002), and isotopic tracer experiments in other stonefly species indicate that most adults remain ≤200 m from their emergence stream (Macneale et al. 2005). Results of previous genetic studies of caddisflies indicate that some macropterous species may not be successful dispersers (Myers et al. 2001). Nevertheless, many limnephilid caddisflies, such as *D. gilvipes*, have longer emergence windows and estimated longer lifespans relative to other caddisflies (Wiggins 2004) that may provide increased time for dispersal and gene flow to occur among populations. However, insects are poikilotherms, so the autumn emergence of *D. gilvipes*, particularly in high-elevation habitats (Erman 1989), may reduce their dispersal potential relative to that of the summer-emerging *C. californica*, *P. californica*, and *H. pacifica*.

Where dispersal-related traits and behaviors appear consistent between species, other factors may explain differences in population genetic structure. For example, *H. pacifica* and *C. californica* are both in the family Perlidae and have nearly identical body size, wing size, and emergence timing and duration (Peckarsky 1979, Sheldon 1980, 1999). However, these species had strikingly different population genetic structure and association of distinct populations with current geographic barriers. These differences might be caused by other, unrecognized differences in dispersal potential, but an alternative hypothesis could be that *H. pacifica* has dispersed at a slower rate than *C. californica* since the last glacial maximum because of different habitat constraints or different corridors of migration. Faster colonization or tolerance of a wider range of habitat conditions during postglacial recolonization may have allowed *C. californica* populations to disperse and differentiate to a greater degree than *H. pacifica*.

Table 3. Haplotype richness and rarefied haplotype richness for concatenated sequences (cytochrome *c* oxidase subunit I and II and *wingless* genes) and mitochondrial (mt) and wingless sample sizes for *Calineuria californica, Dicosmoecus gilvipes, Pteronarcys californica, Pteronarcys princeps* by river system and sample region in the western USA.

Species/region	Stream	N_{mt}	$N_{wingless}$	Haplotype richness	Rarefied haplotype richness
D. gilvipes					
Northwestern Washington	Skykomish	8	2	3	2.3
Central Washington	Wenatchee	9	3	3	2.3
Northeastern Oregon	Walla Walla	7	1	2	1.8
Western Montana	St Regis	9	3	2	2.5
Central Oregon	Big Elk	5	0	3	2.3
	Alsea	7	3	4	3.5
	Santiam	5	1	4	4.0
	McKenzie	7	2	3	2.4
Southern Oregon	Umpqua	7	2	3	2.4
	Evans	8	1	7	4.6
Interior northern California	Sacramento	9	2	5	4.1
Coastal northern California	Eel	10	2	9	4.8
Sierra Nevada	Bucks Creek	16	4	4	1.9
	Prosser	7	5	4	3.5
Coastal Northern California	Lagunitas	7	1	4	3.1
Central coast California	Big Sur	10	1	5	3.5
	Big Creek	8	3	4	2.4
P. californica					
Western Montana	St Regis	11	3	3	1.7
Southern Montana	Yellowstone	10	3	1	1.0
Eastern Oregon	Walla Walla	8	3	2	1.6
Central Oregon	Alsea	6	1	3	3.0
	Santiam	11	2	4	3.2
Interior northern California	Sacramento	2	0	2	
	Big Chico	7	2	3	2.7
Coastal northern California	Eel	10	2	4	3.9
	Austin	9	4	3	2.6
Central coastal California	Coyote	10	5	3	2.6
	Big Sur	8	1	5	4.5
	Big Creek	10	4	3	2.6
P. princeps					
Northwestern Washington	Chuckanut	2	0	2	
Coastal northern California	Smith	1	0	1	
Sierra Nevada	Schneider	4	1	2	2.0
	Sagehen	7	4	4	3.1
	Convict	6	2	1	1.0
C. californica					
Northwestern Washington	Chuckanut	1	1	1	
	Skykomish	4	4	1	1.0
Northeastern Oregon	Walla Walla	4	0	3	3.0
Central Oregon	Alsea	11	1	6	3.0
	Big Elk	2	0	1	
Southern Oregon	Evans	10	2	4	2.2
Interior Northern California	Sacramento	2	0	2	
	Moore	10	0	5	3.1

Table 3 (*Continued*)

Species/region	Stream	N_{mt}	$N_{wingless}$	Haplotype richness	Rarefied haplotype richness
Coastal northern California	Eel	9	0	7	3.7
	Russian	15	1	9	3.3
	Atascadero	7	0	6	3.8
	Navarro	2	0	1	
	Lagunitas	5	0	3	2.6
Sierra Nevada	Sagehen	7	3	5	4.0
	Spanish	4	4	4	4.0
	Mammoth	5	3	2	1.7
Central coastal California	Little Butano	5	3	3	2.8
	Gazos	4	0	2	2.0
	Scott	10	2	4	3.2
	Big Creek	9	5	2	2.0
	Big Sur	11	1	5	2.6
Southern California mountains	San Gabriel	21	7	6	2.7
H. pacifica					
Northwestern Washington	Chuckanut	17	3	3	2.4
	Oyster	9	4	2	1.5
Western Montana	St. Regis	4	3	3	3.0
Southern Montana	Yellowstone	8	4	1	1.5
Central Oregon	Alsea	9	2	4	2.3
	Big Elk	5	0	2	1.8
Interior northern California	Sacramento	12	2	3	1.4
	Klamath	4	1	2	2.0
Coastal northern California	Smith	1	1	1	
	Eel	8	4	3	2.4
	Atascadero	4	2	2	1.8
	Lagunitas	9	2	3	1.4
Sierra Nevada	American	8	1	5	3.2
Central coastal California	Scott	11	6	4	2.5
Southeastern Arizona	Ash	11	3	3	2.6

Cryptic biodiversity

In some cases, genetic markers can identify cryptic diversity among certain geographic regions that may not be reflected in morphologically based taxonomic keys (Pfrender et al. 2010, Zhou et al. 2010), and this may be the case within the species *C. californica*. In our study, concatenated gene trees and individual mitochondrial gene trees support 5 distinct *C. californica* populations (3–10% divergence from each other), but the more conservative nuclear gene trees corroborated strong support only for a distinct Sierra Nevada/northwestern Washington clade within *C. californica* (0.5–2% divergence). This intraspecific diversity in *C. californica* parallels that which was found in *Pteronarcys*. *Pteronarcys princeps* occurs in the Sierra Nevada Mountains, whereas *P. californica* occurs at lower elevations. We hypothesize that in the high altitudes of the Sierra Nevada and northern reaches of its range in northwest Washington, *C. californica* may show cryptic intraspecific diversity. In the case of a common mayfly, cryptic lineages in alpine streams aligned with life-history differences (Leys et al. 2017). Therefore, *C. californica* may be a strong candidate for future morphological or life-history studies to investigate it as a possible species complex with geographic distinctions.

Gene selection and study site distribution

Assessments of population genetic structure rely on the target genes and the distribution of study sites to accurately represent the actual connectivity of populations within the species. For example, previous investigators have demon-

strated diversity among riverine insect populations based on mitochondrial DNA alone (Schultheis et al. 2012, Previšić et al. 2014). However, results of other studies indicate that inclusion of both mitochondrial and nuclear genes can provide a more comprehensive analysis of population connectivity (Elbrecht et al. 2014) because they differ in mutation rates, and also because nuclear genes undergo recombination, whereas mitochondrial genes do not. Our use of both mitochondrial and nuclear genes enabled a comparison of gene trees for each gene to corroborate phylogeographic conclusions.

Uneven sampling within the range of each species may affect the number and distribution of haplotypes and alleles found at each site and in each region. In particular, additional specimens of *C. californica* from the northern extent of the species range in Washington state or Montana would enhance our ability to compare haplotypes and alleles between northern and southern populations, but *C. californica* is less common in these regions than elsewhere. *Hesperoperla pacifica* also may have additional genetic structure in Arizona and the Sierra Nevada Mountains that more sampling could clarify.

Implications for conservation of biodiversity with climate change

Despite being widespread with large ranges, each study species has genetic structure that suggests vulnerability to losses of intraspecific genetic diversity under climate-change scenarios. *Calineuria californica*, *D. gilvipes*, and *P. californica* had strong genetic structure that may increase risk to future losses of intraspecific biodiversity (Bálint et al. 2011, Hotaling et al. 2017). In addition, the distribution of genetic diversity within species suggests similar refugial and expansion patterns in response to previous climate shifts among at least 3 of the study species. Thus, these species may respond in like manner to future climate changes. Because they are widespread and numerically important in river ecosystems along the Pacific Coast, the resilience of these species is likely to be important for stream food webs. For example, *D. gilvipes* accounted for 55 to 96% of the macroinvertebrate biomass in an Oregon stream (Tait et al. 1994), and their abundance can have strong effects on stream communities (Power et al. 2008).

We identified geographically and genetically isolated populations of *H. pacifica* and *C. californica* at high elevations (>1800 m) in the southern latitudes of their species range. These populations are vulnerable to range contraction and extirpation. For example, predicted increases in air and, consequently, water temperatures may alter biodiversity including greater losses of intraspecific biodiversity (Giersch et al. 2016, Jordan et al. 2016) in high-elevation than in lowland streams (Null et al. 2013, Elsen and Tingley 2015). Investigations of small, montane mammals demonstrated upelevation range shifts that correspond with increased minimum temperatures over the past century (Moritz et al. 2008). Populations near mountaintops are of particular concern because dispersal to higher elevations to remain within physiological requirements is physically constrained (Grayson 2005, La Sorte and Jetz 2010). Therefore, vulnerability may be highest where we find isolated, high-elevation, and genetically distinct populations. Sierra Nevada Mountain populations are not as geographically isolated, but they also differ from populations in lowland streams and probably are at risk from thermal or hydrologic changes that result in range contraction.

In conclusion, we identified intraspecific genetic diversity in populations of large-bodied, co-occurring riverine insect species. Genetic indications of both concordance in population structure and common glacial refugia suggest the importance of Pleistocene glaciation for species dispersal and phylogeography, even for widespread species. Last, we identified cryptic diversity in the range of each species, particularly in mountain streams, that may be the intraspecific biodiversity most vulnerable to global climate change.

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Author contributions: MGP, PMO, and VHR designed the study. MGP identified study sites, collected specimens, sequenced DNA, and analyzed the data. MGP, PMO, and VHR interpreted the data. MGP drafted the manuscript. PMO and VHR provided critical revision for the manuscript.

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