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Incorporation of DNA barcoding into a large-scale biomonitoring program: opportunities and pitfalls

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Abstract. Taxonomic identification of benthic macroinvertebrates is critical to protocols used to assess the biological integrity of aquatic ecosystems. The time, expense, and inherent error rate of species-level morphological identifications has necessitated use of genus- or family-level identifications in most large, statewide bioassessment programs. Use of coarse-scale taxonomy can obscure signal about biological condition, particularly if the range of species tolerances is large within genera or families. We hypothesized that integration of deoxyribonucleic acid (DNA) barcodes (partial cytochrome c oxidase subunit I sequences) into bioassessment protocols would provide greater discriminatory ability than genus-level identifications and that this increased specificity could lead to more sensitive assessments of water quality and habitat. Analysis of DNA barcodes from larval specimens of Ephemeroptera, Plecoptera, and Trichoptera (EPT) taxa collected as part of Maryland's Biological Stream Survey (MBSS) revealed ~2 to 3× as many DNA-barcode groups or molecular operational taxonomic units (mOTUs) as morphologically identified genera. As expected, geographic distributions for several mOTUs were tighter than for the parent genus, but few mOTUs showed closer associations with water-quality variables or physical-habitat features than did the genus in which they belonged. The need for improved protocols for the consistent generation of DNA barcodes is discussed.

Key words: DNA barcoding, taxonomic resolution, bioassessment, benthic macroinvertebrate, EPT, water quality, streams, environmental sensitivity.

The US Clean Water Act of 1972, as amended in 1977, directs states and tribes to restore and maintain the biological integrity of the nation's waters. To aid states and tribes in assessing the aquatic community, the US Environmental Protection Agency (EPA) actively develops, evaluates, and promotes the use of biological indicators, defined as numerical values (e.g., an index or model output) derived from biological measures with known statistical properties that convey useful information for environmental decision making (Klemm et al. 1990). Use of biological indicators in scientifically rigorous biological monitoring and assessment programs is an effective way to characterize the current status of aquatic communities and to track or predict changes in the condition of those communities. All US states use information from the benthic macroinvertebrate community to develop indicators of the condition of aquatic communities, albeit at different levels of taxonomic

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resolution (Carter and Resh 2001). Benthic macroinvertebrates, particularly larval forms of many aquatic insects, are both key components of aquatic food webs and sensitive to changes in pollution and habitat and, therefore, indicative of the condition of the entire aquatic community (Cranston 1990, Rosenberg and Resh 1996). Macroinvertebrate incidence and abundance data are used to inform different types of biological indicators, including biotic indices (e.g., Hilsenhoff 1987, Wallace et al. 1996), multimetric indices (e.g., Karr et al. 1986), and measures of taxonomic completeness (e.g., Moss et al. 1987, Cao et al. 2003, Hawkins 2006).

The difficulty of taxonomic identifications complicates use of macroinvertebrates in bioassessments, particularly for aquatic insect larvae that often lack species-specific morphological characters, such as well-developed genitalia (most species keys of insects are based on adult males). The time, expense, and inherent error rate of species-level identifications, particularly for early instars or difficult groups, has necessitated the use of genus- or family-level identifications in most statewide bioassessment programs. Identification of larvae at taxonomic levels higher than species is more feasible, but taxonomic identification error rates at the family or genus level can still be high (Stribling et al. 2008, Haase et al. 2010) and contribute to imprecision in assessing the condition of aquatic communities. This coarser-scale taxonomy also can obscure signal about ecological condition to some degree. Physiological tolerances, behaviors, and life-histories often vary among congeners (Resh and Unzicker 1975, Cranston 1990, Barbour et al. 1999, Lenat and Resh 2001, Waite et al. 2004; Fig. 1), and thus, species provide distinct perspectives on water quality and habitat condition. Conflation of sensitive and tolerant species within a taxon results in reduced environmental sensitivity of the biological indicator.

The extent to which reduced sensitivity of the biological indicator resulting from taxonomic lumping to genus or family levels affects management decisions is debatable (Guerold 2000, Hawkins et al. 2000, Hewlett 2000, Bailey et al. 2001, Lenat and Resh 2001, Waite et al. 2004, Chessman et al. 2007) and is dependent on the management context. The effects of the practice probably are less important in general assessments of aquatic ecosystem condition than when diagnosing stressors or classes of stressors affecting the condition of an aquatic community. Use of the macroinvertebrate community for stressor diagnosis remains an important management goal (e.g., Norton et al. 2000, Stranko et al. 2005, Liess et al. 2008).

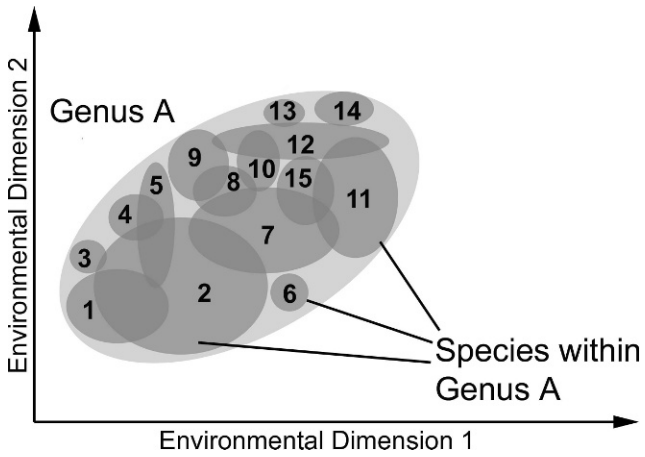

FIG. 1. A conceptual diagram describing the environmental tolerance of a bioindicator. The tolerance of the genus-level bioindicator (light gray) encompasses all tolerance ranges of the 15 species (dark gray) within that genus. Some species within the genus are expected to have overlapping environmental tolerances.

Incorporation of diagnostic deoxyribonucleic acid (DNA) signatures or DNA barcodes (Hebert et al. 2003) into bioassessment protocols holds great potential to reduce limitations of morphological taxonomic identifications for larval aquatic insects and other macrobenthos. Identifications based on a standard stretch of mitochondrial DNA (i.e., the Folmer region of cytochrome c oxidase subunit I [COI]) lessens the burden on the existing (but diminishing) sophisticated and specialized morphological expertise required for species-level identifications. DNA-based taxonomic identifications provide an alternative method that can discriminate among morphologically cryptic species (Zhou et al. 2010), immature life stages (Zhou et al. 2007) and females (Pilgrim and Pitts 2006), and damaged specimens (Savolainen et al. 2005). Thus, a fully populated reference library that matches species taxonomy with DNA barcodes could allow nearly all specimens to be identified to species (Ball et al. 2005, Ball and Armstrong 2006, Hajibabaei et al. 2006a, Hogg et al. 2009). Last, availability of a standardized, objective method for precise taxonomic discriminations could facilitate use of biodiversity information for diagnostic stressor identification methods.

To evaluate potential incorporation of DNA barcoding into bioassessment and the logistical requirements for such a program, researchers from the US Environmental Protection Agency (EPA) and Smithsonian Institution partnered with the Maryland Department of Natural Resources (MD-DNR) during the 2007 Maryland Benthic Stream Survey (MBSS). Aquatic insects (Ephemeroptera, Plecoptera, and

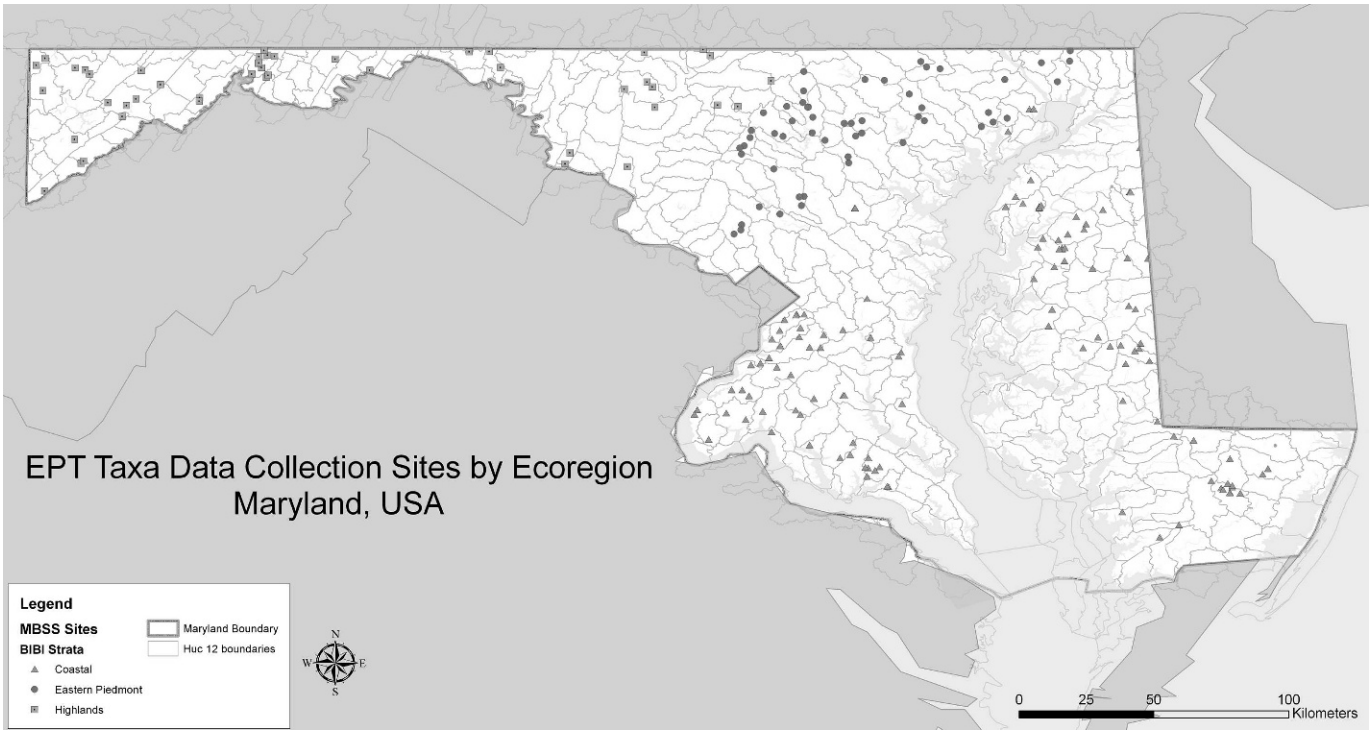

FIG. 2. Map of 2007 Maryland Biological Stream Survey (MBSS) sites used in our study. Sites are coded based on the geographical strata used in Maryland's Benthic Index of Biotic Integrity (BIBI). HUC (Hydrologic Unit Code) 12 denotes subwatershed-level boundaries within Maryland.

Trichoptera [EPT] specimens) collected during the MBSS were processed for DNA-barcode analyses to address several questions about the potential role of barcoding in stream bioassessment: 1) Is DNA barcoding an effective tool to add to an existing aquatic monitoring program? 2) Does DNA barcoding improve taxonomic resolution for identifying benthic insect larvae and, if so, how does this improvement affect assessment of environmental condition? 3) What measures must be taken to integrate DNA barcoding successfully into future bioassessment programs?

Methods

Benthic macroinvertebrates were collected as part of the 2007 MBSS according to sampling protocols developed by MD-DNR (Stranko 2007). Habitat and water-chemistry data also were collected according to MD-DNR protocols (Kazyak 2001). Collection sites ($n = 206$) were distributed across the state and included multiple watersheds and 3 geographical strata within Maryland (Coastal Plains, Eastern Piedmont, and Highlands; Fig. 2). Macroinvertebrate collections were stored in 95% nondenatured ethanol and transported to the laboratory for further processing. The 2007 MBSS protocol required only that specimens be placed in 95% ethanol and not allowed to dry. In the laboratory, EPT specimens were sorted, processed, and identified morphologically to genus (in 95% ethanol throughout) according to standard operating procedures developed by MD-DNR (Boward and Friedman 2000). After morphological identification, all specimens, regardless of stage of development, were processed for DNA analysis. Some taxa were common at certain sites, and in these cases, a maximum of 10 individuals per taxon per site were chosen for DNA analysis.

Specimens were distributed into 96-well plates and batches of 4 to 5 plates were alternately sent to 2 DNA-barcoding laboratories (US EPA in Cincinnati, Ohio, and the Smithsonian's Laboratory for Analytical Biology in Suitland, Maryland). DNA was extracted from a subsection of specimen tissue (typically a leg) in a 96-well-plate format, either Qiagen DNeasy 96 (Qiagen, Valencia, California) or AutoGenprep 965 (AutoGen, Holliston, Massachusetts). A portion of the COI gene was amplified by polymerase chain reaction (PCR) with universal primers (Folmer et al. 1994), and products were visualized on agarose gels. Unsuccessful COI amplifications were repeated at least once with a modified protocol (use of additional DNA template in the reaction, additional PCR cycles, or different COI

primers [degenerate Folmer primers, Meyer 2003; or lepidopteran primers, Hebert et al. 2004]). DNA sequencing of the PCR product was done in both forward and reverse directions for each specimen (BigDye Terminator v3.1; Applied Biosystems, Foster City, California) and run on a capillary sequencer (3730xl DNA Analyzer; Applied Biosystems).

Raw sequences were reviewed and edited using Sequencher (v 4.7; Gene Codes Corp, Ann Arbor, Michigan). Ambiguous bases at the ends of each sequence were removed. Forward and reverse sequences from each specimen were combined to form a contiguous sequence (contig) and ambiguous bases within the body of the contig were resolved by manually assigning base calls when possible (i.e., if the read in one direction showed a clear, single base pair [bp] peak that did not conflict with the ambiguity in the opposite direction). Consensus or single-direction sequences that were >400 bp in length (a slight compromise between the typical 500-bp requirement for DNA barcodes and the ~130 bp length of mini-barcodes; Hajibabaei et al. 2006b), obtained a PHRED (a base-calling program for DNA sequencing; available from CodonCode Corporation at http://www.phrap.com) sequence-quality score >90%, and contained <1% ambiguous bases were submitted to the Barcode of Life Data system (BOLD) (Ratnasingham and Hebert 2007) along with relevant collection information (stream, site code, latitude, longitude, elevation, collector, identifier, collection date, life stage, and taxonomic data). BOLD was used as a work bench for evaluating the sequences generated in our study (all data and subprojects are publically available within 'AMI Maryland Dept. Nat. Res. EPT study' [AMIMD]). All sequences used in our study have been deposited in GenBank (accession numbers HQ150087 to HQ152783 and HQ260989 to HQ261231).

Sequence data were aligned and analyzed in MEGA (version 4.1; Tamura et al. 2007) (pairwise deletion) to generate Kimura-2-parameter genetic distances and cluster sequences with neighbor-joining. DNA-barcode groups were designated as molecular operational taxonomic units (mOTUs = putative species; Floyd et al. 2002, Blaxter 2004) if they were separated by <2% minimum genetic distance and were distinct from other genetic clusters (i.e., the existence of a barcode gap; Meyer and Paulay 2005). Development of a complete DNA-barcode library based on morphologically resolved adults of the EPT species of Maryland is ongoing with the expectation of linking unidentified barcode groups to nominal species in the near future.

We evaluated whether mOTUs within a genus had narrower environmental requirements than the genus as a whole by comparing the incidence of each mOTU at different sites with site-specific habitat and water-chemistry data collected by MBSS personnel. The site data were evaluated with principal components analysis (PCA) using varimax rotation in SYSTAT (version 11; Systat Software, Chicago, Illinois) to reduce dimensionality (see Appendix 1 for definitions of the habitat and water-chemistry variables and the PCA output of these variables). The site data and taxon collection data were then analyzed with multiresponse permutation procedures (MRPP) in PC-ORD (version 4.34; McCune and Medford 1999). MRPP is a nonparametric method that tests whether pairwise distances between PCA scores for members within the group (e.g., environmental characteristics of sites in which a DNA-barcode group was observed) are smaller than those for the larger group (e.g., all sites where the genus containing the barcode group was observed). For comparison, the MRPP analysis also was done on genera to determine whether genus-level discriminations were associated with significantly narrower environmental requirements (i.e., significantly smaller average pairwise distance between sites) compared to all sites where EPT were collected. Several genera in each order were chosen for MRPP analysis with the first 3 principal components (PCs) of the site data. Genera were chosen for MRPP analysis if they contained multiple barcode groups (most with ≥ 5) and ≥ 2 of those mOTUs were found at multiple sites.

Results

A total of 5947 EPT specimens from the 2007 MBSS bioassessment were identified morphologically to genus. Of 4140 specimens processed for DNA analysis, 2338 (56.5%) yielded COI sequences that were >400 bp (2197 sequences were >500 bp), achieved a PHRED sequence quality score >90%, and possessed <1% ambiguous bases. To date, sequences from 1000 Ephemeroptera, 475 Plecoptera, and 863 Trichoptera larval specimens collected in Maryland in 2007 have been deposited in BOLD. DNA barcodes were not obtained for a few rare genera (Ephemeroptera: *Danella*, *Tricorythodes*, *Siphlonurus*; Plecoptera: *Cultus*, *Yugus*, *Oemopteryx*; Trichoptera: *Helicopsyche*, *Parapsyche*, *Oxyethira*, *Agarodes*). These taxa were represented by ≤ 3 specimens across all sites combined. Aside from the failures of amplification for these rare taxa, successful generation of DNA barcodes was not consistently difficult for any other group. Thus, failure to generate DNA barcodes did not appear to be taxon-specific.

DNA sequence analysis revealed the presence of many distinct genetic groups (i.e., mOTUs that were

Table 1. List of Ephemeroptera, Plecoptera, and Trichoptera (EPT) families, genera, and number of deoxyribonucleic acid (DNA) barcode groups (BG) among which the genetic distance was >2% collected for Maryland Biological Stream Survey (MBSS) in 2007.

Ephemeroptera			Trichoptera			Plecoptera		
Family	Genus	BG	Family	Genus	BG	Family	Genus	BG
Ameletidae	*Ameletus*	3	Brachycentridae	*Micrasema*	2	Capniidae	*Allocapnia*	1
Baetidae	*Acentrella*	2	Calamoceratidae	*Heteroplectron*	2	Chloroperlidae	*Paracapnia*	1
	Acerpenna	2	Glossosomatidae	*Glossosoma*	1		*Alloperla*	1
	Baetis	4	Goeridae	*Goera*	1		*Haploperla*	1
	Centroptilum	2	Hydropsychidae	*Cheumatopsyche*	9	Leuctridae	*Sweltsa*	1
	Plauditus	1		*Diplectrona*	3	Nemouridae	*Leuctra*	11
Caenidae	*Caenis*	2		*Hydropsyche*	7		*Amphinemura*	3
Ephemeridae	*Ephemera*	1	Hydroptilidae	*Leucotrichia*	1		*Ostrocerca*	1
Ephemerellidae	*Drunella*	1	Lepidostomatidae	*Lepidostoma*	2	Peltoperlidae	*Prostoia*	5
	Ephemerella	12	Leptoceridae	*Ceraclea*	1		*Peltoperla*	1
	Eurylophella	7		*Oecetis*	1	Perlidae	*Tallaperla*	1
	Serratella	2		*Triaenodes*	4		*Acroneuria*	3
Heptageniidae	*Cinygmula*	1	Limnephilidae	*Ironoquia*	3		*Eccoptura*	1
	Epeorus	3		*Platycentropus*	2	Perlodidae	*Perlesta*	1
	Leucrocuta	2		*Pycnopsyche*	7		*Clioperla*	2
	Maccaffertium	12	Molannidae	*Molanna*	1	Pteronarcyidae	*Isoperla*	11
	Stenacron	3	Odontoceridae	*Psilotreta*	3	Taeniopterygidae	*Pteronarcys*	1
Isonychiidae	*Isonychia*	2	Philopotamidae	*Chimarra*	3		*Strophopteryx*	2
Leptophlebiidae	*Leptophlebia*	1		*Dolophilodes*	1			
	Paraleptophlebia	1		*Wormaldia*	3			
Metretopodidae	*Siphloplecton*	1	Phryganeidae	*Ptilostomis*	1			
			Polycentropodidae	*Nyctiophylax*	1			
				Polycentropus	5			
			Psychomyiidae	*Lype*	1			
				Psychomyia	3			
			Rhyacophilidae	*Rhyacophila*	4			
			Uenoidae	*Neophylax*	5			

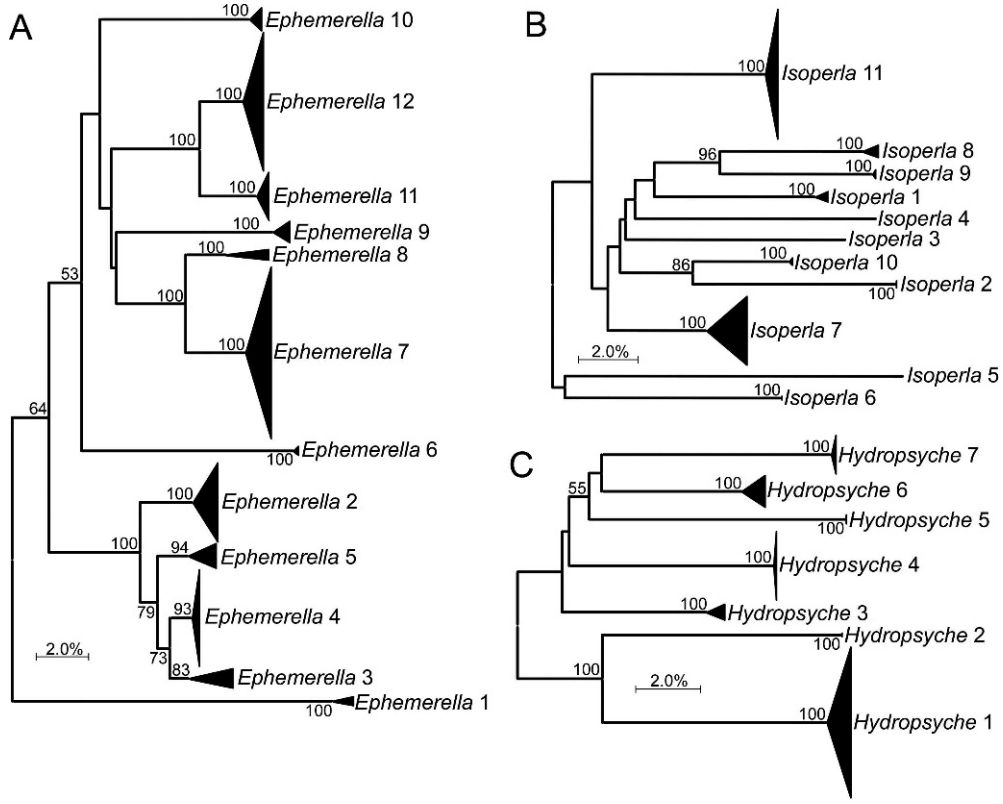

FIG. 3. Representative neighbor-joining trees of molecular operational taxonomic unit (mOTU)-rich genera: *Ephemerella* (A), *Isoperla* (B), and *Hydropsyche* (C). mOTUs are defined as having a minimum genetic distance >2%. Numbers before each node are bootstrap support values (>50%) based on 1000 pseudoreplicates. The width of the triangles is proportional to the genetic diversity within the mOTU; the height of the triangles is proportional to the number of individuals found in that mOTU.

>2% divergent from each other) within morphologically determined genera (Table 1). A total of 190 mOTUs were identified within the 66 EPT genera identified by morphology. Some genera were represented by a single mOTU (Ephemeroptera: $n = 7$, Plecoptera: $n = 11$, Trichoptera: $n = 10$), but most were represented by multiple barcode groups. Fourteen Ephemeroptera genera were represented by 58 mOTUs, whereas 7 Plecoptera and 17 Trichoptera genera possessed 37 and 67 distinct mOTUs, respectively. The COI sequences of specimens identified morphologically as *Ephemerella* could be resolved into 12 distinct genetic groups (Fig. 3A). Within Plecoptera, *Isoperla* specimens could be resolved into 11 groups (Fig. 3B), and within Trichoptera, *Hydropsyche* specimens fell into 7 distinct groups (Fig. 3C). As demonstrated by the neighbor-joining trees (Fig. 3A–C), genetic distances between these highly supported (bootstrap values from 83–100%) groups are >2% threshold proposed for identifying unique DNA mOTUs (Meyer and Paulay 2005). This threshold is more conservative than the 1% threshold used in previous Ephemeroptera work (Ball et al. 2005), but even at this larger distance, these mOTUs are expected to conform largely to existing species (Zhou et al. 2009).

Table 2 summarizes the number of EPT taxa (families, genera, and species) historically reported in Maryland (Stribling et al. 1998), and the number observed in the 2007 MBSS collection that were identified morphologically to genus. The number of genetic groups identified by DNA barcoding for each EPT order was 2 to 3× greater than the number of genera identified, and for Ephemeroptera and Trichoptera, was greater than the number of species that have been documented in Maryland. Not all genetic groups depicted recognized species or subspecies, and we refer to them as mOTUs until the association of COI barcodes from confirmed, vouchered adult specimens of each species can be made.

Multiple mOTUs within a genus often occurred together at the same site. For example, at site JONE-109, 8 genera of EPT taxa were identified morphologically, however, 12 distinct genetic groups were observed (Fig. 4). Three genera, *Ephemerella*, *Cheumatopsyche*, and *Diplectrona* were represented by 2, 2, and 3 genetic groups, respectively.

TABLE 2. Taxonomic groups previously observed in Maryland (Stribling et al. 1998) and those observed from our study (2007 Maryland Biological Stream Survey). mOTU = molecular operational taxonomic unit based on deoxyribonucleic acid (DNA) barcodes for which genetic distance was >2%.

	Number of families observed		Number of genera observed		Number of species observed	mOTUs
Order	Historical	2007 (barcoded)	Historical	2007 (barcoded)	Historical	2007
Ephemeroptera	12	11 (9)	32	24 (21)	42	65
Plecoptera	9	9	32	21 (18)	76	48
Trichoptera	18	19 (17)	41	32 (27)	66	77

We used PCA of the 22 environmental variables collected at each site (n = 183 after removing sites with missing data) to investigate any relationship between mOTUs and environmental data. PCA resolved 6 PCs with eigenvalues >1 that together explained ~74% of the variation. The first 3 PCs represented ~52% of the variation. PC 1 included loadings related to geography (e.g., elevation, latitude, longitude), PC 2 was associated with physical-habitat measures (e.g., velocity/depth diversity, instream habitat, epifaunal substrate), and PC 3 included loadings associated with water chemistry (e.g., conductivity, acid neutralizing capability, pH) (see Appendix 2 for loadings, variable definitions, and the variables that influenced PCs 4–6).

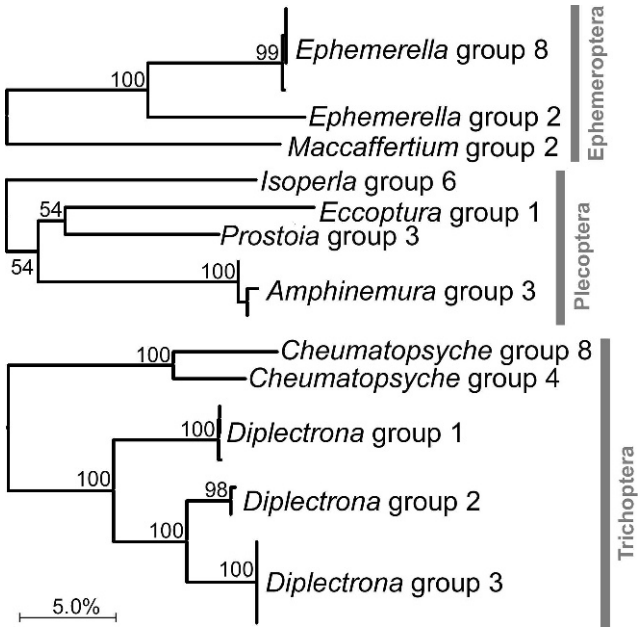

FIG. 4. An example of Ephemeroptera, Plecoptera, Trichoptera (EPT) molecular operational taxonomic unit (mOTU) diversity occurring at single site (JONE-109) depicted in 3 neighbor-joining trees separated by order. Numbers before each node are bootstrap support values (>50%) based on 1000 pseudoreplicates.

Results for the MRPP analysis for genera showed that, after sequential Bonferroni correction of α levels to account for multiple tests, very few genera had significantly narrower geographic (PC 1), physical (PC 2), or chemical (PC 3) environmental requirements than did EPT taxa as a whole (Table 3). The mayfly genus *Ephemerella*, stonefly genera *Amphinemura* and *Leuctra*, and caddisfly genera *Diplectrona* and *Pycnopsyche* were significantly associated with PC 1, representing geography-related variables. *Ephemerella* and the caddisfly genera *Cheumatopsyche* and *Hydropsyche* were significantly associated with PC 2, representing physical-habitat variables. *Pycnopsyche* and the stonefly genus *Isoperla* were significantly associated with PC 3, representing water-chemistry-related variables. Mayfly genera *Eurylophella* and *Maccaffertium* and the stonefly genus *Prostoia* were not significantly associated with any of the first 3 PCs. MBSS tolerance values (Table 3) did not appear to influence which genera resided in significantly more similar sites as represented by PC 2 or PC 3, the 2 PCs most relevant for environmental-quality characterization.

The MRPP results revealed a number of mOTUs with environmental requirements that were significantly narrower than those of the parent genus (Table 3). However, most of these cases involved the PC associated with geography (PC 1). A total of 14 of 52 (26.9%) mOTUs were collected from sites that were significantly more similar on PC 1 than were the sites of their parent genus: *Ephemerella* groups 2, 5, 7, 9, and 11, *Maccaffertium* group 4, *Amphinemura* group 1, *Isoperla* group 4, *Cheumatopsyche* groups 3 and 9, and *Hydropsyche* groups 1, 4, 5, and 7. Only 1 (1.9%) mOTU (*Ephemerella* 12) was from sites that were significantly more similar on PC 2 (related to physical habitat) than were the sites of their parent genus. Three (5.8%) mOTUs resided at sites that were significantly more similar on PC 3 (related to water chemistry) than were the sites of their parent genus: *Amphinemura* group 1, *Hydropsyche* group 5, and *Pycnopsyche* group 6. Site similarity of mOTUs and

TABLE 3. Results of multiresponse permutation procedures testing the breadth of environmental responses of barcode groups (mOTUs for which genetic distance was >2%) and genera against the breadth of responses of Ephemeroptera, Plecoptera, and Trichoptera taxa as a whole. Genus-level Maryland Biological Stream Survey (MBSS) estimates of tolerance (scale 0–10: 0 = intolerant, 10 = tolerant; Bressler et al. 2004) to habitat degradation are provided. Values that were statistically significant values after correcting for the false discovery rate are in bold.

Genera	MBSS tolerance value	No. of sites	PC1	PC2	PC3
Ephemeroptera					
Ephemerella	2.3	75	**0.00026**	**0.00189**	0.03177
Eurylophella	4.5	22	0.11459	0.01855	0.08712
Maccaffertium	4.6	55	1.0000	0.08370	0.12867
Plecoptera					
Amphinemura	3.0	39	**0.000008**	0.13214	0.28000
Isoperla	2.4	34	0.18762	0.03478	**0.00129**
Leuctra	0.4	17	**0.00000005**	0.11787	0.06316
Prostoia	4.5	12	0.17996	1.0000	0.39127
Trichoptera					
Cheumatopsyche	6.5	82	0.26931	**0.00948**	0.12609
Diplectrona	2.7	29	**0.00013**	0.10527	0.17465
Hydropsyche	7.5	60	0.25253	**0.00003**	0.05334
Pycnopsyche	3.1	21	**0.00366**	1.0000	**0.00030**
mOTU					
Ephemerella 2		20	**0.00357**	1.0000	0.20197
Ephemerella 3		8	0.03181	0.46810	0.53832
Ephemerella 4		25	0.01265	0.67625	0.07895
Ephemerella 5		7	**0.00839**	0.91762	0.32774
Ephemerella 6		5	0.14974	0.33349	0.07885
Ephemerella 7		28	**0.00428**	0.05560	0.18660
Ephemerella 8		4	0.87668	0.37425	0.34135
Ephemerella 9		4	**0.00172**	0.38155	0.09095
Ephemerella 10		36	0.18678	0.51140	1.0000
Ephemerella 11		10	**0.00007**	1.0000	0.01222
Ephemerella 12		3	0.04988	**0.00730**	0.17011
Eurylophella 3		3	0.12848	0.78464	0.44492
Eurylophella 4		6	0.49247	0.44135	0.86596
Eurylophella 7		12	1.0000	0.24487	0.45904
Maccaffertium 2		7	0.08639	0.81883	0.27897
Maccaffertium 4		3	**0.00878**	1.0000	1.0000
Maccaffertium 6		3	0.41143	0.58382	0.74445
Maccaffertium 7		27	0.09418	1.0000	0.66561
Maccaffertium 10		7	0.66763	0.90193	0.23794
Maccaffertium 11		4	0.44226	0.67769	0.76137
Maccaffertium 12		14	0.09555	0.09969	0.46659
Amphinemura 1		6	**0.00213**	0.19212	**0.00333**
Amphinemura 2		14	0.38261	0.87643	0.13337
Amphinemura 3		32	0.85101	1.0000	0.96416
Isoperla 2		3	0.08731	0.19568	0.78717
Isoperla 4		17	**0.00775**	1.0000	0.65111
Isoperla 7		24	0.13875	0.45262	0.97779
Isoperla 9		3	0.92482	0.51404	0.32601
Leuctra 6		7	0.79602	0.92383	0.23059
Leuctra 9		4	0.06927	1.0000	0.34553
Prostoia 3		8	1.0000	0.51254	1.0000
Prostoia 5		3	0.22644	0.55193	0.78944
Cheumatopsyche 1		28	0.01458	0.16643	0.08093
Cheumatopsyche 3		28	**0.00054**	1.0000	0.65668
Cheumatopsyche 4		32	1.0000	1.0000	0.35568
Cheumatopsyche 5		3	0.29983	0.23419	0.53411
Cheumatopsyche 7		12	0.14842	1.0000	0.28079
Cheumatopsyche 8		7	0.67284	0.26038	0.04438

TABLE 3. Continued.

Genera	MBSS tolerance value	No. of sites	PC1	PC2	PC3
Cheumatopsyche 9		13	**0.00201**	0.58790	0.95042
Diplectrona 1		7	0.21538	1.0000	1.0000
Diplectrona 2		17	0.07966	1.0000	0.76106
Diplectrona 3		11	0.16093	0.57048	0.18051
Hydropsyche 1		18	**0.00994**	0.79437	0.20277
Hydropsyche 2		7	0.14094	0.48151	0.40779
Hydropsyche 3		14	0.29247	0.69547	0.41556
Hydropsyche 4		7	**0.00662**	0.68802	0.72959
Hydropsyche 5		4	**0.00001**	0.32034	**0.00168**
Hydropsyche 7		36	**0.00423**	1.0000	0.67739
Pycnopsyche 2		5	0.21789	0.82382	0.44272
Pycnopsyche 4		7	0.26198	1.0000	0.20440
Pycnopsyche 5		3	0.17556	0.38022	0.43835
Pycnopsyche 6		5	0.04361	0.27731	**0.00858**

parent genera for *Diplectrona*, *Eurylophella*, *Leuctra*, and *Prostoia* mOTUs and parent genera did not differ on any PC. The significant difference in site similarity for *Amphinemura* group 1 vs the genus *Amphinemura* for PC2 is illustrated in Fig. 5A. Sites where *Amphinemura* 1 occurred were tightly spaced relative to sites where the genus as a whole occurred. The lack of significant difference in site similarity between any of the barcode groups within *Diplectrona* and the parent genus is shown in Fig. 5B. These 3 barcode groups had largely overlapping distributions in PC space.

Discussion

Delineation of genetically distinct larval insect groups allows biological discrimination at a resolution that is exceedingly difficult and expensive to achieve with morphological taxonomy. We provided a mechanism to delimit these genetically distinctive groups by incorporating DNA-barcode analysis of larval aquatic insects (EPT taxa) into existing bioassessment protocols used by the state of Maryland. The number of genetically distinct mOTUs identified was nearly 3× the number of genera identified by morphological analysis. This large increase in the number of operational units available for incorporation into bioassessments represents new data that could further improve biomonitoring. We are not advocating creation of a new taxonomic level called the mOTU. Scientific rigor requires that the systematics, ecology, and environmental assessment communities continue to work together to link larval DNA barcodes to valid species names where possible and to improve the taxonomy and understanding of evolutionary ecology where it is not. A multidisciplinary effort focused on building this reference database at a national or continental scale would greatly advance the ecological understanding needed for science-based resource management.

The increased taxonomic discrimination provided by DNA barcoding could alter how some of the metrics of Maryland's updated Benthic Index of Biotic Integrity (Southerland et al. 2005) are scored. Number of Ephemeroptera taxa is one of these metrics. The increased number of Ephemeroptera taxa (as mOTUs) that were identified could have implications for how this metric is calculated. For additional metrics that include categories based on % composition of the invertebrate community (e.g., % scrapers, % swimmers), DNA barcoding could help assign specimens to the appropriate guild, habit, or trophic level by placing larval specimens in nominal species. Many aquatic insect genera generally can be classified as clingers, swimmers, sprawlers, collectors, scrapers, shredders, predators, etc., but some genera (or even families) cannot be categorized so broadly. For example, species in the diverse trichopteran genera *Polycentropus* and *Rhyacophila* are predominantly predatory, but some exhibit alternative feeding modes (Wiggins 1996). DNA barcoding could help to identify members of these genera to species so they could be placed in the proper feeding guild, thereby improving the use of the metric.

DNA barcoding increased the number of distinct groups identified, but the results from the MRPP analysis initially suggested that the increased taxonomic resolution of the mOTUs does not necessarily translate to significantly increased resolution of environmental condition. However, a subset of mOTUs demonstrated significantly narrower environmental tolerances than their parent genera, and most of those mOTUs were associated with PC 1, i.e.,

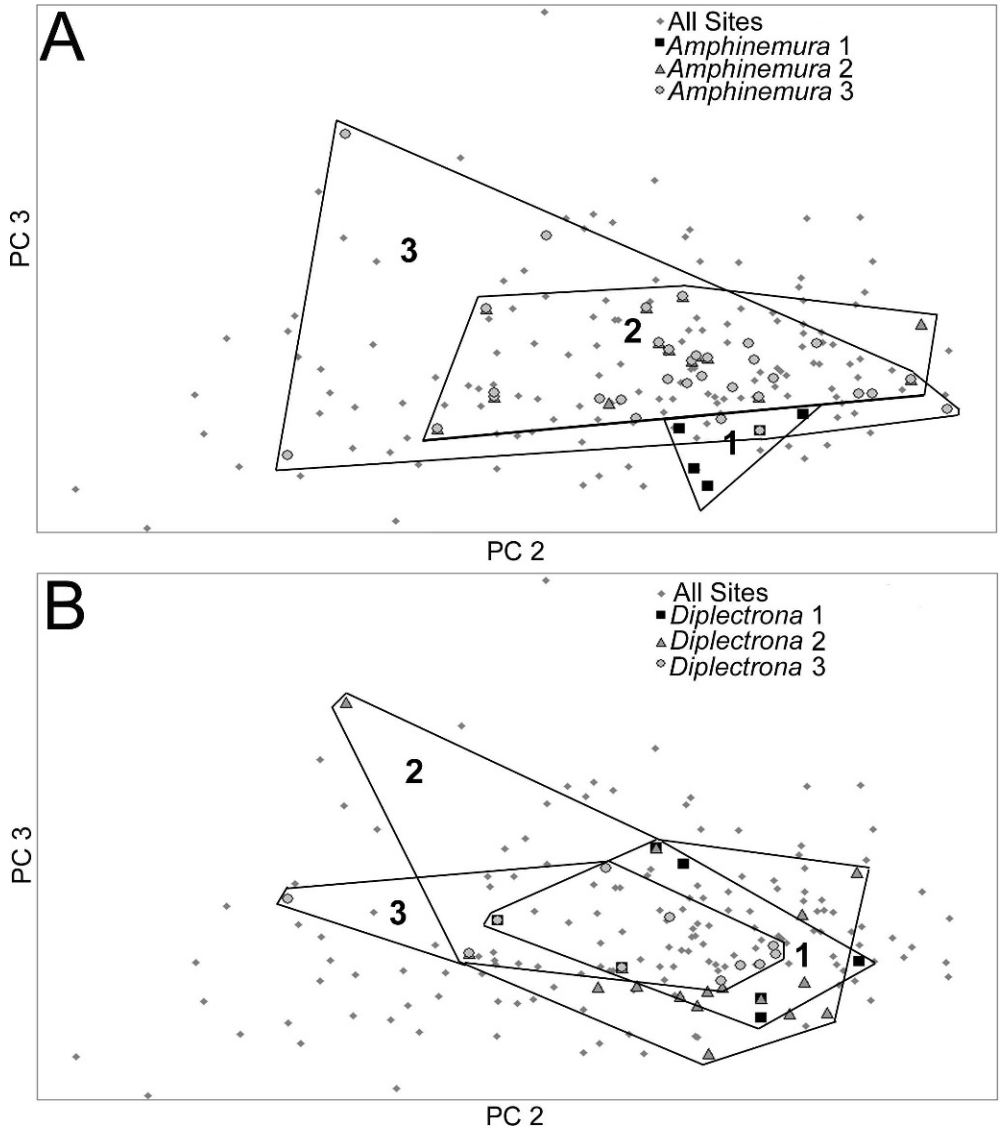

FIG. 5. Principal components analysis showing sites sampled during the Maryland Biological Stream Survey in 2007 plotted on principal component (PC) 2 (predominantly physical-habitat quality measures) and PC 3 (predominantly water-chemistry measures). Sites where the stonefly genus *Amphinemura* (A) and the caddisfly genus *Diplectrona* (B) occurred are highlighted. The polygons outline all sites at which each molecular operational taxonomic unit (mOTU) was collected.

with many geography-related loadings (latitude, longitude, elevation, and water temperature) that are of relatively less use for environmental assessments. Other loadings were highly or moderately correlated with PC 1 (dissolved O_2, riffle quality, absence of dissolved organic C), but could not be disassociated from geographic variables. Just 4 of the mOTUs demonstrated significantly constrained habitat (PC 2) or water-quality-related (PC 3) tolerances.

MRPP analysis of genus-level environmental associations suggested that this analysis coupled with Bonferroni correction for multiple tests may be very stringent. Many of the genera did not show significantly narrower environmental requirements for PC 2 or PC 3 in comparison to all EPT taxa, a result that is surprising given the range of environmental tolerance values (Table 3). For example, in comparison to all EPT taxa together, neither the plecopteran genus *Leuctra*, with a very low MBSS tolerance value of 0.4 (scale: 0 = completely intolerant, 10 = completely tolerant), nor the trichopteran genus *Diplectrona*, with a tolerance value of 2.7, had significantly narrower tolerances represented by principal components PC 2 and PC 3. In addition, many of the rarer barcode

groups that occurred at 1 or 2 sites and could be important diagnostic indicators of environmental condition could not be included in the MRPP analyses.

Our results suggest that taxonomic resolution at the scale of mOTUs would only marginally improve the ability to use bioindicators to assess environmental condition, but we do not conclude that the finer taxonomic discriminations availed by DNA-barcode analysis are of little value to bioassessments. Our results should be considered preliminary given the relatively small size of the EPT data set, and several other potential uses for DNA barcoding relative to bioassessment remain to be explored (see below). Certainly, other important indicator organisms, such as aquatic Diptera, especially chironomids—a family that is notoriously difficult to identify, but often contains more taxa occupying more ecological niches than EPT taxa within a given region—should be evaluated further. Application of DNA barcodes for identifying different species of chironomids for bioassessment is an area of active investigation (Sharley et al. 2004, Pfenninger et al. 2007).

Relatively few environmental variables were explored in our analysis, and associations with many potential environmental stressors remain unexplored. Many relevant stressors, such as metals, pesticides, legacy and emerging contaminants/pollutants, and even climate, land use, and water quantity should be assessed in future studies.

Beyond species-level identifications of aquatic insect larvae, DNA barcoding could have other important roles in terms of bioassessment and environmental conservation. As DNA-barcoding methods become more prevalent, the amount of georeferenced genetic data available for benthic invertebrates will continue to grow. Levels of genetic diversity in these data could be monitored for temporal and spatial changes, possibly as a sentinel for stressor-induced ecological changes that may occur prior to local extirpations.

Incorporation of DNA barcoding into bioassessments provides a potential platform for a uniform system of taxonomic identification that can be applied across regions, countries, and continents. One of the main challenges for aquatic bioassessments is the inability to compare and combine assessment data from different agencies that use different criteria or metrics (Hawkins 2006). DNA barcoding alone will not resolve this problem, but incorporation of a standard method for identification of aquatic-insect larvae would be an important first step toward the data uniformity necessary to evaluate broad-scale trends with bioassessment data.

The potential for DNA barcoding to improve the quality and utility of bioassessments is large, but methodological issues must be addressed before DNA barcoding can be applied in large-scale bioassessments. In our study, only 57% of specimens subjected to DNA-barcoding analysis yielded COI sequences that could be used to assign the specimen to a distinct genetic group. Successful barcoding depends on DNA template in sufficient quality and quantity for PCR, as well as primers that uniformly and consistently amplify the DNA-barcoding locus across taxa (Hebert et al. 2003). In follow-up analyses, we subjected a subset of the DNA templates to PCR with primers for the 16S ribosomal subunit, an alternative mitochondrial locus, and found very similar patterns of amplification success across taxa. We concluded that the quality and quantity of DNA recovery was the major limitation to success. We used 2 different DNA extraction methods, often with different tissue subsections of the same sample extracted by 2 independent laboratories, with no differences in amplification success. Furthermore, DNA recovery rates were consistently low, and we identified a relationship between DNA barcode success and DNA template concentration. Follow-up experiments with freshly collected whole specimens subjected to the same extraction protocols used in our study resulted in DNA concentrations that were generally an order of magnitude higher and achieved DNA barcode success rates >98% (data not shown). We conclude that specimen preservation, either in the field, storage, or during morphological examination, must be improved.

Existing bioassessment protocols were not designed to preserve specimens to ensure consistent DNA preservation. Many protocol changes necessary for better DNA preservation, such as using larger volumes of ethanol for sample fixing and storage and ensuring that adequate ethanol exchange takes place, can be incorporated easily into current methods, especially where the use of ethanol is already part of an existing protocol. Future work should address improving collection protocols for bulk collection of specimens for genetic work, including removal of large detritus, the size and frequency of additional ethanol washes, cold storage of samples prior to genetic work, and potentially new solutions for DNA preservation in aquatic specimens.

DNA barcoding for bioassessment can provide greater taxonomic discrimination at comparable effort to traditional morphological methods. With continuing technological improvements, the cost of DNA barcoding will continue to fall, and advances in automation will increase the number of samples that

can be processed substantially. In turn, this efficiency will allow use of larger sample sizes and improve power to assess differences in the integrity of aquatic communities.

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APPENDIX 1. Definitions of the habitat variables from Maryland Biological Stream Survey included in this work. BIBI = Benthic Index of Biotic Integrity.

Variable name	Definition
BIBI Stratum	Geographical strata used in Maryland's BIBI; includes Coastal Plains, Eastern Piedmont, or Highlands
Velocity/depth diversity	Variety of velocity/depth regimes present at a site; 4 categories include slow-shallow, slow-deep, fast-shallow, and fast-deep; sites possessing habitat in all 4 categories receive an optimal rating
Instream habitat	Perceived value of habitat to the fish community; 4 categories classify % cobble, boulder, submerged logs, undercut banks, snags, root wads, aquatic plant, or other stable habitat (scale 0–20)
Epifaunal substrate	Perceived value of hard and stable substrates usable by benthic macroinvertebrates; 4 categories classify colonization potential (scale 0–20)
Pool quality	Variety and spatial complexity of slow- or still-water habitat within the sample segment (scale 0–20)
Riffle quality	Depth, complexity, and functional importance of riffle/run habitat in the sample segment (scale 0–20)
Embeddedness	Fraction of surface area of larger particles that is surrounded by fine sediments on the stream bottom (%)
Acid neutralizing capacity	Overall measure of stream acidification and acid sensitivity (μeq/L)
Aesthetic rating	Visual appeal of the site and presence/absence of human refuse (scale 0–20)
Shading	Degree and duration of shading at a site during summer, including any effects of shading caused by landforms (%)

APPENDIX 2. Loadings for each environmental variable on the first 6 principal components of a principal components analysis. BIBI = Benthic Index of Biotic Integrity. Bold indicates loadings $> |0.500|$.

Variable	PC1	PC2	PC3	PC4	PC5	PC6
BIBI stratum	**0.895**	0.154	0.131	0.177	0.019	−0.017
Elevation	**0.853**	0.136	−0.161	−0.085	−0.142	0.202
Longitude	**0.852**	0.164	0.009	0.074	−0.283	0.169
Latitude	**0.740**	0.149	0.270	0.154	0.286	−0.179
Temperature	**−0.611**	−0.177	0.141	0.442	0.042	0.003
Dissolved O_2 concentration	**0.526**	**0.511**	0.205	−0.160	0.162	−0.099
Velocity/depth diversity	0.187	**0.867**	0.047	0.131	0.102	0.059
Instream habitat	0.179	**0.836**	−0.172	0.212	−0.046	0.043
Epifaunal substrate	0.227	**0.785**	−0.091	0.188	−0.068	−0.212
Pool quality	−0.170	**0.768**	−0.125	0.292	0.106	0.234
Riffle quality	0.442	**0.682**	0.021	0.005	0.142	−0.119
Embeddedness	−0.297	**−0.593**	−0.283	0.075	0.093	0.335
Conductivity	−0.010	−0.156	**0.801**	−0.039	−0.039	0.240
Acid neutralizing capacity	0.186	−0.173	**0.761**	0.096	0.112	0.119
Aesthetics	0.20	−0.059	**−0.704**	0.209	−0.003	−0.032
pH	0.301	0.256	**0.678**	0.339	0.089	−0.242
Acreage of watershed	0.011	0.201	−0.026	**0.817**	0.021	−0.051
Stream order	0.008	0.343	−0.045	**0.764**	−0.003	−0.015
NO_3^- concentration	−0.145	0.232	0.076	−0.191	**0.808**	−0.049
Shading	−0.081	0.137	−0.034	−0.448	**−0.712**	−0.070
SO_4^{2-} concentration	0.157	−0.025	0.284	−0.063	−0.012	**0.846**
Dissolved organic C concentration	−0.475	−0.363	−0.420	0.115	−0.185	0.055
Eigenvalue	4.328	4.346	2.757	2.152	1.484	1.203
% variation explained	19.67	19.75	12.53	9.78	6.75	5.48