Gill, B.A., J.B. Sandberg, & B.C. Kondratieff. 2015. Evaluation of the morphological species concepts of 16 western Nearctic *Isoperla* species (Plecoptera: Perlodidae) and their respective species groups using DNA barcoding. *Illiesia*, 11(11):130-146. Available online: http://illiesia.speciesfile.org/papers/Illiesia11-11.pdf

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EVALUATION OF THE MORPHOLOGICAL SPECIES CONCEPTS OF 16 WESTERN NEARCTIC *ISOPERLA* SPECIES (PLECOPTERA: PERLODIDAE) AND THEIR RESPECTIVE SPECIES GROUPS USING DNA BARCODING

Brian A. Gill[1], John B. Sandberg[2], & Boris C. Kondratieff[3]

[1] Department of Biology, Colorado State University, Fort Collins, CO 80523, U.S.A.
E-mail: gillbriana@gmail.com

[2] Aquatic Bioassessment Lab – California Dept. of Fish & Wildlife & CSUC Research Foundation, California State University, Chico, CA 95929-055, U.S.A.
E-mail: jsandberg@csuchico.edu

[3] Department of Bioagricultural Sciences and Pest Management, Colorado State University, Fort Collins, CO 80523, U.S.A.
E-mail: Boris.Kondratieff@colostate.edu

ABSTRACT

We evaluate support for the morphologically based species concepts for 16 western Nearctic *Isoperla* Banks 1906 species (Plecoptera: Perlodidae) and their respective species groups using the barcode fragment of the mitochondrial gene Cytochrome c Oxidase subunit I (COI) and phylogenetic analyses. Species identifications and assignments to groups were based solely on current morphological species concepts in the literature. COI was sequenced for each species and pairwise genetic distances calculated to understand the magnitude and distribution of genetic variation within this group. COI data were analyzed using both distance (neighbor-joining) and character based (Bayesian inference) phylogenetic approaches. We found for seven morphologically defined taxa that intraspecific genetic variation at COI exceeded the traditional arbitrary 2% sequence divergence threshold sometimes used to delimit species. However, despite these relatively high intraspecific divergence values, we consistently found diagnostic gaps in divergence values between morphologically defined species. Phylogenetic analyses strongly supported the monophyly of all individuals assigned the same species identification based on morphology. Morphological species groups originally proposed by Szczytko & Stewart (1979) were only partially supported with two monophyletic species groups; however, one ambiguous paraphyletic/polyphyletic species group and two polyphyletic species groups were also recovered. Based on current evidence, we propose no changes to the conceptualization of the species groups presented in Szczytko & Stewart (1979) at this time, but rather point out that the present arrangement of species within groups is in need of revision.

Keywords: *Isoperla*, Perlodidae, Plecoptera, DNA barcoding, Phylogenetics

INTRODUCTION

There are 26 recognized species of western Nearctic *Isoperla* Banks 1906a (Table 1) excluding eastern species with minor distribution ranges extending west of the Mississippi River (Baumann & Lee 2009, Kondratieff & Baumann 2002, Stewart & Stark 2002, Szczytko & Stewart 1979, Szczytko & Stewart 1984, Szczytko & Stewart 2002). Szczytko & Stewart (1979) provided the first revision of the western Nearctic *Isoperla* treating 20 species. Subsequently, Szczytko & Stewart (1984), Bottorff et al. (1990), Szczytko & Stewart (2002), Szczytko & Stewart (2004), Baumann & Lee (2009), Sandberg & Kondratieff (2013), Szczytko & Stewart (2013) have contributed to the taxonomic knowledge of the adults of this genus in the region. Szczytko & Stewart (1979) divided 20 western Nearctic *Isoperla* species into five species groups based on morphological characters in the order originally presented, *I. quinquepunctata*, *I. phalerata*, I. *sobria*, *I. marmorata*, and *I. sordida* groups. Four western *Isoperla* species have been recently treated in Szczytko & Kondratieff (2015).

Sequence data provide an additional line of evidence for testing morphological based species concepts. DNA barcoding, for animals most frequently using a 658 bp fragment of the mitochondrial Cytochrome c Oxidase subunit I (COI) gene, was envisioned as a standardized approach for species identification (Hebert et al. 2003a, b). This approach has worked well for a large number of insect groups. However, the question remains, is there a defined level of sequence divergence for the COI barcode fragment that is sufficient for delimiting closely related species. Initially, thresholds such as 2% divergence criterion (Ball et al. 2009, Zhou et al. 2009) or 10X the intraspecific divergence (Hebert et al. 2004a) were proposed. More recently, the standard repository for DNA barcode data known as the Barcode of Life Database (BOLD) added the Barcode Index Number (BIN) feature, which uses refined single linkage (RESL) clustering to assign records to BINs (putative species; Ratnasingham & Hebert 2013). These BINs are based on levels of sequence divergence among specimens (distance based), and delimit species based on gaps between the values of intraspecific and interspecific genetic distances among specimens in the database. The BIN system implements a sliding threshold for species delimitation that can vary by group.

Validation of the DNA barcoding approach has come mainly through analysis of divergence levels across many species to assess congruence between morphological species concepts and levels of sequence divergence. For some taxa, enough sequence data have accumulated to potentially identify group-specific trends in levels of intra- and interspecific divergence values, but to our knowledge no formal meta-analysis of these data exists for the Plecoptera. We contend that relatively high levels of intraspecific divergence, above 2% divergence, are not uncommon among the Plecoptera (Zhou et al. 2009, 2010, Mynott et al. 2011, Sweeney et al. 2011, Boumans & Baumann 2012, Avelino-Capistrano et al. 2014, Elbrecht et al. 2014, Gill et al. 2015). This trend may be driven by the low vagility of some Plecoptera species (Boumans & Baumann 2012) and prompts caution in the interpretation of DNA barcode data for these species. Studies with wide taxon sampling and geographical coverage will be especially helpful in testing the generality of this emerging trend.

Despite the difficulty in defining strict thresholds for species delimitation within the stoneflies, in the context of expertly applied morphological taxonomy, DNA barcode data can be useful for testing phylogenetic hypotheses especially when coupled with powerful character based phylogenetic approaches (parsimony, maximum likelihood, and Bayesian inference). However, several important caveats require mention. First, the phylogenetic signal useful for resolving relationships among species may be lost in DNA barcodes because mitochondrial genes generally evolve faster and accumulate more substitutions than nuclear genes (Galtier et al. 2009). Loss of phylogenetically informative character states from sequence data occurs when differentiation of the sequence is masked by reversals to ancestral states by random chance (saturation). Second, when analyzing COI data in a phylogenetic framework we assume that the COI gene tree is concordant with the evolutionary

Table 1. Systematic list of 26 western Nearctic *Isoperla* species excluding eastern species with minor distribution ranges extending west of the Mississippi River.

Western Nearctic Species & Complexes	Published Distribution	Male Characters			
		SP	DS	V	TS
***I. quinquepunctata* Complex**					
1. *I. acula* Jewett	USA: CA	A	PF	R	P
2. *I. jewetti* Szczytko & Stewart	USA: CO, TX		A	L	
3. *I. longiseta* Banks	CAN: AB, BC, MB, PQ, SK; USA: CO, IA, ID, IL, KS, MN, MO, MT, NM, SD, UT, WY				
4. *I. mormona* Banks	CAN: BC; USA: AZ, CA, CO, ID, MT, NM, OR, UT, WA, WY		PF	T	
5. *I. quinquepunctata* (Banks)	CAN: AB, BC, SK; MEX: BJ; USA: CA, CO, ID, MT, NE, NM, NV, OR, SD, UT, WY		A	L	
***I. phalerata* Complex**					
6. *I. phalerata* (Smith)	USA: CO, ID, NM, OR, SD, UT, WY	A	A	L	A
7. *I. pinta* Frison	CAN: AB, BC; MEX: BJ; USA: CA, CO, ID, MT, OR, UT, WA, WY				
***I. sobria* Complex**					
8. *I. baumanni* Szczytko & Stewart	USA: CA	A	P	L	A
9. *I. gravitans* (Needham & Claassen)	USA: OR, WA				
10. *I. miwok* Bottorff & Szczytko	USA: CA			V	
11. *I. sobria* (Hagen)	CAN: AB, BC, YK; USA: AK, AZ, CA, CO, ID, MT, NM, NV, OR, UT, WA, WY				
12. *I. tilasqua* Szczytko & Stewart	USA: OR			L	
***I. marmorata* Complex**					
13. *I. fulva* Claassen	CAN: AB, BC; USA: AZ, CA, CO, ID, MT, NM, NV, OR, UT, WA, WY	P	P	L	A
14. *I. marmorata* (Needham & Claassen)	USA: CA, NV, OR, WA				
15. *I. roguensis* Szczytko & Stewart	USA: CA, OR				
***I. sordida* Complex**					
16. *I. adunca* Jewett	USA: CA	P	A	PE	A
17. *I. bifurcata* Szczytko & Stewart	USA: CA, ID, OR, WA				
18. *I. denning* Jewett	USA: CA				
19. *I. fusca* Needham & Claassen	CAN: AB, BC, YK; USA: ID, MT, OR, WA, WY				
20. *I. petersoni* Needham & Christenson	CAN: AB, BC, SK, YK; USA: AK, CO, ID, MT, UT, WY				
21. *I. rainiera* Jewett	USA: OR, WA				
22. *I. sordida* Banks	CAN: AB, BC; USA: AK, CA, ID, MT, OR, WA				
23. *I. umpqua* Szczytko & Stewart	USA: OR				P
Unassigned					
24. *I. decolorata* (Walker)	CAN: BC, MB, NT, SK, YK; USA: AK	A	A	PE	A
25. *I. katmaiensis* Szczytko & Stewart	USA: AK				
26. *I. laucki* Baumann & Lee	USA: CA	P	P		

Abbreviations. Male Characters: **SP**=Sclerotized aedeagal process, **DS**=Distinct aedeagal spinule patch, **V** =Vesicle shape, **TS**=Distinct tergal spinule or stout setal patch. Male Character States: A=Absent, L=Lobe, P=Present, R=Rectangular, T=Trapezoidal, PE=Pedunculate, PF=Fine spinulae present, V=Vestigial.

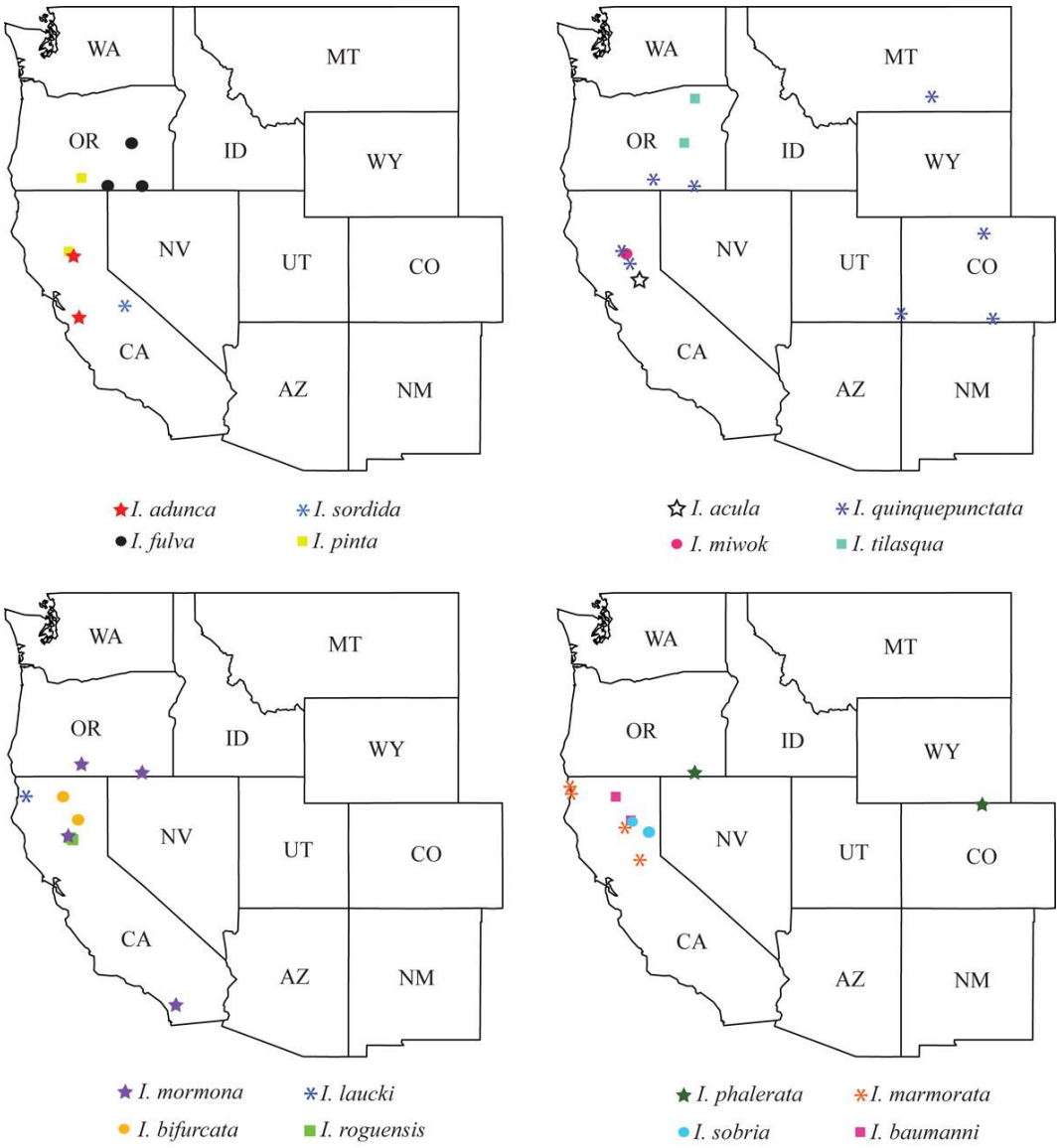

Fig. 1. Geographic locations of western *Isoperla* species populations included in phylogenetic analyses.

history of the species. This assumption may be violated due to hybridization or incomplete lineage sorting (Moritz & Cicero 2004, Hebert & Gregory 2005, DeWalt 2011), though the frequency of these problems remains unclear.

Here, we accept the aforementioned caveats and use COI as additional information useful for testing existing morphologically based species concepts and species group designations for 16 *Isoperla* species. We explore the appropriate strategy for distance-based species delimitation and assess support for species and species groups using both distance-based (neighbor-joining; NJ) and character-based (Bayesian inference; BI) methods in hopes that these data could be used to guide future analyses. Finally, we compare these results with the morphological and behavioral characters from recent studies (Szczytko & Stewart 1979, Sandberg

2011a, b, Sandberg & Kondratieff 2013).

MATERIALS AND METHODS

Much of the California and Oregon material for this study was collected during previous studies (Sandberg 2011a, b, Sandberg & Kondratieff 2013). Despite efforts to maximize geographical coverage of populations of species in our analysis (see Table 1 for published distributions of species; see Fig. 1 for populations sampled for this study), we acknowledge that some widely distributed taxa are under sampled because of the wide geographical extent of their distributions and the limited availability of fresh specimens for sequencing. All specimens sequenced for this study (Table 2) were expertly identified based on morphology. For adult male specimens, aedeagal characters were examined following Szczytko & Stewart (1979) and Sandberg & Kondratieff (2013). Specimen data in the form of a comma delimited file is available at Western USA Isoperla.csv.

DNA Barcoding.

Tissue from 103 specimens (80 adult, 23 larval), representing each species, was sent to the Canadian Center for DNA Barcoding (CCDB) for sequencing. Standard DNA barcoding protocols were used (Ivanova et al. 2005, Hajibabaei et al. 2005, Ivanova et al. 2006a, b). A cocktail of the primers LCO1490/HCO2198 (Folmer et al. 1994) and Lep-F1/Lep-R1 (Hebert et al. 2004b) was used for PCR and bidirectional sequencing. Edited sequences were posted to the Barcode of Life Database (BOLD; http://www.boldsystem.org; Ratnasingham & Hebert 2007).

Calculation of Genetic Distances and Phylogenetic Analyses.

Sequences deemed barcode compliant on BOLD (minimum length 500 bp, < 1% ambiguous base calls, no stop codons or gaps) were downloaded and aligned using MUSCLE (Edgar 2004) in MEGA v. 6 (Tamura et al. 2013). We estimated all pairwise genetic distances, mean intraspecific, and mean interspecific distances among morphologically defined species using the Kimura-2-parameter substitution model (Kimura 1980) with pairwise deletion option. From these data we calculated maximum intraspecific genetic distance and minimum interspecific genetic distance for each species to assess the presence of consistent barcode gaps capable of partitioning intraspecific and interspecific genetic variation separately. For this we looked at the "nearest neighbor distance" defined as the minimum genetic distance from one morphologically defined species to the most closely related taxon included in this study.

Neighbor-joining analysis was conducted in MEGA v. 6 with the same nucleotide substitution model and settings for missing data as used for calculating genetic distances. Nodal support was assessed using 10,000 bootstrap replicates. BIN information for each record was downloaded from BOLD November 30, 2014 and displayed on the resultant tree along with locality information using TreeGraph2 (Stöver & Müller 2010).

For the BI approach, we determined that GTR+I+G was the appropriate model of nucleotide substitution using jModelTest2 (Guindon & Gascuel 2003, Darriba et al. 2012) based on Aikake's Information Criteria (AIC). We then used MrBayes v 3.2 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003) to execute two simultaneous runs with random starting trees and four Markov chains for 10 million generations sampled every 1000 generations. The first 25% of sampled trees were discarded as burn-in. We ensured that our two 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). A 50% majority rule consensus tree was constructed from the post burn-in trees. Nodal support was assessed based on posterior probabilities. The resultant BI tree with BIN and locality information for each record was visualized using TreeGraph2 (Stöver & Müller 2010).

Morphological and Behavioral Characters.

Morphological and behavioral characters from the literature (Szczytko & Stewart 1979, Sandberg 2011a, b, Sandberg & Kondratieff 2013) were considered in light of the results of our genetic

Table 2. Identification and USA locality information of DNA barcoded *Isoperla* and *Cascadoperla* specimens. Process ID identifiers are for the Barcode of Life Database (BOLD). Stage is either Adult (A) or Larval (L). Sex was only assigned to adult specimens.

BOLD Process ID	Species	State	County	Waterbody	Latitude	Longitude	Stage	Sex
STONE001-13	*I. acula*	CA	El Dorado	Deadman Cr.	38.65385	-120.82995	A	M
STONE002-13	*I. acula*	CA	El Dorado	Deadman Cr.	38.65385	-120.82995	A	M
STONE003-13	*I. acula*	CA	El Dorado	Deadman Cr.	38.65385	-120.82995	A	F
STONE004-13	*I. acula*	CA	El Dorado	Deadman Cr.	38.65385	-120.82995	A	F
STONE005-13	*I. adunca*	CA	Butte	Campbell Cr. trib.	39.59779	-121.54646	A	M
STONE006-13	*I. adunca*	CA	Butte	Campbell Cr. trib.	39.59779	-121.54646	A	M
STONE007-13	*I. adunca*	CA	Butte	Campbell Cr. trib.	39.59779	-121.54646	A	F
STONE008-13	*I. adunca*	CA	Butte	Campbell Cr. trib.	39.59779	-121.54646	A	F
STONE055-13	*I. adunca*	CA	Stanislaus	Orestimba Cr.	37.28900	-121.22008	L	
STONE056-13	*I. baumanni*	CA	Plumas	Domingo Spr.	40.36093	-121.34669	A	M
STONE057-13	*I. baumanni*	CA	Plumas	Domingo Spr.	40.36093	-121.34669	A	F
STONE075-13	*I. baumanni*	CA	Siskiyou	McCloud R.	41.24020	-122.02441	A	F
STONE009-13	*I. bifurcata*	CA	Plumas	Domingo Spr.	40.36093	-121.34669	A	M
STONE010-13	*I. bifurcata*	CA	Plumas	Mosquito Spr. Cr.	40.36113	-121.34004	A	F
STONE011-13	*I. bifurcata*	CA	Plumas	Mosquito Spr. Cr.	40.36113	-121.34004	A	F
STONE058-13	*I. bifurcata*	CA	Plumas	Domingo Spr.	40.36093	-121.34669	A	F
STONE076-13	*I. bifurcata*	CA	Siskiyou	McCloud R.	41.24020	-122.02441	A	M
STONE012-13	*I. fulva*	OR	Harney	Trout Cr.	43.80484	-118.91082	A	M
STONE013-13	*I. fulva*	OR	Harney	Trout Cr.	42.17439	-118.43199	A	F
STONE077-13	*I. fulva*	OR	Lake	Deep Cr.	42.18564	-119.99512	A	M
STONE014-13	*I. laucki*	CA	Humboldt	Dragsaw Spr.	41.24468	-123.69180	A	M
STONE015-13	*I. laucki*	CA	Humboldt	Dragsaw Spr.	41.24468	-123.69180	A	F
STONE016-13	*I. laucki*	CA	Humboldt	Dragsaw Spr.	41.24468	-123.69180	A	F
STONE064-13	*I. laucki*	CA	Humboldt	Dragsaw Spr.	41.24468	-123.69180	L	
STONE017-13	*I. marmorata*	CA	El Dorado	Greenwood Cr.	38.82587	-120.94571	A	M
STONE018-13	*I. marmorata*	CA	El Dorado	Greenwood Cr.	38.82587	-120.94571	A	M
STONE019-13	*I. marmorata*	CA	El Dorado	Greenwood Cr.	38.82587	-120.94571	A	F
STONE020-13	*I. marmorata*	CA	El Dorado	Greenwood Cr.	38.82587	-120.94571	A	F
STONE021-13	*I. marmorata*	CA	Tehama	Big Chico Cr.	40.06367	-121.60387	A	F
STONE022-13	*I. marmorata*	CA	Tehama	Big Chico Cr.	40.06367	-121.60387	A	F
STONE078-13	*I. marmorata*	CA	Del Norte	Wilson Cr.	41.60490	-124.10066	A	M
STONE079-13	*I. marmorata*	CA	Humboldt	Prairie Cr.	41.34854	-124.02988	A	F
STONE027-13	*I. mormona*	OR	Harney	Trout Cr.	42.17439	-118.43199	A	M
STONE028-13	*I. mormona*	OR	Harney	Trout Cr.	42.17439	-118.43199	A	F
STONE061-13	*I. mormona*	CA	San Diego	Doane Cr.	33.33674	-116.89696	L	
STONE065-13	*I. mormona*	CA	Butte	Big Chico Cr.	39.76340	-121.79120	A	M
STONE081-13	*I. mormona*	OR	Klamath	Sprague R.	42.44096	-121.18719	A	M
STONE023-13	*I. miwok*	CA	Butte	Campbell Cr. trib.	39.59779	-121.54646	A	M
STONE024-13	*I. miwok*	CA	Butte	Campbell Cr. trib.	39.59779	-121.54646	A	M
STONE025-13	*I. miwok*	CA	Butte	Campbell Cr. trib.	39.59779	-121.54646	A	F
STONE026-13	*I. miwok*	CA	Butte	Campbell Cr. trib.	39.59779	-121.54646	A	F
STONE066-13	*I. phalerata*	OR	Harney	Trout Cr.	42.17439	-118.43199	A	M
STONE067-13	*I. phalerata*	CO	Larimer	Dale Cr.	40.96235	-105.36741	A	F
STONE029-13	*I. pinta*	CA	Butte	Butte Cr.	39.71388	-121.71884	A	M
STONE030-13	*I. pinta*	CA	Butte	Butte Cr.	39.71388	-121.71884	A	M
STONE031-13	*I. pinta*	CA	Butte	Butte Cr.	39.71388	-121.71884	A	F
STONE032-13	*I. pinta*	CA	Butte	Butte Cr.	39.71388	-121.71884	A	F
STONE068-13	*I. pinta*	OR	Klamath	Sprague R.	42.44096	-121.18719	A	M
STONE033-13	*I. quinquepunctata*	CA	Butte	Butte Cr.	39.71388	-121.71884	A	M
STONE034-13	*I. quinquepunctata*	CA	Butte	Butte Cr.	39.71388	-121.71884	A	F
STONE035-13	*I. quinquepunctata*	OR	Harney	Trout Cr.	42.17439	-118.43199	A	M

STONE036-13	*I. quinquepunctata*	OR	Harney	Trout Cr.	42.17439	-118.43199	A	M
STONE037-13	*I. quinquepunctata*	OR	Harney	Trout Cr.	42.17439	-118.43199	A	F
STONE038-13	*I. quinquepunctata*	OR	Harney	Trout Cr.	42.17439	-118.43199	A	F
STONE069-13	*I. quinquepunctata*	CA	Yuba	Yuba R.	39.22054	-121.37593	A	F
STONE070-13	*I. quinquepunctata*	CA	Yuba	Yuba R.	39.22054	-121.37593	A	M
STONE080-13	*I. quinquepunctata*	OR	Harney	Trout Cr.	42.17439	-118.43199	L	
STONE082-13	*I. quinquepunctata*	OR	Lake	Crooked Cr.	42.41125	-120.29129	A	M
STONE084-13	*I. quinquepunctata*	OR	Harney	Trout Cr.	42.17439	-118.43199	L	
SIERA051-14	*I. quinquepunctata*	CO	Larimer	unnamed stream	40.35556	-105.27972	L	
SIERA052-14	*I. quinquepunctata*	CO	Las Animas	Purgatoire R.	37.13944	-104.87972	A	F
SIERA053-14	*I. quinquepunctata*	MT	Bighorn	Bighorn R.	45.64448	-107.65913	A	F
SIERA054-14	*I. quinquepunctata*	CO	Montezuma	McElmo Cr.	37.32472	-109.03583	A	F
STONE039-13	*I. roguensis*	CA	Butte	Butte Cr.	39.71388	-121.71884	A	M
STONE040-13	*I. roguensis*	CA	Butte	Butte Cr.	39.71388	-121.71884	A	F
STONE041-13	*I. roguensis*	CA	Butte	Butte Cr.	39.71388	-121.71884	A	F
STONE071-13	*I. roguensis*	CA	Butte	Butte Cr.	39.71388	-121.71884	A	M
STONE042-13	*I. sobria*	CA	Plumas	spr. fed trib. (Corral)	39.89349	-120.51617	A	M
STONE043-13	*I. sobria*	CA	Plumas	spr. fed trib. (Corral)	39.89349	-120.51617	A	M
STONE044-13	*I. sobria*	CA	Plumas	spr. fed trib. (Corral)	39.89349	-120.51617	A	F
STONE045-13	*I. sobria*	CA	Plumas	spr. fed trib. (Corral)	39.89349	-120.51617	A	F
STONE072-13	*I. sobria*	CA	Plumas	Mosquito Spr. Cr.	40.36113	-121.34004	A	M
STONE073-13	*I. sobria*	CA	Plumas	spr. fed trib. (Corral)	39.89349	-120.51617	A	M
STONE046-13	*I. sordida*	CA	Mono	Coldwater Cr.	37.59306	-118.99226	A	F
STONE047-13	*I. sordida*	CA	Mono	Coldwater Cr.	37.59306	-118.99226	A	F
STONE048-13	*I. sordida*	CA	Mono	Coldwater Cr.	37.59306	-118.99226	A	F
STONE074-13	*I. sordida*	CA	Mono	Coldwater Cr.	37.59306	-118.99226	A	M
STONE049-13	*I. tilasqua*	OR	Umatilla	Meacham Cr.	45.50283	-118.42206	A	F
STONE050-13	*I. tilasqua*	OR	Umatilla	Meacham Cr.	45.50283	-118.42206	A	F
STONE051-13	*I. tilasqua*	OR	Harney	Trout Cr.	43.80484	-118.91082	A	M
STONE052-13	*I. tilasqua*	OR	Harney	Trout Cr.	43.80484	-118.91082	A	M
STONE053-13	*I. tilasqua*	OR	Harney	Trout Cr.	43.80484	-118.91082	A	F
STONE054-13	*I. tilasqua*	OR	Harney	Trout Cr.	43.80484	-118.91082	A	F
SIERA062-14	*C. trictura*	CA	Siskiyou	McCloud R.	41.24012	-122.02479	A	F

analyses. Specifically, we sought morphological and behavioral support for novel relationships elucidated by phylogenetic analyses.

RESULTS

Sequencing.

The CCDB returned sequences for 86 of the 103 (83%) submitted specimens. All complete failures (producing no reads) were larval specimens of *Isoperla denningi* Jewett 1955 (N=17). One *I. denningi* specimen was returned with a partial sequence (656 bp) but was excluded from our analysis due to suspect quality. The failure to sequence 17 out of 18 submitted *I. denningi* specimens, preserved similarly to all other submitted material, suggests that the standard primers employed by CCDB are not suitable for this taxon and that alternative primers need to be used in the future. Additionally, one *I. quinquepunctata* (Banks 1902) specimen was <500 bp and was omitted based upon missing data. All sequences for the 84 specimens used for this study have been made publicly available on BOLD (http://www.barcodinglife.com) under the project names STONE and SIERA.

Genetic Distances.

Intraspecific genetic distances ranged from 0.00 to 7.40% with a mean of 2.18% (SE = 0.12%), exceeding an arbitrary 2% divergence criterion in 7 of the 16 *Isoperla* species studied (43.75% of taxa including *I. quinquepunctata*, *I. mormona* Banks 1920, *I. adunca* Jewett 1962, *I. baumanni* Szczytko & Stewart 1984, *I. bifurcata* Szczytko and Stewart 1979, *I. marmorata* (Needham & Claassen 1925), *I. fulva* Claassen 1937 (Table 3). Interspecific

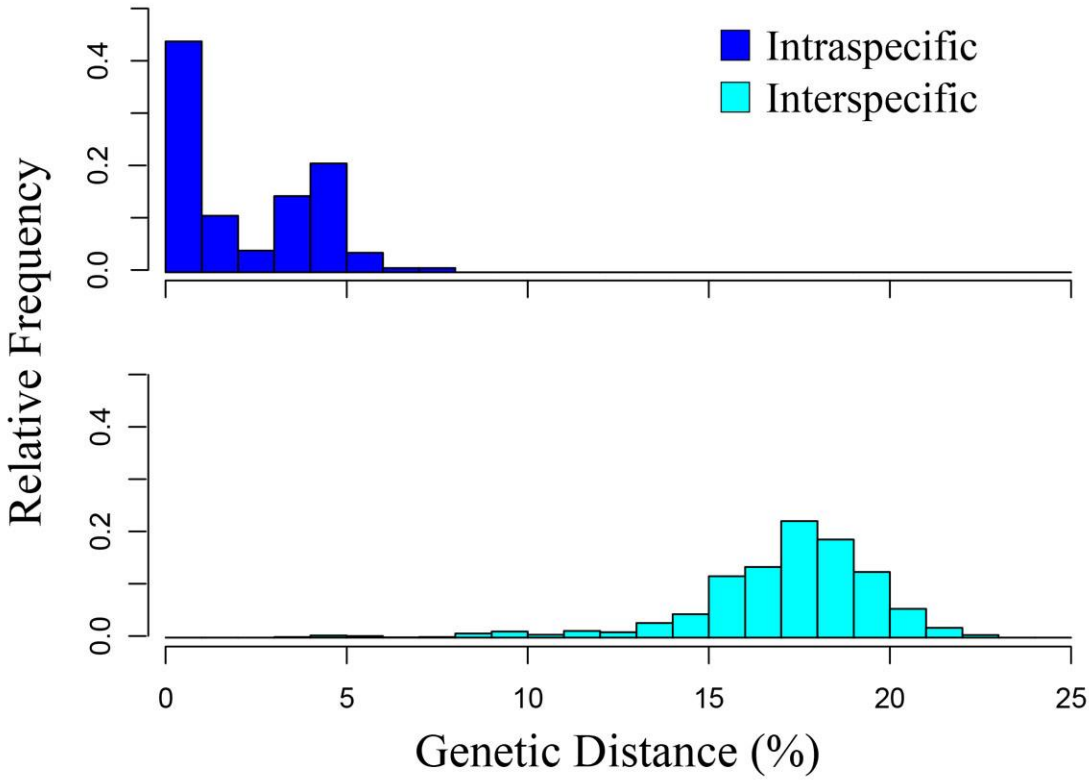

Fig. 2. Relative frequency histogram of intraspecific and interspecific pairwise genetic distances for all taxa studied. Distances determined using the Kimura-2-parameter model of nucleotide substitution with pairwise deletion option.

distances ranged from 3.80 to 23.00% with a mean of 17.18% (SE = 0.05%) (Fig. 2, Table 4). Intraspecific genetic distances never exceeded the distance to the nearest neighbor species. However, we note that within several species, BOLD defined multiple BIN groups when intraspecific distances were relatively large (*I. quinquepunctata*, *I. mormona*, *I. adunca*, *I. baumanni*, *I. bifurcata*, *I. marmorata*).

Phylogenetic Analyses.

The monophyly of groups of individuals assigned the same species identification was well supported by both the NJ (Fig. 3) and BI approaches (Fig. 4). Deeper relationships among species were generally resolved with higher support using character-based BI instead of distance-based NJ (Figs. 3 vs. 4). The *I. phalerata* and *I. marmorata* species groups were well supported as monophyletic, while the *I. sobria* and *I. sordida* groups appear polyphyletic and the *I. quinquepunctata* group is ambiguously para- or polyphyletic.

DISCUSSION

Distance-based Species Delimitation.

The 16 western North American *Isoperla* species analyzed show a wide range of variability in intraspecific divergence values and some of these values are quite high (5-7%; Table 3). However, these values are consistent with other values

Table 3. Maximum intraspecific genetic distance (% divergence), nearest neighbor taxon, nearest neighbor distance (% divergence), and number of individuals genotyped for each of the 16 *Isoperla* species studied here determined using COI with the Kimura-2-parameter model and pairwise deletion option.

Species	N	Maximum Intraspecific Distance (%)	Nearest Neighbor	Nearest Neighbor Distance (%)
I. acula	4	1.0	*I. mormona*	12.9
I. adunca	5	7.7	*I. phalerata*	14.2
I. baumanni	3	2.2	*I. tilasqua*	3.8
I. bifurcata	5	3.6	*I. sordida*	7.4
I. fulva	3	2.8	*I. roguensis*	5.1
I. laucki	4	1.7	*I. bifurcata*	11.2
I. marmorata	8	5.1	*I. roguensis*	8.6
I. mormona	5	5.7	*I. acula*	12.9
I. miwok	4	0.5	*I. pinta*	14.9
I. phalerata	2	1.7	*I. pinta*	8.3
I. pinta	5	0.9	*I. phalerata*	8.3
I. quinquepunctata	15	5.1	*I. phalerata*	15.7
I. roguensis	4	1.3	*I. fulva*	5.1
I. sobria	6	1.1	*I. tilasqua*	11.8
I. sordida	4	0.3	*I. bifurcata*	7.4
I. tilasqua	6	0.8	*I. baumanni*	3.8
Total/Mean (%)	83	2.6		9.5

reported in the Plecoptera DNA barcoding literature (Zhou et al. 2009, 2010, Mynott et al. 2011, Sweeney et al. 2011, Boumans & Baumann 2012, Avelino-Capistrano et al. 2014, Gill et al. 2015, Stark et al. 2015). Indeed, Plecoptera researchers seem to be increasingly reporting high intraspecific genetic divergence values from across the order. For example, Zhou (2009, 2010) reports values of 3.13% for an unknown species of *Isoperla* and 2.54% for the nemourid *Amphinemura palmeni* (Koponen 1917) (as *A. linda* Ricker (1952) from subarctic Canada. Several species of gripopterygids of the genus *Riekoperla* McLellan 1971 were shown by Mynott et al. (2011) to have intraspecific divergence values up to 5.8%. Sweeney et al. (2011) also report high intraspecific divergence values for *Perlesta* Banks 1906b (Perlidae) and no gap between intraspecific and interspecific divergence values for a subset of representatives of this genus. Boumans & Baumann (2012) report up to 5.6% divergence in *A. wui* (Claassen 1936) and 2.8% in *A. standfussi* (Ris 1902). Avelino-Capistrano et al. (2014) report intraspecific divergence values for *Kempnyia* Klapálek 1914 (Perlidae) ranging from 0-15%. Recently, Gill et al. (2015) also found mean percent divergence values between 3-6% among populations of *Moselia infuscata* (Claassen 1923) (Leuctridae).

When we reviewed the distribution of divergence values for all pairwise comparisons of individuals (Fig. 2), we observed overlap in divergence values between individuals of species with high intraspecific divergences and others with low interspecific divergences. This distribution is driven by a combination of high intraspecific variability in some taxa and low interspecific distances among others (Table 3). Fortunately, the observed variability in intra- and interspecific divergence values does not entirely preclude species delimitation using DNA barcoding, but rather the use of any single strict divergence criterion for these taxa. A strict divergence criterion applied to this dataset would result in considerable over-lumping (combination of species with low

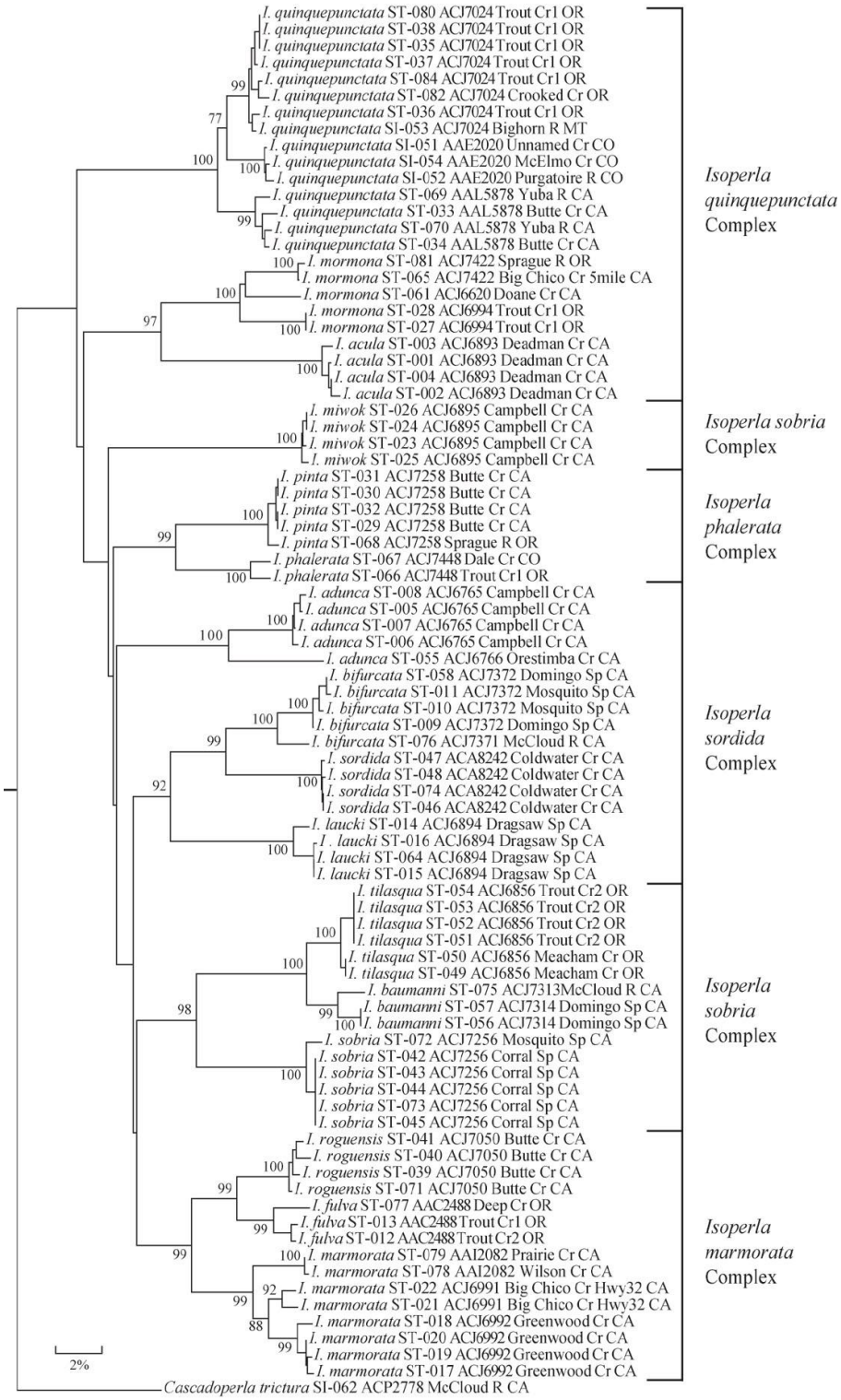

Fig. 3. Neighbor-joining tree of 16 western Nearctic *Isoperla* species based on a 658 bp fragment of COI estimated using the Kimura-2-paramter model with pairwise deletion option. Nodal support values are bootstrap percentages from 10,000 replicates. Locality and BIN are provided for each record. Scale indicates % divergence.

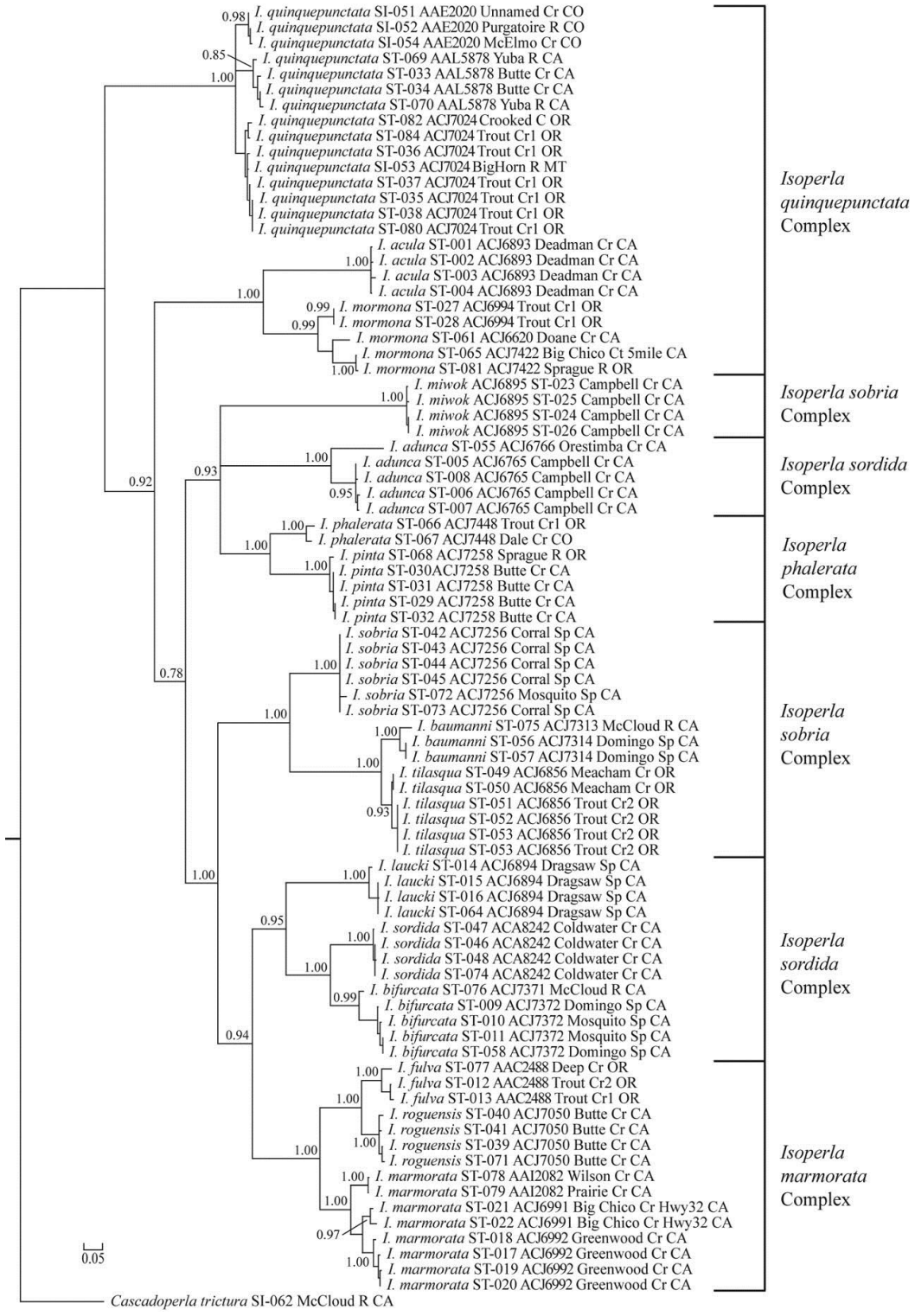

Fig. 4. Fifty percent majority rule consensus tree from Bayesian analysis of 16 western Nearctic *Isoperla* species based on a 658 bp fragment of COI. Nodal support values are posterior probabilities. Locality and BIN are provided for each record. Scale indicates estimated number of substitutions per site.

interspecific genetic distances) or splitting of species (separation of species with high intraspecific genetic distances). Yet, intraspecific genetic distances never exceeded interspecific distances to the nearest neighbor taxon. Consequently, despite high intraspecific variability and some instances of low interspecific distances, genetic variation within *Isoperla* can still be partitioned based on the presence of gaps between the maximum intraspecific distance and the distance to the nearest neighbor. Moreover, species with high intraspecific variability tend to have high genetic distances to their nearest neighbor taxon.

The distribution of intra- and interspecific genetic variation observed here suggests that a sliding threshold for species delimitation might best explain the data. The RESL algorithm was able to separate all of the species included in the research; there were no shared BIN identifications among different morphologically defined taxa. Frequently, the RESL algorithm over divides species into multiple BINs when intraspecific distances are large (see Figs. 3 or 4). This could be a consequence of diverse population genetic structure, incomplete geographic sampling, or a combination of both. These scenarios cause the RESL algorithm to detect gaps within species when COI haplotypes from different populations of the same species are divergent (no intermediate haplotypes). The strong geographic signature of BINs across our trees supports this hypothesis.

Support for Morphological Taxonomic Hypotheses.

Both the distance-based NJ and character-based BI approaches strongly supported the monophyly of groups of specimens corresponding to 16 morphologically defined *Isoperla* species. There were no conflicts between NJ and BI approaches in the relationships among species (Figs. 3 & 4). The deepest relationships among the *Isoperla* species were weakly supported using NJ, but power to resolve the backbone of the tree increased using the character-based BI approach allowing for generally well-supported resolution of internal nodes. Several of Szczytko & Stewart's (1979) species groups were fully supported by our molecular data and the others were only partially supported. Here, we iteratively consider the morphological and behavioral features of these taxa in the context of our genetic results.

The *I. phalerata* species group (*I. phalerata* (Needham 1917) and *I. pinta* Frison 1937) is well supported as monophyletic, agreeing closely with the original morphological arrangement of Szczytko & Stewart (1979). The species group has morphologically distinct characters unique from all other western *Isoperla* species including barbed male paraprocts and a larval checkerboard pronotal pattern. Likewise, the unique male diphasic drumming interval call pattern supports monophyly of the species group.

The *I. marmorata* species group (*I. fulva*, *I. roguensis* Szczytko & Stewart 1984, and *I. marmorata*) is also well supported as monophyletic by our molecular analysis. We note that within *I. marmorata*, there appears to be tentative support for three cryptic or sibling species (Figs. 3 & 4). These groups seem to be supported morphologically by slight to extreme pigment differences as noted previously by Sandberg & Kondratieff (2013). These slight population differences require further investigation.

The *I. quinquepunctata* species group (*I. acula* Jewett 1962, *I. jewetti* Szczytko & Stewart 1976, *I. longiseta* Banks 1906b, *I. mormona*, *I. quinquepunctata*) is either paraphyletic or polyphyletic (ambiguous). Specifically, *I. acula* and *I. mormona* may be more closely related to the other *Isoperla* included in this study than to *I. quinquepunctata*. This is consistent with the morphological observations that *I. acula* and *I. mormona* share similar aedeagal characters lacking in *I. quinquepunctata* (Table 1). For both *I. acula* and *I. mormona*, the apical half of the aedeagus is conical and ventrally curved with constricted tip and distinct but fine setae are present. If possible additional molecular studies of the two other species of the species group, *I. longiseta* and *I. jewetti* (but presumed extinct), should be conducted. It is likely that the species group as it is currently defined will be reconstituted as two or more groups as more information is gathered.

The *I. sobria* species group (*I. baumanni*, *I. gravitans* (Needham & Claassen 1925), *I. miwok*

Table 4. Mean percent sequence divergence among species determined using COI with the Kimura-2-parameter model with pairwise deletion. Species abbreviations: *I. acu* = *Isoperla acula*, *I. adu* = *Isoperla adunca*, *I. bau* = *Isoperla baumanni*, *I. bif* = *Isoperla bifurcata*, *I. ful* = *Isoperla fulva*, *I. lau* = *Isoperla laucki*, *I. mar* = *Isoperla marmorata*, *I. miw* = *Isoperla miwok*, *I. mor* = *Isoperla mormona*, *I. pha* = *Isoperla phalerata*, *I. pin* = *Isoperla pinta*, *I. qui* = *Isoperla quinquepunctata*, *I. rog* = *Isoperla roguensis*, *I. sob* = *Isoperla sobria*, *I. sor* = *Isoperla sordida*, and *I. til* = *Isoperla tilasqua*.

I. acu	19.2															
I. adu	17.9	20.4														
I. bau	20.5	19.3	17.0													
I. bif	19.9	17.8	16.8	17.7												
I. ful	20.0	18.1	18.2	17.6	16.1											
I. lau	18.1	17.6	16.3	19.7	12.5	16.6										
I. mar	20.1	18.6	17.5	16.5	14.6	10.2	15.4									
I. miw	18.6	21.7	16.9	18.3	17.7	18.2	18.1	18.3								
I. mor	20.2	13.9	19.7	19.4	18.7	18.1	19.3	19.1	21.2							
I. pha	17.9	19.4	15.0	16.2	17.5	13.4	16.0	15.9	16.2	16.0						
I. pin	17.7	17.6	15.8	16.6	18.2	15.3	17.2	17.3	15.2	16.1	08.6					
I. qui	17.1	19.8	18.2	21.5	18.8	17.8	18.5	18.1	18.3	17.6	16.8	17.0				
I. rog	20.5	19.5	18.0	18.0	15.2	05.7	15.6	09.5	18.5	19.3	15.3	17.1	17.6			
I. sob	19.4	17.5	16.4	12.3	16.1	15.1	15.7	15.0	16.8	18.4	14.2	14.0	19.0	15.7		
I. sor	20.0	17.8	17.2	16.8	08.7	15.3	13.6	13.7	19.5	17.5	14.8	17.2	19.4	14.6	16.1	
I. til	19.6	20.3	15.8	04.3	17.9	17.1	19.3	16.9	17.8	18.6	15.4	15.7	20.6	17.6	12.0	16.0
	C. tri	*I. acu*	*I. adu*	*I. bau*	*I. bif*	*I. ful*	*I. lau*	*I. mar*	*I. miw*	*I. mor*	*I. pha*	*I. pin*	*I. qui*	*I. rog*	*I. sob*	*I. sor*

Bottorff & Szczytko 1990, *I. sobria* (Hagen 1874), *I. tilasqua* Szczytko & Stewart 1979) appears polyphyletic. *Isoperla miwok* appears to be more closely related to *I. adunca* and the *I. phalerata* species group (*I. phalerata* and *I. pinta*) than to *I. sobria*, *I. tilasqua* and *I. baumanni.* The grouping of *I. baumanni*, *I. tilasqua,* and *I. sobria* is well supported by the molecular data and also morphologically by similar aedeagal characters (Table 1). For *I. baumanni* and *I. tilasqua*, the aedeagal apex is expanded and tip inverted, the median posterior margin has a distinct lobe with a fine wide posteroventral setal patch and a fine narrow setal patch above the lobe. *Isoperla baumanni* and *I. tilasqua* are most reliably separated by pigment and internal aedeagal differences (Sandberg & Kondratieff 2013). *Isoperla sobria* is to some extent similar, possessing only a distinct setal patch on the posterior aedeagal margin. The aedeagus of *I. miwok* is distinct from those of *I. baumanni*, *I. tilasqua,* and *I. sobria*, having paired median longitudinal patches of distinct setae along the basal posterior margin (Table 1). Additionally, the larval lacinia of *I. miwok* is measurably distinct from other *I. sobria* group species, having only 1-2 widely spaced submarginal row (A) setae where the others possess 2-7 closely spaced setae. Whether this unique lacinial character in conjunction with our molecular evidence supports removal of *I. miwok* from the *I. sobria* species group requires further study.

The *I. sordida* species group (*I. adunca*, *I. bifurcata*, *I. denningi*, *I. fusca* Needham & Claassen 1925, *I. petersoni* Needham & Christenson 1927, *I. rainiera* Jewett 1954, *I. sordida* Banks 1906c, *I. umpqua* Szczytko & Stewart 2013) also appears polyphyletic (based upon our results for three of eight species). According to our molecular analysis, *I. adunca* is more closely related to *I. miwok* and the *I. phalerata* group species than to *I. laucki* Baumann & Lee 2009, *I. sordida* and *I. bifurcata*. Species in the *I. sordida* group appear distinct from other western Nearctic *Isoperla* species groups based on the presence of a pedunculate vesicle (Table 1). However, this synapomorphy needs to be tested when assigning other *Isoperla* species with pedunculate vesicles to the *I. sordida* group. The larval lacinia of *I. adunca*

with its continuous submarginal row setae is distinct from all other known western species. Perhaps *I. adunca* should be removed from the *I. sordida* species group.

Lastly, *I. laucki* (species group unassigned) may be more closely related to *I. bifurcata* and *I. sordida* than to the 13 other *Isoperla* species of this molecular study (Figs. 3 & 4). Alternatively, Sandberg & Kondratieff (2013) indicated that *I. laucki* might be more similar to *I. marmorata* based upon shared aedeagal spinule patches and sclerotized processes. Our molecular phylogenies conflicted with this inference.

In conclusion, the *I. marmorata* and the *I. phalerata* species groups proposed of Szczytko & Stewart (1979) were supported by our DNA barcode data as monophyletic, while the *I. quinquepunctata*, *I. sobria*, and *I. sordida* groups were found to be para- or polyphyletic. Based on current evidence, we propose no changes to the conceptualization of the species groups presented in Szczytko & Stewart (1979) at this time, but rather point out that the present arrangement of species within species groups is in need of revision. Future molecular studies should strive to increase taxon and population sampling, especially for widely distributed species, and utilize multiple genetic markers. We hope that this analysis will help to guide future investigations.

ACKNOWLEDGEMENTS

We thank two anonymous referees for comments that improved this manuscript. Special thanks and gratitude for contributions made early in the project are given to Brian White of San Diego, California. We especially thank Jon J. Lee (Eureka, California), Larry E. Serpa (Fairfax, California), and William Isham (Carlsbad, California) for providing *Isoperla* specimens for use in this project. We thank Mark Simmons (Colorado State University) for help interpreting phylogenetic results. The Aquatic Bioassessment Lab, Chico, California, provided funding for DNA barcoding. Brian A. Gill is supported by an NSF Graduate Research Fellowship.

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Received 19 March 2015, Accepted 20 August 2015, Published 10 September 2015