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Cryptic biodiversity in streams: a comparison of macroinvertebrate communities based on morphological and DNA barcode identifications

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Abstract: Species-level identifications are difficult or impossible for many larval aquatic macroinvertebrates. We described the taxonomic composition of macroinvertebrate communities from 5 coastal streams in 3 neighboring catchments in southern California. We compared taxonomic identifications based on deoxyribonucleic acid (DNA) barcoding (cytochrome *c* oxidase subunit I [COI]) with morphological identifications of the same specimens. We examined 5870 individuals, and barcodes with sequence lengths >350 base pairs (bp) for 91% of those specimens. We used the naturally occurring gaps in divergence frequencies for each order (usually 2% level of genetic divergence) to delimit putative species for all taxonomic groups except *Simulium* (3%) and *Baetis* (1%). We identified 200 species across these 5 streams. We identified 104 more species via barcodes than via morphology (200 vs 96, a 108% increase). Richness increases were greatest for Chironomidae (60 more species), Ephemeroptera (10 species), Acari (10 species), and Trichoptera (6 species). Forty-five percent of the genera/species identified morphologically represented >2 species. Many (86) species identified with barcodes were represented by only 1 or 2 specimens and were found at only 1 stream. Thus, species rarity (either spatially or numerically) appears to be a common characteristic of these streams. Barcoding increased total richness at each site by 12 to 40 taxa over morphology alone, and increased the difference between reference and impact sites in terms of lost taxa. These results suggest that macroinvertebrate biodiversity in streams has been underestimated substantially in the past, as has the biodiversity lost in response to environmental stress. The potential of DNA barcoding will not be fully realized until we can assign traits, such as habitat preference, ecological function, and pollution tolerance, at the species level.

Key words: DNA barcoding, cytochrome *c* oxidase, COI gene, mitochondrial DNA, freshwater, macroinvertebrates, water-quality monitoring, community structure, species richness, taxonomy

Species are the basic unit of ecology and ecosystems. Species are the building blocks of ecological structure and function, the currency used to attach value and assess change in conservation biology, and the basis of environmental advocacy and regulation. However, ecologists have rarely, if ever, had complete knowledge of the species composition of any given habitat or set of habitats. For example, the few relatively thorough inventories of stream macroinvertebrates suggest that at least several hundred to >1000 macroinvertebrate species can exist in a section of stream or river (e.g., Morse et al. 1980, 1983, Zwick 1998, Humpesch and Fesl 2005, J. C. Morse [Clemson University], personal communication). Unfortunately, most stream invertebrates can be identified only on the basis of morphological characters apparent in adult males or (in some cases) relatively mature juveniles, neither of which is often collected. Thus, many small, juvenile, or damaged individuals commonly collected in stream macroinvertebrate samples are identifiable only to the level of genus or higher. In addition, many genera in a given stream are represented by ≥2 morphologically cryptic species, which can greatly complicate data interpretation (e.g., Zurwerra

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et al. 1987, Funk et al. 1988, 2008, Jackson and Resh 1992, 1998, Duan et al. 2000, Hogg et al. 2005, Williams et al. 2006, Pauls et al. 2010, Kim et al. 2012, Anderson et al. 2013). Thus, although the scientific literature and environmental regulations often refer to aquatic macroinvertebrate species, in practice, we are generally unsure what macroinvertebrate species are actually present in a stream or river.

The difficulty of attaching a species name to each stream macroinvertebrate collected is evident in the published literature and in the protocols designed for stream monitoring. For example, even in studies that take macroinvertebrate identifications to the lowest taxonomic level possible, authors generally leave ≥50% of the individuals examined at the genus or higher level (e.g., Waite et al. 2004, Arscott et al. 2006, Sweeney et al. 2011). Moreover, to save time and reduce inconsistencies among personnel, dates, and sites, most state sampling protocols in the USA require only genus or family identifications or a combination of taxonomic efforts (e.g., some mayflies to species, caddisflies and stoneflies to genus, and chironomid midges to family or "genus" based on gross morphology; Carter and Resh 2001, Richards and Rogers 2006). Even where indicator species are monitored and communicated to the public, the "species" is actually a genus or a complex of closely related species (e.g., *Hexagenia* in the Mississippi River or Great Lakes; Fremling 1991, Webb et al. 2012). When investigators have been able to differentiate between closely related, congeneric species, they have observed some differences in both pollution tolerance and functional traits (Lenat 1993, Schmidt-Kloiber et al. 2006). Our intent is not to disparage the current system for monitoring streams and rivers but, rather, to point out that to date we have been unable take full advantage of the specimens collected during environmental assessments. These bioassessments also are missed opportunities to add to our species-specific knowledge base because new ecological and regulatory information has gone unrecognized. The numerous analyses and discussions over the last 4 decades that examined the information lost or gained depending on the taxonomic resolution in stream macroinvertebrate data (e.g., Resh and Unzicker 1975, Bowman and Bailey 1997, Lenat and Resh 2001, Arscott et al. 2006, Jones 2008, Greffard et al. 2011, Monk et al. 2012) is evidence that the value of species identifications has long been of interest. This issue remains unresolved to some degree today because of our inability to identify most individuals to species.

Genetic methods developed over the last several decades have helped and can help further with species identifications and the clarification of species boundaries for aquatic macroinvertebrates. One such molecular taxonomic method is referred to as deoxyribonucleic acid (DNA) barcoding and uses a 658-base pair (bp) region (the Folmer region) of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene. Genetic distinctness based on DNA barcodes has helped identify or confirmed morphologically distinct species and has provided insights into boundaries among morphologically indistinct species (e.g., Hebert et al. 2004a, b, Monaghan et al. 2005, Ward et al. 2005, Hajibabaei et al. 2006, Smith et al. 2006, Burns et al. 2008, Zhou et al. 2010, 2011, Renaud et al. 2012, Webb et al. 2012). Species we have defined based on barcodes are described as putative. However, for stream macroinvertebrates, limited data suggests that agreement is good among species designated by DNA barcoding and those based on morphological, ecological, or behavioral data (Zhou et al. 2010, 2011, Sweeney et al. 2011, Renaud et al. 2012, Webb et al. 2012, Anderson et al. 2013). The purpose of our study was to examine how our perception of macroinvertebrate community structure changes when it is based on species-level taxonomy (using barcodes) vs genus/species- or higher-level taxonomy associated with state-of-the-art traditional morphology. We examined 2 questions: 1) How much does macroinvertebrate taxon richness and rarity at a site and across a region change when specimens are identified by DNA barcoding vs morphology alone, and 2) How do barcode identifications affect the assessment of "lost taxa" in response to environmental stress?

METHODS

Sampling sites

We compared macroinvertebrate assemblages collected from 5 streams in the Los Angeles region (Ventura and Los Angeles Counties, California; Stein et al. 2013). Two sites (West Fork [WF], lat 34.2410°N, long 117.8690°W, and East Fork [EF], lat 34.2300°N, long 117.7800°W) of the San Gabriel River drain mountainous watersheds (469 and 536 m asl, respectively) covered primarily by evergreen forests and shrub/scrub. Two of the sites (Big Tujunga Wash, lat 34.2740°N, long 118.3150°W; Arroyo Seco, lat 34.2050°N, long 118.1660°W) are tributaries of the Los Angeles River. Both of these watersheds are transitional (395 and 344 m asl, respectively) between the mountains and lowlands, with predominantly shrub/scrub land cover and some urban development. Conejo Creek (lat 34.2010°N, long 119.0010°W) is a tributary of Calleguas Creek in Ventura County. This lowland watershed (32 m asl) is highly modified with extensive agricultural and urban development. Thus, the 5 study streams represent a range of environmental conditions, with WF and EF San Gabriel draining relatively natural watersheds, Big Tujunga Wash and Arroyo Seco with some urban development near the sampling sites, and Conejo with more-extensive agricultural and urban development (Stein et al. 2013). A 6th stream (Arroyo Simi in the Calleguas Creek watershed) also was sampled and included in the analyses by Stein et al. (2013), but this site was not included in our paper because the macroinvertebrate samples were improperly preserved for molecular analysis and barcode success was low (11%).

We sampled 2 reaches at each site: one 150-m-long reach upstream of where stream banks had been physically

stabilized by armoring (i.e., primarily bank stabilization with hard substrates, such as concrete walls, boulders, or gabions) and 1 within the 100- to 200-m reach where armoring had occurred on 1 or both stream banks. This armoring severely changes in-stream habitat by constraining the lateral movement of the reach and affects pool scour and meander development (Stein et al. 2013). Our focus was on the macroinvertebrate faunas that characterized each of these streams, so we combined the specimens collected at the 2 reaches into a single sample from each stream. Analyses of the effects of stream-bank armoring on the macroinvertebrate community were presented by Stein et al. (2013, 2014).

Field collections

We collected macroinvertebrates in June and July 2010 using the multihabitat method described in Ode (2007). Each reach was divided into 11 equidistant transects, and a 30- to 60-s kick sample with a 500-µm mesh D-frame net was collected at an objectively chosen location along each transect (i.e., 25, 50, or 75% of the way across the stream), for a total of 0.9 m^2 of streambed sampled per reach. The 11 subsamples were composited into 1 container and specimens were preserved immediately in 95% ethanol. Samples were drained and replenished with 95% ethanol within 24 to 48 h of collection to preserve tissue for DNA analysis.

Identifications, barcoding and data interpretation

We sorted ~1200 macroinvertebrates from each site (600/reach) and identified them morphologically following the taxonomic standards of the Southwestern Association of Freshwater Invertebrate Taxonomists (i.e., mainly to genus, including chironomids; level 2 in Richards and Rogers 2006). Some noninsect groups (e.g., oligochaetes, ostracods) were left at higher taxonomic levels, such as class or order (Table 1). Morphological identifications were provided by personnel at the California Department of Fish and Game Aquatic Bioassessment Laboratory at California State University Chico.

We sent tissue from each specimen (typically legs where possible or an anterior body part [e.g., chironomids, worms]) to the Canadian Centre for DNA Barcoding (CCDB) at the University of Guelph. Genomic mitochondrial DNA was extracted and the 658-bp barcoding region of the COI gene was amplified and sequenced using highly automated protocols established at the CCDB by Ivanova et al. (2006; http://www.ccdb.ca/resources.php). Sequences and detailed information about all specimens including photographs are stored on GenBank and Barcode of Life Data systems (BOLD) web sites (Ratnasingham and Hebert 2007; http://www.barcodinglife.com/, projects CFWIA to CFWII). For specimens that failed to barcode, another tissue sample was sent to EMP (US Environmental Protection Agency, National Exposure Research Laboratory, Cincinnati, Ohio) for a 2nd attempt at obtaining a barcode.

Of the 5870 individuals submitted for barcoding, COI sequences ≥350 bp from 5349 specimens (91% of total) were exported from BOLD and brought into MEGA 5.05 (Tamura et al. 2007) and aligned using ClustalW with default parameters. We used pairwise comparisons to assess frequency of % genetic divergence for major macroinvertebrate groups (i.e., by orders), and neighbor-joining (NJ) trees with pairwise deletion and Kimura-2-parameter distance to identify the genetically distinct Molecular Operational Taxonomic Units (MOTUs) or barcode species present (Fig. S1A–I). Bootstrap values on NJ trees were based on 500 replications.

We used the gap in divergence frequencies beginning at ~2% for each of the macroinvertebrate groups (Figs 1A–G, 2) to distinguish the difference in genetic structure within a species (<2% divergence) vs between species (>2% diver-

Table 1. Richness measured for 5 California (CA) streams and White Clay Creek (Pennsylvania [PA]) based on morphology (M) and barcode (B) identifications for Ephemeroptera, Plecoptera, Trichoptera, Chironomidae, oligochaete worms, and other macroinvertebrates (e.g., other Diptera, Coleoptera, Acari, Corixidae, Odonata, Mollusca, *Prostoma*, Ostracoda). WF = West Fork, EF = East Fork.

	Total		Ephemer-optera		Plecop-tera		Trichop-tera		Chirono-midae		Oligo-chaeta		Others	
Stream	M	B	M	B	M	B	M	B	M	B	M	B	M	B
WF San Gabriel	61	95	8	12	0	0	10	12	19	37	1	1	23	33
EF San Gabriel	51	91	7	15	1	1	8	10	16	35	1	4	18	26
Big Tujunga Wash	26	38	2	4	0	0	1	2	19	25	0	0	4	7
Arroyo Seco	21	45	3	6	1	1	0	0	12	29	1	1	4	8
Conejo	30	46	4	5	0	0	1	1	12	25	1	1	12	14
5 CA streams	96	200	13	23	2	2	12	18	31	91	1	6	37	60
White Clay[a]	88	180	10	19	3	9	14	17	42	93	1	8	18	34

[a] Data modified from Sweeney et al. 2011.

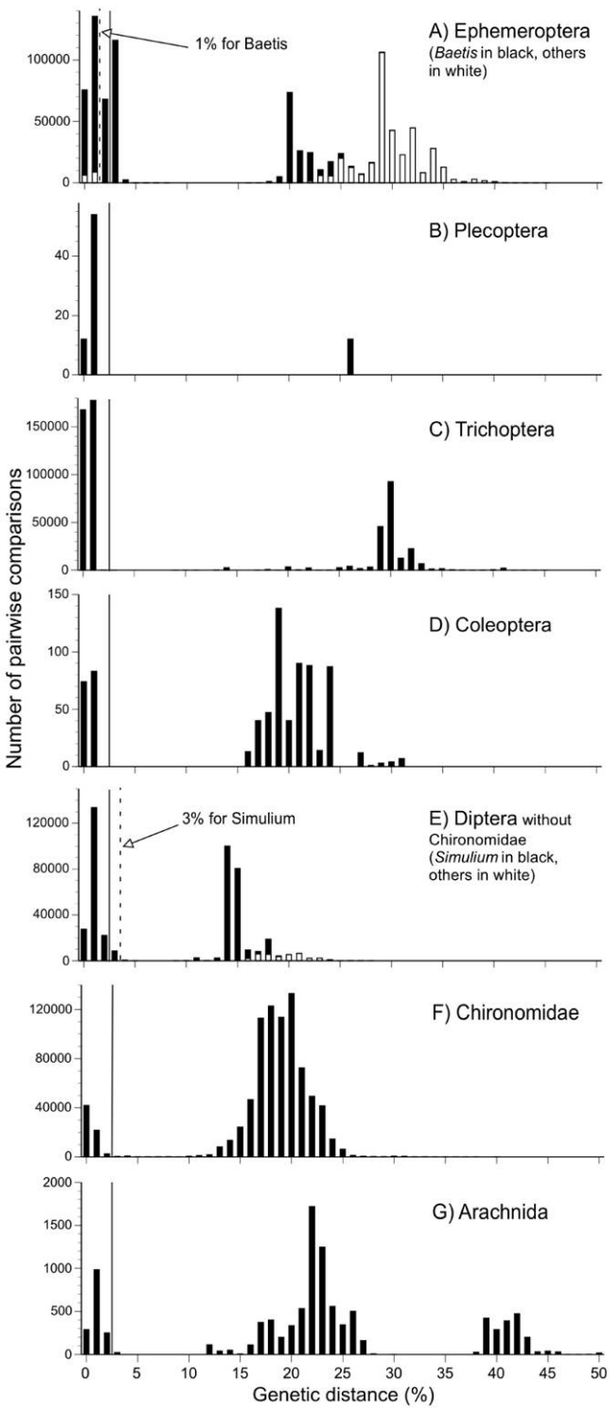

Figure 1. Number of pairwise comparisons vs % genetic distance for Ephemeroptera (A), Plecoptera (B), Trichoptera (C), Coleoptera (D), Diptera without chironomids (E), Chironomidae (F), and Arachnida (G) collected from 5 California streams in June 2010. Solid and dashed vertical lines indicate divergence used to separate molecular operational taxonomic units (MOTUs): 2% for all groups except *Baetis* (1%) and *Simulium* (3%).

gence) (cf. Hebert et al. 2003a, b, Meyer and Paulay 2005, Rivera and Currie 2009, Sweeney et al. 2011, and others). MOTUs or barcode species were delimited using 2% divergent distance for all taxa groups except *Baetis* (1%) and *Simulium* (3%) (Figs 1A–G, 2). The break between intra- and interspecific divergence for the blackfly *Simulium* began at ~3% (Fig. 1E), which split *Simulium* into 6 species (Fig. S1F). *Baetis* was complicated in that 3 species were delimited morphologically (*Baetis tricaudatus*, *Baetis adonis*, and *Baetis* sp.), but barcodes in the NJ tree distinguished 6 clusters, 4 of which are similar genetically (*B. adonis* 1 vs *B. tricaudatus* 1, *B. adonis* 2 vs *B. tricaudatus* 3; Figs 2, S1A). The above approach was applied to most specimens collected, and most barcode species were defined based on individuals with full (658 bp) sequences. Additional examination of delimitation challenges and analytical options for *Simulium*, *Baetis*, and *Eukiefferiella* can be found in White et al. (2014).

If a short sequence (<350 bp) had <2% divergence match to a long sequence (>350 bp), then it was given the designated MOTU name associated with that >350 bp sequence. To make the barcode data comparable to the morphology data (i.e., include all individuals identified morphologically), specimens that did not have a barcode (i.e., no sequence or a short sequence <350 bp that did not match a >350 bp sequence) were assigned the morphology-based designation. These individuals were counted as a new OTU if they had not been barcoded and it was the only time it occurred in the sample, but if it had been sampled as a barcoded taxon then it did not add to richness measures. This approach to assigning names to individuals that did not have a barcode was conservative and may have underestimated barcode richness, but it affected relatively few individuals (392 of 5870 [6.7%] across 5 sites, including 278 [70.9%] at Conejo) and allowed us to include all individuals collected in the analyses.

RESULTS

Biodiversity revealed by barcoding: taxonomic resolution and cryptic species

We identified a total of 200 species across the 5 streams (Table 1), 191 based on barcodes and 9 based on morphology because no or inadequate barcodes were obtained. Total richness for individual streams was far less than the regional total, and ranged from 91 and 95 species at EF and WF San Gabriel, respectively, to 38 species at Big Tujunga Wash, 45 species at Arroyo Seco, and 46 species at Conejo. Most (167) of these species were insects. Among the 191 species delimited based on barcodes, most were defined with complete (652–658 bp) COI sequences and >1 individuals. Of the 55 species represented by only 1 specimen, only 8 had sequence lengths <652 bp (407–634 bp). Different species in the same genus were relatively distinct genetically, with a mean divergence of 13.3% (range = 2.0–

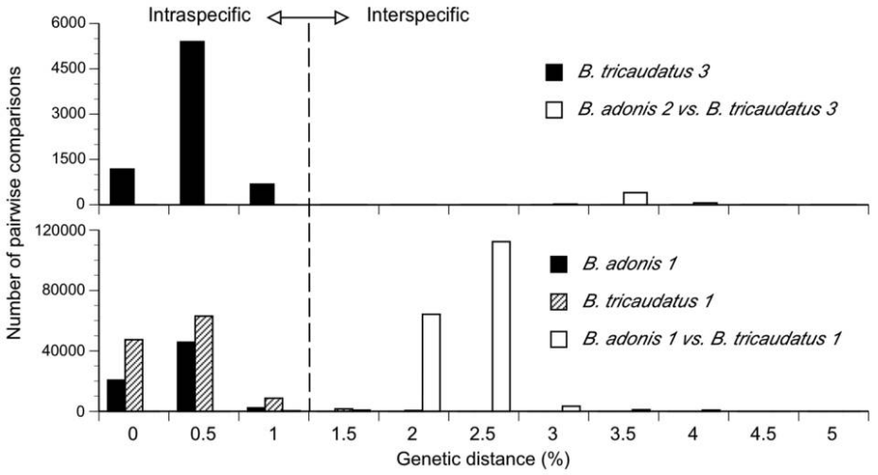

Figure 2. Number of pairwise comparisons vs % genetic distance for pairs of genetically similar *Baetis* molecular operational taxonomic units (MOTUs) (*Baetis adonis* 2 vs *Baetis tricaudatus* 3 and *B. adonis* 1 vs *B. tricaudatus* 1); numbers after a name indicates our MOTU designations (see neighbor-joining [NJ] trees in Fig. S1A–I). All individuals were collected from 5 California streams in June 2010. *Baetis adonis* 2 had only 4 individuals so intraspecific variation (maximum = 0.002%) is not shown.

29.7%). Thus, interspecific differences were, on average, 5× greater than the 2% threshold used to delimit most species. Valid species names could be assigned to only 34 (18%) of the 191 barcoded species (Fig. S1). Eleven names were based on our morphological identifications, whereas 23 names came from the barcode library in BOLD or GenBank. The remaining barcode MOTUs did not closely match (i.e., >2% difference) any species with sequences in the barcode library in BOLD or were a match to a BOLD specimen identified only to genus or higher.

The objective of the morphological identifications initially used in our study was not to identify individuals to the lowest taxonomic level possible, but rather, to a predetermined, standard level that balanced availability of keys, effort, and information gained (i.e., Richards and Rogers 2006). The standard level in most cases was genus, but species-level identifications were standard for some genera (especially *Baetis*, but also *Diphetor*, *Calineuria*, *Ordobrevia*, *Eubrianax*, and *Psephenus*, which are all monotypic in California). Of the 96 morphological taxa identified among these 5 streams, 18 (19%) were species and 69 (72%) were genera, and only 9 (9%) were family or higher. Of the 5870 specimens examined morphologically, 22% were identified to species, 74% to genus, and only 4% identified to family or higher. Barcodes revealed that many of the genera and species identified morphologically might actually represent >1 species. For example, of the 18 species or species groups initially identified based on morphology alone, 6 (*Ephemerella maculata*, *Drunella coloradensis*, *Baetis adonis*, *Diphetor hageni*, *Microtendipes pedellus* grp., *Microtendipes rydalensis* grp.) represented 2 species based on barcodes, whereas *Baetis tricaudatus* represented 3 species. In addition, among the 69 genera with no species identified morphologically, 16 represented 2 species based on barcodes, and another 16 had 3 to 10 species. If all specimens were identified only to genus or higher (as in many stream-monitoring programs), 19 of 84 genera would have been represented by 2 species, and an additional 19 genera would have been represented by 3 to 10 species (based on barcodes). Many of the genera with multiple species were chironomids (19 of 38), but other groups, such as caddisflies (6 genera with multiple species), mayflies (5 genera), mites (4 genera), and nonchironomid Diptera (3 genera) were represented. *Polypedilum* (10 species), *Eukiefferiella* (9 species), *Cricotopus*, and *Tanytarsus* (8 species each) were especially speciose.

Barcodes also allowed identification of 100 specimens (1.7% of total) that were too small to be identified morphologically beyond family and allowed us to group these specimens with conspecifics (e.g., Ephemerellidae to *Serratella micheneri*, Heptageniidae to *Ecdyonurus criddlei*, Hydropsychidae to *Hydropsyche* 1 or 2, Libellulidae to *Paltothemis lineatipes*, Empididae to *Neoplasta*, Stratiomyidae to *Caloparyphus/Euparyphus* 1, 2, or 3). Barcodes also detected individuals that initially had been misidentified based on morphology so they could be re-examined and properly grouped with conspecifics. For example, the initial identifications of 228 midges (15% of the chironomids) and 49 *Baetis adonis*, *Baetis tricaudatus*, or *Baetis* CA 1 (4.7% of the *Baetis*) were changed to reflect the correct genus/species. Based on morphological misidentifications, several chironomid genera (*Tanytarsus* vs *Micropsectra*, *Eukiefferiella* vs *Cardiocladius*, *Eukiefferiella* vs *Orthocladius*, *Orthocladius* vs *Cricotopus*) appeared to be especially challenging for the taxonomists. However, in our study, chironomid identifications to genus were based pri-

marily on gross body morphology rather than detailed head-capsule characteristics that are essential elements in chironomid keys.

Biodiversity and rare species detected by barcoding

Many species (86 of 200, 43%) were represented by only 1 (29%) or 2 (14%) of the 5870 specimens examined and, therefore, generally were found at only 1 stream. Similarly, 45 to 63% of the species at a site were represented by only 1 or 2 of the ~1200 specimens examined at each site. Over half (121) of the species were found at only 1 of the 5 sites. Thus, many of the macroinvertebrate taxa in these streams were relatively rare in samples within and among sites. Many of the differences among streams presumably reflect the environmental stressors associated with urban or agricultural development at Big Tujunga Wash, Arroyo Seco, and Conejo that eliminated some pollution-sensitive species and created new opportunities for some pollution-tolerant species. However, even 2 neighboring streams (WF and EF San Gabriel) with limited anthropogenic influences supported faunas that differed in the species present. For example, 47 of the 95 (49%) barcode species at WF San Gabriel were not collected at EF San Gabriel (i.e., unique taxa), and 44 of 91 (48%) barcode species at EF San Gabriel were not collected at WF San Gabriel (Fig. 3). In addition, 47 of the 95 (49%) barcode species at WF San Gabriel and 43 of 91 (46%) barcode species at EF San Gabriel were represented by only 1 or 2 of the ~1200 specimens examined (~0.1–0.2% of the specimens at each site; Fig. 3). Thus, whether rarity is defined spatially (at only 1 site) or numerically (only 1–2 specimens per site), almost ½ of the macroinvertebrate taxa at the 2 San Gabriel sites would be considered rare.

High frequency of rare species increases the number of individuals requiring examination when describing a

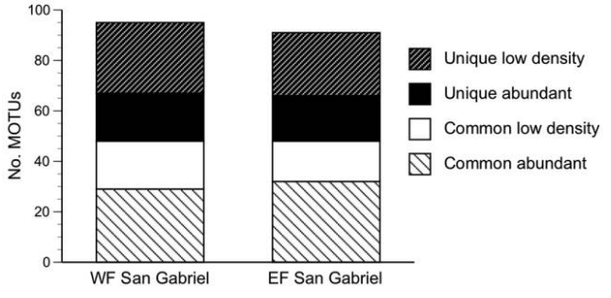

Figure 3. Molecular operational taxonomic units (MOTUs) for the West Fork (WF) and East Fork (EF) of the San Gabriel River sorted into 4 groups based on presence and abundance: unique low density (at only 1 site and ≤2 individuals), unique abundant (at only 1 site and ≥3 individuals), common low density (at both sites but ≤2 individuals at specified site), and common abundant (at both sites and ≥3 individuals at specified site).

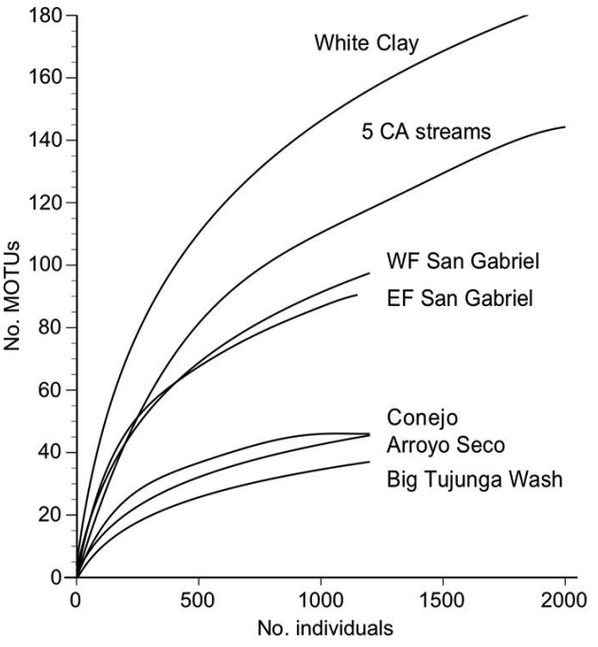

Figure 4. Species accumulation curves for all barcode molecular operational taxonomic units (MOTUs) in 5 California (CA) streams collectively and individually and 1 Pennsylvania stream (White Clay Creek). Values were based on resampling 1000 times. WF = West Fork, EF = East Fork.

local or regional fauna. Species accumulation curves for individual sites approached horizontal asymptotes for Big Tujunga Wash, Arroyo Seco, and Conejo (Fig. 4), results suggesting that the sampling effort (~1200 individuals) had produced a reasonably accurate representation of the spring/summer macroinvertebrate faunas for these sites. Examining additional specimens would not add much to the total number of species found at these sites, even for chironomid midges (Fig. 5). The accumulation curves for WF and EF San Gabriel also began to level out toward horizontal asymptotes, but increasing the number of individuals examined from 1000 to 1200 at either stream added 3 to 4 additional species (primarily chironomid midges; Figs 4, 5). The accumulation curve for 5 streams combined was similar to the curves for WF and EF San Gabriel (i.e., increasing the number of individuals examined from 5500 to 5700 added 3 to 4 additional species; Fig. 4). This result indicates that we have a reasonably good representation of the regional fauna in the spring/summer, although some species in this region probably were not collected in our study.

Implications of cryptic biodiversity revealed by barcodes

If we assume that morphological data yielded a conservative estimate of species richness in our study (i.e.,

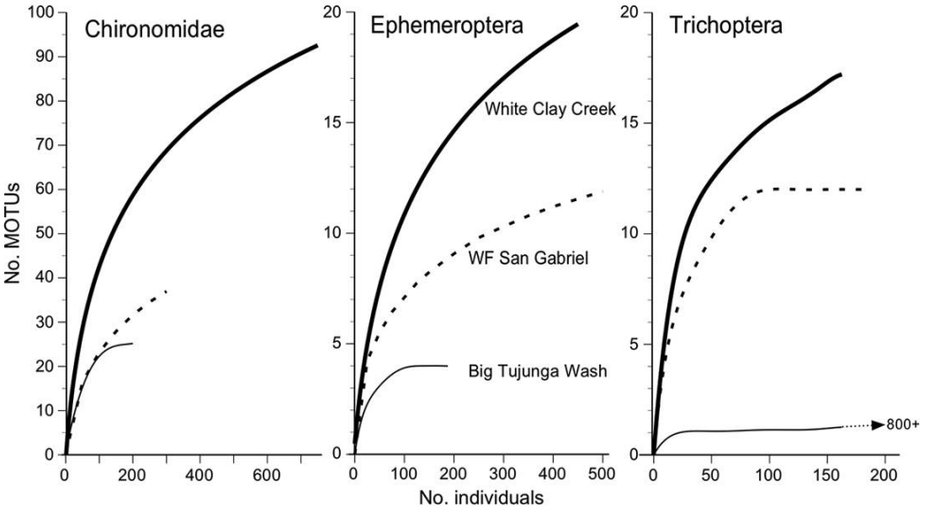

Figure 5. Species accumulation curves for barcode molecular operational taxonomic units (MOTUs) of 3 common groups in 3 creeks: White Clay Creek, WF San Gabriel River, and Big Tujunga Wash. In Big Tujunga Wash >800 Trichoptera were examined but the asymptote was reached and the line cropped after a resampling of 150 individuals. MOTU values were based on resampling 1000 times.

assume a genus was represented by only 1 species unless otherwise indicated), barcodes identified 104 more species than were identified with morphology alone across the 5 streams (96 taxa vs 200 species), a 108% increase (Table 1). Richness increases were greatest for Chironomidae (60 additional species, 194% increase), followed by Ephemeroptera (10 species, 77% increase), Acari (10 species, 200% increase), and Trichoptera (6 species, 50% increase). The increase in total richness per stream ranged from 34 species at WF San Gabriel and 40 at EF San Gabriel to 12 species at Big Tujunga Wash, 24 species at Arroyo Seco, and 16 species at Conejo (Table 1). The increase was greater for the WF and EF San Gabriel sites, which are in watersheds with much less intensive land use than the other 3 watersheds. If we use the WF San Gabriel as a reference site, then barcoding increased the species loss from environmental stress by 22 species (63%) at Big Tujunga Wash, 10 species (25%) at Arroyo Seco, and 18 species (58%) at Conejo (Table 1). Thus, relying on morphology alone underestimated the loss of biodiversity in response to environmental stress in these streams by 25 to 63%.

Barcode identifications also found more subtle differences among sites that reflected species replacements within a genus rather than just lost species. This effect was observed between reference sites, between potentially impaired sites, and between reference and potentially impaired sites. For example, the dominant species of *Simulium* and *Orthocladius* at EF San Gabriel were not dominant at WF San Gabriel (Fig. 6B, C). The dominant species of *Orthocladius* at Arroyo Seco was not dominant at Big Tujunga Wash (the 2 similarly situated degraded sites) (Fig. 6C). In addition to the species losses at Big Tujunga Wash and Arroyo Seco relative to WF and EF San Gabriel (Table 1), the dominant species changed for several genera (e.g., *Baetis* and *Simulium* [Fig. 6A, B], *Eukiefferiella*, *Rheotanytarsus*). None of these differences among sites would have been apparent had the comparisons relied on morphology to produce only genus-level identifications. As a result, the similarity (Jaccard index) between sites decreased when genus data were converted to species (i.e., barcodes) (Table 2). For example, the similarity between WF and EF San Gabriel decreased from 58 to 35.

DISCUSSION

Species identifications based on barcodes

Barcodes from the benthic macroinvertebrates collected from the 5 streams identified >100 more taxa than were identified based on morphology alone and resulted in putative species-level designations for 93% of the individuals examined. This percentage is a significant increase in the number of individuals identified to species relative to the results of most studies of benthic macroinvertebrates. Carter and Resh (2001) found that larval Ephemeroptera, Plecoptera, and Trichoptera were left at the genus or higher level in 54 to 56% of state monitoring programs, and chironomid midges were left at the genus or higher level in 70% of the programs. In 3 studies, investigators attempted to identify as many specimens as possible to species based on morphology. Waite et al. (2004) took 25% of the specimens from 490 streams to species, Arscott et al. (2006) took 36% of the specimens from 60 streams to species, and Sweeney et al. (2011) took 46% of the specimens from 1 stream to species.

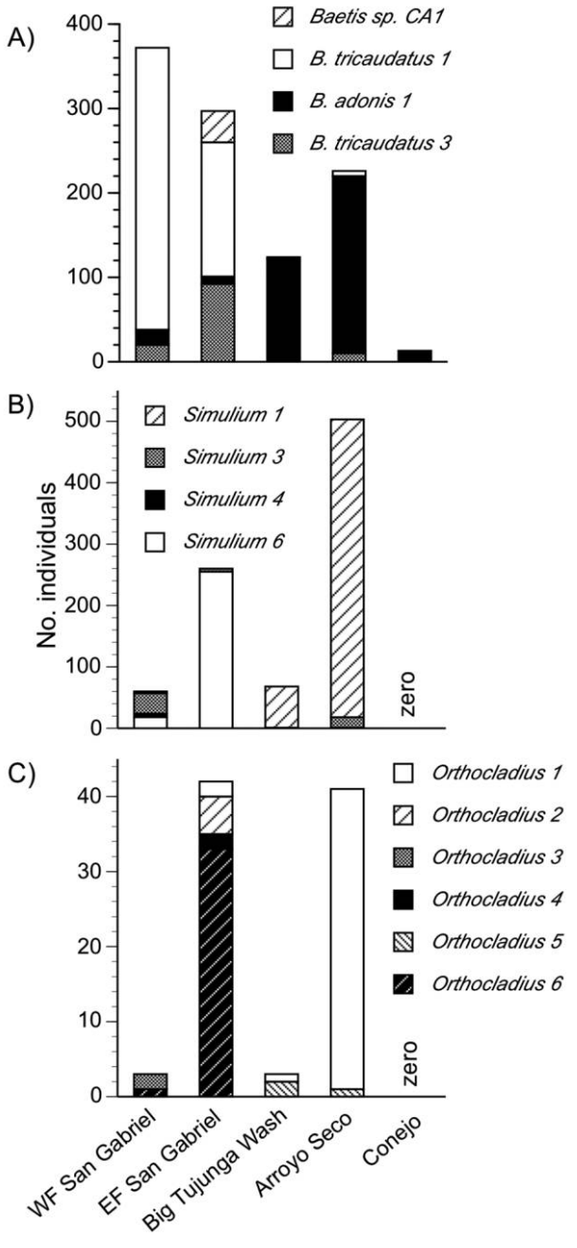

Figure 6. Differences among the 5 California streams for *Baetis* (A), *Simulium* (B), and *Orthocladius* (C) containing multiple species. Conejo Creek had no *Simulium* or *Orthocladius*.

Genetic divergence among species in a genus averaged 13% across all macroinvertebrate groups examined. Thus, most of the species we identified had genetic divergences that far exceeds our 2% cut off for delimiting species and were well differentiated from their nearest neighbors. Genetic differences between nearest neighbors approached or marginally exceeded our 2% cut off for delimiting species in only 8 species pairs or groups among the 191 MOTUs. The genetic differentiation we observed among species was similar in magnitude to results of more focused taxonomic studies that found good agreement between morphological and barcode species and numerous morphologically cryptic species that were genetically distinct (e.g., Monaghan et al. 2005, Stahls and Savolainen 2008, Pauls et al. 2010, Zhou et al. 2010, 2011, Lucentini et al. 2011, Mynott et al. 2011, Harvey et al. 2012, Kim et al. 2012, Larson et al. 2012, Renaud et al. 2012, Webb et al. 2012). We recognize the importance of discussions of species delimitation and species concepts (e.g., Agapow et al. 2004, Sites and Marshall 2004, DeSalle et al. 2005, Pons et al. 2006) and concerns about taxonomic inflation (e.g., Isaac et al. 2004, Zachos et al. 2013), but we think that the vast majority of the species identified in our study are distinct species and that when barcode libraries are more complete, many will match up well with morphologically defined species known to occur in this region. For example, 306 mayfly species, 379 stonefly species, 746 caddisfly species, and 516 chironomid species, most of which are not in the barcode library of BOLD and GenBank, are currently listed on the California inventory maintained by the Southwest Association of Freshwater Invertebrate Taxonomists (http://www.safit.org/ste.html).

Questions concerning species delimitation, species concepts, and taxonomic inflation may become an issue for stream macroinvertebrates in the future as barcode libraries expand to include more species, and species are better represented by specimens from sites across a wider geographic range (Bergsten et al. 2012, Webb et al. 2012, Anderson et al. 2013). However, the biggest impediment to accurate identification of benthic macroinvertebrates is that diagnostic morphological characters for species frequently are unknown or nonexistent for the life stages most commonly collected from streams or rivers (i.e., immature larvae of species identified by adult male characters). We think that COI barcodes and other genetic data are additional characters that may facilitate morphological identifications by helping to associate larvae with named adults and to differentiate between intra- and interspecific variation in morphology. We do not view barcodes as a replacement for morphological characters, but as a tool to help resolve species by confirming and directing morphological effort and by pushing taxonomic resolution beyond the limits of morphology for many specimens (e.g., larval chironomid midges, mayflies, stoneflies, caddisflies). We also recognize that COI barcodes may not be able to resolve all species relationships (e.g., Shaw 2002, Whitworth et al. 2007, Alexander et al. 2009, Chen et al. 2012), and that other DNA methods may occasionally give different results or give rise to questions about delimiting/distinguishing closely related species. That said, our study provides additional evidence that barcodes have the potential to increase greatly the frequency and accu-

Table 2. Similarity between sites as measured with the Jaccard index. WF = West Fork, EF = East Fork.

Stream	Taxonomic level	Conejo	EF San Gabriel	Arroyo Seco	WF San Gabriel
Big Tujunga Wash	Genus	26	23	38	25
	Species	13	9	34	14
Conejo	Genus	.	14	20	16
	Species	.	3	10	5
EF San Gabriel	Genus	.	.	28	58
	Species	.	.	18	35
Arroyo Seco	Genus	.	.	.	19
	Species	.	.	.	13

racy of species identifications from commonly collected benthic macroinvertebrate samples.

Biodiversity and rare species detected by barcoding

Barcodes have been used primarily to discriminate or describe relationships among closely related species or a number of species within a genus or family (Hebert et al. 2004a, Smith et al. 2006, Burns et al. 2008, Pauls et al. 2010, Anderson et al. 2013, but see also Hebert et al. 2004b, Ward et al. 2005, Hajibabaei et al. 2006, Dincă et al. 2011, Webb et al. 2012). It is unusual for a single barcode study to examine numerous individuals across a wide range of unrelated species within a small region as we have done here. In the most similar effort, investigators examined 1579 benthic macroinvertebrates from 2 sites in a small stream (White Clay Creek [WCC]) in southeastern Pennsylvania (PA) at about the same altitude but >3500 km east of the streams in our study (Sweeney et al. 2011). A comparison of the results of these 2 studies provides interesting insights into the structure of stream macroinvertebrate communities and the frequency of morphologically cryptic species. First, in both studies, many species that could not be or were not resolved based on morphological characters present in the specimens collected were identified based on barcodes (104 more species [108% increase] for California (CA); 86 more species [78% increase] for PA). Many species identified with barcodes were rare spatially (at only 1 site) or numerically (represented by 1 or 2 specimens). Rare taxa (species or genera) repeatedly have been a subject of interest in the study of stream macroinvertebrate communities (e.g., Cao and Williams 1999, Lenat and Resh 2001, Nijboer and Verdonschot 2004, Arscott et al. 2006, Van Sickle et al. 2007, Heino and Soininen 2010, Poos and Jackson 2012, Heino 2013, and references therein). Little agreement exists among investigators regarding the value/contribution of rare taxa. Some authors have concluded they are redundant, whereas others have found them informative, especially if the goal is to detect subtle changes or if they are diagnostic of different stream conditions. Many of these authors analyzed data sets in which most the specimens were not identified to species, and therefore, the full impact of species identifications has not yet been examined.

Second, the total number of species was greater at the PA site than at any individual CA site and was comparable to the total number of species in the 5 CA streams combined (Table 1). Species accumulation curves for each site showed the potential differences in the number of specimens needed to describe the fauna at a site or for a region (e.g., WCC > CA, WF San Gabriel > Arroyo Seco) (Figs 4, 5). Differences between CA and PA are evidence that these macroinvertebrate communities are structured differently, with more species at WCC relative to any of the CA sites at similar sampling efforts. Thus, sampling efforts recommended to characterize a local fauna (e.g., Vinson and Hawkins 1996, Cao et al. 2007, Cao and Hawkins 2011) may vary depending on whether sampling focuses on more or less speciose habitats, regions, or taxonomic groups (e.g., Chironomidae vs Ephemeroptera, Plecoptera, Trichoptera).

Implications of cryptic biodiversity revealed by barcoding

Our results and those of Sweeney et al. (2011) clearly show that reliance on morphology has limited our perception of macroinvertebrate biodiversity in streams and rivers and of the loss of biodiversity in response to environmental stressors. Using barcodes in the identification process would greatly increase the number of species collected at a site, especially reference sites and, therefore, would produce a more accurate estimate of the biodiversity lost at an impaired site. Increased frequency of species identifications will be an important step toward generating the data and insights needed to put discussions regarding protection and management of streams based on data involving aquatic macroinvertebrates on the same level with ongoing, similar conversations involving plants, fish, amphibians, reptiles, birds, and mammals (all of which are species based). At present, we cannot discuss in a robust

fashion macroinvertebrate species' presence or absence, rarity or abundance, stability or variability for streams and rivers anywhere in the world. We cannot truly understand abiotic or biotic interactions if we do not know the species involved. Last, connections to and impacts of our scientific communications are limited and possibly inaccurate if the names we use are incorrect or too general.

Species-level identifications will become more informative as we replace our current understanding of stream macroinvertebrates (largely genus- or family-based) with knowledge of the basic biology for individual species. We need to know if species have narrow or broad environmental requirements in natural settings and how this translates into sensitivity to or tolerance of anthropogenic changes in water or habitat quality (Heino 2013). Use of species-based biology will be a major change in functional traits-based approaches to understanding macroinvertebrate community structure and applications to biomonitoring that refer to species traits but actually use genus- or family-level characteristics (e.g., Poff 1997, Poff et al. 2006, Heino et al. 2007, Statzner and Bêche 2010, Heino 2013) that (often unknowingly) homogenize species-level differences (e.g., Janzen et al. 2012). Species-specific insights may not be needed to identify highly impaired stream sites because the level of impairment often involves loss of entire genera or families. However, species-level data would improve our ability to identify, with confidence, subtle changes in macroinvertebrate communities and, hence, water quality in streams and rivers. This ability would be invaluable for detecting and assessing impacts to streams and rivers in a more timely fashion (before serious degradation occurs) and would provide more rapid measurement of the rate and degree of stream and river recovery in response to improved watershed management or proactive stream and river restoration.

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