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Morphological taxonomy, DNA barcoding, and species diversity in southern Rocky Mountain headwater streams

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Abstract: Elevation gradients allow scientists to observe changes in fauna over a range of abiotic conditions. A variety of trends in aquatic insect diversity patterns across elevation have been reported. However, many of these studies are confounded because they include streams at lower elevations, which are often larger in size and more polluted than their higher-elevation counterparts. Moreover, such studies always relied solely on morphological delineation of taxa, thereby potentially overlooking cryptic diversity. We reduced these limitations by sampling only minimally impacted wadeable streams across an elevation gradient and by combining morphological taxonomy with deoxyribonucleic acid (DNA) barcoding to identify taxa. We collected numerically abundant Ephemeroptera, Plecoptera, and Trichoptera (EPT) from single streams at ~200-m elevation intervals across >1000-m transects in 3 watersheds draining the eastern slope of the Colorado Rocky Mountains. Based on morphology alone, we identified 49 numerically abundant EPT morphospecies across 26 sites. Using DNA barcoding, we found 69 distinct lineages that probably represent distinct species. EPT species richness was highest at mid-elevations, and rates of turnover along elevation transects showed no consistent elevation trend or trend among ecological zones defined by vegetation. β-diversity across sites at comparable elevations in different watersheds showed a negative trend with increasing elevation that was marginally significant for DNA barcode taxa ($p = 0.051$) but not for morphospecies. Furthermore, significant ($p < 0.05$) differences in taxon richness, turnover, and lateral β-diversity values generated by DNA barcoding underscore the ability of molecular tools to quantify patterns in aquatic insect diversity across elevations.
Key words: diversity, elevation, DNA barcoding, taxonomy, aquatic insect, EPT, southern Rocky Mountain

Elevation gradients provide unique opportunities to study how communities respond to changes in abiotic conditions within relatively small geographic areas. Typically, ecologists have found that species richness decreases with increasing elevation (MacArthur 1972, Brown and Gibson 1983, Begon et al. 1990, Brown 1988, Stevens 1992), a pattern analogous to that of latitudinal diversity, where species richness decreases with increasing latitude for most taxonomic groups (Stevens 1989). However, Vinson and Hawkins (2004) and Pearson and Boyero (2009) pointed out that taxa of the orders Ephemeroptera, Plecoptera, and Trichoptera (EPT) provide notable exceptions to this general latitudinal pattern. Elevation transects are latitudinal analogs, so these taxa also may exhibit different richness trends across elevation gradients. A variety of elevation trends in diversity have been shown for terrestrial and aquatic taxa, including plants (e.g., Bhattarai and Vetaas 2003), vertebrates (e.g., Rahbek 1997), and invertebrates (e.g., Janzen 1976), leading some investigators to conclude that the negative trend might not be a general one, but rather might be caused by a small number of empirical studies demonstrating a compelling trend (Rahbek 1995).

Stream ecologists have found a variety of trends in stream insect richness along elevation gradients (Allan 1975, Minshall et al. 1985, Ward 1986, Perry and Schaeffer 1987, Flowers 1991, Lang and Reymond 1993, Ormerod et al. 1994, Suren 1994, Brewin et al. 1995, Tate and Heiny 1995, Grubaugh et al. 1996, Jacobsen et al. 1997,

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Monaghan et al. 2000, Jacobsen 2003). Thus far, nearly every pattern in richness across elevations has been reported for lotic insect taxa, from hump-shaped (Minshall et al. 1985, Brewin et al. 1995, Grubaugh et al. 1996), to increasing (Lang and Reymond 1993, Tate and Heiny 1995) or decreasing with elevation (Allan 1975, Ward 1986, Perry and Schaeffer 1987, Ormerod et al. 1994, Suren 1994, Jacobsen et al. 1997, Monaghan et al. 2000, Jacobsen 2003), to cases where no trend is evident (Flowers 1991). In the Colorado Rocky Mountains, positive (Tate and Heiny 1995) and negative (Allan 1975, Ward 1986, Perry and Schaeffer 1987) patterns of richness with elevation have been reported.

β-diversity along elevation gradients also is important for understanding regional-scale diversity patterns. β-diversity is a measure of the similarity of communities among multiple sites, and turnover is a specific form of β-diversity that is a measure of the similarity of adjacent sites (Whittaker 1960, 1972, Tuomisto 2010). Both β-diversity and turnover can explain the degree of heterogeneity of biota and habitats across a region (Wilson and Shmida 1984). However, few investigators have examined turnover along elevation gradients, and most have focused on terrestrial taxa (Wilson and Shmida 1984, Rahbek 1997, Mena and Vázquez-Domínguez 2005, Finn et al. 2013). Some investigators have found that, with increasing elevation, loss of aquatic taxa increases while gain of taxa remains low, a pattern suggesting that rates of turnover may be generally lower at lower elevations (Allan 1975, Ward 1986, Jacobsen 2004). Finn et al. (2013) found higher rates of turnover among high and mid-elevation than among low-elevation communities. Others have hypothesized that turnover, or faunal replacement, should be highest in regions of transition between distinct vegetation zones (ecozones; Dodds and Hisaw 1925).

Jacobsen (2004) argued that many studies of stream insect diversity patterns across elevation had inappropriate sampling designs and identified several reasons why designs might obscure detectable patterns. Examples include sampling too few sites or human-impacted sites, sampling an insufficient elevation gradient, and failure to control for stream order (Jacobsen 2004). In addition, inconsistent taxonomic resolution of stream insect identification can confound comparisons (Jacobsen 2004).

Dexoyribonucleic acid (DNA) barcoding has the potential to improve our understanding of diversity patterns by addressing the common problem of inconsistent taxonomic identification in studies of stream insects (Baird and Sweeney 2011). Species-level units can be delimited by pairwise comparison of mitochondrial cytochrome *c* oxidase subunit I gene (COI) sequences (Hebert et al. 2003), a technique that has been used effectively to aid in the description of communities and to reveal hidden diversity (Zhou et al. 2009, 2010, 2011, Sweeney et al. 2011). Specimens that typically are difficult to identify morphologically, such as early instars or adult females, can be associated with expertly identified adult male material, thereby increasing the taxonomic resolution of descriptions of stream insect communities (e.g., Zhou et al. 2007, Pauls et al. 2010). Moreover, researchers doing DNA barcoding can query their sequences against online databases, such as the Barcode of Life Data System (BOLD) (Ratnasingham and Hebert 2007) to aid in identification.

To understand elevation trends in stream insect diversity, we used an integrated taxonomic approach combining morphological taxonomy and DNA barcoding for species-level identifications of taxa, controlled for changes in stream size across elevations by sampling only wadeable tributaries to a mainstem river, and sampled only minimally impacted sites. We addressed several questions about stream insect diversity: 1) How does the richness of the orders Ephemeroptera, Plecoptera, and Trichoptera (EPT) change across elevation? 2) How broadly or narrowly are EPT species distributed across elevation? 3) Does species turnover increase linearly with elevation, or is turnover highest between distinct ecozones defined by elevation and vegetation? 4) How similar are communities at comparable elevations across 3 adjacent watersheds? 5) How does species-level assessment with DNA barcoding affect our interpretation of these elevation trends in aquatic insect diversity?

METHODS

Study area and collection

We selected sites in watersheds of 3 major rivers draining the eastern slope of Colorado Rocky Mountains (Fig. 1): the Cache La Poudre (CLP), Big Thompson (BT), and Saint Vrain (SV). Starting at the base of the mountain front (1500 m asl), we selected minimally im-

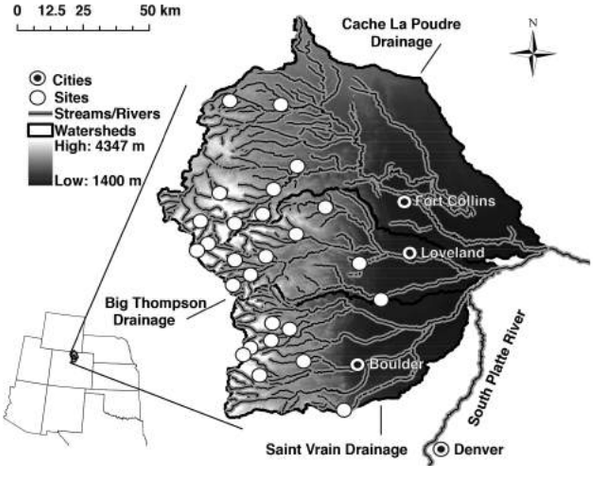

Figure 1. Map of study area showing collection localities and elevation in the Cache La Poudre, Big Thompson, and Saint Vrain watersheds in northern Colorado, USA.

pacted wadeable streams in different watersheds and proceeded upward within each watershed to find comparable tributaries at every 200 m of elevation. We chose the 200-m increment to capture changes in species composition and to detect major environmental gradients across elevation. No trend related to elevation was apparent in stream size (i.e., larger streams were not sampled preferentially at lower elevations). If minimally impacted sites were not available at a particular elevation, no sites were included for that elevation.

We sampled a total of 26 sites (Fig. 1) between 27 June 2011 and 10 August 2011: 8 sites in CLP from 1992 to 3397 m asl, 10 in BT from 1556 to 3478 m asl, and 8 in SV from 2015 to 3348 m asl. These sites spanned 5 ecozones defined primarily by vegetation: plains (1500 to 1650 m asl), foothills (1650 to 2440 m asl), lodgepole pine (*Pinus contorta* Douglas) (2440 to 3050 m asl), spruce–fir (3050 to 3300 m asl), and alpine (>3300 m asl). Plains and foothill ecozone designations were adapted from Ward (1986), whereas montane zones followed descriptions by Peattie (1936) and used by Finn and Poff (2005). We sampled 2 plains, 9 foothill, 7 lodgepole pine, 4 spruce–fir, and 4 alpine sites.

At each site, we collected immature EPT individuals (aquatic larvae) for a standardized period of 2 h from all available microhabitats with a 500-μm kick net and by haphazardly picking up rocks within a 100-m reach. We sorted the collected material coarsely in the field and defined dominant taxa by numerical abundance. In many instances, taxa could be identified only to the family or generic level in the field. In these cases, we sampled hierarchically by collecting more specimens of taxa identified at higher levels (i.e., family) and fewer when we were confident of a lower-level identification (i.e., monotypic species). This collection method ensured that we had adequate and representative material for morphological analysis in the laboratory. We also used a beating sheet and aerial net to collect adult specimens from riparian vegetation until no new taxa were found. We preserved all specimens initially in ≥95% EtOH, which was replaced within 24 h (Baird et al. 2011).

Identification

In the laboratory, we sorted numerically abundant EPT taxa to the lowest possible taxonomic level using available taxonomic literature (see Merritt et al. 2008). The aquatic insect fauna of the southern Rocky Mountains is relatively well known (Ward et al. 2002), so we made generic- and many species-level identifications. An exception was very immature Chloroperlidae, which we left at the family level. Adult Ephemeroptera and Plecoptera were identified by BCK, and Trichoptera were identified by D. E. Ruiter (Grants Pass, Oregon). Expertly identified material for all taxa and stages was available for comparison at the C. P. Gillette Museum of Arthropod Diversity, Colorado State University.

We used a numerical threshold for dominance to screen taxa. We required that ≥10 individuals/morphospecies be found at ≥1 study site for that taxon to be considered numerically abundant and, therefore, eligible for DNA barcoding. In consequence, we excluded 18 larval taxa from our analysis that were collected in much lower numbers (mean ± SE, 3 ± 0.52) than those we identified as numerically abundant EPT taxa (21 ± 3). We selected up to 5 individuals from each numerically abundant morphospecies and sampling site for barcoding. This subsampling protocol is comparable to that used in other barcoding studies (e.g., Ward et al. 2005) and increased or maximized geographic and taxonomic coverage and our ability to detect cryptic species, while minimizing cost.

We used standard protocols from the Canadian Center for DNA Barcoding (CCDB) for extraction (Ivanova et al. 2006), polymerase chain reaction (PCR), and sequencing (Ivanova et al. 2005, Hajibabaei et al. 2005, deWaard et al. 2008). For PCR, we first used the primer sets LCO1490/HCO2198 (Folmer et al. 1994) and LepF1/LepR1 (Hebert et al. 2004) to amplify a 658 base pair (bp) fragment of the COI gene. If these primer sets failed for Ephemeroptera and Plecoptera, we switched to the degenerate Folmer primer set (Meyer 2003), which amplifies the same gene. If the standard primer sets failed for Trichoptera, we used COI 1709Fg (Zhou et al. 2007) and COI 2191R (Kjer et al. 2001), which amplify a smaller 441-bp fragment of the same Folmer region. Following PCR, we visualized successful amplicons on a 2% agarose gel. PCR products were cleaned using ExoSAP-IT® (Affymetrix, Santa Clara, California) according to manufacturer's protocol. Purified PCR products were cycle-sequenced using Big Dye v3.1 dye termination kit, purified using Sephadex, and sequenced bidirectionally on an ABI 3730 sequencer (Applied Biosystems, Foster City, California).

Analyses

Species delimitation We trimmed and assembled COI sequences in Sequencher 5.0.1 (Gene Codes, Ann Arbor, Michigan) and made them publicly available with associated collection and taxonomy information on the Barcode for Life Data system (BOLD; Ratnasingham and Hebert 2007) under the project name Diversity of Rocky Mountain Stream Insects (DRMSI). We aligned sequences in MEGA (version 5; Tamura et al. 2011) using the ClustalW algorithm with default parameters. We checked sequences manually. We calculated pairwise genetic distances among all specimens using Kimura's 2-parameter model with 1000 bootstrap replicates (Kimura 1980), and plotted the frequency of these distances by order to visualize the barcode gap and establish a threshold for species delimita-

tion (Fig. 2). Consequently, we chose a 2% divergence criterion, a threshold demonstrated here to be exceeded only rarely by members of the same species and historically congruent with morphological identification of aquatic insect taxa (Avise 2000, Zhou et al. 2007, 2009, Ball et al. 2009).

We examined each group of immatures to determine final identification. In cases where we collected an associated adult specimen (expertly identified) that clustered with other specimens at a higher level of identification (e.g., an adult male of the chloroperlid *Sweltsa lamba* (Banks) clustered with *Sweltsa* sp. nymphs), we changed the higher-order identifications to reflect the species-level identity (in the above example, the identification of *Sweltsa* sp. nymphs would have been changed to *Sweltsa lamba*). Otherwise, we queried sequences for each group in BOLD (Ratnasingham and Hebert 2007) to match our sequence to a specimen in the database. When we could make a match with confidence, we changed the identity of the appropriate specimens accordingly. Otherwise we designated group members as having insufficient data to determine their identification (ISD) or as cryptic species. Specimens gained status as cryptic species only if COI sequences were available for all sympatric congeneric species in our sequence library or in BOLD (Ratnasingham and Hebert 2007), and the sequences of these specimens showed >2% divergence from these known taxa. We used the appendices of Ward et al. (2002) to determine the set of known taxa for Colorado.

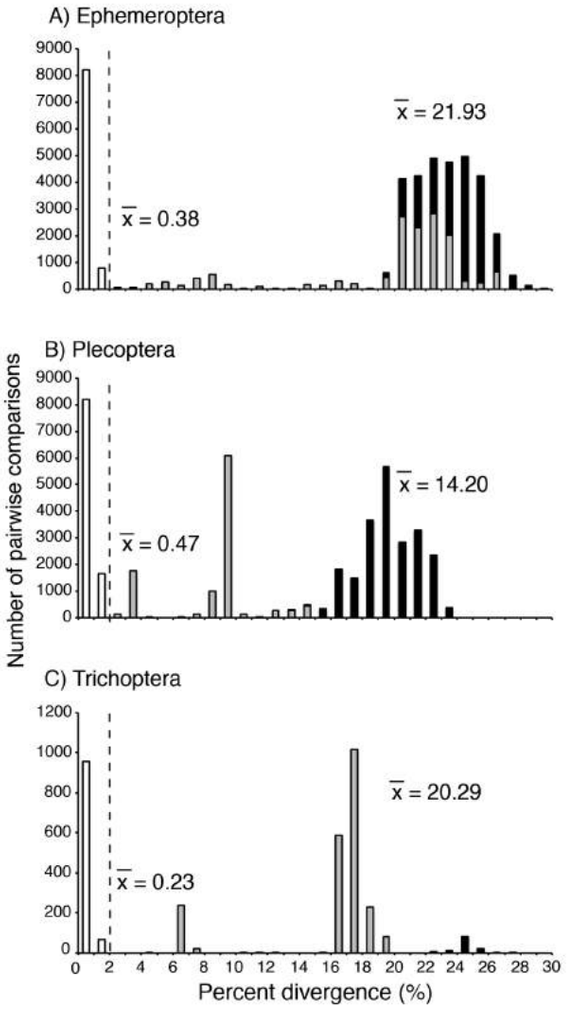

Figure 2. Number of pairwise comparisons vs genetic divergence among specimens calculated using Kimura's 2-parameter model for Ephemeroptera (A), Plecoptera (B), and Trichoptera (C). White portions of bars represent comparisons among members purported to be of the same barcode species designation, gray portions are comparisons among members of the same purported genus, and black portions are comparisons among members of the same purported family. The 2 mean values for each order are average % genetic divergence for intraspecific and interspecific comparisons, respectively. Vertical dashed line indicates the value of genetic divergence (2%) used to separate barcode taxa.

Richness across elevations We estimated numerically abundant EPT richness at the genus/species (morphospecies) and barcode-taxon (genetic lineages presumably representing true species) levels for each site. We calculated morphospecies richness according to the methods of Sweeney et al. (2011), who synonymized higher-level with lower-level identifications if more-developed (i.e., later-instar) or later life-stage (i.e., adult) specimens were available (e.g., records for adult males of heptageniid mayfly *Cinygmula mimus* (Eaton) and immature/damaged *Cinygmula* sp. at a locality, but only *C. mimus* [the lowest level identification] counted for richness). This approach prevents inflation of richness values by the presence of immature or damaged specimens and integrates identifications from the collection of both adults and immatures. We estimated richness of barcode taxa as the number of taxa differing by >2% sequence divergence.

We plotted estimated richness as a function of elevation and used polynomial regression to determine sequentially the best fit for the data at the morphospecies and barcode-taxon levels. We compared morphospecies and barcode-taxon richness values with a paired *t*-test. After checking our data for normality and homogeneity of variance, we compared mean richness of sites in the 5 ecozones with analysis of variance (ANOVA) followed by Least Significant Difference (LSD) tests to compare means among ecozones.

Elevation ranges We estimated elevation ranges for each taxon by subtracting the minimum elevation collection record from the maximum elevation collection record for each taxon at the lowest morphological and

barcode-taxon levels. We used a Wilcoxon rank-sum test to test whether elevation ranges differed between morphospecies and barcode taxa. For this analysis, we included all morphological taxa (e.g., elevation ranges were calculated for both *Sweltsa borealis* (Banks) and *Sweltsa* sp. specimens).

Species turnover We calculated species turnover between consecutive sites ascending each elevation transect with Whittaker's species turnover index ($\beta_W = [\gamma - \alpha]/\alpha$; Whittaker 1972, Tuomisto 2010), which measures the similarity between 2 sites based on presence/absence data (Koleff et al. 2003). For our study, γ is the total taxonomic richness within an elevation and α is the mean of the taxonomic richness among sites within an elevation band. We used β_W because it is independent of species richness and reflects differences in community composition (Koleff et al. 2003, Tuomisto 2010). β_W is used commonly in studies along elevation gradients (Mena and Vázquez-Domínguez 2005). On a scale from 0 to 1, higher β_W values indicate a higher turnover rate of taxa between sites. We calculated pairwise β_W values for morphospecies and barcode-taxon data sets and plotted values as a function of elevation. We used linear regression to test for a linear trend in β_W across elevations and polynomial regression and goodness-of-fit tests to test whether higher-order polynomial regressions could better describe the data. Because we calculated β_W based on adjacent pairs of sites, taxonomic data for some sites are unavoidably used in >1 calculation. Thus, we violated the assumption of independence of data points, and we used this test to describe the likelihood of a pattern in β_W, not to draw strict inference.

We separated β_W values into 2 groups, those between sites within an ecozone and those between sites in different ecozones. We used a *t*-test to test for a difference in β_W values from sites within vs spanning ecozones. Last, we compared β_W values for morphospecies and barcode taxa with a Wilcoxon signed-rank test.

Among-watershed β-diversity We used Whittaker's true β-diversity index ($\beta = \gamma/\alpha$) (Whittaker 1960, Tuomisto 2010) to test for similarity among sites in different watersheds but at comparable elevations. Higher β-diversity values for an elevation band indicate fewer shared taxa among sites in different watersheds at that elevation. We excluded the highest- and 2 lowest-elevation sites (of 26 sites total) from this comparison because we lacked multiple sites at an equivalent elevation.

We calculated β-diversity values for morphospecies and barcode-taxon data sets, plotted them together against elevation, and tested for linear trends in β-diversity with linear regression. We used polynomial regression and a goodness-of-fit tests to evaluate the alternative that a higher-order function could better describe the relationship between β-diversity and elevation. We used a paired *t*-test to compare values of β-diversity with and without barcoding.

RESULTS

Sequencing

We amplified DNA barcodes ≥500 bp for all 1224 specimens sequenced and used to delimit barcode taxa. More than 99% of these sequences met the barcode-compliance criteria elected by the Consortium for DNA Barcoding and used to evaluate the quality of records uploaded to the BOLD database (Ratnasingham and Hebert 2007). The <1% (4 sequences) that did not meet the barcode-compliance criteria were based on a single high-quality read (>500 bp, 0 ambiguous base calls) and were not divergent from other compliant records. No relationship between final consensus sequence lengths and insect order was indicated.

Species richness across elevations

EPT richness showed a hump-shaped trend in all 3 watersheds when plotted by watershed and compositely at the morphospecies and barcode-taxon levels (Fig. 3A–D). Barcode-taxon and morphospecies richness differed significantly (paired *t*-test, $t_{25} = 2.59$, $p = 0.016$), but showed similar patterns within (Fig. 3A–C) and among (Fig. 3D) watersheds. Mean EPT richness across all sites was 11 based on morphospecies and 12 based on barcode taxa. Site richness ranged from 0 to 19 morphospecies and 0 to 19 barcode taxa. The site with the highest morphospecies richness was in the CLP at 2411 m asl (19 morphospecies), and the site with the highest barcode-taxon richness was in the SV at 2388 m asl (19 lineages probably representing distinct species).

A quadratic equation fit the plot of richness vs elevation significantly better than a linear regression for morphospecies (goodness-of-fit test, $R^2 = 0.65$, $F_{2,23} = 41.88$, $p < 0.0001$) and barcode taxa ($R^2 = 0.63$, $F_{2,23} = 38.50$, $p < 0.0001$). A cubic equation did not significantly improve the fits ($F_{3,22} = 1.43$, $p = 0.261$; $F_{3,22} = 1.73$, $p = 0.190$, respectively).

Morphospecies and barcode-taxon richness differed among ecozones (ANOVA, $F_{4,21} = 5.77$, $p = 0.003$; $F_{4,21} = 5.55$, $p = 0.003$, respectively). Mean morphospecies and barcode-taxon richness differed between the plains and the foothills, lodgepole pine, and spruce–fir ecozones and between the alpine and the foothills, lodgepole pine, and spruce–fir ecozones (Fisher's LSD, all $p < 0.05$), but not between the plains and alpine ecozones or among the foothills, lodgepole pine, and spruce–fir ecozones. These results reflected the higher richness values at mid-elevations. Only 2 sites were in the plains ecozone, so plains diversity might have been underestimated.

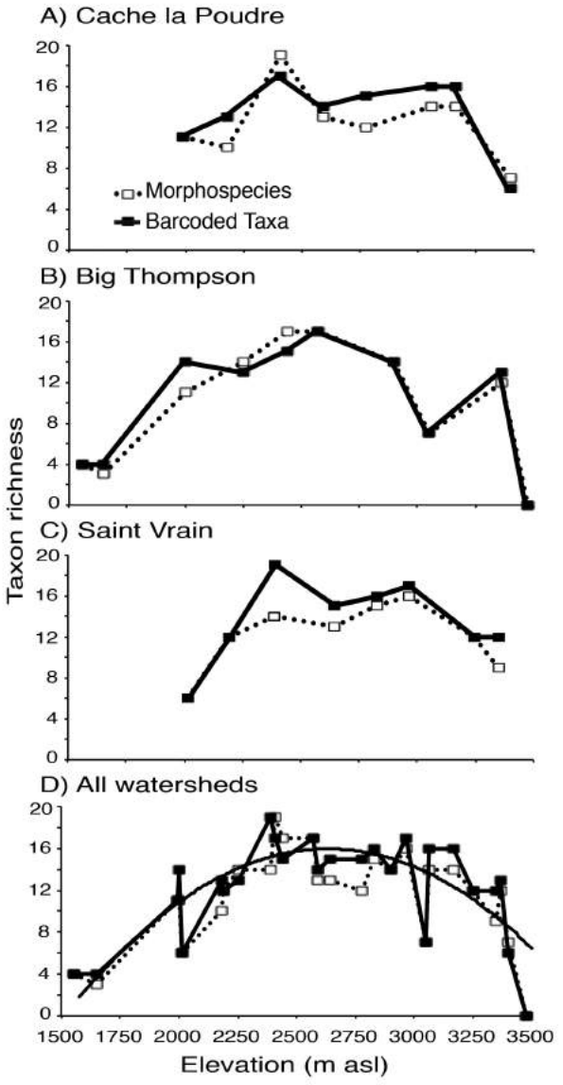

Figure 3. Morphospecies and barcode taxon richness by elevation for the Cache La Poudre (A), Big Thompson (B), and Saint Vrain (C) watersheds in Colorado, USA, and from all watersheds combined (D). A 2nd-order polynomial function was fit to the data from all watersheds.

Elevation ranges

Elevation ranges for EPT taxa varied from narrow to wide (Table 1). The median elevation ranges were 838 m for morphospecies and 553 m for barcode taxa, and elevation ranges differed between morphospecies and barcode taxa (Wilcoxon rank-sum test using normal approximation, $p = 0.007$; Fig. 4).

Species turnover

Morphospecies and barcode-taxon β_W was not linearly related to elevation when data from all watersheds were pooled (linear regression, $F_{1,21} = 0.68$, $p = 0.4178$, positive trend, $R^2 = 0.03$; $F_{1,21} = 0.69$, $p = 0.414$, positive trend, $R^2 = 0.03$; respectively) or within watersheds (CLP: $F_{1,5} = 0.71$, $p = 0.437$, negative trend, $R^2 = 0.12$; $F_{1,5} = 0.17$, $p = 0.699$, positive trend, $R^2 = 0.03$; BT: $F_{1,7} = 2.11$, $p = 0.189$, positive trend, $R^2 = 0.23$; $F_{1,7} = 1.43$, $p = 0.272$, positive trend, $R^2 = 0.17$; SV: $F_{1,5} = 2.45$, $p = 0.178$, negative trend, $R^2 = 0.33$; $F_{1,5} = 2.48$, $p = 0.176$, negative trend, $R^2 = 0.33$; respectively) (Fig. 5A–C). Higher-order polynomial regression did not improve the fit.

Morphospecies and barcode-taxon β_W between sites within ecozones did not differ from values spanning adjacent ecozones (*t*-tests, $t_{21} = 0.13$, $p = 0.897$; $t_{21} = 0.49$, $p = 0.629$; respectively). Along the CLP and BT transects, β_W differed between morphospecies and barcode taxa (Wilcoxon sign-rank tests, $p = 0.047$, $p = 0.031$, respectively).

β-diversity across watersheds

Morphospecies β-diversity within elevation bands was negatively related to elevation, but the relationship between barcode-taxon β-diversity within elevation bands and elevation was only marginally significant (linear regression, $F_{1,6} = 2.15$, $p = 0.193$, $R^2 = 0.26$; and $F_{1,6} = 5.92$, $p = 0.051$, $R^2 = 0.50$; respectively; Fig. 6). A 2nd-order polynomial did not significantly improve the fit for either morphospecies or barcode taxa. β-diversity differed between morphospecies and barcode taxa (paired *t*-test, $t_7 = -2.68$, $p = 0.032$).

DISCUSSION

Richness across elevations

Richness of numerically abundant EPT morphospecies and barcode taxa showed a hump-shaped pattern along the elevation gradient (Fig. 3A–D). This pattern is consistent with our finding that richness values were significantly higher in foothill, lodgepole pine, and spruce–fir ecozones than in the low-elevation plains or high-elevation alpine regions. Thus, our results indicate higher species richness at mid-elevations in these Rocky Mountain streams. These results are in agreement with those of a published meta-analysis showing that the most common richness trend across elevation (after controlling for sampling effort and area sampled) is a hump-shaped distribution (Rahbek 1995). Colwell and Hurtt (1994) predicted mid-elevation peaks in species richness under a model that assumed random placement of different elevation ranges and no biological gradient. Comparable hump-shaped trends have been found for stream insect richness across elevations in the Nepalese Himalayas (Brewin et al. 1995), along the Salmon River in Idaho (Minshall et al. 1985), and in the southern Appalachians (Grubaugh et al. 1996). However, our results differ from results of other studies of Colorado streams in which both positive and negative trends in richness with elevation have been

Table 1. Morphospecies (MS, bolded) and deoxyribonucleic acid (DNA)-barcode identified (BT, *) species list for all sites. Numbers of morphospecies barcoded, final numbers of specimens representing each barcode taxon, and elevation ranges are presented for both morphospecies and DNA-barcode taxa. In the cryptic species column, Y (cryptic species) or ISD (insufficient genetic data) indicates taxa for which a specific identification could not be determined based on morphology or DNA barcodes. N = number.

Order	Family	Genus	Species	*N* MS barcoded	Final *N* barcoded	Range MS	Range BC taxon	Cryptic species
Ephemeroptera	**Ameletidae**	***Ameletus***	**sp.**	**50**	–	**2252–3364**	–	
			*celer**	–	22	–	2775–3364	
			*doddsianus**	–	14	–	2252–2590	
			sp. A*	–	2	–	2643	(ISD)
			sp. B*	–	10	–	2643–3348	(ISD)
			sp. C*	–	1	–	3060	(ISD)
	Baetidae	***Baetis***	***bicaudatus****	**75**	75	**2252–3364**	2252–3364	
			flavistriga*	**8**	8	**1556–1650**	1556–1650	
			magnus	**10**	–	**2015–3060**	–	
			sp. A*	–	16	–	2015–3060	Y
			sp. B*	–	26	–	2001–2590	Y
			sp. C*	–	1	–	2388	Y
			tricaudatus*	**46**	13	**1556–2830**	1556–2181	
		Diphetor	*hageni**	–	14	–	2189–2590	
		Fallceon	***quilleri***	**24**	–	**1556–2590**	–	
			sp. A*	–	7	–	1556–1650	Y
			sp. B*	–	3	–	1650	Y
	Ephemerellidae	***Drunella***	***coloradensis****	**44**	2	**2388–3348**	3166	
			doddsii*	**25**	25	**2411–2964**	2411–2964	
			grandis*	**25**	26	**1992–2830**	1992–2830	
			sp. A*	–	42	–	2388–3348	Y
		Ephemerella	***dorothea infrequens****	**40**	4	**2001–3060**	2001–2181	
			sp. A*	–	36	–	2001–3060	Y
			tibialis*	**27**	27	**1992–2830**	1992–2830	
	Heptageniidae	***Cinygmula***	**sp.**	**73**	–	**2181–3397**	–	
			*mimus**	–	8	–	2181–2830	
			sp. A*	–	29	–	2388–3166	(ISD)
			sp. B*	–	19	–	2775–3397	(ISD)
			sp. C*	–	17	–	2443–3348	(ISD)
		Epeorus	***albertae****	**13**	8	**1992–3397**	1992–2181	
			*deceptivus**	–	5	–	3060	
			longimanus*	**55**	48	**1992–3060**	1992–2964	
			sp. A*	–	6	–	1992–2015	Y
		Rhithrogena	***robusta****	**30**	29	**2411–3364**	2643–3364	
			sp. A*	–	2	–	2181–2411	(ISD)
	Leptohyphidae	***Tricorythodes***	***explicatus****	**5**	5	**1556**	1556	
	Leptophlebiidae	***Paraleptophlebia***	***heteronea****	**19**	19	**1992–2830**	1992–2830	
	Siphlonuridae	***Siphlonurus***	***occidentalis****	**21**	21	**2015–2590**	2015–2964	
Plecoptera	**Chloroperlidae**	***Alloperla***	***pilosa****	**10**	18	**2900–3397**	2900–3397	
			thalia*	**5**	5	**2181**	2181	
		Genus	**sp.**	**38**	–	**2189–3397**	–	
		Suwallia	sp. A*	–	14	–	2189–3364	(ISD)
			sp. B*	–	8	–	2189–3364	(ISD)

Table 1 (*Continued*)

Order	Family	Genus	Species	*N* MS barcoded	Final *N* barcoded	Range MS	Range BC taxon	Cryptic species
		Sweltsa	***borealis****	**33**	71	**2443–3397**	2443–3397	
			coloradensis*	**22**	23	**1992–2411**	1992–2411	
			lamba*	**66**	79	**2252–3397**	2252–3397	
			sp.	**44**	–	**2443–3397**	–	
		Triznaka	***pintada****	**12**	16	**1992–2590**	2001–2590	
			signata*	**14**	10	**1992–2181**	1992–2181	
	Leuctridae	***Paraleuctra***	***vershina***	–	–	**2189–3364**	–	
	Nemouridae	***Malenka***	*coloradensis**	–	2	–	2590	
			flexura*	**15**	15	**2573–3364**	2573–3364	
		Podmosta	***decepta****	**7**	7	**2643–2775**	2643–2775	
			delicatula*	**20**	18	**2411–2964**	2411–2964	
		Zapada	**sp.**	**38**	–	**2573–3364**	–	
			*oregonensis**	–	10	–	2643–3249	
			oregonensis group*	–	28	–	2573–3397	
	Perlidae	***Hesperoperla***	***pacifica****	**36**	36	**2001–3249**	2001–3060	
	Perlodidae	***Isoperla***	***fulva****	**25**	26	**2001–2964**	2001–2964	
			sobria*	**15**	15	**2388–3364**	2388–3364	
		Kogotus	***modestus****	**57**	56	**2189–3249**	2189–3249	
		Megarcys	***signata****	**29**	6	**2643–3397**	2643–2775	
			sp. A*	–	23	–	2900–3397	Y
		Pictetiella	***expansa****	**5**	5	**3348**	3348	
Trichoptera	**Brachycentridae**	***Brachycentrus***	***americanus****	**5**	1	**2001**	2001	
			sp. A*	–	4		2001	Y
	Hydropsychidae	***Arctopsyche***	***grandis****	**12**	12	**2411–2830**	2411–2830	
		Hydropsyche	***oslari****	**11**	10	**1992–2001**	1992–2001	
			*slossonae**	–	1		1992	
	Lepidostomatidae	***Lepidostoma***	**sp.**	**13**	–	**2001–2388**	–	
			*unicolor**	–	13	–	2001–2388	
	Limnephilidae	***Hesperophylax***						
			*designatus**	–	15	–	1992–2015	
			occidentalis	**15**	–	**1992**	–	
	Rhyacophilidae	***Rhyacophila***	***angelita****	**18**	17	**2189–3166**	2189–3060	
			brunnea*	**36**	10	**2189–3166**	2189–3060	
			harmstoni*	**5**	5	**2900–3249**	2900–3249	
			hyalinata*	**17**	18	**2900–3348**	2900–3348	
			sp. A*	–	26	–	2252–3166	(ISD)
	Uenoidae	***Neothremma***	***alicia****	**11**	11	**2443–2573**	2443–3166	
Total				1224	1224			

found (Allan 1975, Ward 1986, Perry and Schaeffer 1987, Tate and Heiny 1995). Theory (Colwell and Hurtt 1994, Rahbek 1995) and a few empirical studies support the idea of a mid-elevation peak in species richness of aquatic insects (Minshall et al. 1985, Brewin et al. 1995, Grubaugh et al. 1996), but discrepancies between our work and studies from the same region (Allan 1975, Ward 1986, Perry and Schaeffer 1987, Tate and Heiny 1995) remain unexplained. These differences might be a consequence of fundamental differences in experimental design between

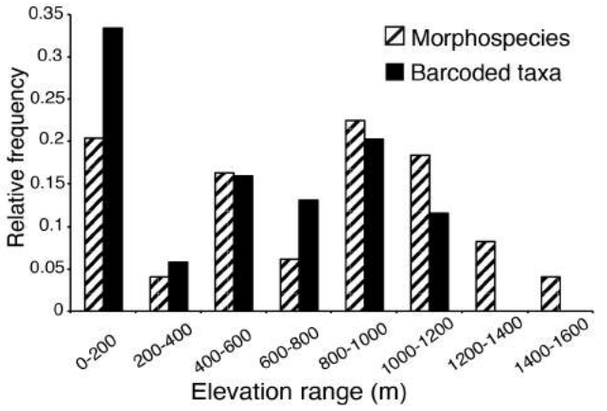

Figure 4. Frequency of a given elevation-range class for morphospecies (genera/species) or species identified using deoxyribonucleic acid (DNA) barcodes.

our study and previous work (i.e., length of elevation transects, control for stream order and human impacts on sites, and taxonomy).

Allan (1975) and Perry and Schaeffer (1987) considered elevation transects that began at relatively high elevations (2610 m and 2315 m asl, respectively) on Colorado's Western Slope and found an inverse richness trend with elevation. We would have found a similar trend had we started our transects at comparable elevations because of large numbers of mid-elevation taxa (Fig. 3). Tate and Heiny (1995) and Ward (1986) studied richness across a range of elevation similar to the range we studied, but they sampled progressively larger streams at lower elevations, as did Allan (1975) and Perry and Schaeffer (1987). Such a design is appropriate when testing how communities change with stream order as proposed in the River Continuum Concept (Vannote et al. 1980) but not when assessing the effect of elevation on richness (Jacobsen 2004) because stream fauna can differ among streams of differing order even at the same elevation (Grubaugh et al. 1996, Vinson and Hawkins 1998). Thus, elevation effects were confounded with stream-order effects in all previous studies of richness along an elevation gradient on the Western Slope of the Rocky Mountains. Moreover, richness values at the lowest-elevation sites in the studies by Perry and Schaeffer (1987) and Tate and Heiny (1995) were likely to be affected by anthropogenic activities, whereas we sampled relatively pristine tributaries to control for the effects of human activity on our assessment of the effects of elevation on richness.

Previous investigators used only morphospecies identifications to assess elevation trends in species richness. However, we supplemented morphologically based identification of a relatively well-known fauna with DNA barcoding. DNA barcoding significantly changed richness values at sites, a difference that is likely to be more marked in less well-known faunas (Sweeney et al. 2011). Barcoding both increased and decreased the number of taxa identified at a given locality. Increases were caused by splitting of morphospecies identified at higher levels (usually genera) and discovery of cryptic diversity (Table 1). Decreases occurred when barcoding synonymized or changed identifications, often in cases of morphological over-splitting (i.e., separation based on tentative characters).

Determining true cryptic diversity in stream insects is challenging. Several taxa identified at higher levels, usually immature specimens identified to genus, could not

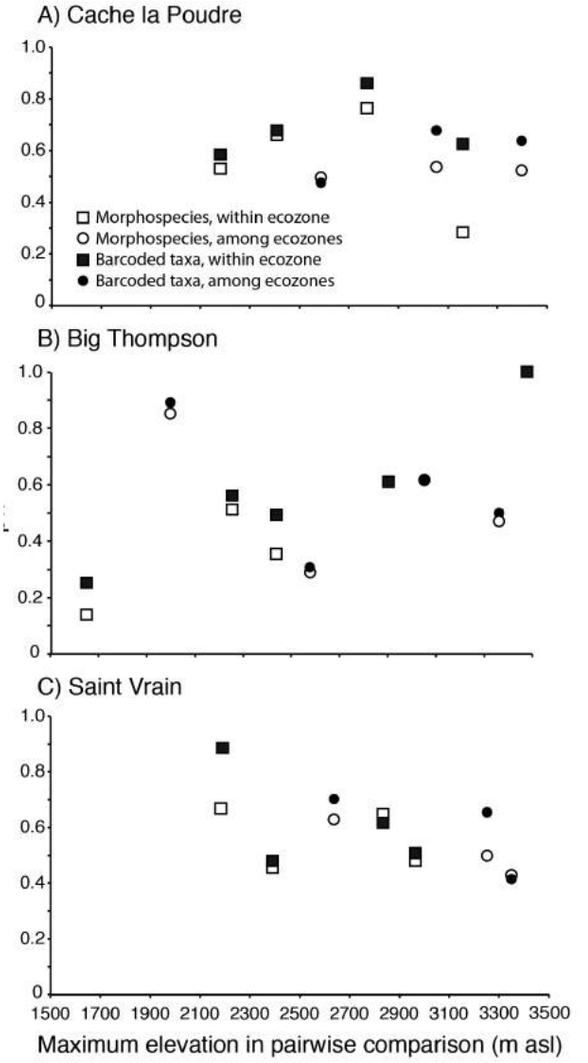

Figure 5. Whittaker's species turnover (β_W) as a function of maximum elevation for pairwise comparisons across an elevation gradient for the Cache La Poudre (A), Big Thompson (B), and Saint Vrain (C) watersheds for morphospecies (genera/species) and species identified using deoxyribonucleic acid (DNA) barcodes.

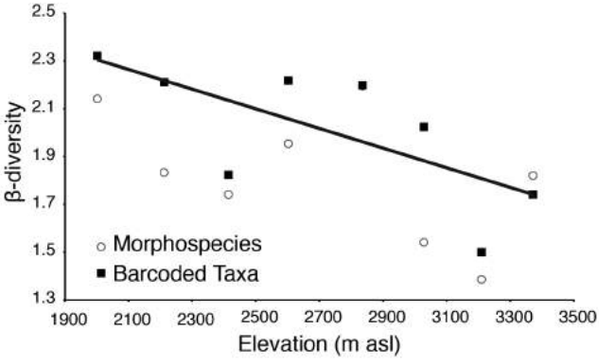

Figure 6. Whittaker's β-diversity as a function of mean elevation for sites of comparable elevation across the 3 watersheds in the study area. The *x*-axis denotes the elevation class for which β-diversity was calculated. A trend line is fitted to the barcode-taxon data showing a marginally significant relationship between β-diversity and elevation.

be positively associated with adult specimens identified to species or with available BOLD records (Ratnasingham and Hebert 2007). In these situations, we designated a taxon as a cryptic species only when reference sequences were available for all sympatric congeneric species from our streams. Otherwise, we designated the taxon as ISD to indicate the absence of sufficient genetic data to differentiate between extant described species and cryptic species. In many cases, taxa were designated ISD because reference sequences were not available on BOLD (Ratnasingham and Hebert 2007).

Our integrative approach increased taxonomic resolution and our ability to characterize patterns of stream insect diversity. However, we acknowledge several caveats in our study. First, our study was based only on numerically abundant EPT taxa. These 3 insect orders constitute a large proportion (~75%) of the fauna in this region (Ward 1986, Zuellig et al. 2012), and thus should be representative of the community as a whole. However, Diptera is also a numerically abundant order in Colorado mountain streams. Diptera followed a richness trend similar to that of Ephemeroptera and Trichoptera in a study by Ward (1986). Thus, the exclusion of Diptera should not affect our overall conclusions, but this hypothesis remains to be tested using DNA barcoding. Second, our data reflect a single intensive sampling event in early summer. Ward (1986) pointed out that year-round sampling in Colorado streams would not have an overall effect on relative taxon richness of Ephemeroptera, but would add Trichoptera taxa at mid-elevations and would reveal more winter/spring-emerging Plecoptera, which had a hump-shaped richness trend when sampled at multiple times (Ward 1986). Thus, although sampling on 1 occasion limited the number of taxa analyzed, the overall richness trend appears to be valid, and year-round sampling probably would further increase richness values only at mid-elevations.

Elevation ranges

Species elevation distributions across the watersheds we surveyed were similar to those reported by Ward (1986). However, some differences arose because of our methods. We probably underestimated elevation ranges for 2 reasons. First, we included only numerically abundant taxa, and elevation ranges might have been larger had we included vagrant individuals and low-density populations at the margins of elevation ranges. Second, we barcoded only 5 individuals per taxon per locality, so our ability to detect true elevation ranges of rare cryptic taxa was limited. However, our approach provided fine-scale taxonomic resolution by using barcoding when estimating species-level elevation ranges.

In agreement with Ward's study (1986), most Plecoptera had broad elevation ranges. However, *S. borealis* generally was restricted to high-elevation sites and *Triznaka signata* (Banks) was found only at lower-elevation sites. Like Ward (1986), we did not observe many broadly distributed Trichoptera taxa. *Arctopsyche grandis* (Banks) and *Hydropsyche* spp. were restricted to high- and low-elevation sites, respectively. Diversity of Trichoptera was low at low elevations, a result that could be related to stream size. We controlled for increasing stream size with decreasing elevation, and the small wadeable streams in our study may support a lower diversity of Trichoptera than the larger low-elevation streams sampled in other studies. Ward (1986) commented on a progressive increase in richness of Ephemeroptera with decreasing elevation. In contrast, we found a variety of taxa with broad elevation ranges that generally spanned mid-elevations, and decreasing richness at low elevations, a finding likely to be related to differences in community structure between small and large low-elevation streams.

DNA barcoding significantly changed estimates of elevation ranges primarily because it increased our ability to detect taxa with small elevation ranges (Fig. 4). Thus, DNA barcoding increased our ability to estimate species distributions and is a tool that may be particularly useful for describing diversity patterns in areas where the taxonomic composition of stream insect communities is poorly characterized.

Species turnover

Based on previous studies reporting loss of taxa with increasing elevation, we expected to see increasing turnover with elevation. However, we found no consistent increase in turnover of morphospecies or barcode taxa (Fig. 5A–C). High variation in turnover across elevations suggests that compositional similarity at adjacent sites may not be determined by the position of taxa along these elevation gradients (Fig. 5A–C). Our results contrast with our interpretation of previous studies that suggested high rates of turnover at mid to high elevations

(Allan 1975, Ward 1986). These discrepancies might be explained by differences in sampling design because differences in stream size could lead to the appearance of increased turnover by confounding elevation and stream-size effects. We might have seen higher turnover at higher elevations had we included non-EPT species. However, we think it reasonable that the absence of a general trend in turnover could be explained by heterogeneous patterning of aquatic insects across the landscape, resulting from a combination of limited dispersal ability, population structure, and isolation by distance (Bilton et al. 2001, Bohonak and Jenkins 2003, Hughes et al. 2009, Patrick and Swan 2011).

Turnover between adjacent sites within ecozones did not differ from turnover between adjacent sites in different ecozones. These results indicate that communities did not change any more at sites near ecozone transitions than at sites within ecozones, and by extension, that terrestrial vegetation may not strongly influence community composition. Allan (1975) found that vegetation zones on the Western Slope of the Rockies did not affect faunal replacement (as hypothesized by Dodds and Hisaw 1925). Turnover differed between morphospecies and barcode taxa in 2 watersheds (CLP and BT), indicating that barcoding can enhance our ability to interpret community variability.

β-diversity

β-diversity among communities in different watersheds (CLP, BT, SV) at comparable elevations tended to be negatively related to elevation (Fig. 6), but the regression slope for β-diversity of barcode taxa as a function of elevation was only marginally different from 0. R^2 values for these regressions were moderately high and suggested more-homogeneous species composition among higher-elevation than among lower-elevation sites. We sampled only numerically abundant EPT taxa, so our findings support the idea that only a small number of these species can tolerate conditions at high elevations (Ward 1994), and therefore, only those taxa are found across all watersheds at these elevations. Finn and Poff (2005) found that weedy traits, such as long-distance dispersal ability and high fecundity, were more common at high-elevation sites, a result indicating that species at high-elevation sites may be filtered by possession of the functional traits necessary for survival in these environments (Poff 1997). In contrast, a larger pool of potential taxa may inhabit lower-elevation streams (Ward 1986).

In contrast to our results, Finn and Poff (2005) found the lowest community similarity among high-elevation streams. This difference between our results and theirs may be a consequence of our use of DNA barcoding or of exclusion of rare taxa and insects from orders other than EPT. In addition, Finn and Poff (2005) sampled streams of increasing order at lower elevations. Thus, changes in community composition with stream order complicate a direct comparison between their results and ours. Moreover, analysis of genetic population structure may show different diversity patterns than analysis of taxonomic community because high levels of genetic differentiation have been found at high elevations under several models of stream insect population structure (Hughes et al. 2009).

β-diversity of barcode taxa tended to be negatively related to elevation and was significantly different than β-diversity of morphospecies. Thus, use of barcoding can change β-diversity disproportionately to changes in α-diversity causing values of β-diversity to differ. Finer delineation of taxa may increase richness estimates and estimates of the degree of heterogeneity between communities at a regional scale, especially when barcoding divides morphospecies into multiple taxa with different elevation ranges.

Conclusions

Our study design and taxonomic approach provided unique and ecologically important views of stream insect diversity patterns across streams in the same region that differ in elevation. Richness data showed strong evidence for a hump-shaped trend (higher richness at mid-level elevations) in numerically abundant EPT taxa richness across elevations. Elevation ranges of taxa were similar to those reported previously, but generally were smaller. We found no consistent trend in species turnover, and β-diversity of sites at comparable elevations in 3 adjacent watersheds tended to be negatively related to elevation.

DNA barcoding was helpful in standardizing disparate levels of taxonomic identification with species-level units. We found evidence that this tool can change how we interpret trends in diversity. Taxon richness, the distribution of elevation ranges, and similarity of communities in adjacent sites along ascending elevation transects and between sites at comparable elevations in different watersheds differed significantly between analyses based on DNA barcoding and analyses based on morphospecies. We argue, as have others, that the approach we used could be even more informative in regions, such as the tropics, where the fauna is relatively unknown (Sweeney et al. 2011).

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