DISSERTATION

DIVERSITY, DISTRIBUTIONS, AND EVOLUTION OF ROCKY MOUNTAIN AND ANDEAN STREAM INSECTS

Submitted by

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ABSTRACT

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Concordant with latitudinal increases in seasonality, the "climate variability hypothesis" (CVH) posits that the breadth of species' thermal tolerances should increase with latitude. Across elevations, the "mountain passes are higher in the tropics hypothesis" (MPHT) postulates that the narrow thermal tolerances of tropical species should limit their dispersal across elevations more than the broad thermal tolerances of temperate species. We consequently expect tropical species to have more limited elevational ranges and higher rates of population isolation, divergence, and speciation than temperate species, which could lead to higher tropical than temperate species richness. Moreover, many tropical species might be cryptic, as they have diverged primarily in physiological and dispersal traits, rather than traits with distinct morphological phenotypes. In this dissertation, I investigate how the CVH might provide a mechanistic explanation for global trends in species richness, cryptic diversity, and elevational distributions. In chapter one, I recapitulate the CVH and MPHT hypothesis and summarize related key literature. In chapter two, I characterize the diversity and distributions of stream insects in the Colorado Rocky Mountains. In chapter three, I compare the species richness and elevational ranges of species from the Colorado Rocky Mountains and the Andes of Ecuador. Lastly in chapter four, I integrate data from physiological, landscape genetic, and biogeographic investigations to evaluate the support for the CVH as a key mechanism determining global trends in species diversity, distributions, and vulnerability to climate change.

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DEDICATION

For my mother, Rosemary, and father, Jay

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1. INTRODUCTION

Unprecedented rates of global warming and associated effects on biodiversity have necessitated the study of species vulnerability to climate change (Hughes 2000, Walther et al. 2002, Root et al. 2003, 2005, Parmesan and Yohe 2003, Parmesan 2006, IPCC Core Writing Team et al. 2014). These assessments require quantification of both species exposure and sensitivity to rapid global warming and increasingly frequent climatic extremes (Williams et al. 2008). To accurately quantify species' exposure to climate change, climate scientists continue to work to refine forecasts of future climate scenarios (IPCC Core Writing Team et al. 2014). At the same time, biologists are working to understand the inherent sensitivities of species to rapid global climate change (Bernardo et al. 2007, Deutsch et al. 2008). Studies are needed to determine the capacity of species to tolerate or adapt to climate change. In this dissertation, I explore one key factor thought to play an important role in determining species sensitivity to climate change, historical exposure to climate variability over evolutionary time (Dobzhansky 1950, Stevens 1989).

The "climate variability hypothesis" (CVH) posits that there is a positive relationship between latitude and the breadth of species' thermal tolerances (Dobzhansky 1950, Stevens 1989). It is well known that annual thermal variation (seasonality) increases with latitude (Dobzhansky 1950, Vannote and Sweeney 1980, Müller 1982, Stevens 1992, Sunday et al. 2011). Accordingly, temperate species are exposed to wide ranges of annual temperatures, whereas tropical species experience relatively stable temperatures year-round. Over evolutionary time, natural selection should favor temperate species capable of tolerating seasonal highs and lows, resulting in species with wide thermal tolerances (Dobzhansky 1950, Stevens 1989).

Alternatively, in the tropics, natural selection should favor adaptation to local thermal conditions, resulting in species with relatively narrow thermal tolerances. Ultimately, narrower tropical than temperate thermal tolerances may make tropical species less able to respond to climate change, especially if tropical species are already living closer to their critical thermal limits (Tewksbury et al. 2008, Deutsch et al. 2008).

To test the CVH, researchers have compared species' critical thermal limits (Snyder and Weathers 1975, van Berkum 1988, Addo-Bediako et al. 2000, Gibert and Huey 2001, Compton et al. 2007, Deutsch et al. 2008, Huey et al. 2009, Calosi et al. 2010), acclimation abilities (Feder 1982, Tsuji 1988, Calosi et al. 2010), and metabolic rates (Tsuji 1988, Bernardo et al. 2007) across latitude. Ectotherms are used in these experiments, because their reliance on external sources of heat makes them particularly responsive to selection pressures associated with climatic seasonality. In support of the CVH, many macrophysiological studies for a diverse set of taxa demonstrate that the breadth of species' thermal tolerances, acclimation abilities, and basal metabolic rates all tend to increase with latitude (Snyder and Weathers 1975, Feder 1982, van Berkum 1988, Tsuji 1988, Addo-Bediako et al. 2000, Gibert and Huey 2001, Compton et al. 2007, Bernardo et al. 2007, Deutsch et al. 2008, Huey et al. 2009, Calosi et al. 2010).

As additional tests of the CVH, researchers have also examined latitudinal differences in dispersal and geographical range sizes. Following the CVH, temperate species with broad thermal tolerances should have higher dispersal ability than tropical species with narrow thermal tolerances (Janzen 1967). In support of this idea, several studies have found greater within-species population genetic differentiation in the tropics than the temperate zone, indicating higher tropical than temperate dispersal limitation (Martin and McKay 2004, Eo et al. 2008). Additionally, a broad thermal tolerance should allow temperate species to successfully inhabit a

larger range of environments than the narrow thermal tolerances of tropical species, leading to smaller tropical than temperate species distributions (Dobzhansky 1950). The resultant trend of increasing geographical range sizes with latitude is well-documented and known as the Rapoport effect (Rapoport 1982, Stevens 1989). Together, studies of latitudinal differences in dispersal and trends in geographical range sizes provide additional evidence in support of the CVH.

The CVH also has important implications for dispersal across elevations and elevational range sizes (Janzen 1967, Ghalambor et al. 2006). In the "mountain passes are higher in the tropics hypothesis" (MPHT hypothesis), Janzen (1967) proposed that narrower tropical than temperate thermal tolerances should make tropical mountains more effective physiological barriers to species dispersal than temperate mountains of comparable height. Fundamentally, the MPHT hypothesis is based on Janzen's observation that elevational climatic zonation is higher in the tropics than the temperate zone. In the temperate zone, we consequently expect that low elevational climatic zonation and broad thermal tolerances should facilitate dispersal and allow species to spread broadly across elevations, whereas in the tropics, high elevational climatic zonation and narrow thermal tolerances should limit dispersal and restrict species to narrow elevational bands.

By extension the MPHT hypothesis (Janzen 1967) also provides a mechanistic basis for latitudinal differences in species richness and cryptic diversity in montane regions (Ghalambor et al. 2006). Resistance to elevational dispersal should result in high levels of population isolation across elevations. Over time, a combination of mutation, genetic drift, and natural selection will cause these populations to diverge from one another genetically. Divergence to the point of reproductive isolation results in new species. Moreover, reproductive isolation driven by physiological and dispersal traits lacking recognizable morphological phenotypes may result in

morphologically indistinguishable but independently evolving lineages (*i.e.* cryptic species). Thus based on the MPHT hypothesis (Janzen 1967), the expectation of higher tropical than temperate resistance to elevational dispersal leads us to predict higher tropical than temperate rates of speciation, species richness, and cryptic diversity (Ghalambor et al. 2006).

Since the publication of the MPHT hypothesis, researchers have been working to test its predictions. In his original paper, Janzen (1967) demonstrated that elevational climatic zonation is indeed higher in the tropics than the temperate zone. Subsequently, researchers have estimated and compared the thermal niches of species from the tropics and temperate zone and found that tropical species do in fact tend to experience a narrower range of temperatures than related temperate species (Kozak and Wiens 2007, Cadena et al. 2012). Numerous studies have also documented that tropical species tend to be more narrowly distributed across elevations than temperate counterparts (Heyer and Ronald 1967, Wake and Lynch 1976, Terborgh 1977, Huey 1978, McCain 2009, Cadena et al. 2012). Furthermore, the trend of increasing species richness from the poles to the equator, or the latitudinal diversity gradient, has been recognized since the early 1800s (Hawkins 2001). Despite this wealth of correlative evidence in support of the MPHT hypothesis (Janzen 1967), to date no study has comprehensively linked latitudinal differences in climate variability to species' physiology, dispersal, elevational range sizes, speciation rates, species richness, and cryptic diversity in a single taxon.

Dissertation objectives:

The main objective of my doctoral research is to evaluate support for the CVH (Dobzhansky 1950, Stevens 1989) and the MPHT hypothesis (Janzen 1967). I focus on how latitudinal difference in climate variability over evolutionary time influence rates of speciation, species richness, cryptic diversity, and elevational range sizes. Together with collaborators, I

hope that my work will also contribute to our collective understanding of global trends in species vulnerability to climate change.

Study system:

To test the CVH (Dobzhansky 1950, Stevens 1989) and MPHT hypothesis (Janzen 1967), I studied mountain stream insects in the Colorado Rocky Mountains (40°N) and the Ecuadorian Andes (0°). The Rocky Mountains exemplify a temperate location with high levels of climatic seasonality and low elevational climatic zonation, whereas the Andes exemplify a tropical location with low levels of climatic seasonality and high elevational climatic zonation. Working in these locations facilitated cross-latitudinal comparisons of the effects of historical climate variability on species diversity and distributions.

I chose to work with mountain stream insects because they are abundant, easily collected, speciose, globally distributed, and ecologically important components of stream ecosystems (Dominguez et al. 2006, Merritt et al. 2008). In particular, stream insects are important links in food webs between primary producers and higher trophic levels (Cummins 1974), provide energy subsidies to surrounding riparian species (Nakano and Murakami 2001), and play key roles in stream nutrient cycling (Wallace and Webster 1996). Stream insects are also ectothermic and consequently expected to respond to selection pressures associated with latitudinal differences in levels of historical climate variability.

Chapter objectives:

In my first study (**Ch.2**), I characterized the diversity and distributions of mountain stream insects in three watersheds along the Colorado Front Range: The Cache La Poudre, Big Thompson, and St. Vrain. Focusing on the collection of mayflies (Ephemeroptera), stoneflies (Plecoptera), and caddisflies (Trichoptera; together "EPT species"), I collected both immature

and adult specimens from a total of 26 streams. I chose study streams that were minimally impacted wadeable tributaries located across large (> 1000 m) elevational gradients every 200 m of elevation gain. In the lab, I identified specimens morphologically to the lowest level possible using available literature (Ward et al. 2002, Dominguez et al. 2006, Merritt et al. 2008). I then DNA barcoded (Hebert et al. 2003) a subset of specimens found at each site. Using identifications based on morphology and DNA barcoding, I looked at elevational trends in species richness, elevational turnover, and among drainage β-diversity.

To date, many different elevational richness trends have been found for mountain stream insects including hump-shaped (Minshall et al. 1985, Brewin et al. 1995, Grubaugh et al. 1996), increasing (Lang and Reymond 1993, Tate and Heiny 1995), decreasing (Allan 1975, Ward 1986, Perry and Schaeffer 1987, Ormerod et al. 1994, Suren 1994, Jacobsen et al. 1997, Monaghan et al. 2000, Jacobsen 2003) and no trend (Flowers 1991). Turnover has been found to increase with elevation (Allan 1975, Ward 1986, Jacobsen 2004, Finn et al. 2013) and among regions of transition such as between distinct elevational vegetation zones (Dodds and Hisaw 1925). Before the study presented in this dissertation, nobody had looked at among drainage β-diversity. Additionally, previous studies of elevational diversity trends of mountain stream insects did not control for increasing stream size with decreasing elevation or address cryptic diversity. My approach of sampling wadeable streams at all elevations and using DNA barcoding (Hebert et al. 2003) to aid in species identification addresses the aforementioned shortcomings, providing a novel perspective on elevational trends in mountain stream insect diversity. Moreover, this work validates my integrative taxonomic approach combining morphology and DNA barcoding for future studies.

In my second study (**Ch. 3**), I compared the species richness and elevational ranges of species from the Colorado Rocky Mountains and the Andes of Ecuador to test the MPHT hypothesis (Janzen 1967). I used the same sampling design and approach for species identifications as in chapter two for my work in Ecuador, though the taxonomic scope of this study was limited to mayflies (Ephemeroptera). Using these data, I examined latitudinal differences in species richness, cryptic diversity, and elevational ranges of species from Colorado and Ecuador. To test the MPHT hypothesis, I tested the predictions that there should be 1) more species, 2) higher cryptic diversity, and 3) smaller elevational ranges in the tropics than temperate zone.

With some exceptions, it is widely accepted that species richness increases from the poles towards the equator (Fischer 1960, MacArthur 1965, Pianka 1966, Rohde 1992, Willig et al. 2003, Hillebrand 2004). Despite numerous hypotheses, the mechanism underlying the latitudinal diversity gradients remains unknown (Willig et al. 2003). Even less understood is the global distribution of cryptic diversity (Bickford et al. 2007), a problem exacerbated by the fact that most studies of cryptic diversity are from temperate regions. Additionally, researchers have compared the elevational distributions of tropical and temperate species and found different results (Kozak and Wiens 2007, McCain 2009, Cadena et al. 2012). Unfortunately, the aforementioned studies fail to address cryptic diversity, tend to analyze data from the literature collected using multiple methods, and do not control for phylogeny. Here, I build on previous research by using DNA barcoding (Hebert et al. 2003) to detect cryptic diversity, using data collected empirically using a consistent sampling design, and using phylogenetic comparative methods (Felsenstein 1985).

In my third and final study (**Ch.4**), I integrated data from my doctoral dissertation work with that of collaborators to comprehensively evaluate support for climate variability as a key mechanism determining global trends in species diversity (Dobzhansky 1950, Janzen 1967, Stevens 1989, Ghalambor et al. 2006). In this paper, I documented the thermal regimes of tropical and temperate streams. I related stream temperature variability to the breadth of species' thermal tolerances. I analyzed latitudinal difference in population genetic differentiation and number of migrants per generation. I then related latitudinal differences in physiology and dispersal to species richness, cryptic diversity, and speciation rates. The result is the most comprehensive assessment of the CVH (Dobzhansky 1950, Stevens 1989) and MPHT hypothesis (Janzen 1967) to date.

Conclusions and significance:

The main objective of my doctoral research is to evaluate support for the CVH (Dobzhansky 1950, Stevens 1989) and the MPHT hypothesis (Janzen 1967). In my first study, I developed an approach to rapidly survey the diversity and distributions of stream insects using integrative taxonomy combining morphology and DNA barcoding (Hebert et al. 2003). Working with a relatively well-known fauna (Ward et al. 2002), I found more species and significantly different β-diversity values using integrative taxonomy than using morphology alone, stressing the importance of addressing cryptic diversity (Bickford et al. 2007) and validating my taxonomic approach. In my second study, I explicitly compared the species richness, cryptic diversity and elevational ranges of mayflies from Colorado and Ecuador to test the MPHT hypothesis (Janzen 1967). Using integrative taxonomy, I found more species, higher cryptic diversity, and smaller elevational ranges in Ecuador than Colorado, results exactly the opposite of those found using morphology alone and strongly supporting the MPHT hypothesis.

In my last study, I found strong evidence for climate variability (Dobzhansky 1950, Janzen 1967, Stevens 1989) as a driver of latitudinal differences in diversity and species vulnerability to climate change. Specifically, I documented how stream temperature range increases with latitude and stream temperature range predicts species' thermal breadths. I also showed that latitude predicts dispersal ability, species richness, and speciation rate. Regarding Colorado and Ecuador specifically, stream temperature range is generally lower in Ecuador than Colorado, resulting in Ecuadorian species having narrower breadths of thermal tolerance than related Colorado species. Together, relatively narrower thermal tolerances and lower dispersal ability of Ecuadorian than Colorado species lead to higher rates of speciation, species richness, and cryptic diversity in Ecuador than Colorado.

Fundamentally, this body of work demonstrates the utility of integrative taxonomy in determining the composition of regional faunas and uncovering cryptic diversity. In doing so, it advances our knowledge of latitudinal and elevational trends in species diversity and distributions. Perhaps most importantly, it also provides support for climate variability (Dobzhansky 1950, Janzen 1967, Stevens 1989) as a mechanism underlying the latitudinal diversity gradient (Fischer 1960, MacArthur 1965, Pianka 1966, Rohde 1992, Willig et al. 2003, Hillebrand 2004), a long-standing challenge for ecologists and evolutionary biologists. Moreover, it contributes to our knowledge of geographic variation in species sensitivity to rapid climate change by characterizing latitudinal differences in species physiology and dispersal ability. As predicted based on the CVH (Dobzhansky 1950, Stevens 1989) and MPHT hypothesis (Janzen 1967), my work supports the idea that the narrow thermal tolerances and low dispersal ability of tropical species may make them more vulnerable to global warming than related temperate species.

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2. MORPHOLOGICAL TAXONOMY, DNA BARCODING, AND SPECIES DIVERSITY IN SOUTHERN ROCKY MOUNTAIN HEADWATER STREAMS[1]

Summary

Elevational 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 three 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 midelevations, 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

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values generated by DNA barcoding underscore the ability of molecular tools to quantify patterns in aquatic insect diversity across elevations.

Introduction

Elevational 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, Brown 1988, Begon et al. 1990, 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 (2003) 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. Elevational transects are latitudinal analogs, so these taxa also may exhibit different richness trends across elevational gradients. A variety of elevational trends in diversity have been shown for terrestrial and aquatic taxa, including plants (Bhattarai and Vetaas 2003), vertebrates (Rahbek 1997), and invertebrates (Janzen et al. 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 elevational gradients (Allan 1975, Minshall et al. 1985, Ward 1986, Perry and Schaeffer 1987, Flowers 1991, Lang and Reymond 1993, Suren 1994, Ormerod et al. 1994, Brewin et al. 1995, Tate and Heiny 1995, Grubaugh et al. 1996, Jacobsen et al. 1997, Monaghan et al. 2000, Jacobsen 2003). Thus far, nearly every pattern in richness across elevation 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 elevational have been reported.

β-diversity along elevational 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 elevational 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 (Jacobsen 2004) argued that many studies of steam 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 elevational gradient, and failure to control for stream order

(Jacobsen 2004). In addition, inconsistent taxonomic resolution of stream insect identifications can confound comparisons (Jacobsen 2004).

Deoxyribonucleic 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 (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 elevational 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 three adjacent

watersheds? and 5) How does species-level assessment with DNA barcoding affect our interpretation of these elevational trends in aquatic insect diversity?

Methods

Study area and collection

We selected sites in watersheds of three major rivers draining the eastern slope of Colorado Rocky Mountains (Fig. 2.1): 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 impacted wadeable streams in different watersheds and proceeded upward within each watershed to find comparable tributaries at every 200 m of elevational. 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 elevation). If minimally impacted sites were not available at a particular elevation, no sites were included for that elevational.

We sampled a total of 26 sites (Fig. 2.1) between 27 June 2011 and 10 August 2011: eight sites in CLP from 1992 to 3397 m asl, ten in BT from 1556 to 3478 m asl, and eight in SV from 2015 to 3348 m asl. These sites spanned five ecozones defined primarily by vegetation: plains (1500 to 1650 m asl), foothills (1650 to 2500 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 two plains, nine foothill, seven lodgepole pine, four spruce–fir, and four 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 (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 (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 (Hajibabaei et al. 2005, Ivanova 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 the 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 v. 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 delimitation (Fig. 2.2). 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 its 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 *Swetlsa 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.

Richness across elevation

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 elevational 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 five ecozones with analysis of variance (ANOVA) followed by least significant difference (LSD) tests to compare means among ecozones.

Elevational ranges

We estimated elevational ranges for each taxon by subtracting the minimum elevational collection record from the maximum elevational collection record for each taxon at the lowest morphological and barcode-taxon levels. We used a Wilcoxon rank-sum test to test whether elevational ranges differed between morphospecies and barcode taxa. For this analysis, we

included all morphological taxa (*e.g.*, elevational ranges were calculated for both *Sweltsa borealis* [Banks] and *Sweltsa* sp. specimens).

Species turnover

We calculated species turnover between consecutive sites ascending each elevational transect with Whittaker's species turnover index ($\beta_W = [\gamma - \alpha]/\alpha$; Whittaker 1972, Tuomisto 2010), which measures the similarity between two sites based on presence/absence data (Koleff et al. 2003). For our study, γ is the total taxonomic richness within an elevational and α is the mean of the taxonomic richness among sites within an elevational 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 elevational 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 elevational. We used linear regression to test for a linear trend in β_W across elevation 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, but not to draw strict inference.

We separated β_W values into two 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 1972, Tuomisto 2010) to test for similarity among sites in different watersheds but at comparable elevations. Higher β-diversity values for an elevational band indicate fewer shared taxa among sites in different watersheds at that elevational. We excluded the highest- and two lowest-elevational sites (of 26 sites total) from this comparison because we lacked multiple sites at an equivalent elevational.

We calculated β-diversity values for morphospecies and barcode-taxon datasets, plotted them together against elevational, 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% (four sequences) that did not meet the barcode-compliance criteria were based on a single high-quality read (> 500 bp, zero 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 elevation

EPT richness showed a hump-shaped trend in all three watersheds when plotted by watershed and compositely at the morphospecies and barcode-taxon levels (Fig. 2.3A–D).

Barcode-taxon and morphospecies richness differed significantly (paired t-test, $t_{25} = 2.59$, p = 0.016), but showed similar patterns within (Fig. 2.3A–C) and among (Fig. 2.3D) watersheds. Mean EPT richness across all sites was 11 based on morphospecies and 12 based on barcode taxa. Site richness ranged from zero to 19 morphospecies and zero 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.* elevational significantly better than a linear regression for morphospecies (goodness-of-fit test, $R^2 = 0.65$, $F_{2,23} = 41.88$, $p < 0.001$) and barcode taxa ($R^2 = 0.63$, $F_{2,23} = 38.50$, $p < 0.001$). 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 $p < 0.050$), 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-elevation. Only two sites were in the plains ecozone, so plains diversity might have been underestimated.

Elevational ranges

Elevational ranges for EPT taxa varied from narrow to wide (Table 2.1). The median elevational ranges were 838 m for morphospecies and 553 m for barcode taxa, and elevational ranges differed between morphospecies and barcode taxa (Wilcoxon rank-sum test using normal approximation, p = 0.007; Fig. 2.4).

Species turnover

Morphospecies and barcode-taxon β_W was not linearly related to elevation when data from all watersheds were pooled (linear regression, $F_{1,21} = 0.68$, $p = 0.418$, 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. 2.5A–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 elevational bands was negatively related to elevation but the relationship between barcode-taxon β-diversity within elevational bands and elevational 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. 2.6). 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 elevation

Richness of numerically abundant EPT morphospecies and barcode taxa showed a hump-shaped pattern along the elevational gradient (Fig. 2.3A–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-elevational plains or high-elevational alpine regions. Thus, our results indicate higher species richness at mid-elevations in these Rocky Mountain streams. These results agree with those of a published meta-analysis showing that the most common richness trend across elevational (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 elevational 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 elevational have been 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-elevational 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 our study and

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

Allan (1975) and Perry and Schaeffer (1987) considered elevational transects that began at relatively high elevation (2610 and 2315 m asl, respectively) on Colorado's Western Slope and found an inverse richness trend with elevational. We would have found a similar trend had we started our transects at comparable elevation because of large numbers of mid-elevational taxa (Fig. 2.3). 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, elevational effects were confounded with stream-order effects in all previous studies of richness along an elevational 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 elevational 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 2.1). 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 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 three 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-elevation 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 one 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-elevation.

Elevational ranges

Species elevational 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 elevational ranges for two reasons. First, we included only numerically abundant taxa, and elevational ranges might have been larger had we included vagrant individuals and low-density populations at the margins of elevational ranges. Second, we barcoded only five individuals per taxon per locality, so our ability to detect true elevational ranges of rare cryptic taxa was limited. However, our approach provided fine-scale taxonomic resolution by using barcoding when estimating species-level elevational ranges.

In agreement with Ward's study (1986), most Plecoptera had broad elevational ranges. However, *S. borealis* generally was restricted to high-elevational sites and *Triznaka signata* (Banks) was found only at lower-elevational 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-elevational sites, respectively. Diversity of Trichoptera was low at low elevation, a result that could be related to stream size. We controlled for increasing stream size with decreasing elevational, and the small wadeable streams in our study may support a lower diversity of Trichoptera than the larger low-elevational streams sampled in other studies.

Ward (1986) commented on a progressive increase in richness of Ephemeroptera with decreasing elevational. In contrast, we found a variety of taxa with broad elevational 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 elevational ranges primarily because it increased our ability to detect taxa with small elevational ranges (Fig. 2.4). 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. 2.5A–C). High variation in turnover across elevation suggests that compositional similarity at adjacent sites may not be determined by the position of taxa along these elevational gradients (Fig. 2.5A–C). Our results contrast with our interpretation of previous studies that suggested high rates of turnover at mid to high elevation (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 elevation 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 two 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. 2.6), but the regression slope for β-diversity of barcode taxa as a function of elevation was only marginally different from zero. R^2 values for these regressions were moderately high and suggested more-homogeneous species composition among higher-elevational than among lower-elevational 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 elevation (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-elevational 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 elevation. 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 elevation 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 elevational 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 elevation) in numerically abundant EPT taxa richness across elevation. Elevational 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 elevation in three 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 elevational ranges, and similarity of communities in adjacent sites along ascending elevational 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).

Table 2.1 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	

Table 2.1 (*Continued*)

Order	Family	*Genus*	*Species*	N MS barcoded	Final N barcoded	Range MS	Range BC taxon	Cryptic species
			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	***Paraleptophlebi***						
			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	

Table 2.1 (*Continued*)

Order	Family	*Genus*	*Species*	N MS barcoded	Final N barcoded	Range MS	Range BC taxon	Cryptic species
		Genus						
			sp.	**38**	-	**2189-3397**	-	
		Suwallia						
			*sp. A**	-	14	-	2189-3364	(ISD)
			*sp. B**	-	8	-	2189-3364	(ISD)
		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	-	28	-	2573-3397	
	Perlidae	***Hesperoperla***						
			pacifica*	**36**	36	**2001-3249**	2001-3060	

Table 2.1 (*Continued*)

Order	Family	*Genus*	*Species*	N MS barcoded	Final N barcoded	Range MS	Range BC taxon	Cryptic species
	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	

Table 2.1 (*Continued*)

Order	Family	*Genus*	*Species*	N MS barcoded	Final N barcoded	Range MS	Range BC taxon	Cryptic species
			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			

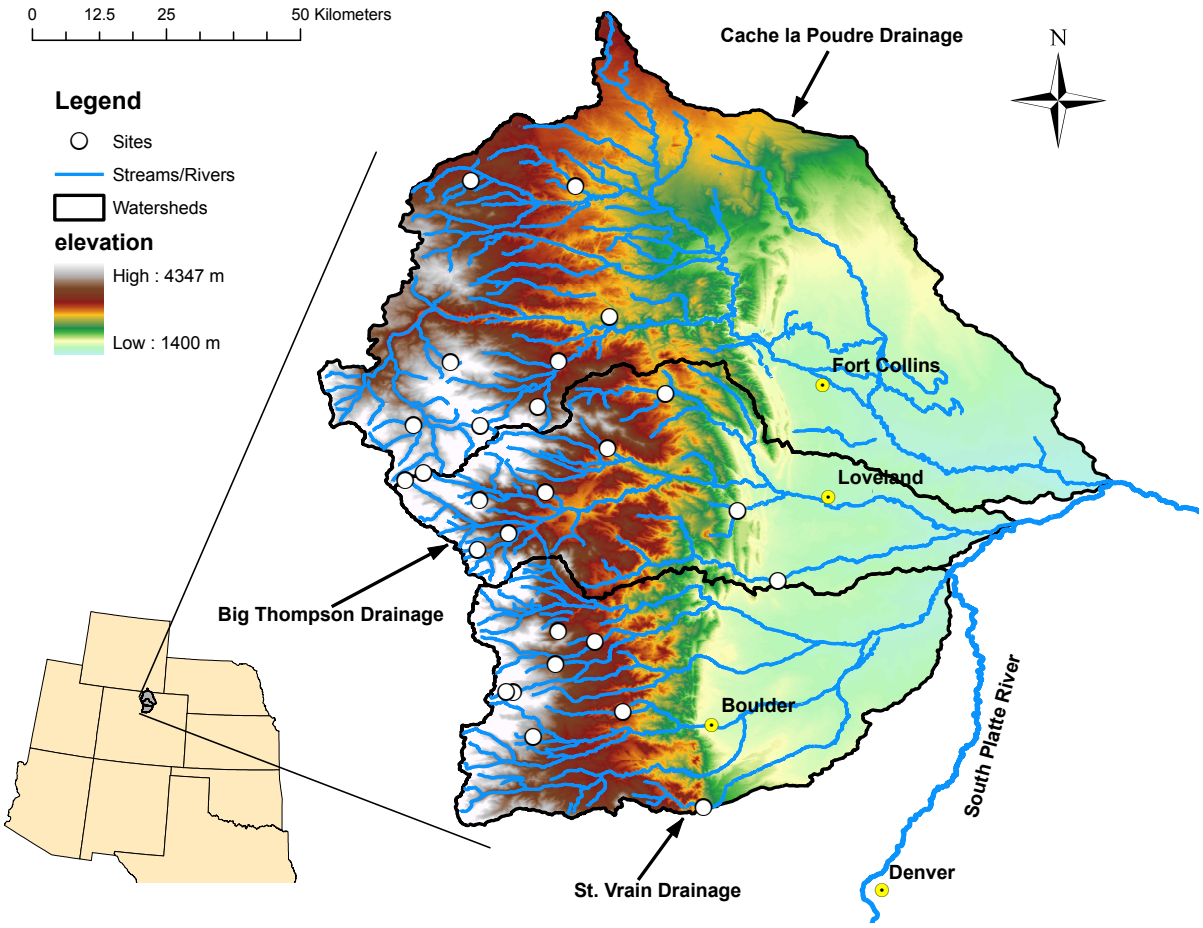

Figure 2.1 Map of study area showing collection localities and elevation in the Cache La Poudre, Big Thompson, and Saint Vrain watersheds in northern Colorado, USA.

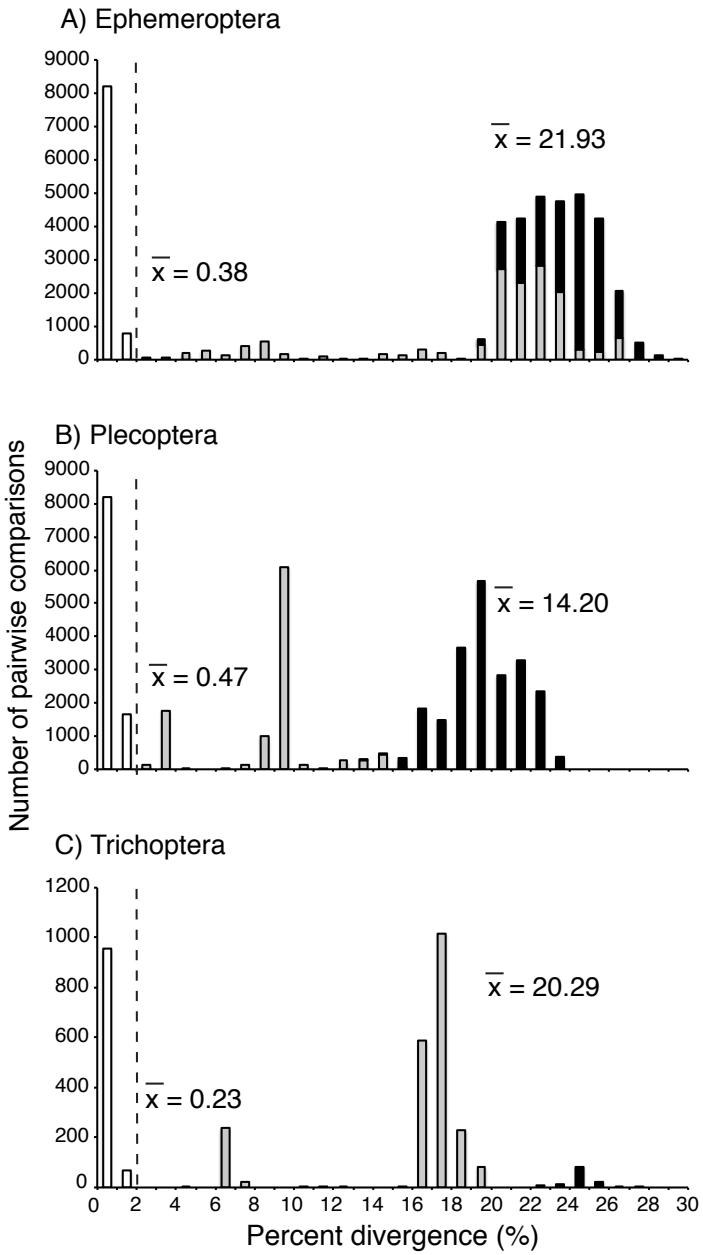

Figure 2.2 Number of pairwise comparisons *vs*. genetic divergence among specimens calculated using Kimura's 2-parameter model for Ephemeroptera (A), Plecoptera (B), and Trichoptera (C). White portions of bars represent comparisons among members purported to be of the same barcode species designation, gray portions are comparisons among members of the same purported genus, and black portions are comparisons among members of the same purported family. The two 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.

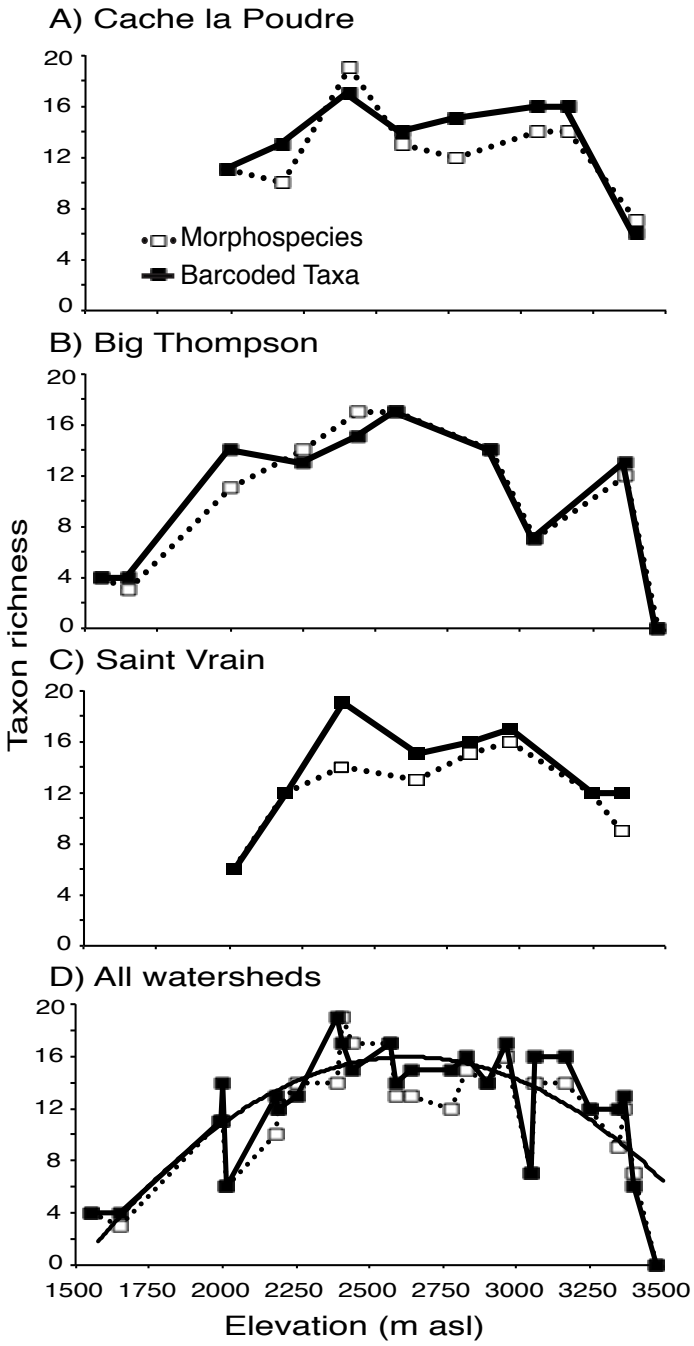

Figure 2.3 Morphospecies and barcode taxon richness by elevation for the Cache La Poudre (A), Big Thompson (B), and Saint Vrain (C) watersheds in Colorado, USA, and from all watersheds combined (D). A 2nd-order polynomial function was fit to the data from all watersheds.

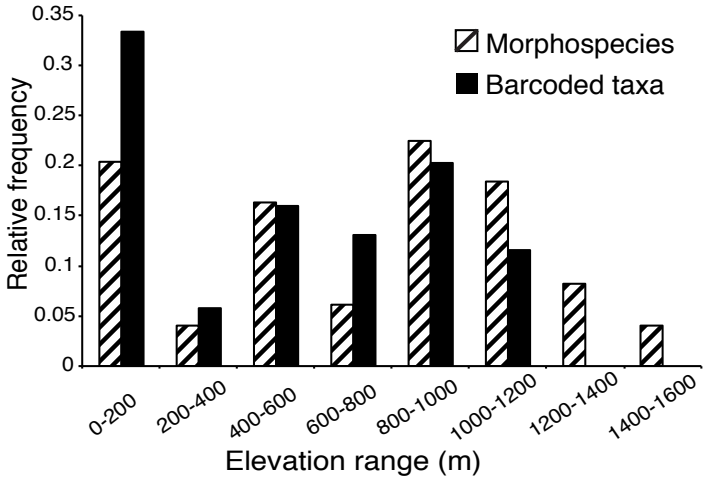

Figure 2.4 Frequency of a given elevation-range class for morphospecies (genera/species) or species identified using deoxyribonucleic acid (DNA) barcodes.

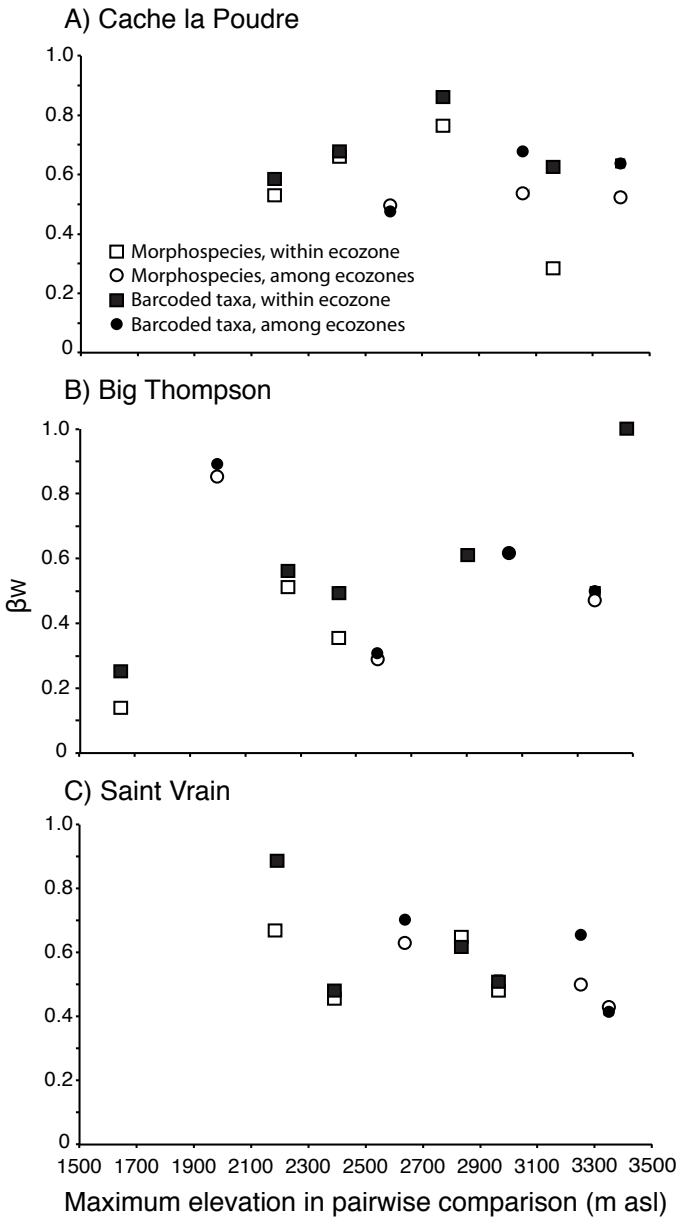

Figure 2.5 Whittaker's species turnover (βW) as a function of maximum elevation for pairwise comparisons across an elevation gradient for the Cache La Poudre (A), Big Thompson (B), and Saint Vrain (C) watersheds for morphospecies (genera/species) and species identified using deoxyribonucleic acid (DNA) barcodes.

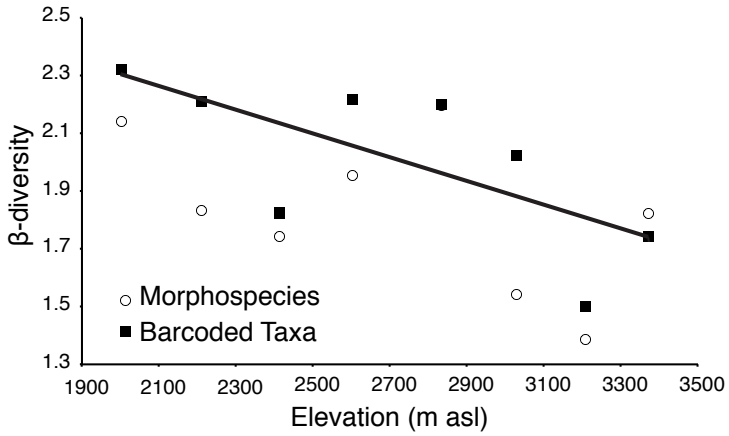

Figure 2.6 Whittaker's β-diversity as a function of mean elevation for sites of comparable elevation across the 3 watersheds in the study area. The x-axis denotes the elevation class for which β-diversity was calculated. A trend line is fitted to the barcode-taxon data showing a marginally significant relationship between β-diversity and elevation.

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3. CRYPTIC SPECIES DIVERSITY REVEALS BIOGEOGRAPHIC SUPPORT FOR THE 'MOUNTAIN PASSES ARE HIGHER IN THE TROPICS' HYPOTHESIS[2]

Summary

The 'mountain passes are higher in the tropics' (MPHT) hypothesis posits that reduced climate variability at low latitudes should select for narrower thermal tolerances, lower dispersal and smaller elevational ranges compared with higher latitudes. These latitudinal differences could increase species richness at low latitudes, but that increase may be largely cryptic, because physiological and dispersal traits isolating populations might not correspond to morphological differences. Yet previous tests of the MPHT hypothesis have not addressed cryptic diversity. We use integrative taxonomy, combining morphology (6136 specimens) and DNA barcoding (1832 specimens) to compare the species richness, cryptic diversity and elevational ranges of mayflies (Ephemeroptera) in the Rocky Mountains (Colorado; approx. 40°N) and the Andes (Ecuador; approx. 0°). We find higher species richness and smaller elevational ranges in Ecuador than Colorado, but only after quantifying and accounting for cryptic diversity. The opposite pattern is found when comparing diversity based on morphology alone, underscoring the importance of uncovering cryptic species to understand global biodiversity patterns.

Introduction

Understanding patterns of diversity and distributions of species is a cornerstone of ecology. Recently, ecologists have recognized the importance of identifying cryptic species in studies ranging from assessing the dynamics of interspecific interactions (Hebert et al. 2004) to accurately predicting biodiversity losses from climate change (Bálint et al. 2011). Cryptic species

[2] Gill, B.A, B.C. Kondratieff, K.L. Casner, A.C. Encalada, A.S. Flecker, D.G. Gannon, C.K. Ghalambor, J.L, Guayasamin, N.L. Poff, M.P. Simmons, S.A. Thomas, K.R. Zamudio, and W.C. Funk. 2016. Cryptic species diversity reveals biogeographic support for the 'mountain passes are higher in the tropics' hypothesis. Proceedings of the Royal Society London B: Biological Sciences 283:20160553.

are taxa that are morphologically indistinguishable and consequently often incorrectly considered as a single nominal species when in fact constituent taxa are genetically divergent and reproductively isolated from each other (Bickford et al. 2007). While the identification of cryptic species is crucial for understanding global biodiversity patterns (Bickford et al. 2007), cryptic species are seldom addressed in studies of large-scale trends in species richness and ranges. Failure to distinguish cryptic species underestimates species richness and distorts our perception of trends in diversity (Vieites et al. 2009, Funk et al. 2012, Gill et al. 2014). Additionally, pooling together cryptic species with distinct characteristics can obscure our understanding of each taxon's individual niche and function (Molbo et al. 2003, Hebert et al. 2004, Blair et al. 2005, Kankare et al. 2005, Stireman et al. 2005, Eastwood et al. 2006, Smith et al. 2006). These problems are further exacerbated by our incomplete understanding of the geographic distribution of cryptic diversity (Bickford et al. 2007), which is likely non-random and thus a potential bias for inferences of species diversity and distributions at large scales. Here we provide evidence that quantifying and accounting for cryptic diversity illuminates biodiversity patterns driven by latitudinal differences in climate variability that would otherwise be missed.

The "climate variability hypothesis" (CVH; Dobzhansky 1950, Stevens 1989) posits that the breadth of a species' thermal tolerance and geographical range size should be proportional to the degree of climate variability it has experienced over evolutionary time. Climate (temperature) variability generally increases with latitude (Dobzhansky 1950, Vannote and Sweeney 1980, Müller 1982, Stevens 1992, Sunday et al. 2011), resulting in temperate organisms experiencing broader temperature ranges than tropical species due to pronounced seasonality. The CVH thus predicts selection for broad thermal tolerances of temperate species and narrower thermal tolerances of tropical species. Narrow thermal tolerance will in turn select against dispersal into

inhospitable climates, resulting in smaller geographical ranges (Dobzhansky 1950, Janzen 1967, Stevens 1989, Ghalambor et al. 2006).

Janzen (1967) extended the CVH to elevational gradients by proposing the 'mountain passes are higher in the tropics' hypothesis (MPHT) as a mechanism to explain high species turnover and smaller elevational ranges at lower latitudes. The MPHT hypothesis proposes that mountains are more effective physiological barriers for tropical than temperate species, because along elevational transects, the annual thermal regimes of sites in the tropics have less overlap than those in the temperate-zone (Janzen 1967). Consequently, the broad thermal tolerances of temperate-zone species should allow them to disperse more broadly across elevational gradients, whereas narrow thermal tolerances of tropical species should restrict their distributions to relatively narrow elevational bands (Janzen 1967, Ghalambor et al. 2006).

By extension, the MPHT hypothesis provides a mechanistic explanation for latitudinal differences in species richness (Ghalambor et al. 2006). The MPHT hypothesis predicts that across tropical elevational gradients, populations with narrow thermal tolerances and limited dispersal ability will have increased isolation, genetic divergence, and speciation, ultimately leading to higher species richness at low latitudes. Moreover, divergence in the traits functioning as the isolating mechanism proposed by the MPHT hypothesis (*i.e.* narrow thermal tolerance and low dispersal ability) may not necessarily be correlated with obvious morphological differences among species at different elevational zones. Consequently, in the tropics, we would expect not only more species, but also more morphologically similar, cryptic species.

The MPHT hypothesis provides a rich framework with which to test hypotheses about latitudinal differences in species richness, levels of cryptic diversity, and elevational ranges (Ghalambor et al. 2006). To date, several studies support predictions from the MPHT hypothesis

(Ghalambor et al. 2006). For example, many groups of organisms show increases in species richness from the poles towards the equator (the latitudinal diversity gradient; Fischer 1960, MacArthur 1965, Pianka 1966, Rohde 1992, Willig et al. 2003, Hillebrand 2004); across elevations, faunal similarity of communities increases with latitude (Huey 1978); tropical faunas generally have high levels of cryptic species diversity (Hebert et al. 2004, Vieites et al. 2009, Funk et al. 2012); tropical species often display small elevational ranges (Lieberman et al. 1996), and the elevational ranges of many species increase with latitude (McCain 2009). In contrast, findings from several other studies appear to contradict the predictions from the MPHT hypothesis. For example, exceptions to the latitudinal diversity gradient exist (Gaston and Blackburn 2000); the majority of cryptic species found to date are temperate (Bickford et al. 2007), and some taxonomic groups do not show differences in the elevational ranges of tropical and temperate species (Kozak and Wiens 2007, Cadena et al. 2012).

Equivocal support for predictions of the MPHT hypothesis could arise from several important limitations that potentially affect the conclusions of previous studies. First, most cross-latitude comparisons of species richness and elevational ranges lack comparable taxonomic resolution across latitudes and do not address cryptic diversity. Tropical and temperate sites vary significantly not only in terms of diversity, but also in taxonomic resolution, with less explored and highly diverse tropical biota potentially including high levels of cryptic diversity (Hebert et al. 2004, Vieites et al. 2009, Funk et al. 2012). Second, all previous studies comparing latitudinal changes in elevational ranges were based on museum collection records, published literature, regional field guides, surveys, and/or online distributional databases (Kozak and Wiens 2007, McCain 2009, Cadena et al. 2012). Such measures of species elevational ranges can provide biased estimates if species occupy different elevations in different parts of their range, or through

time (Cadena et al. 2012). Third, when data are combined from studies using different sampling designs, any associated sampling biases can only be corrected for *post hoc*. Lastly, previous studies (Kozak and Wiens 2007, McCain 2009, Cadena et al. 2012) have not used comparative phylogenetic approaches to distinguish effects of shared phylogenetic history on elevational range size values. The ideal test of patterns predicted by the MPHT hypothesis would use consistent criteria for species delimitation, identify cryptic species *a priori,* estimate ranges empirically using standardized sampling methods, and control for phylogeny.

Here, we test the MPHT hypothesis by comparing species richness, levels of cryptic diversity, and elevational ranges of stream-dwelling mayflies (Ephemeroptera) from north temperate (the Rocky Mountains, Colorado, USA) and tropical (the Andes, Napo, Ecuador; Fig. 3.1) latitudes. Using standardized methods, we conducted a broad scale sampling effort along multiple large elevational transects (~ 2,000 m) in both Colorado and Ecuador. Because many tropical mayfly species remain undescribed (Brittain 1982, Dominguez et al. 2006, Sartori and Brittain 2014) and have not previously been represented with species level taxonomy in cross-latitudinal comparisons, we used an integrative taxonomic approach, combining morphological taxonomy and DNA barcoding (Hebert et al. 2003), to delimit species and detect cryptic diversity. We then used a comparative phylogenetic approach to control for phylogenetic signal while testing for a relationship between elevational ranges and latitude. Our study extends the MPHT hypothesis by linking latitudinal differences in climate variability to cryptic diversity and finding support for several key predictions of the MPHT hypothesis, thus demonstrating the importance of accounting for cryptic diversity to make correct inferences about global biodiversity patterns.

Materials and Methods

Study area and collection

In the Colorado Rockies, we established elevational transects in three distinct watersheds all draining into the South Platte River and spanning an elevational gradient from 1,556 to 3,478 m asl (1,922 m total). In the Andes in Ecuador, we established two transects in watersheds draining into the Napo River and spanning an elevational gradient from 1,664 to 4,248 m asl (2,584 m total). Within each transect, we selected distinct wadeable tributaries to the river main stem every 200 m of elevational gain for sampling.

In Colorado, we sampled a total of 26 sites from 27 June to 10 August 2011. The following year in Ecuador, we sampled 26 sites from 17 January to 25 February 2012. At each site, we collected mayfly immatures using a standard D-frame kick-net (500-µm mesh) for approximately two hours within a 100-m reach of the stream. We emptied the contents of kick-nets into pans and sorted specimens of numerically abundant taxa in the field to the lowest taxonomic level possible. On the same date at each site, we collected adult mayfly specimens from the riparian area using an aerial net and a beating sheet until no new taxa were found. We preserved all specimens immediately in ≥ 95% ethanol, which was replaced with fresh ethanol within 24 hours (Baird et al. 2011).

Morphological and molecular species identification

We identified 6,136 specimens morphologically (Colorado: 4,035; Ecuador: 2,101) to the lowest taxonomic level possible (MTUs) using available literature (Ward et al. 2002, Dominguez et al. 2006, Merritt et al. 2008, Domínguez and Fernández 2009). Fewer specimens were examined in Ecuador than Colorado not because of differences in sampling effort, but rather because mayfly densities were naturally lower in Ecuador than Colorado, a potential

consequence of latitudinal differences in resource availability (Stout and Vandermeer 1975), the relative strengths of direct (Fox 1977, Dudgeon 1993, Flowers and Pringle 1995) and indirect interspecific interactions (Flecker 1992), and/or predictability of the hydrologic regime (Dudgeon 1993, Flecker and Feifarek 1994). The level of taxonomic resolution achieved for MTUs depended on latitude, life stage, and sex, and collections were consequently classified at various taxonomic levels. Expertly identified material was available for comparison for Colorado taxa at the C.P. Gillette Museum of Arthropod Diversity, Colorado State University. For taxa from Ecuador, a regional collection of immatures was available for comparison at the Aquatic Ecology Laboratory at the Universidad San Francisco de Quito.

To establish species-level taxonomic units from MTUs, we used DNA barcoding (Hebert et al. 2003). For each MTU defined at each site, we DNA barcoded up to ten specimens, if available. For Colorado, we utilized five previously published DNA barcodes per MTU per site if available from Gill et al. (2014) and when possible sequenced five additional specimens for this analysis (1,188 specimens total). For Ecuador, we DNA barcoded ten individuals per MTU per site if available (644 specimens total). We used standard high-throughput DNA barcoding protocols from the Canadian Center for DNA Barcoding (Hajibabaei et al. 2005, Ivanova et al. 2005, 2006). Primers are listed in Appendix 1.1. DNA barcode sequences were trimmed, assembled, and checked manually in Sequencher v. 5.3 (Gene Codes, Ann Arbor, MI, USA). Sequences were uploaded to the Barcode of Life Database (BOLD; Ratnasingham and Hebert 2007) and made publicly available in the dataset "Richness and Elevational Ranges of Mayflies" (DS-RERM; doi:dx.doi.org/10.5883/DS-RERM). Sequences were automatically screened by BOLD for common contaminants and stop codons. Refined single linkage (RESL) clustering (Ratnasingham and Hebert 2013) was used to assign sequences to "barcode index numbers"

(BINs), taxonomic units putatively equivalent to biological species. We examined concordance between MTUs and BINs and reexamined specimens as necessary in instances of conflict. All taxonomic and locality information for both MTU and BIN designations is provided in Extended Data File S3.1. We used BIN identifications as preliminary units for phylogenetic analysis.

Phylogenetic analyses

Parsimony

We aligned all available DNA barcode sequences representing each BIN from each family independently of the others in MAFFT v. 7 (Katoh 2002) using strategy G-INS-i with offset value 0.1 and all other options set as default. For each alignment, we included one sequence from each genus from all other mayfly families included in our study as outgroups. We examined alignments for potentially erroneous base calls, gaps, and unusually divergent sequences. In cases of potentially erroneous base calls and gaps, we reexamined the original sequencing chromatograms. All alignments are available from the Colorado State University Digital Repository (CSUDR): http://hdl.handle.net/10217/170247.

We conducted equally weighted parsimony tree searches using each data matrix. Up to 50 trees were held within each of 10,000 random-addition-sequence (RAS) tree-bisection-reconnection (TBR) searches that also implemented 100 ratchet iterations (Nixon 1999), which alternated between equal character weighting and each character having a 10% chance of being upweighted and a 5% chance of being downweighted. A strict consensus tree was then calculated by using TBR-collapsing (Goloboff and Farris 2001). Parsimony jackknife (Farris et al. 1996) analyses were conducted using TNT v. 1.1 (Goloboff et al. 2008) with the removal probability set to approximately e^{-1} (0.37). One-thousand jackknife pseudoreplicates were performed using the same search strategy, albeit with only 100 RAS + TBR + ratchet searches per

pseudoreplicate. Jackknife values were mapped onto the strict consensus trees using SumTrees v. 3.3.1 (Sukumaran and Holder 2010), after which the trees were examined and printed using TreeGraph v. 2.0.54-364 (Stöver and Müller 2010).

We reduced well-supported tip clades (≥ 63% bootstrap (Felsenstein 1985) or jackknife (Farris et al. 1996); support achieved by one uncontradicted synapomorphy) down to a single terminal per species. In many cases, saturation prevented robust resolution of higher-level relationships among the species in our study. Consequently, we constrained higher-level relationships in our tree using previous studies of mayfly systematics. We used decision rules modified from (Poff et al. 2006) listed in Appendix 1.1 to prioritize which studies to use when multiple sources of information about species relationships were available. Detailed documentation of nodal support based on our phylogenetic analysis of COI and constraints from the literature is provided in Extended Data File S3.2 and Fig. S3.1.

Bayesian inference

We randomly choose one DNA barcode sequence from among the set of longest sequences for each BIN (supported by our parsimony analysis) and one specimen of *Anacroneuria* Klapálek (Plecoptera) for an outgroup. We aligned these sequences in MAFFT v. 7 (Katoh 2002) using the same settings as described above (alignments available from CSUDR: http://hdl.handle.net/10217/170247). We determined that the HKY+G was the appropriate model of nucleotide substitution using jModelTest2 (Guindon and Gascuel 2003, Darriba et al. 2012) based on Akiake's Information Criteria. We ran four simultaneous analyses in parallel (Altekar et al. 2004) with four chains for 50,000,000 generations in MrBayes v. 3.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003) through the CIPRES Science Gateway (Miller et al. 2010). We ran this analysis with and without constraints from the literature. We ensured

that our four simultaneous independent runs converged and reached stationarity by checking that the average standard deviation of split frequencies was < 0.01, that effective sample sizes for parameters were > 200, and by plotting the −ln likelihood scores against generation time in Tracer v 1.6 (Rambaut et al. 2014). We discarded the first 12,500,000 trees (25%) as burn-in and used the remaining trees to construct fifty percent majority rule consensus trees.

Determination of elevational ranges

For each species supported by our phylogenetic analysis, elevational ranges were interpolated between the highest and lowest elevation collection localities. Species found at only one locality were assigned a value of zero. The elevational ranges of Ecuadorian species were truncated at an upper limit of 3500 m asl, allowing us to compare the same elevational interval from ~ 1500-3500 m in both Colorado and Ecuador (McCain 2009). Species found only above 3500 m asl were excluded from further analyses. Because this truncated sampling range could artificially reduce estimates of tropical ranges, we ran analyses with and without truncating Ecuadorian elevational ranges and removing taxa found over 3500 m asl. Our results were qualitatively the same with and without truncation and the removal of high elevation taxa (for a summary of non-truncated results see Table S3.1).

Trees for comparative phylogenetic analysis

We trimmed our parsimony supertree to include only species found within the desired 1500–3500 m asl elevational interval. Because the parsimony supertree had numerous soft polytomies and no branch lengths, we randomly resolved polytomies 1,000,000 times resulting in 1,000,000 alternative topologies. Arbitrary branch lengths were assigned to each tree using one of two common methods. For half of the alternative topologies (500,000), we set all branch lengths equal to one. For the remaining alternative topologies (500,000), branch lengths were set

according to Grafen's (1989) method where lengths are set equal to the number of descendant tips minus one.

We trimmed our Bayesian 50% majority rule consensus trees to include only species found within the desired 1500–3500 m asl elevational interval. We left polytomies unresolved. We used estimates of relative divergence for branch lengths. All trees are available from CSUDR: http://hdl.handle.net/10217/170247.

Comparative phylogenetic analysis

We used PGLS (Grafen 1989) fit with an Ornstein–Uhlenbeck model (OU; Hansen 1997, Butler and King 2004) of trait evolution to control for phylogenetic signal while comparing the elevational ranges of species from Colorado and Ecuador using the gls function in the R package nlme (Pinheiro et al. 2015). We used maximum likelihood estimation to determine the appropriate value of the model parameter α. We dummy coded the explanatory variable latitude "0" for Ecuador and "1" for Colorado and the response variable was elevational range (m). Consequently, significantly positive regression slopes would support the one-sided hypothesis that Colorado species have larger ranges than Ecuador species. For non-ultrametric trees, variance heterogeneity was modeled with the option "weights" in gls (Paradis et al. 2004). To summarize regression results (available from CSUDR: http://hdl.handle.net/10217/170247), we provide regression parameter estimates and summary statistics for one-sided hypothesis tests in Table 3.1. For parsimony trees, we model averaged (Burnhan and Anderson 2002) regression parameters (slope, intercept, standard errors).

Results

Latitudinal differences in species richness

The use of integrative taxonomy, combining morphological study, DNA barcoding, and phylogenetic analyses of DNA barcodes, revealed higher species richness in Ecuador than Colorado streams (Fig. 3.2A). However, based on morphology alone, we identified more morphological taxonomic units (MTUs) in Colorado than Ecuador, a disparity caused by high levels of cryptic tropical diversity. DNA barcoding and subsequent phylogenetic analyses of DNA barcodes for a subset of morphologically identified specimens from each MTU and locality resulted in the recognition of 95 distinct species (Ecuador: 54; Colorado: 41), an increase in the number of taxa identified in both locations, but a disproportionate increase in Ecuador (350%) compared to Colorado (46%) (Fig. 3.2A; MTUs *vs.* species).

Latitudinal comparison of elevational ranges

We found strong support for larger elevational ranges in Colorado mayfly species compared to related species from Ecuador, a pattern only apparent after delimiting species and elevational ranges using DNA barcoding (Fig. 3.2B). Using almost all (> 99%) parsimony supertree topologies and both constrained and unconstrained Bayesian trees, we consistently found that Colorado elevational ranges were significantly larger (~ 200 m wider; ~61% greater) than those of species from Ecuador (Table 3.1). In most cases, we found limited evidence for phylogenetic signal in elevational range size estimates (Table 3.1; Fig. 3.2C).

Discussion

The MPHT hypothesis predicts that reduced climate variability in the tropics will result in reduced dispersal and smaller ranges across elevational gradients than in the temperate zone (Janzen 1967, Ghalambor et al. 2006). If true, substantial elevational climatic zonation in tropical

mountain systems should lead to increased opportunities for speciation and an accumulation of species at low latitudes (Ghalambor et al. 2006) many of which may be cryptic. Using integrative taxonomy to delimit species and identify cryptic diversity, we found higher mayfly species richness in Ecuador than Colorado. After controlling for phylogeny, we also found strong evidence that the elevational ranges of mayfly species are smaller in the Andes than in the Rocky Mountains. Collectively, our results indicate that many tropical mayflies previously identified morphologically as taxa with large elevational ranges, are in fact collections of different cryptic species with small elevational ranges. Below, we discuss the implications of these results in more detail.

The large increases in the number of mayfly species we detected using integrative taxonomy underscores the need for multipronged approaches in understudied and potentially highly cryptic tropical taxa. To date, large-scale ecological studies of stream insects in the Neotropics have relied primarily on morphological species descriptions and most have limited their analyses to the family or genus levels (*e.g.*, Jacobsen et al. 1997). By applying DNA barcoding methods (Hebert et al. 2003), we standardized our comparisons of diversity and elevational ranges of the mayfly fauna in both Colorado and Ecuador. This approach had two important advantages over identifications based solely on morphology: separating species for which keys do not exist, and distinguishing cryptic species. Cryptic species are common in the tropics (Hebert et al. 2004, Vieites et al. 2009, Funk et al. 2012) and if undetected, pooling of multiple cryptic species will lead to underestimates of species richness and overestimates of species distributions. In our study, overcoming tropical taxonomic limitations using integrative taxonomy allowed for a robust latitudinal comparison of mayfly species richness and distributions.

We found higher mayfly species richness in Ecuador than Colorado (Fig. 3.2A) supporting the hypothesis that higher levels of elevational climatic zonation along tropical gradients and reduced dispersal of tropical species may promote speciation (Ghalambor et al. 2006). Assuming extinction rates are similar across latitude, high rates of tropical speciation should lead to higher species richness at lower latitudes (Ghalambor et al. 2006). Mechanistically, this accumulation of species is thought to occur in one of two ways. Populations might become isolated on distinct sides of mountain passes, leading to divergence and eventual speciation (allopatric speciation; Ghalambor et al. 2006, Cadena et al. 2012). Alternatively, populations might also adaptively diverge and speciate along a single elevational gradient (parapatric speciation; Kozak and Wiens 2007, Cadena et al. 2012). Though a few studies have attempted to draw generalizations about latitudinal differences in modes of speciation (Kozak and Wiens 2007, Cadena et al. 2012), it seems that both allopatric (*e.g.*, Cadena et al. 2012) and parapatric (*e.g.*, Kozak and Wiens 2007) mechanisms operate in the tropics and that the taxon-specific balance between dispersal and selection (Gavrilets 2004, Cadena et al. 2012) determines the extent of parapatric speciation along single elevational gradients. Our results provide evidence that high species richness of tropical mayflies may arise not only via allopatric speciation, but also by parapatric speciation along single elevational gradients because limited thermal tolerance restricts the dispersal ability of these species (Brittain 1982, Sartori and Brittain 2015).

Consistent with the predictions of the MPHT hypothesis, we found that mayflies from Colorado had significantly larger (~ 200 m wider; ~61% greater) elevational ranges than mayflies from Ecuador (Fig. 3.2B; Table 3.1). Our results are consistent with those observed for many terrestrial vertebrate species, in which tropical taxa had smaller elevational distributions

than temperate species (McCain 2009). In contrast, latitudinal differences were not found in two other studies (Kozak and Wiens 2007, Cadena et al. 2012), which focused more on latitudinal differences in modes of speciation than on comparing elevational ranges. Discrepancies among the aforementioned studies are likely driven by differences in procedures for selection and analysis of elevational range size data. McCain (2009) found that many tropical vertebrate species had smaller elevational distributions than related temperate species. In doing so, McCain demonstrated the need to control for strong effects of mountain height, sampling scale (local *vs*. regional), and percentage of height sampled in studies compiling data from multiple published sources. Additionally, none of the aforementioned studies addressed cryptic diversity, and given the high occurrence of cryptic species in the tropics, this limitation is clearly important. Here, we avoided these problems by using standardized protocols across latitude for species delimitation, detection of cryptic diversity, and determination of elevational ranges.

While the observed latitudinal differences in elevational ranges provide indirect evidence for some of the predictions of the MPHT hypothesis, the mechanisms responsible for these patterns remain untested. Variation in thermal tolerance is commonly invoked as an explanation for climate-based latitudinal differences in elevational range sizes (Janzen 1967, Ghalambor et al. 2006, Bozinovic et al. 2011), but several other mechanisms could also be operating. First, species interactions could modify distributions by elevational niche partitioning (Ghalambor et al. 2006, Kozak and Wiens 2007, McCain 2009, Cadena et al. 2012). Smaller elevational ranges of tropical species could result from higher levels of tropical interspecific competition (Schemske et al. 2009). Second, latitudinal differences in rates of growth and development could limit available time for downstream larval drift and upstream adult flight (the recolonization cycle; Müller 1954), with shorter-lived and faster-developing tropical species moving less than

temperate species (Brown et al. 1996, Dudgeon 2000). More limited dispersal of tropical species would result in smaller tropical elevational ranges. Lastly, latitudinal differences in mayfly emergence periodicity could limit the dispersal of tropical species. While temperate species generally emerge simultaneously or over short periods (synchronously), tropical mayflies generally emerge year-round (asynchronously; Brittain 1982, Sartori and Brittain 2015). Thus, for tropical mayflies finding a mate at a distant site might be challenging if the timing of emergence among sites is not consistent. This stochasticity could result in selection for philopatry, resulting in smaller tropical elevational ranges.

Consistent with previous surveys (Brown et al. 1996), we found high within-latitude variability in elevational ranges (three orders of magnitude), even among closely related species (Fig. 3.2C). Interspecific variation in elevational ranges could result from variation in physiological and dispersal traits, which reflect species-specific differences in levels of genetic variation available for selection to act on and/or genetic constraints on physiological adaptation. Additionally, other ecological factors could act in concert with species-specific physiological and dispersal traits to explain observed within-latitude variation in elevational ranges. Mayflies can occupy multiple trophic groups including benthic detritivory, suspension feeding, algivory, and predation (Merritt et al. 2008). Consequently, species distributions could be restricted by food availability, which is regulated by an independent suite of factors. Mayfly species also differ significantly in behavior; the clade includes swimmers, clingers, climbers, burrowers, or sprawlers (Merritt et al. 2008), and those modes require specific microhabitats with variable distributions determined by hydro-geomorphic processes. Moreover, some mayfly species may have specific requirements for oviposition sites (Encalada and Peckarsky 2006); consequently, the availability of suitable sites for eggs may influence species distributions. Thus, species-

specific differences in traits provide a viable explanation for the observed within-latitude variation in elevational ranges.

In conclusion, using integrative taxonomy, we found higher species richness, higher cryptic diversity, and smaller elevational ranges in Ecuador than Colorado, providing strong support for several key predictions of the MPHT hypothesis. Our results implicate climate variability along tropical and temperate elevational gradients as an important selective pressure determining large-scale biogeographic trends for higher cryptic diversity and smaller elevational range sizes in the tropics. Moreover, large increases in the number of species detected and the effects of addressing cryptic diversity on estimates of latitudinal differences in elevational ranges underscores the general importance of uncovering cryptic species in cross-latitudinal and large-scale biogeographic studies.

Table 3.1 Summary of results from phylogenetic generalized least squares regression (PGLS) fit with an Ornstein-Uhlenbeck model of trait evolution (maximum likelihood used to determine α). PGLS was used to control for shared evolutionary history while comparing elevational range breadths across latitude. The explanatory variable "latitude" was dummy coded "0" for Ecuador and "1" for Colorado and the response variable was elevational range breadth (m). Consequently, significantly positive regression slopes support the 1-sided hypothesis that Colorado species have larger elevational ranges than species from Ecuador. For parsimony trees, regression parameters (slope, intercept, standard errors) are model averaged and values of alpha and hypothesis tests are presented as medians. Constraints on phylogenies are detailed in Appendix S3.1 and Fig. S3.1.

Phylogenetic Method	Constraints	Branch Lengths	Slope (±SEM)	Intercept (±SEM)	Alpha (Median)	1-sided p CO > EC (Median)	$p < 0.05$ (%)	$p < 0.10$ (%)
Parsimony	Y	Equal	195 (±82)	329 (±54)	(8.39)	(0.008)	99.99	100.00
Parsimony	Y	Grafen's	191 (±84)	333 (±56)	(112.10)	(0.010)	99.72	99.99
Bayesian	Y	Relative Divergence	179	326	17.93	0.018	100.00	100.00
Bayesian	N	Relative Divergence	179	327	20.42	0.018	100.00	100.00

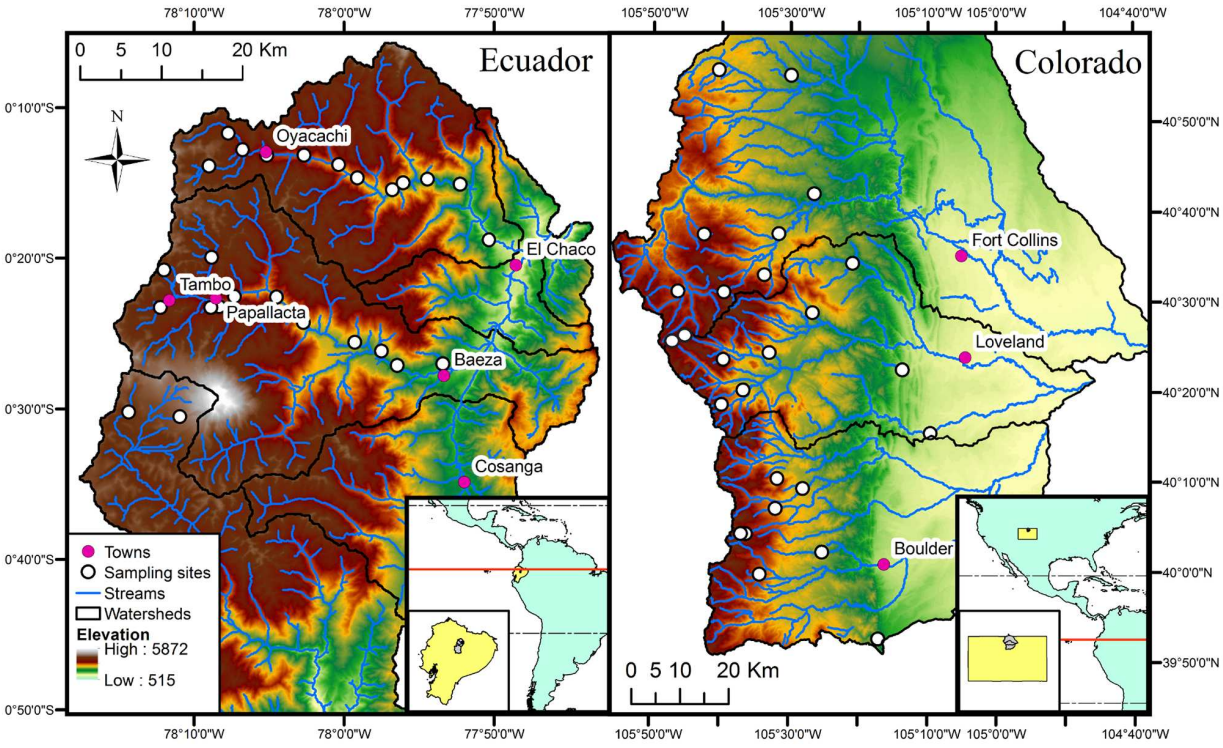

Figure 3.1 Site maps for Ecuador (a; approx. 0°) and Colorado (b; approx. 40°N). Sites were selected every 200 m of elevational gain starting at 1500 m asl. Smallest insets show positions of study area within country of Ecuador or state of Colorado. Larger insets show latitudinal position of study areas relative to equator (red line).

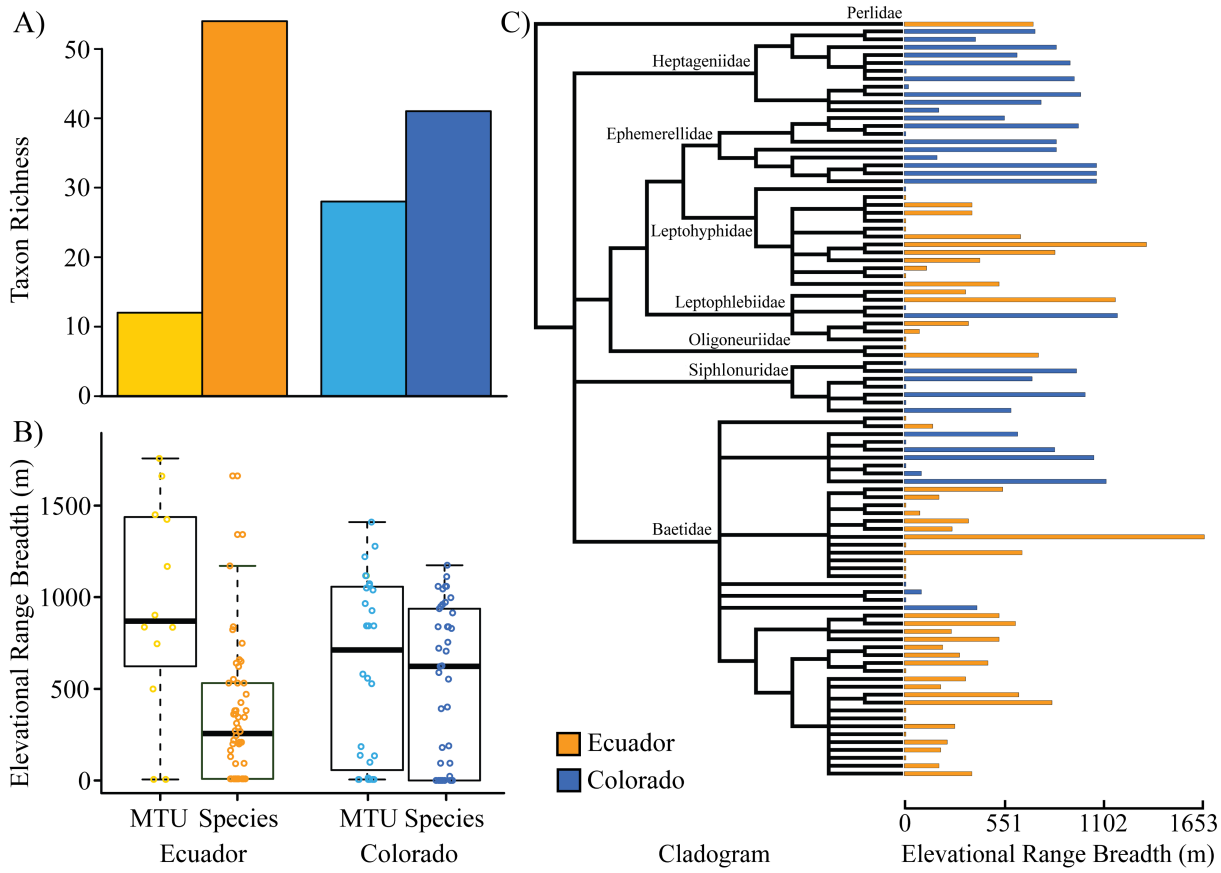

Figure 3.2 (A) Taxon richness by latitude determined for morphological taxonomic units (MTUs) and species defined using integrative taxonomy (Ecuador: MTU n = 12, species n = 54; Colorado: MTU n = 28, species n = 41), (B) Elevational range breadths (m) by latitude determined for MTUs and species and (C) Cladogram of species relationships (left) with elevational range breadths (right) colored by latitude.

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4. NARROW THERMAL TOLERANCE AND LOW DISPERSAL DRIVE DIVERSIFICATION IN TROPICAL MOUNTAINS[3]

Summary

Many different ecological and evolutionary hypotheses have been proposed to explain the high species richness of tropical mountains. Recent theoretical developments have connected latitudinal differences in environmental conditions to species traits to global trends in biodiversity. Specifically, low levels of climate variability in the tropics are thought to favor species with narrow thermal breadths and low dispersal ability, thus promoting population isolation and genetic divergence. Over time, these conditions should promote high rates of tropical speciation, particularly in areas of high topographic relief such as mountains. Yet, despite a wealth of correlative evidence from independent studies of latitudinal differences in species' thermal breadths, population genetic structure, and elevational ranges, researchers have not mechanistically linked climate variability to global trends in montane diversity using integrative methods in a single taxon. Here, we show that thermal tolerance and dispersal ability have diverged in tropical and temperate species in three orders of mountain stream insects. Tropical species had overall narrower thermal tolerances and lower dispersal than related temperate species, resulting in significantly higher levels of genetic diversification and cryptic diversity. Those processes were reflected in higher tropical speciation rates and a greater

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Author Contributions: N.R.P., B.A.G., and A.A.S. collected, analyzed, and synthesized data for the study. B.A.G., K.L.C., and M.M.G performed field collections and/or lab work. A.B., P.M, and M.S. contributed population genetic and phylogenetic analyses. N.R.P., B.A.G., A.A.S, W.C.F., and K.R.Z. wrote the paper, with contributions from all authors.

accumulation of species richness over time, thus linking latitudinal differences in climate to functional traits to speciation to high montane species richness in the tropics.

Introduction

Analyses of global trends in species richness indicate that montane regions in the tropics are some of the most biodiverse ecosystems on earth, with not only high species richness but also many endemic species (Myers et al. 2000, Orme et al. 2005, Ricketts et al. 2005). Yet despite the development of numerous ecological and evolutionary hypotheses to explain the high species richness of tropical mountains (Willig et al. 2003, Mittelbach et al. 2007, Graham et al. 2014, Fine 2015), we have had surprisingly limited success in demonstrating the mechanisms underlying this trend.

One hypothesis with potential to mechanistically explain global trends in montane species richness is the "climate variability hypothesis" (CVH; Dobzhansky 1950, Stevens 1989). Concordant with latitudinal increases in climatic seasonality, the CVH posits that the breadth of species' thermal tolerances should increase with latitude. It follows that across elevations, lower tropical climatic seasonality should result in greater tropical elevational climatic zonation (Janzen 1967). Together, narrower tropical species' thermal tolerances and higher elevational climatic zonation should limit species dispersal within and among tropical elevational gradients, resulting in reduced gene flow and increased population genetic differentiation (Ghalambor et al. 2006). Over time, these conditions should promote speciation leading to a build-up of species in the tropics, many of which may be cryptic if tropical montane species diverge primarily in physiological and dispersal traits lacking morphological phenotypes.

Several studies have provided independent lines of evidence in support of the CVH: many tropical species have narrower thermal tolerances (Addo-Bediako et al. 2000, Sunday et al.

2011), show greater population genetic isolation (Martin and McKay 2004), and occupy smaller elevational ranges (McCain 2009), resulting in greater species turnover across elevational gradients (Huey 1978). Yet, comprehensive support for the CVH as the definitive mechanism underlying global trends in montane species richness has been difficult to achieve, because doing so requires the integration of a large volume of diverse data on physiological traits, gene flow, species diversity, and macroevolutionary rates for taxa from both tropical and temperate regions (Ghalambor et al. 2006, Graham et al. 2014).

Here, we test the CVH using a trait-based analysis of thermal tolerance and dispersal ability and explore the role of these traits in driving global trends in species richness in three orders of aquatic insects: Mayflies (Ephemeroptera), stoneflies (Plecoptera), and caddisflies (Trichoptera) found in tropical (Ecuador) and temperate (Colorado) montane streams. We first characterized the thermal breadth and realized dispersal in a subset of focal species in each insect order from each latitude. We then assessed latitudinal differences in species richness, cryptic diversity, and rates of diversification to relate climatic variability to species traits to global trends in montane biodiversity.

Methods and results

In both the Ecuadorian Andes and the Colorado Rockies, we measured stream temperature ranges and confirmed that thermal variation was indeed lower in Ecuador than Colorado ($t(8) = 4.367$, $p = 0.002$). We then measured the minimum and maximum critical thermal limits (CT_{Min} and CT_{Max}; Lutterschmidt and Hutchison 1997) for a total of nine tropical and 14 temperate morphologically-defined species (morphospecies; MTUs) to estimate each taxon's thermal breadth ($CT_{Max}-CT_{Min}$; Huey and Stevenson 1979). We found that tropical morphospecies had narrower thermal breadths than their temperate relatives (PGLS, $t(46) =$

−5.347, P < 0.001); Shah et al. 2017). Furthermore, stream temperature range was a strong predictor of thermal breadth (PGLS, t (48) = 7.550, P < 0.001; Table S4.1), providing a direct connection between latitudinal differences in climate variability and critical thermal limits (Fig. 4.1A).

We next characterized realized dispersal for a total of four tropical and four temperate morphospecies. We used double digest restriction site associated DNA (ddRAD) sequencing (Peterson et al. 2012) to genotype several hundred individuals per taxon (222−877) at hundreds to thousands of SNP loci (419−4544; Table S4.2). Clustering of genotypes into genetic demes revealed that the extent of genetic diversification across morphospecies' ranges was lower in all taxa from Colorado (mean = 2.75, SD = 1.5) than in those from Ecuador (mean = 11.5, SD = 3.9; Fig. S4.2). Furthermore, estimates of pairwise population genetic differentiation (F_{st}) and number of migrants per generation (N_em) for each taxon showed overall lower levels of dispersal in tropical taxa (Fig. 4.1B–C).

We then applied linear modeling of landscape resistance parameters to assess the effect of geographic distance and elevation among sites on genetic differentiation. The best model showed significant interactions between latitude and geographic distance ($F_{1,1154} = 1.86$; $p = 0.012$) and latitude and elevational difference ($F_{1,1154} = 33.20$; $p < 0.001$), indicating that elevation and distance have greater effects on genetic differentiation in the tropics than the temperate zone (Table S4.3). Combined, the results of our traits based analyses link latitudinal differences in physiology to lower tropical than temperate realized dispersal and population genetic differentiation.

Next, we estimated the regional species richness and cryptic diversity of our Colorado and Ecuador study areas. We examined specimens using both morphology alone (11,433

specimens examined: Ecuador: 4,511; Colorado: 6,922) and using integrative taxonomy (Padial et al. 2010), by combining morphology and DNA barcoding (3,980 specimens barcoded: Ecuador: 1,495; Colorado: 2,485). DNA barcoding allowed us to capture potential cryptic species that can bias estimates of species richness (Gill et al. 2016). Integrative taxonomy revealed higher species richness in Ecuador than Colorado for the Ephemeroptera (54 *vs*. 41 species) and Trichoptera (71 *vs*. 35), but not the Plecoptera (14 *vs*. 36). Nevertheless, for all three insect orders, we observed larger proportional increases in the number of taxa determined using integrative taxonomy *vs*. morphology in Ecuador than Colorado (Ephemeroptera: +350% *vs*. +40%; Plecoptera: +56% *vs*. +3%; Trichoptera: +137% *vs*. −3%), indicating higher levels of cryptic diversity in the tropics than temperate zone.

Lastly, to better understand the influence of time for speciation to occur on diversity in the tropics *vs*. the temperate zone, we estimated a phylogeny for the 251 species found in our regional diversity surveys (Figs. S4.4–4.7) and used stochastic character mapping (Huelsenbeck et al. 2003) to infer the ancestral location (temperate or tropical) for each of our three major insect orders (Fig. S4.8). We found that the Ephemeroptera and Trichoptera have longer and similar histories in the tropics, while the Plecoptera more recently colonized the tropics (Zwick 2000), potentially explaining their reduced tropical diversity and underscoring the need to consider the relative age of tropical *vs*. temperate lineages in downstream analyses. We then fit a series of binary state speciation and extinction (BiSSE) models (Maddison et al. 2007) to assess latitudinal differences in rates of speciation (λ) and extinction (μ), finding that the best model supported higher rates of speciation in Ecuador than Colorado, but no difference in extinction rates (Fig. 4.1D–E, Fig. S4.9, and Table S4.1). Thus, collectively, our work connects latitudinal

differences in thermal physiology (Fig. 4.1A) and realized dispersal (Fig. 4.1B–C) to diversification (Fig. 4.1D–E) and species richness and cryptic diversity (Fig. 4.1F).

Discussion

In summary, while numerous studies of biological diversification have sought a general mechanism to explain the origins of the extraordinary species diversity of tropical montane regions (Willig et al. 2003, Mittelbach et al. 2007, Graham et al. 2014, Fine 2015), none have provided empirical evidence mechanistically linking environmental factors to species traits to macroevolution (Ghalambor et al. 2006). Here, using an interdisciplinary approach, we found strong support for the CVH (Dobzhansky 1950, Stevens 1989) in which physiological and genetic differentiation result from latitudinal differences in climatic variability, with clear consequences for diversification rates and ultimately species richness. Specifically, we found a consistent pattern of narrower thermal breadths, lower realized dispersal, higher population differentiation, higher speciation rates, higher species richness, and higher cryptic diversity within the tropics than temperate zone, providing some of the strongest evidence to date that species' traits can have profound effects on diversification.

Previous tests of hypotheses to explain global trends in species richness have correlated ecological, historical, and evolutionary factors with global trends in species richness (Willig et al. 2003, Mittelbach et al. 2007, Graham et al. 2014, Fine 2015). Likewise, previous tests of the CVH have correlated latitudinal changes in thermal tolerance (Addo-Bediako et al. 2000, Sunday et al. 2011), population genetic isolation (Martin and McKay 2004), and trends in species distributions with climatic variability. However, these tests have not been combined for specific taxonomic groups, precluding the important link between mechanisms operating at the level of individuals and populations, and the resulting regional patterns of species richness. Our trait-

based approach reveals that the accumulation of aquatic insect species in montane regions in the tropics can be explained by the combined influence of local adaptation for thermal tolerance and reduced dispersal rates, which in turn result in isolation of populations, genetic differentiation, and high rates of speciation.

We propose that rates of diversification globally, may be more predictable than previously thought (Jablonski et al. 2006) if traits based approaches are employed. Comparing organismal traits with functional consequences for population isolation and dispersal, and how those vary with environmental gradients, will allow us to predict the processes driving genetic divergence among populations for many different organisms in many different habitats (Smith et al. 2014, Zamudio et al. 2016). The relevant traits will certainly vary among taxonomic groups and specific environments, but consistently important traits will be those affecting population isolation and rates of diversification.

Bridging the gap between traits, their function in variable environments, and diversification rates, has important implications for biodiversity conservation. Tropical ectotherms are predicted to persist closer to the edge of their evolved thermal tolerance (Deutsch et al. 2008), and thus paradoxically, the same traits that promote high speciation and diversity in the tropics are the ones that make them especially vulnerable to rapid changes in thermal environments. Mechanistic, trait-based approaches provide a predictive framework for understanding the vulnerability of species in the face of environmental change (Williams et al. 2008).

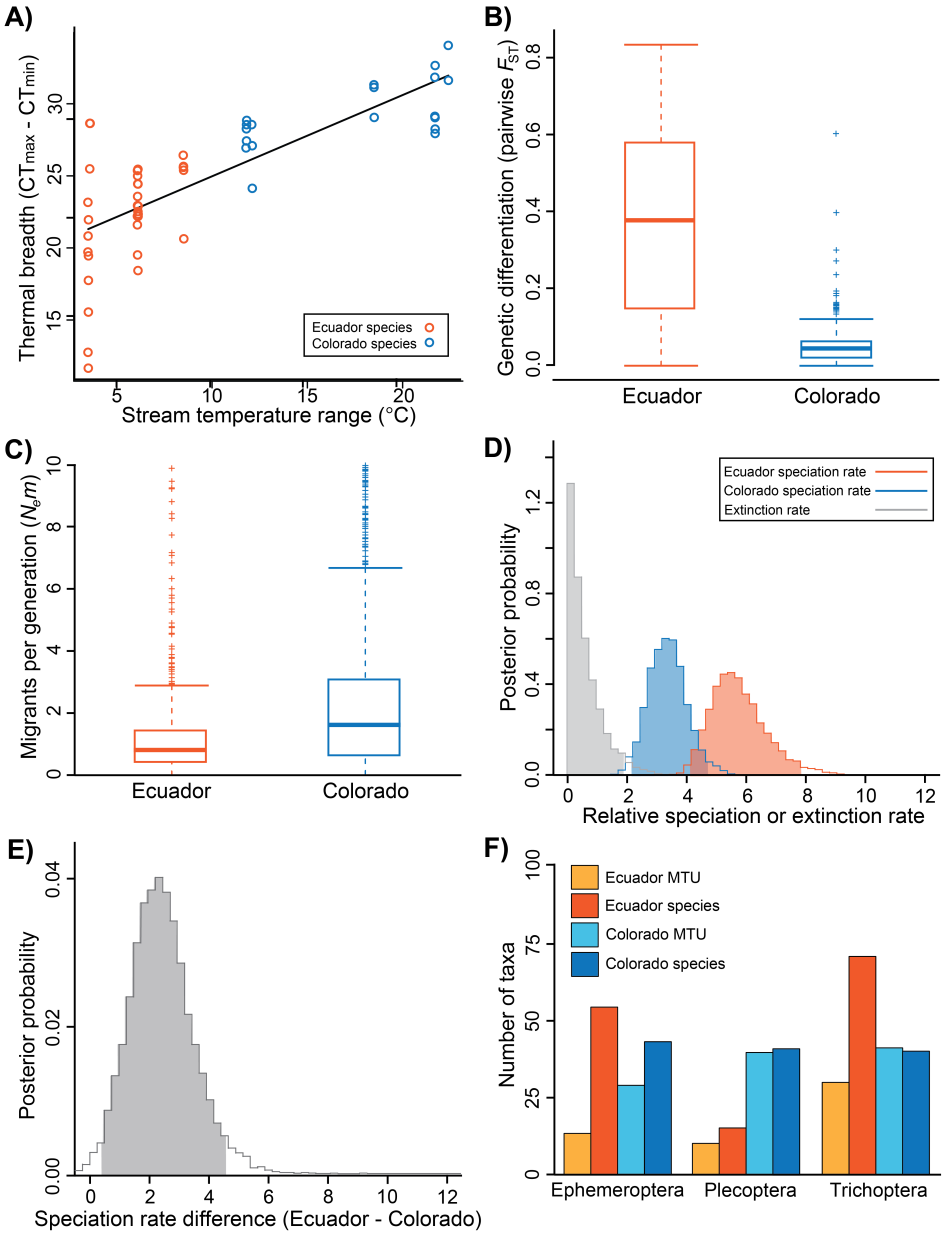

Figure 4.1 Physiological and dispersal trait variation lead to different speciation rates and species richness in tropical and temperate stream invertebrates. A) Thermal breadth *vs*. stream temperature range shows broad thermal tolerance in higher latitude streams where stream temperature ranges are wider. B-C) Levels of subpopulation differentiation and dispersal based on genomics, quantified as pairwise population F_{st} and the number of migrants (N_em) per generation, indicate higher levels of connectivity among sites and more extensive movement and in Colorado. D-E) Speciation and extinction rates estimated with best fit BiSSE model show that speciation is higher in the tropics than temperate zone, and that extinction is equal among regions. F) The number of cryptic species identified with COI barcoding over those identified with morphological taxonomy was much higher in Ecuador (orange bars) relative to Colorado (blue bars).

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APPENDIX 1.1

Supplemental Methods

COI primers

For PCR, we first used a primer cocktail combining LCO1490/HCO2198 (Folmer et al. 1994) and LepF1/LepR1 (Hebert et al. 2004) for all taxa. If these primers failed, we tried the degenerate primer sets dgLCO-1490/dgHCO-2198 (Meyer 2003) and jgLCO1490/jgHCO2198 (Geller et al. 2013), which are designed to amplify the same gene region.

Decision rule criteria for constraints

We used the following decision rules modified from Poff et al. (2006) to determine which information to use in cases when multiple studies describing a phylogenetic relationship were available: 1) well-supported (≥ 63% bootstrap (Felsenstein 1985) or jackknife support (Farris et al. 1996)) molecular phylogenetic studies with many characters were preferred over less well supported molecular phylogenetic studies with fewer characters, 2) well-supported molecular phylogenetic analyses were preferred over morphology-based phylogenetic analyses not reporting any measure of support, 3) well-supported morphology-based phylogenetic analyses were preferred over weakly supported molecular phylogenetic analyses, and 4) current classifications were used when neither strongly supported molecular or morphological phylogenetic analyses were available. Detailed documentation of nodal support based on our phylogenetic analysis of COI and constraints from the literature is provided in Fig. S3.1.

Table S3.1 Summary of results from phylogenetic generalized least squares regression (PGLS) for non-truncated elevational ranges fit with an Ornstein-Uhlenbeck model of trait evolution (maximum likelihood used to determine α). PGLS was used to control for shared evolutionary history while comparing elevational range breadths across latitude. The explanatory variable "latitude" was dummy coded "0" for Ecuador and "1" for Colorado and the response variable was elevational range breadth (m). Consequently, significantly positive regression slopes support the 1-sided hypothesis that Colorado species have larger elevational ranges than species from Ecuador. For parsimony trees, regression parameters (slope, intercept, standard errors) are model averaged and values of alpha and hypothesis tests are presented as medians.

Phylogenetic Method	Constraints	Branch Lengths	Slope (±SEM)	Intercept (±SEM)	Alpha (Median)	1-sided p CO > EC (Median)	$p < 0.05$ (%)	$p < 0.10$ (%)
Parsimony	Y	Equal	192 (±80)	333 (±51)	8.52	0.009	100.00	100.00
Parsimony	Y	Grafen's	193 (±82)	333 (±53)	120.69	0.021	100.00	100.00
Bayesian	Y	Relative	180	334	55.72	0.015	100.00	100.00
Bayesian	N	Relative	179	334	55.61	0.015	100.00	100.00

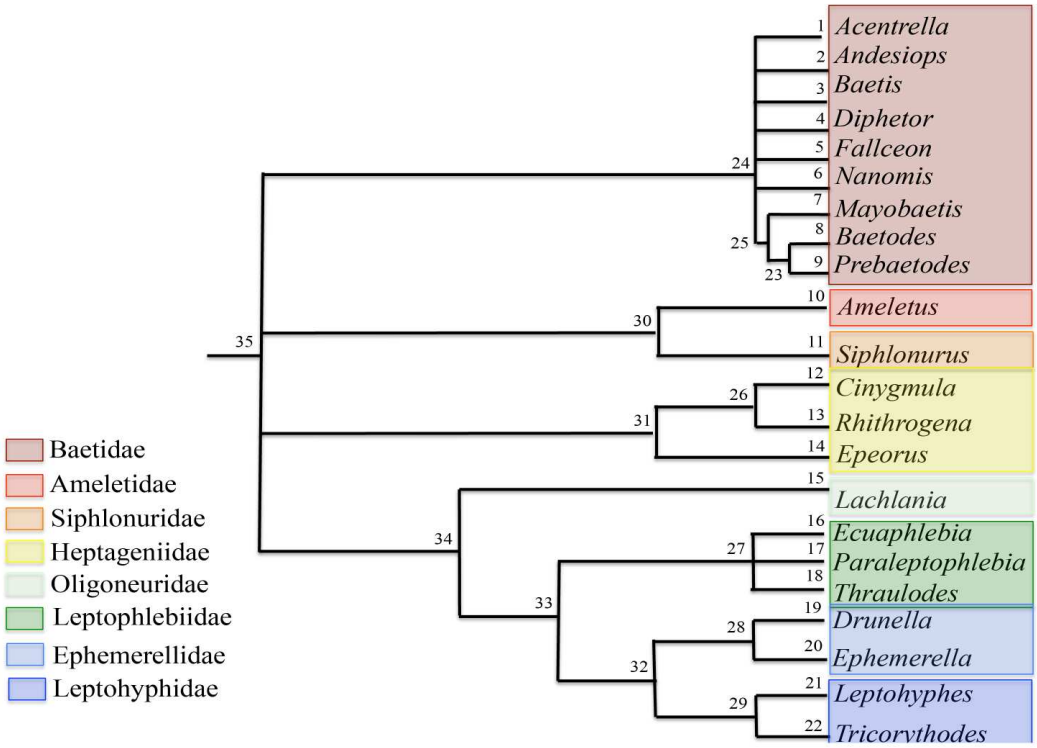

Figure S3.1 Diagram of constraints used in phylogenetic analyses. Node numbers reference Extended Data File S3.2.

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APPENDIX 2.1

Supplemental methods

Study area and collection

We collected aquatic larvae from the orders Ephemeroptera (mayflies), Plecoptera (stoneflies), and Trichoptera (Caddisflies) for physiological experiments, landscape genomics, and regional diversity surveys. We sampled montane streams in the Colorado Rockies and Ecuadorian Andes (Extended Data File S4.1). To collect insects, we used Hess, Surber, and D-frame nets with 500 μm mesh, and searched under submerged stones. We collected samples from wadeable, minimally impacted tributaries, at approximately 200 m elevational intervals, from elevations of 1556 to 3478 m asl in Colorado, and 1664 to 4248 m asl in Ecuador. For tests of thermal tolerance and measurements of gene flow, we subsampled phylogenetically paired taxa with sufficient sample sizes across sites. For estimates of regional diversity, we supplemented larval collections with adults collected from streamside vegetation. All specimens used for genetic analyses were field preserved in 100% ethanol, which was replaced after 24 hours to ensure long-term preservation.

Measuring thermal tolerance

To measure thermal limits, we followed procedures described in Shah et al. 2017. Insects from each stream were placed in 1 L mesh-enclosed containers in a large insulated cooler with filtered stream water. To allow insects to acclimate to laboratory conditions and reach a post-absorptive state, we acclimated specimens for 48 h on a 12:12 h light-dark cycle at the average native stream temperature prior to experiments. Flow was generated within the cooler using an aquarium pump, and leaf litter was removed to prevent feeding.

For both CT_{Max} and CT_{Min} experiments, we placed insects in individual chambers set in water baths held at the acclimation temperature. We measured CT_{Max} in up to 12 individuals (mean = 8.34, min = 4) per species per stream site, and CT_{Min} in up to nine individuals (mean = 8.5, min = 5) per species per stream site. To measure CT_{Max}, we ramped the temperature of the water bath up at a rate of 0.3°C per minute (Dallas and Rivers-Moore 2012) using a titanium aquarium heating rod and a temperature controller (16C-2, Dwyer Instruments Inc., Michigan City, Indiana). We used air stones to maintain adequate oxygenation (> 70%). We recorded CT_{Max} as the temperature at which insects lost the ability to right themselves after being flipped on their back (Angilletta 2009). For CT_{Min}, we ramped the temperature down at a rate of −0.2°C per minute (the rate decreased slightly as temperature neared 4°C) using a thermoelectric cooler Peltier plate (225 W) and prevented ice-formation in the bath by vigorously circulating the water with a pump (302 L per hour). As with CT_{Max}, we used the loss of righting response as an indicator that CT_{Min} had been reached. After all experiments, insects were immediately returned to normal stream temperature for recovery. Normal swimming activity usually resumed within a few minutes. Insects that did not recover were excluded from the analysis. Following experimentation, all insects were stored in 96% ethanol, dried for 24 h at 56°C, and weighed to obtain individual body mass estimates.

Genomic sequence data collection

SNP genotyping was performed using double digest restriction-site associated DNA sequencing (ddRAD-seq; Peterson et al. 2012). We performed sample demultiplexing, alignment, and SNP calling for each taxon in Stacks v 1.19 (Catchen et al. 2013), with parameters as described in Polato et al. 2017. An initial set of SNPs was filtered to remove loci with minor allele frequencies < 0.01 for coalescent model based analyses in ∂a∂i (Gutenkunst

et al. 2009). For frequency based analyses, a more stringent filter was applied to remove loci with minor allele frequencies < 0.05, and those that were typed in < 60% of individuals for a given taxon. Loci showing evidence of bias based on sequencing library or linkage disequilibrium were also excluded.

DNA barcoding

We identified specimens morphologically to the lowest level possible using available literature (Merritt et al. 2008, Domínguez and Fernández 2009). For each morphospecies identified at each site, we DNA barcoded (Hebert et al. 2003) up to ten specimens using standard protocols from the Canadian Center for DNA Barcoding (Hajibabaei et al. 2005, Ivanova et al. 2005, 2006). For this study, we analyzed a total of 3,980 sequences including 1,220 previously published records from Gill et al. 2014, 1,832 records from Gill et al. 2016, and 928 specimens sequenced for this study. We trimmed, assembled, and manually checked raw chromatograms in the program Sequencher v. 5.3 (Gene Codes, Ann Arbor, MI, USA). We uploaded specimen, locality, and sequence data to the Barcode of Life Database (BOLD; Ratnasingham and Hebert 2007) to the publicly available dataset "DS-TTADDD: Thermal tolerance and dispersal drive diversification" (DOI:dx.doi.org/10.5883/DS-TTADDD). We used refined single linkage clustering to delimit groups of specimens into species (Ratnasingham and Hebert 2013).

Testing for latitudinal differences in thermal tolerance

We used phylogenetic generalized least squares regression (PGLS; Grafen 1989) fit with an Ornstein–Uhlenbeck model (Hansen 1997, Butler and King 2004) of trait evolution to control for evolutionary history while comparing physiological trait values across latitude. In these models, latitude was the main predictor, elevation and dry weight were included as covariates, and thermal breadth was the response. We also used PGLS to test for a relationship between

stream temperature range and thermal breadth, while controlling for phylogeny and dry weight. In all models, the parameter α was estimated using maximum likelihood.

Testing for latitudinal differences in genomic variation and gene flow

For each taxon, we computed pairwise chord distance (Dc) with the dist.genpop function in the Adegenet package (Jombart 2008) in R. We then identified the number of genetic clusters in the data using K-means clustering with the find.clusters function. This analysis performs successive K-means clustering, increasing the number of clusters (K) in each model. We used the Bayesian Information Criterion (BIC) statistic to assess the models for the various K values. The curve of BIC statistics for each K was smoothed using a lowess approach to select the optimal model based on the value of K after which further increases led to an increase in BIC (Fig S4.1).

To determine the extent to which sub-population differentiation was influenced by the landscape, and if geographic distance or elevation differences between sites disproportionately affected differentiation of taxa at the different latitudes, we used linear mixed effects models that accounted for the correlated nature of pairwise genetic distance measurements using the maximum-likelihood population effect (Clarke et al. 2002, Van Strien et al. 2012). Mixed effects models were fit using the lme and gls functions in the R package nlme, and correlation matrices were generated using the corMLPE function (https://github.com/nspope/corMLPE; N. Pope pers. comm.). The centered geographic and elevational distances among sites were used as the independent variables, and chord genetic distance (Dc) was used as the dependent variable. Taxonomic order nested within latitude was included as a random effect, resulting in a covariance matrix for each order.

We estimated migration rates among sites along the temperate and tropical elevational gradients for each taxon at each latitude to test the hypothesis that gene flow among sites is more

restricted in the tropics. Migration rates between population pairs were inferred in the program ∂a∂i (Gutenkunst et al. 2009), which uses a diffusion approximation approach on the folded two-dimensional allele frequency spectrum (AFS) estimated over all SNPs. Provided with a parameterized demographic model, ∂a∂i calculates the expected AFS for that model under random genetic drift and then maximizes the similarity to the observed AFS over the parameter values that the model can take on, using a maximum likelihood approach. Since little information is known about the actual demographic histories of the individual populations, we assumed a simple population split model for each analysis. This model features an ancestral population of constant effective size N_0 that splits at time Ts into two subpopulations of constant effective size N_1 and N_2, linked by constant symmetric migration occurring at rate m after the split (Fig S4.2).

Only the inferred estimates for migration rates m were relevant in our analysis, whereas we did not make use of the estimates for split times and effective population sizes also obtained by ∂a∂i. Note that while our simple split model is likely a severe oversimplification for the true demographic history of any given population pair, the migration rates inferred under this model, when compared over many different pairs, should still provide valuable information about general trends in the magnitude of migration.

Overall, migration rates among populations in Colorado were higher than those in Ecuador ($p < 0.001$). This result agrees with the range of F_{st} values observed in Colorado and Ecuador, computed with both ∂a∂i and Adegenet. The range of F_{st} values in Colorado were lower overall, indicating higher levels of interbreeding among insects from the temperate sites.

Historical context for latitudinal differences in species richness

To examine latitudinal differences in taxon richness, we determined the total number of taxa (denoted as 'species' below) identified in Ecuador and Colorado using both morphology

(Morphological Taxonomic Units; MTUs), and integrative taxonomy (combining morphology and DNA barcoding). To determine the relative levels of cryptic diversity at tropical and temperate sites, we looked at the increase in the number of taxa identified following DNA barcoding of morphologically identified specimens (relative to MTUs). Final identifications based on morphology and integrative taxonomy utilizing DNA barcoding are available as Extended Data File S4.2.

We used a Bayesian phylogenetic approach to estimate a phylogeny for the species included in our study. We utilized our DNA barcode data to estimate a tree that was constrained according to well recognized patterns in aquatic insect systematics (Leach 1815, Kolenati 1848, Walsh 1862, McLachlan 1875, Wallengren 1891, Ulmer 1906, 1920, Banks 1907, 1916, Bengtsson 1912, Needham and Murphy 1924, Ross 1938, 1944, Schmid 1955, 1957, Flint 1974, Flint, O.S. 1981, Waltz and McCafferty 1985, 1987, Domínguez 1988, McCafferty and McCafferty 1991, Lugo-Ortiz and McCafferty 1999, 1996, Terry and Whiting 2003, Wang and McCafferty 2004, Holzenthal et al. 2006, Ogden et al. 2009, Malm and Johanson 2011). In determining constraints, a set of decisions rules was used to determine which information to use in cases of multiple available studies describing phylogenetic relationships (Poff et al. 2006, Gill et al. 2016). We randomly choose among available sequences for each species determined using DNA barcoding and aligned them in MAFFT v.7 (Katoh 2002) using strategy G-INS-i with offset value 0.1 and all other options set as default. Once aligned, we used jModelTest2 (Guindon and Gascuel 2003, Darriba et al. 2012) to select the appropriate nucleotide substitution model which was GTR + Γ. We then conducted six runs each with 100,000,000 generations of Bayesian MCMC sampled every 2,500 generations in BEAST v. 2.3.2 (Bouckaert et al. 2014) through the CIPRES Science Gateway (Miller et al. 2010). We modeled lineage specific

substitution rates using a relaxed clock with log-normally distributed rates (Thorne et al. 1998, Kishino et al. 2001, Thorne and Kishino 2002), and diversification using a birth-death tree prior (Yang and Rannala 1997, Popovic 2004, Gernhard 2008). For each run, we plotted the −ln likelihood scores against generation time in Tracer v. 1.6 (Rambaut et al. 2014) and examined the effective sample sizes for parameters to ensure that each analysis reached stationarity. We combined the trees from each run in LogCombiner v. 2.3.2 (Bouckaert et al. 2014), discarded 25% as burn-in, and resampled every 22,500 trees. We summarized a total of 20,000 post burn-in trees sampled from all six runs in TreeAnnotator v 2.3.2 (Bouckaert et al. 2014) to create the maximum clade credibility (MCC) tree. We collapsed weakly supported nodes on the MCC tree (< 0.95 posterior probability) and re-scaled this tree to a height of one for subsequent analyses. Final trees are presented as supplementary figures for all taxa (Fig S4.3) and the Ephemeroptera (Fig S4.4), Plecoptera (Fig S4.5), and Trichoptera (Fig S4.6) separately.

The amount of time a clade has been present in a particular environment could affect the extent of its diversification (Smith et al. 2014), we used stochastic character mapping (Huelsenbeck et al. 2003, Bollback 2006) to determine the most probable location of the ancestors of each insect order to approximate the relative time that each group has been in a given location. We used pattern based as opposed to events based methods for reconstructing historical biogeography because the species included in this study are endemic to different continents (no species occurs in both Colorado and Ecuador). We used the R package phytools (Revell 2014) to simulate 10,000 stochastic character maps for "location" coded as "tropical" or "temperate" using an equal rates model transition matrix. The analysis showed that the Plecoptera are more recent arrivals to the tropics than either the Ephemeroptera or Trichoptera, potentially explaining the low species richness of the Plecoptera in Ecuador (Fig S4.7).

Testing for latitudinal differences in diversification rates

For taxa from all three orders together, we tested for latitudinal differences in diversification rates using binary state speciation and extinction models (BiSSE; Maddison et al. 2007) in the R package diversitree (Fitzjohn 2012). We used the version of BiSSE designed to handle polytomies (Fitzjohn et al. 2009) and fit the full model with six free parameters and compared it to models with speciation, extinction, and dispersal rates constrained to be equal. Model selection using AIC showed that a BiSSE model with different parameters for speciation and dispersal for both Colorado and Ecuador and a single extinction rate for both latitudes provided the best fit to the data (Table S4.1; Fig. S4.8). Using the best model, we ran 10,000 generations of Bayesian MCMC to estimate the posterior probability densities and 95% credibility intervals for each parameter and compare parameters among latitudes. This model provides strong evidence for higher rates of speciation in Ecuador than Colorado, similar extinction rates at both latitudes, and some evidence for higher levels of dispersal of taxa from Ecuador to Colorado than from Colorado to Ecuador (Fig. S4.9).

Table S4.1 Results of PGLS fit with an Ornstein–Uhlenbeck model of trait evolution for thermal breath. Latitude was dummy coded "0" for Colorado and "1" for Ecuador. Consequently, for models including latitude, negative regression slopes indicate a decrease in the estimated parameter value from Colorado to Ecuador.

		Estimate	Std. Error	t-value	p-value
Thermal Breadth	(Intercept)	38.645	4.565	8.465	<0.001
	Latitude	-26.885	5.028	-5.347	<0.001
	Elevation	-0.004	0.002	-2.144	0.037
	Dry Weight	104.759	38.574	2.716	0.009
	Latitude X Elevation	0.008	0.002	3.937	<0.001
	α	315.315			
Thermal Breadth	(Intercept)	19.253	0.942	20.440	<0.001
	Stream Temperature Range	0.564	0.075	7.550	<0.001
	Dry Weight	74.524	42.013	1.774	0.082
	α	367.907			

Table S4.2 Phylogenetically paired taxa within three focal orders (Ephemeroptera (E), Plecoptera (P), Trichoptera (T) of aquatic insects included in genomic comparisons across latitudes. Sample sizes and ddRAD sequencing results for the samples used in the genomics analysis.

	Taxon	Order	N inds	Mean coverage depth	Total # of merged stacks	N loci MAF > 0.01	N loci MAF > 0.05	inds retained	N inds filtered	N loc filtered
Colorado	*Baetis bicaudatus*	E	606	71	7,026	10,058	7,767	99%	603	3,100
	Baetis tricaudatus	E	316	62	5,354	9,141	7,098	78%	245	1,272
	Hesperoperla pacifica	P	404	39	15,717	17,436	13,520	95%	383	4,544
	Rhyacophila brunnea	T	399	46	6,175	8,847	7,366	56%	222	870
Ecuador	*Andesiops peruviensis*	E	990	64	5,447	8,450	6,963	89%	877	1,378
	Baetodes sp	E	401	66	7,164	7,801	6,461	69%	275	848
	Anacroneuria sp	P	598	30	14,104	23,977	21,260	65%	391	1,021
	Smicridea sp	T	614	23	16,027	9,748	7,891	52%	319	419

Table S4.3 Linear model results using genetic distance (Dc) as the response variable, and geographic distance and elevational difference as predictors. Taxonomic order was nested within latitude as a random effect. Geographic distance and/or Latitude were significant predictors of genetic differences, and the significant interactions between latitude and both geographic and elevation distance showed that temperate and tropical populations in temperate and tropical locations do not respond equally to elevational differences in terms of differentiation.

		Estimate	Std. Error	t-value	p-value
All Taxa	(Intercept)	0.219	0.011	20.255	<0.001
	Geographic Distance	0.001	0.000	2.542	0.011
	Latitude	0.099	0.011	9.331	<0.001
	Elevation Difference	-0.000	0.000	-0.731	0.465
	Geo. Dist. x Elevation	-0.001	0.001	-2.511	0.012
	Latitude x Elev. Dif.	0.000	0.000	5.762	<0.001

Table S4.4 Binary state speciation and extinction (BiSSE) models for latitudinal differences in speciation, extinction, and dispersal. In the "Full BiSSE" model speciation, extinction, and dispersal rates are estimated independently for both Colorado and Ecuador (six parameters). In each of the "Equal" models, one set of parameters (speciation, extinction, or dispersal rates) is constrained to be equal. "Equal" models are compared to the "Full BiSSE" model to test the null hypothesis that a set of model parameters should be constrained to be equal. Based on AIC, the best-fit model for this dataset is that in which speciation and dispersal rates are independently estimated for Colorado and Ecuador and extinction rates are set equal.

Model	Degrees of Freedom	Log Likelihood	AIC	Chi Squared	p-value
Full BiSSE	6	-47.699	107.400		
Equal Speciation	5	-50.531	111.060	5.663	0.017
Equal Extinction	5	-47.773	105.550	0.148	0.701
Equal Dispersal	5	-49.830	109.660	4.261	0.040

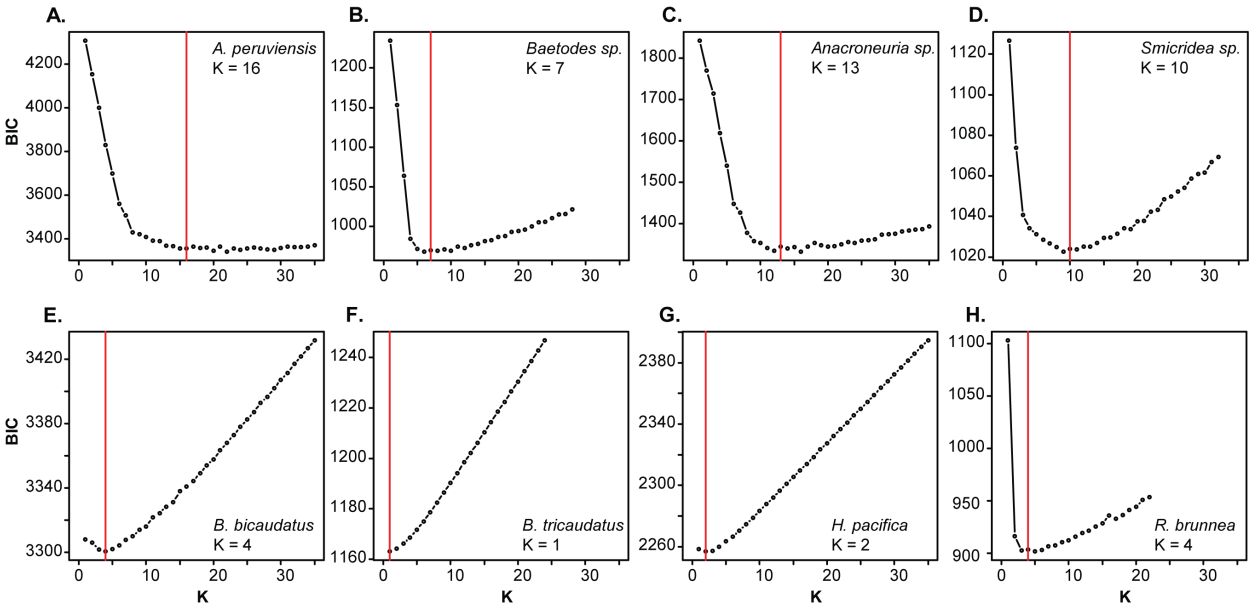

Figure S4.1 Plots of Bayesian information content (BIC) for each proposed number of population subclusters (K) for each tropical (upper) and temperate (lower) taxon. Values of K that minimized BIC were higher in all tropical orders

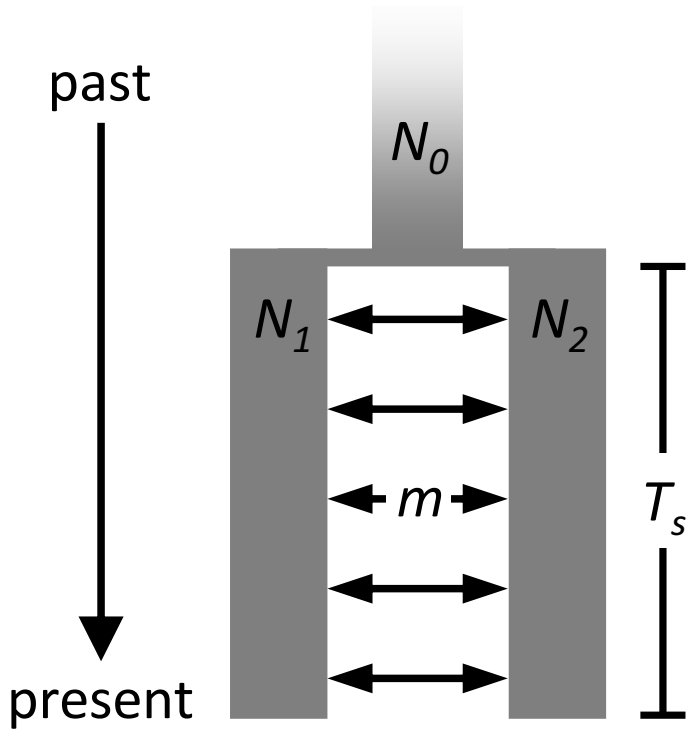

Figure S4.2 The demographic model used for each *∂ahe* analysis. In this model the ancestral population of constant effective size N_0 splits at time T_s into two subpopulations of constant effective size N_1 and N_2, linked by constant symmetric migration occurring at rate m after the split.

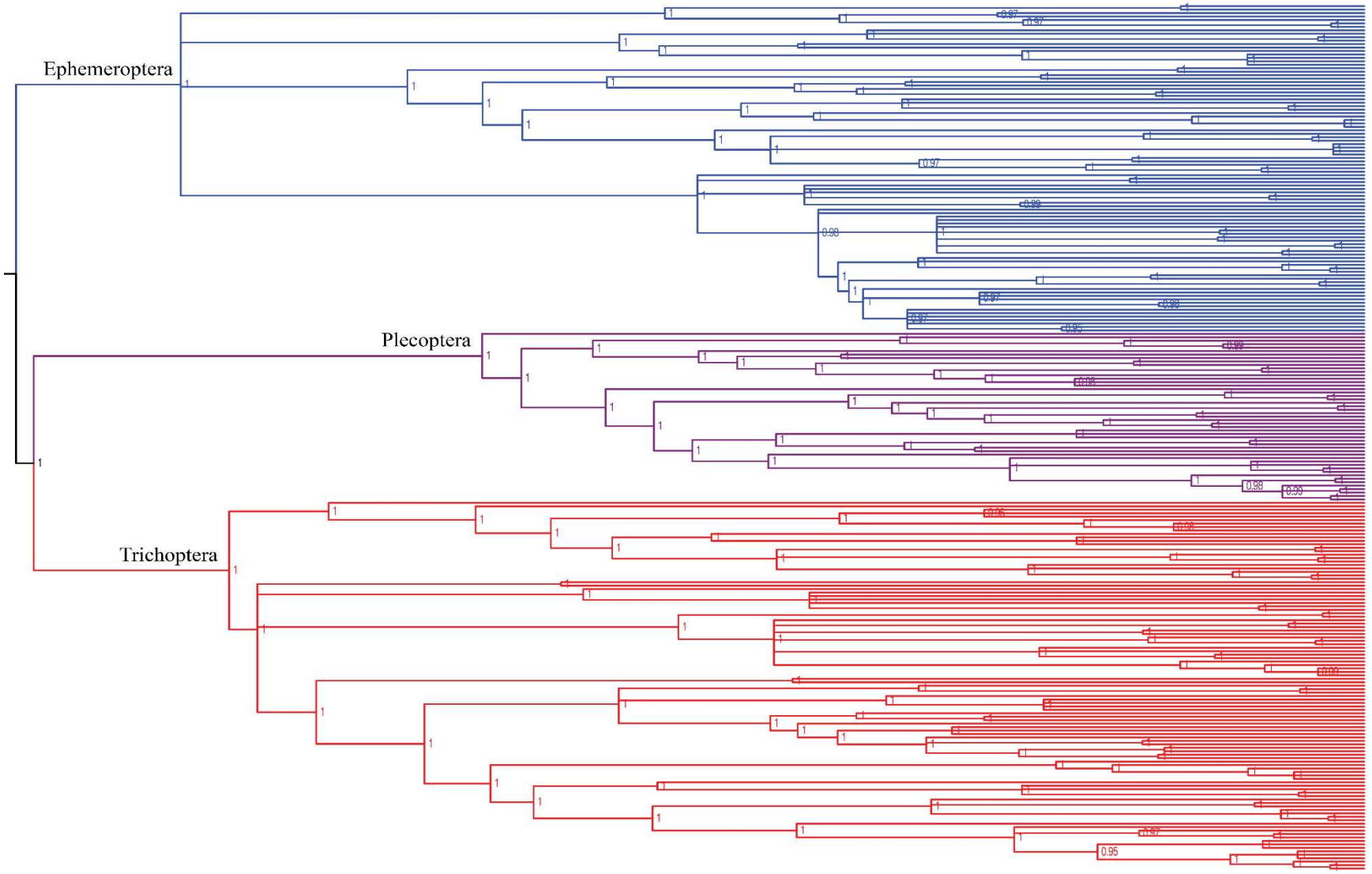

Figure S4.3 Bayesian maximum clade credibility tree representing all taxa included in this study from the orders Ephemeroptera - (blue), Plecoptera (purple), and Trichoptera (red). Nodes with less than 0.95 posterior probability support are collapsed.

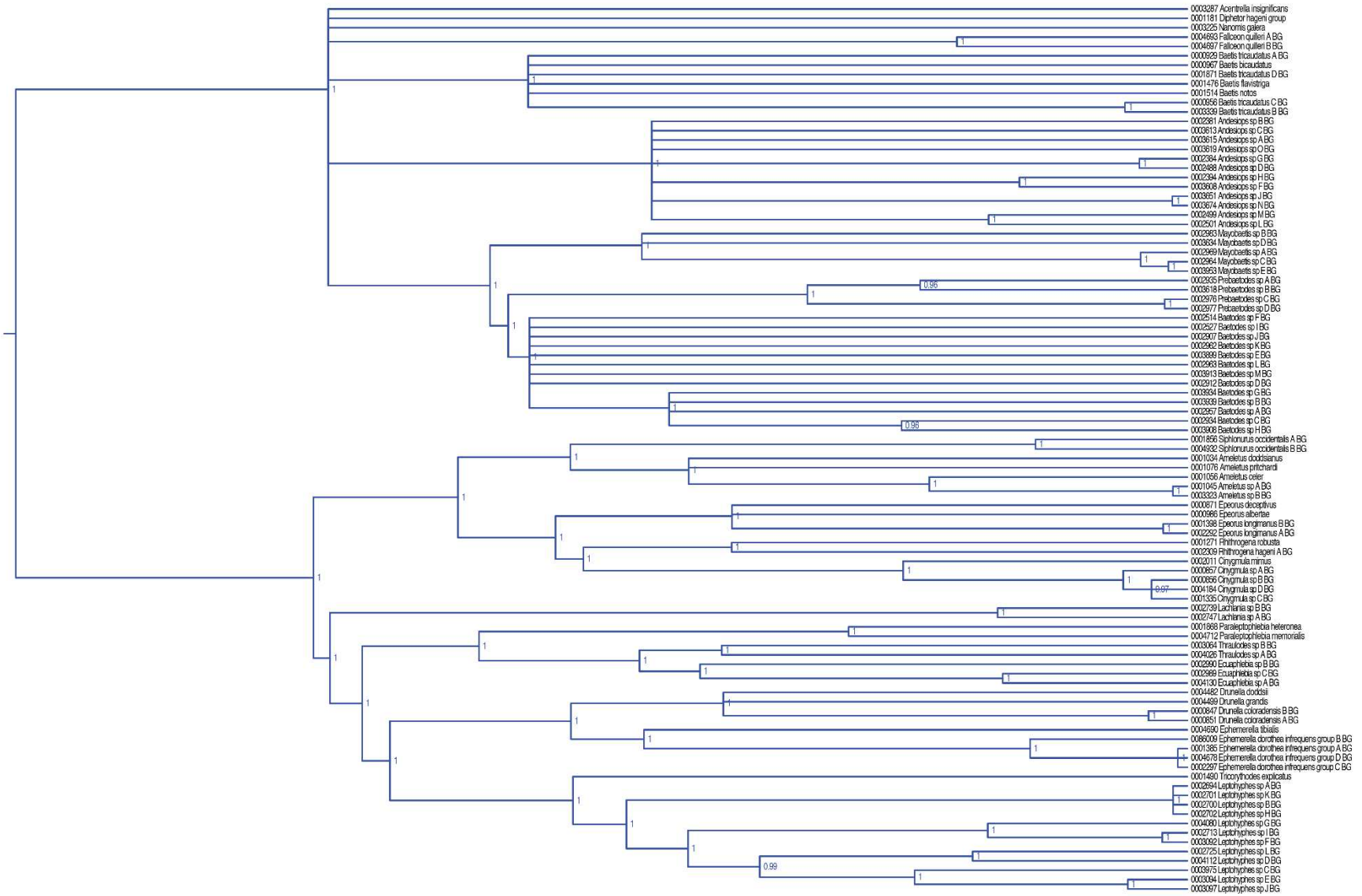

Figure S4.4. Bayesian maximum clade credibility tree representing all taxa included in this study from the order Ephemeroptera. Nodes with less than 0.95 posterior probability support are collapsed

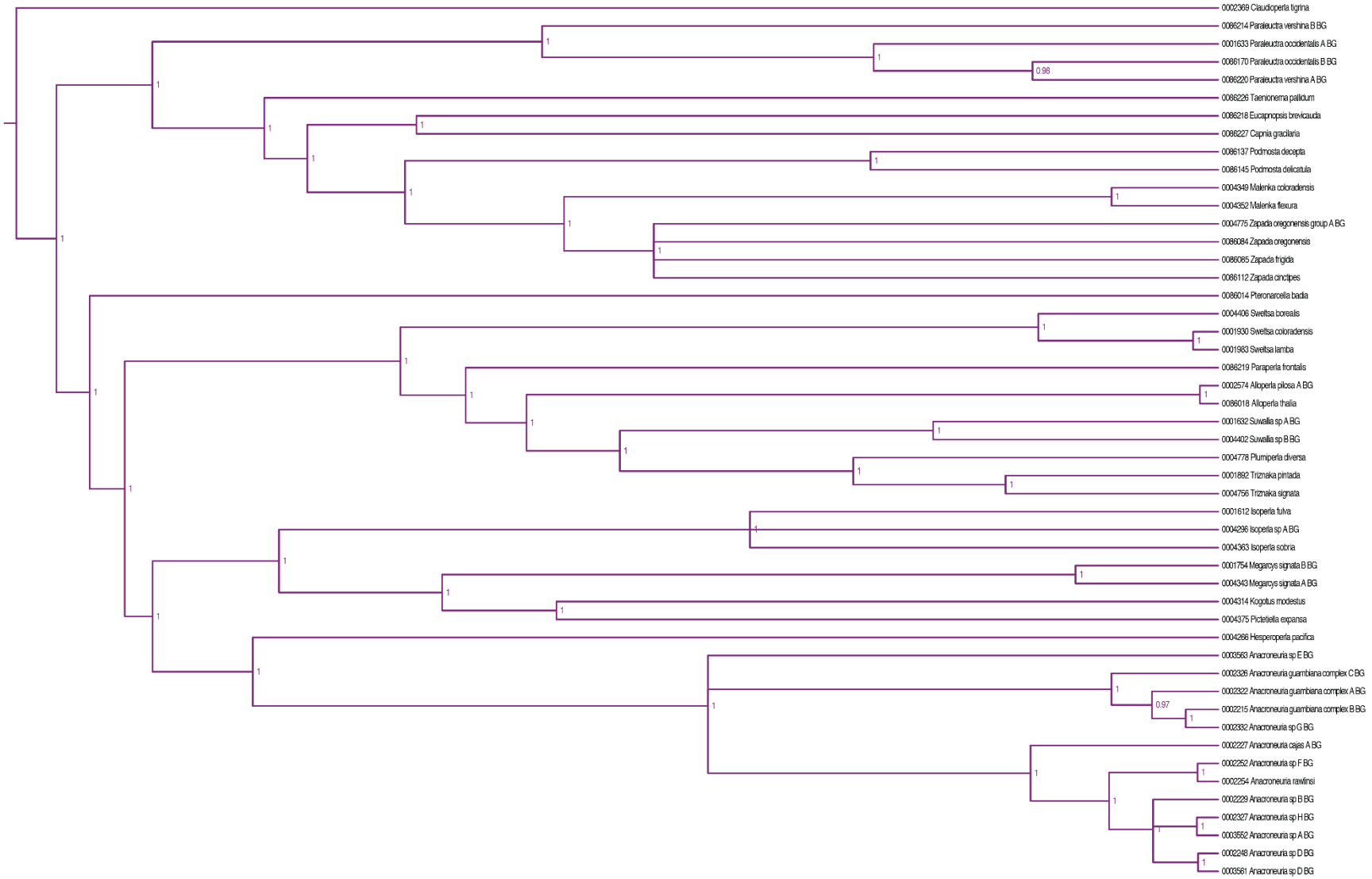

Figure S4.5 Bayesian maximum clade credibility tree representing all taxa included in this study from the order Plecoptera. Nodes with less than 0.95 posterior probability support are collapsed.

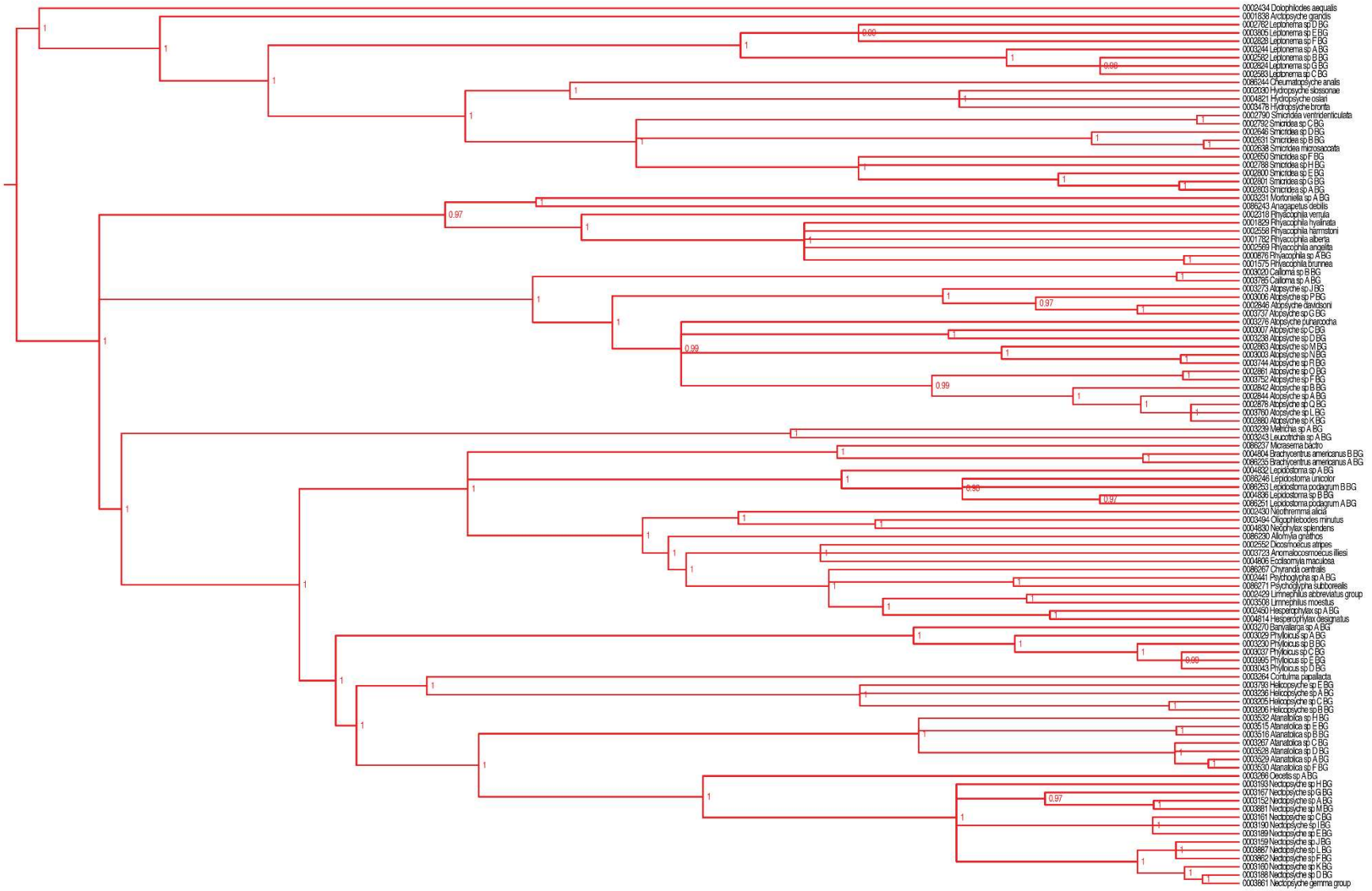

Figure S4.6 Bayesian maximum clade credibility tree representing all taxa included in this study from the order Trichoptera. Nodes with less than 0.95 posterior probability support are collapsed.

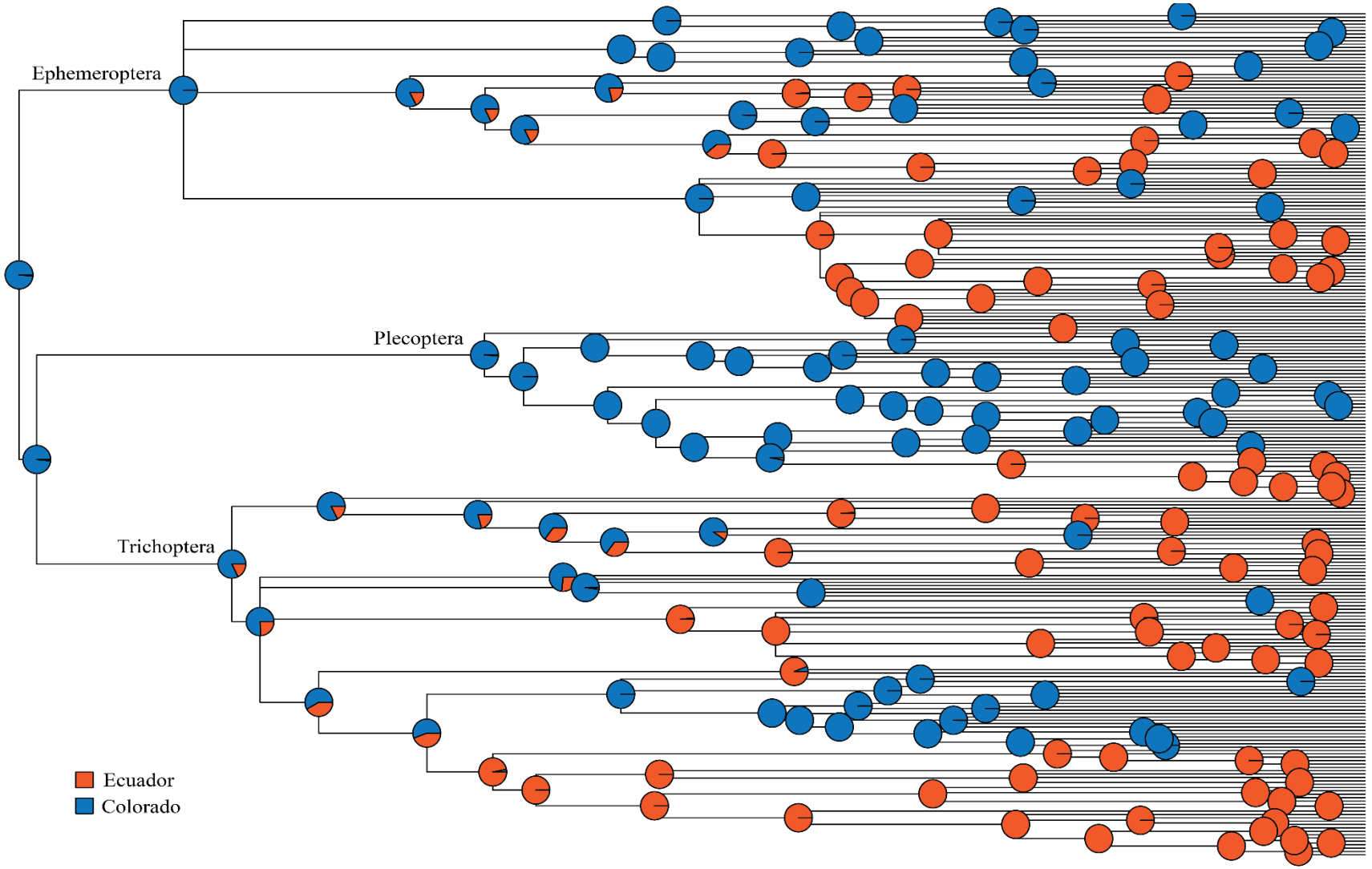

Figure S4.7 Summary of 10,000 stochastic character maps simulated using an equal rate transition matrix used to estimate historical location of each major insect order included in this study. Analysis indicates more recent arrival of the Plecoptera than either Ephemeroptera or Trichoptera to Ecuador, potentially explaining contemporary diversity trends.

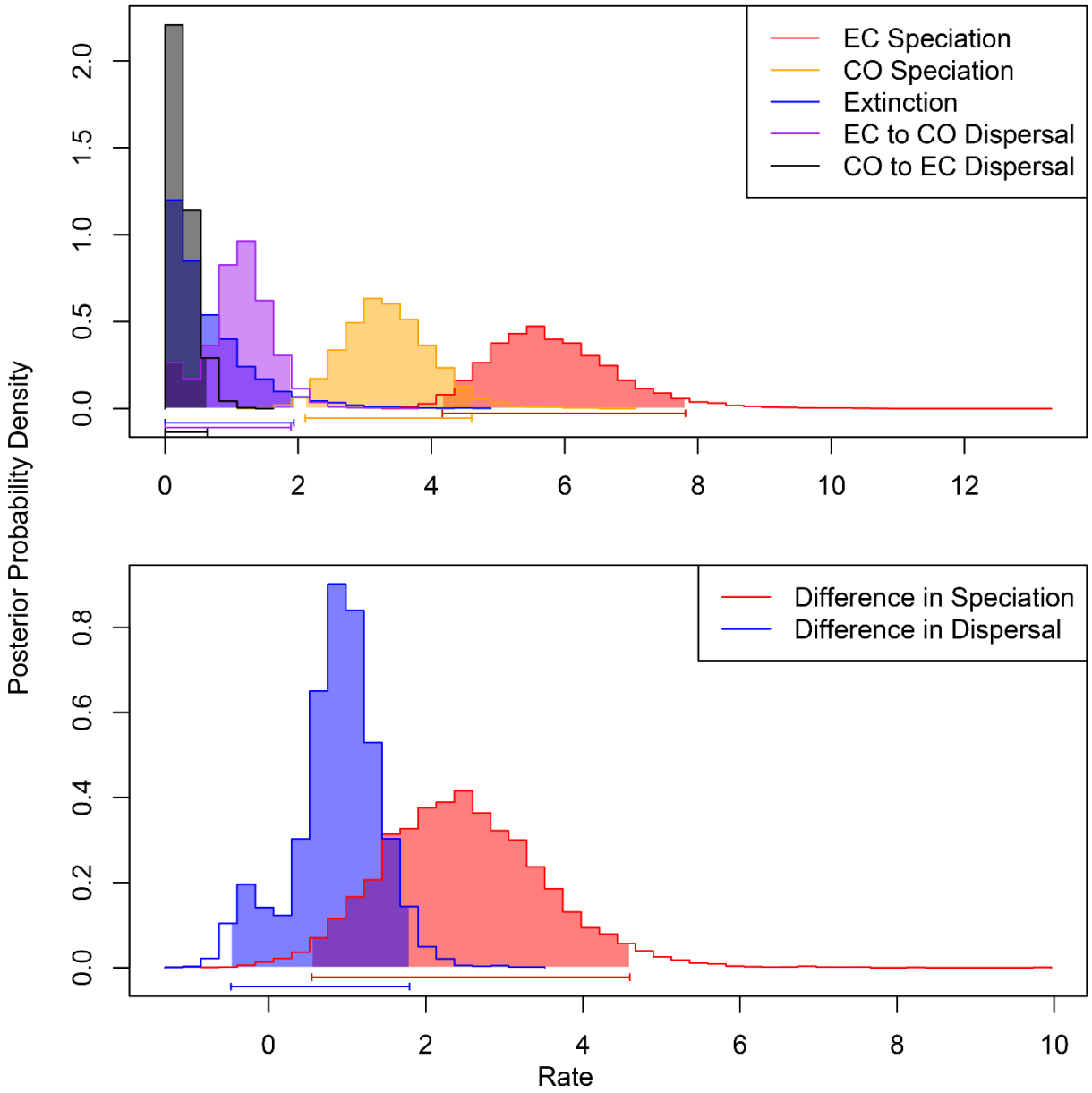

Figure S4.8 Posterior probability density based on 10,000 generations of Bayesian MCMC for rates of speciation, extinction, and dispersal estimated using a BiSSE model in which speciation and dispersal rates are estimated independently for Colorado and Ecuador and extinction rates are set to be equal. 95% credibility interval for probability density for Ecuador speciation rate – Colorado speciation rate is positive and not overlapping zero indicating higher tropical than temperate speciation and thus diversification.

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