Species delimitation in the ground beetle subgenus *Liocosmius* (Coleoptera: Carabidae: *Bembidion*), including standard and next-generation sequencing of museum specimens

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The species of subgenus *Liocosmius* Casey of genus *Bembidion* Latreille are delimited and documented using DNA sequences from eight genes, morphological data, and geography. The subgenus consists of six known species, three of which are described as new: *Bembidion orion* Cooper and Maddison (California), *B. darlingtonielum* Cooper and Maddison (California), and *B. cooperi* Maddison (New Mexico and Arizona). The group ranges from British Columbia south to Baja California, and east to Colorado and New Mexico, with the centre of diversity in California. DNA from a pinned specimen collected in 1968 was sequenced using an Illumina platform, resulting in accurate data of all eight genes studied. Identification tools and descriptions of each species are provided.

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ADDITIONAL KEYWORDS: Bembidiini – *Bembidion* – DNA – Illumina – morphology – museum specimens – species delimitation – systematics.

INTRODUCTION

Members of *Bembidion* Latreille subgenus *Liocosmius* Casey are delicate, spotted ground beetles (Figs 1, 2) living on sandy banks of creeks and rivers (Fig. 3) in western North America. They range from southern British Columbia to northern Mexico, east to Colorado and New Mexico, and are considered in the literature to consist of three species (Lindroth, 1963; Bousquet, 2012). However, in California some specimens appear to be intermediate between two of those forms, casting doubt on the three-species model for the group. As DRM and KWC attempted to resolve this uncertainty, we independently discovered several undescribed species (KWC through morphological studies beginning in the 1960s, and DRM through molecular and morphological studies beginning in 2003). This paper, which reviews the described species and reports on three new species, is the result of the merger of our efforts. Our investigation into species boundaries within *Liocosmius* is a combination of field observations, classical morphological methods, and DNA sequencing.

One of the most promising tools for understanding species boundaries is next-generation sequencing of DNA from pinned specimens in museums, as it can allow sampling of forms that would be difficult to obtain otherwise (Knapp & Hofreiter, 2010; Bi et al., 2013; Nachman, 2013; Staats et al., 2013). Obtaining DNA sequences has proven vital in species delimitation studies in other bembidiines, as DNA contains a particularly clear signal of species boundaries, and has revealed several cryptic species (e.g., Maddison, 2008; Maddison & Arnold, 2009; Maddison & Swanson, 2010). The gene fragments we
typically studied were chosen in part because of reliable polymerase chain reaction (PCR) primer sites that are often more than 600 bases apart; for our standard PCR-based protocols to be successful, there must be enough fragments of that length present in museum samples. However, with the DNA degradation expected in such specimens (Dillon, Austin & Bartowsky, 1996; Erkens et al., 2008; Staats, Cuenca, Richardson, Vrielink-van Ginkel, Petersen, Seberg & Bakker, 2011), many pinned carabids are left with DNA fragments shorter than 400 bases (unpublished data). As next-generation sequencing methods, such as those performed by Illumina machines, are designed to sequence short fragments of DNA, extracts from museum specimens can be perfectly suited to be sequenced by these methods (Knapp & Hofreiter, 2010; Bi et al., 2013; Nachman, 2013; Staats et al., 2013).

Because of the importance of DNA sequences for this study, we conducted field work to acquire new specimens, preserved in ethanol, from a wide geographic

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**Figure 1.** Habitus of *Bembidion* (*Liocosmius*) adults. Scale bar 1.0 mm. A, *Bembidion horni*, USA: New Mexico: Grant Co., Gila River at route 211, Gila, DRM voucher V100778; B, *B. orion*, USA: California: El Dorado Co., Strawberry Creek at Sciots Camp, DRM voucher DNA3070; C, *B. mundum*, USA: California: Lake Co., North Branch Cache Creek at hwy 20 DRM voucher V100777; D, *B. darlingtoniellum*, USA: California: Lake Co., North Branch Cache Creek at hwy 20, DRM voucher V100775. Photographs reprinted with permission, copyright David Maddison, released under a Creative Commons CC-BY 3.0 licence.
range. However, one apparent species from the Sierra Nevada of California, discovered by KWC morphologically, proved elusive in Nature, and in 2011 an attempt was made to sequence genes from a pinned museum specimen. Our first attempts, using PCR and Sanger sequencing, proved somewhat successful, but to a lesser extent than desired. Additional sequences were sought by sequencing DNA from the pinned specimen on an Illumina HiSeq machine. This approach yielded data from many genes, allowing us to confirm its distinctive status. Later field work provided specimens for PCR/Sanger sequencing, and confirmed the accuracy of the Illumina data.

From our molecular and morphological studies we have discovered three previously unrecognized species, bringing the total known diversity in the group to six species. In this paper we present the evidence for these species, and provide tools to aid in their identification.

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MATERIAL AND METHODS

SPECIMENS EXAMINED AND DEPOSITORIES

We examined approximately 1600 specimens of *Liocosmius*; they are from or will be deposited in the collections listed below. Each collection’s listing begins with the coden used in the text.

| Code | Institution                                      |
|------|--------------------------------------------------|
| BMNH | The Natural History Museum, London               |
| CAS  | California Academy of Sciences, San Francisco    |
| CSCA | California State Collection of Arthropods,       |
|      | Sacramento                                       |
| CNC  | Canadian National Collection, Ottawa            |
| CMNH | Carnegie Museum of Natural History, Pittsburgh   |

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**Species of Bembidion (Liocosmius)**

EMEC Essig Museum Entomology Collection, University of California, Berkeley
FFPC Foster F. Purrington, 3028 Brantwood Drive, Zolfo Springs, FL 33890 U.S.A.
MCZ Museum of Comparative Zoology, Harvard University, Cambridge
MNHN Muséum National d’Histoire Zoologique, Paris
OSAC Oregon State Arthropod Collection, Oregon State University, Corvallis
UAIC University of Arizona Insect Collection, University of Arizona, Tucson
USNM National Museum of Natural History, Smithsonian Institution, Washington, DC
ZMUM Zoological Museum, Moscow State University, Moscow

All type specimens mentioned have been examined.

**Collecting and Storage Methods**

Specimens were collected by hand or using an aspirator; specimens were found in their habitat after splashing the soil with water, or after treading the soil and waiting for the beetles thus disturbed to appear on the surface.

Specimens for morphological studies were killed and preserved in *Acer* sawdust to which ethyl acetate was added. Specimens collected specifically for DNA sequencing were killed and stored in 95% or 100% ethanol, with best results obtained when the abdomen was slightly separated from the rest of the body to allow better penetration, or when the reproductive system was dissected out through the rear of the abdomen within a few minutes of the beetle’s death in ethanol. Ethanol was decanted from vials and refilled at least two times within the first few weeks after death. Storage was then at −20 °C.

We do not know the methods used to kill the pinned specimens whose DNA was sequenced. They were collected between 1968 and 1976 by KWC or Fred Andrews, pinned, and were housed in standard museum drawers in either KWC’s house in Riverside, California, or CSCA in the years until 2011, when their DNA was extracted.

**Morphological Methods**

Basic methods for studying adult structures, and terms used, are given in Maddison (1993). Measurements were taken using Microvision’s Cartograph software on images from a JVC KY-F75U camera attached to a Leica Z6 lens. Body length was measured from the front of the labrum to the elytral apex. For the pronotal width to length ratio (PW/PL), width was measured at the position of the middorsal seta, and length was measured along the midline. The PW/PL ratio was measured for at least six specimens from diverse localities per species. Genitalia, when studied, have been mounted in Euparal between two small coverslips attached to archival-quality heavyweight watercolour paper.

Photographs of body parts were taken with a Leica Z6 lens and JVC KY-F75U camera, with the exception of the genitalic close-ups (Figs 12, 14), which were taken with the same camera attached to a Leica DM5500 compound scope. For pronotal, elytral, and genitalic images, a stack of photographs at different focal planes was taken using Microvision’s Cartograph software. These TIFF images were then merged using the PMax procedure in Zerene Systems’ Zerene Stacker; the final images thus potentially have some artefacts caused by the merging algorithm.

**Taxon Sampling for DNA Studies**

Four to eight genes were sequenced for 47 specimens of subgenus *Liocosmius* (Table 1) which had been killed and preserved in 95–100% ethanol. In addition, five pinned museum specimens were sequenced for one or more genes (Table 1).

Five other species of *Bembidion* served as outgroups: *B. (Trechonepha) iridescens LeConte, B. (Trechonepha) trechiforme LeConte, B. (Hirmoplataphus) recticollis LeConte, B. (Hydrium) obliquulum LeConte, and B. (Melomalus) planatum LeConte. These were chosen as they represent potential near-relatives of subgenus *Liocosmius* (Maddison, 2012).

**DNA Sequencing**

The genes studied, and abbreviations used in this paper, are: 28S or 28S rDNA: 28S ribosomal DNA; 18S or 18S rDNA: 18S ribosomal DNA; COI: cytochrome oxidase I; wg: wingless; CAD: carbamoyl phosphate synthetase domain of the *rudimentary* gene; ArgK: arginine kinase; Topo: topoisomerase I; MSP or MSP-300: Muscle-Specific Protein 300. MSP-300 is a gene potentially involved in embryogenesis (Rosenberg-Hasson, Renert-Pasca & Volk, 1996; Technau & Roth, 2008; Xie & Fischer, 2008), and was chosen by DRM during analysis of several *Bembidion* transcriptomes (unpublished data) as potentially useful for phylogenetics in *Bembidion*.

For most specimens, DNA was extracted from muscle tissue or male accessory glands and testes using a Qiagen DNeasy Blood and Tissue Kit. For pinned specimens, DNA was extracted using the same kit, but from the whole, dried specimen, with the abdomen detached to allow greater penetration of buffers and enzymes. Pinned specimens were processed in a laminar flow hood in a designated clean room containing no PCR products, and with practices designed to reduce risk of contamination (e.g., entry to clean room is only allowed if one’s clothes have been cleaned since the last exposure in the distant room containing PCR products).
Four of the pinned specimens (numbers 2827, 2829, 2830, and 2831) whose DNA was sequenced were collected by KWC between 1968 and 1976; the other (number 2826) was collected by Fred Andrews in 1975. For these specimens DNA extraction was done in 2011, or 35–43 years after they were killed.

Except for pinned specimens 2826, 2827, 2829, and 2830, all specimens shown in Table 1 have been sequenced for 28S, COI, CAD, and Topo. Those specimens also sequenced for *wg*, ArgK, MSP, and 18S are evident in Figs 4 and 5. Pinned specimens 2826, 2827, 2829, and 2830 were sequenced for a short region (190–280 bases) of 28S or a short region (122–136 bases) of COI.

With the exception of the Illumina data from specimen DNA2831, gene fragments were amplified by PCR and Sanger sequenced. PCR amplification was conducted on an Eppendorf Mastercycler Thermal Cycler.

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**Table 1.** Sampling of members of *Bembidion* subgenus *Liocosmius*. Four-digit numbers at the start of each row are D.R. Maddison DNA voucher numbers. The three specimens whose numbers are underlined are holotypes; numbers in parentheses indicate pinned, dried, museum specimens. “*” indicates that some sequences for these specimens are from Maddison (2012); the remainder are newly sequenced.

| Bembidion horni Hayward | USA: NM: Catron Co., Largo Creek, 2270 m 34.1725°N 108.5382°W 1408* USA: CA: San Diego Co., Pine Valley Ck, Noble Canyon, 32.8486°N 116.5254°W 1972 USA: AZ: Graham Co., Gila River at Safford, 32.8472°N 109.7158°W 2121 USA: NM: Grant Co., Gila River at route 211, Gila, 1370 m, 32.9691°N 108.5872°W 2122, 2123 USA: CA: Riverside Co., Mount San Jacinto, 'Poppit Flats' (probably Poppet Flat, approximately 33.85°N 116.85°W) (2829) USA: CA: San Benito Co., San Benito River, 754 m, 36.3570°N 120.7855°W 3570 USA: CO: Mesa Co., route 65 W of Fleming Point, 1591 m, 39.1957°N 108.2185°W 3602
| Bembidion orion Cooper and Maddison, sp. nov. | (2826) USA: CA: Alpine Co., 2.9 mi SW Silvercreek Cpgd (approximately 38.575°N, 119.806°W) (2831) USA: CA: Oakhurst, Big Creek, 1520 m (approximately 37.479°N, 119.637°W) 3071 USA: CA: El Dorado Co., Lily Lake, 2020 m, 38.8736°N 120.0821°W 3070, 3079, 3080, 3104 USA: CA: El Dorado Co., Strawberry Creek at Sciot's Camp, 1760 m, 38.7835°N 120.1463°W
| Bembidion mundum (LeConte) | 1329 USA: WA: Whatcom Co., Nooksack River 2.3 km S of Deming, 70 m 48.808°N 122.2019°W 1973 USA: CA: Tulare Co., Kern River at Sequoia National Forest, 35.9668°N 118.4856°W 1979 USA: CA: Del Norte Co., Six Rivers NF, South Fork Smith River, 30 m, 41.7971°N 124.0553°W 1981 USA: Nevada: Lyon Co., Carson River near Weeks, 390 m, 39.2866°N 119.2778°W 2001 USA: OR: Tillamook Co., Nestucca River at Highway 101, 15 m, 45.24°N 123.861°W 2079, 2080*, 2168 USA: CA: Yolo Co., Cache Creek at road 57, 129 m, 38.8238°N 122.1840°W 2689 USA: CA: Lake Co., North Branch Cache Creek at hwy 20, 305 m, 38.9881°N 122.54°W (2830) USA: CA: Mono Co, Walker, at River (approximately 38.516°N, 119.457°W)
| Bembidion darlingtoniellum Cooper and Maddison, sp. nov. | 1414 USA: CA: Yolo Co., Putah Creek, 38.5067°N 122.0427°W 2081, 2167 USA: CA: Yolo Co., Cache Creek at road 57, 129 m, 38.8238°N 122.1840°W 3718 USA: CA: San Benito Co., Laguna Creek at Coalinga Rd, 790 m, 36.3682°N 120.8402°W 2600, 2612, 2613, 2617 USA: CA: Lake Co., North Branch Cache Creek at hwy 20, 305 m, 38.9881°N 122.5400°W
| Bembidion festivum festivum Casey | 2303 USA: CA: San Luis Obispo Co., Salinas River at Hi Mtn Road, 425 m, 35.2942°N 120.3893°W (2827) USA: CA: San Timoteo Canyon, El Casco (approximately 33.981°N, 117.118°W) 2846 USA: CA: Ventura Co., Apache Canyon, 1158 m 34.7722°W, 119.3481°N
| Bembidion festivum hilare Casey | 1980, 2000* USA: CA: Humboldt Co., Shively, at bridge crossing Eel River, 40 m, 40.4434°N 123.9862°W 2077, 2078* USA: CA: Yolo Co., Cache Creek at road 57, 129 m, 38.8238°N 122.1840°W 2688 USA: OR: Douglas Co., Umpqua River along Cougar Ck Rd, 75 m, 43.4723°N 123.5211°W 2690 USA: CA: Lake Co., North Branch Cache Creek at hwy 20, 305 m, 38.9881°N 122.5400°W
| Bembidion cooperi Maddison, sp. nov. | 1426 USA: NM: Catron Co., San Francisco River, 5.7 mi N of Alma, 1525 m 33.4519°N 108.9253°W 2120 USA: NM: Catron Co., Reserve, San Francisco River, 1750 m, 33.7167°N 108.7571°W 2115, 2116, 2132, 2133 USA: NM: Grant Co., Gila River at route 211, Gila, 1370 m, 32.9691°N 108.5872°W 2169, 2170, 2171, 2172 USA: AZ: Yavapai Co., Oak Creek nr Baldwins Crossing, 1215 m, 34.8233°N 111.7990°W

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Figure 4. Maximum likelihood trees for four genes. Outgroups not shown. Branch length is shown proportional to relative divergence, as estimated by RAxML; scale bar indicates 0.1 units. Circles indicate sequences from PCR/Sanger sequencing of pinned specimens, and stars from Illumina sequencing of pinned specimen DNA2831. A, 28S; B, COI; C, CAD; D, Topo.
ProS, using TaKaRa Ex Taq and the basic protocols recommended by the manufacturer. Primers and details of most cycling reactions used are given in Maddison (2012). The MSP gene was amplified with a hemi-nested reaction, each using cycling protocol C9 in Table A1 of Maddison (2012); the first primer pair was MSP1F (CGAGAYGARGTYGATAARATGATGCA) and MSP1R (TCWACCAGATCCATCCACTTGACCAT), followed by MSP2F (GCYGGACAAAAGGARATYAAYCARTGG) and MSP1R. These primers were designed based upon Bembidion sequences, but have proven useful throughout carabids. For pinned specimens, a smaller fragment of 28S was amplified, either a 250-base piece using the primers 28S1F (GAAACCGTTCAGGGGTAAACCTGAG) and 28Ss1R (TGTGCTACTACCGTGGCAGCC), or a 190-base portion of that piece, using the primers 28S1F and 28S2R (CTCCACCGYRGCCGTARATGGC). For these same specimens, a 136-base piece of COI was amplified using the primers COS2F (AGAATATTAATTCGAGCWGAAYTAGG) and COS2R (GAAATGCTATATCWGGRGCTCC). The cycling protocol for these short 28S and COI reactions was protocol C9 of Table A1 of Maddison (2012) except with annealing temperatures of 60 °C, 55 °C, and 50 °C. Amplified products were cleaned, quantified, and sequenced at the University of Arizona’s Genomic and Technology Core Facility using a 3730 XL Applied Biosystems automatic sequencer. Assembly of

Figure 5. Maximum likelihood trees for four genes. Outgroups not shown, except for the ArgK gene tree. Branch length is shown proportional to relative divergence, as estimated by RAxML; scale bar indicates 0.1 units. Stars indicated sequences from Illumina sequencing of pinned specimen DNA2831. A, MSP; B, wg; C, 18S; D, ArgK.
multiple chromatograms for each gene fragment and initial base calls were made with Phred (Green & Ewing, 2002) and Phrap (Green, 1999) as orchestrated by Mesquite’s Chromaseq package (Maddison & Maddison, 2011a, 2011b) with subsequent modifications by Chromaseq and manual inspection. Multiple peaks at a single position in multiple reads were coded using IUPAC ambiguity codes; these data were not phased (Clark, 1990).

For DNA2831, a pinned specimen of Bembidion orion collected on 29 June 1968, a next-generation sequencing approach was used. The extracted DNA was included as one of six multiplexed samples on a 100-base paired-end reaction in an Illumina HiSeq 2000 lane at Oregon State University’s Center for Genome Research and Biocomputing. Approximately 65 million reads were produced for this sample. The reads were then subject to two assembly methods in CLC Genomics Workbench version 6.0.2: (1) a reference-based assembly in which the references were eight sequences (one for each gene) from voucher DNA2167 (a Bembidion darlingtoniellum, Table 1); (2) a de novo assembly using CLC’s default values (including auto-detect of paired distances, automatic bubble size, automatic word size, and scaffolding), followed by BLASTing (Altschul et al., 1997) the sequences of DNA2167 against a database created from that assembly. The reference-based assembly yielded data for all eight genes which were of comparable length to the sequences obtained with PCR and Sanger sequencing. The de novo assembly yielded sequences for all eight genes that were longer than those obtained with Sanger sequencing, but with some missing data in the centre of the wingless gene. For each gene, the final Illumina-based consensus sequences were obtained by combining results from the two assembly approaches. For all genes except wingless and ArgK, the sequences from the two assembly methods were identical. For wingless, the only difference was synonymous, a C versus a T at a third position. For ArgK, there were three non-synonymous and two synonymous differences; for all three non-synonymous differences, the amino acid of the reference-based assembly matched that present in both the reference sequence (DNA2167) and other B. orion, with the de novo assembly showing a distinct amino acid. Any sites that differed between the two assemblies were coded using the ambiguity code for the combined bases.

ALIGNMENT

Alignment of the protein-coding sequences was simple, as there have been no evident insertions or deletions (indels) in the history of the sequenced specimens since their common ancestor. 18S showed only a single base insertion in B. festivum. 28S showed a richer history of insertions and deletions. An alignment of 28S was performed by Opal version 2.1 (Wheeler & Kececioglu, 2007), using default parameter values. In addition to scattered single-base indels, the resulting alignment showed three isolated regions of multiple-base indels. The alignments in these regions were not ambiguous enough to warrant exclusion.

MOLECULAR PHYLOGENETIC ANALYSIS

Models of nucleotide evolution where chosen with the aid of jModelTest version 2.1.2 (Guindon & Gascuel, 2003; Darriba et al., 2012). Among the models supported by GARLI and BEAST, the models chosen by the Bayesian Information Criterion are GTR+I (28S, uwg), GTR+Γ (CAD, COI, Topo), and HKY+I (ArgK, MSP, 18S).

Likelihood analyses were conducted using GARLI version 2.0 (Zwickl, 2006). For each gene, a search for the maximum likelihood tree was conducted using 100 search replicates, and maximum likelihood bootstrap analyses with 500 bootstrap replicates.

Although the main effort of this paper is species delimitation, not inference of a species phylogeny, we have conducted a preliminary inference of the species tree using simultaneous inference of gene trees and the species tree. For this, a *BEAST analysis (Heled & Drummond, 2010; Drummond et al., 2012) was conducted of the 47 Liocosmius specimens and six outgroups for which 28S, CAD, Topo, and COI sequences were all available. A Yule process prior was used for node ages, with the default Jeffrey’s (1/x) prior on the birth rate; the tree model for COI was set to a mitochondrial ploidy type, and autosomal nuclear for the other genes. An uncorrelated lognormal relaxed clock model was used for rates of evolution on each branch. In order to explore the sensitivity of results to assumptions, three analyses were done that differed in priors used for the relaxed clock model. For the first, a uniform [0, 100] prior for the mean clock rate was used, and an exponential prior for the standard deviation of the clock rate (with default parameter values). For the second, a Gamma prior was used for the mean clock rate, and an exponential prior for the standard deviation of the clock rate, both with default parameter values. For the third, a Gamma prior was used for both the mean clock rate and standard deviation of the clock rate, with default parameter values. *BEAST analyses were run until effective sample sizes (ESS, as measured in Tracer (Rambaut & Drummond, 2013), with burn-in of 10%) were all at least 200 (except for COI.treeLikelihood, which reached at least 150). The first 10% of trees were discarded as the burn-in sampling. The first analysis was run for 400 million generations (sampled every 10 000 generations; thus, 36 000 trees were analyzed); the second for 1 billion generations (sampled every 50 000
generations; thus, 18,000 trees were analyzed); the second for 2.03 billion generations (sampled every 20,000 generations; thus, about 91,000 trees were analyzed). Sampling thoroughness was measured by ESS values and by examining plots of likelihood values and other parameters in Tracer 1.6 (Rambaut & Drummond, 2013). The results are presented both as majority-rule consensus trees (as calculated by Mesquite (Maddison & Maddison, 2011b)) and as maximum clade credibility trees (as calculated by TreeAnnotator, a program distributed with BEAST).

**SPECIES DELIMITATION**
We consider as species separately evolving metapopulation lineages (De Queiroz, 2007). We delimit these lineages using evidence provided by patterns of gene trees, morphological data, and geographic data that suggests a lack of gene flow between entities so delimited, but presence of gene flow within such entities. We have not conducted a formal coalescence-based analysis (e.g., Jones & Oxelman, 2014), in part as the signal in the gene trees appears so clear that such an analysis is not required, and in part as no current analysis allows us to incorporate the morphological and geographical data.

**ARCHIVED DATA**
Sequences have been deposited in GenBank with accession numbers KJ624149 through KJ624398.Aligned data containing the entire (untrimmed) sequences for each specimen as well as files containing the inferred trees for each gene have been deposited in Dryad (data available from the Dryad Digital Repository: http://doi.org/10.5061/dryad.m863j). A list of specimens examined with localities and months of capture is also included in the Dryad archive.

**RESULTS OF MOLECULAR ANALYSES**
The DNA sequences obtained from the Illumina-sequenced, pinned specimen DNA2831 are both extensive and match well those of PCR/Sanger sequenced ethanol-preserved material (as indicated by the branch length patterns in Figures 4–5, and the information presented in Table 2). The assembled sequences for DNA2831 include over 10,000 bases of the nuclear ribosomal gene complex, almost 14,000 bases of the mitochondrial genome, and longer pieces for all nuclear protein-coding genes than we sequenced with PCR/Sanger sequencing (Table 2). The assembled regions include between 95 and 100% of the sequences obtained from PCR/Sanger sequencing (Table 2). Of the 7080 bases that overlap with those obtained from other specimens of B. orion, 7073 have exact matches between the Illumina sequences of the pinned specimen and the PCR/Sanger sequences of ethanol-preserved specimens. Two of the bases that differ are both synonymous differences (one in COI and one in ArgK); the other five are the ArgK sites mentioned above in which the de novo assembly differs from the reference-based assembly. The 254 bases of 28S and 136 bases COI that were PCR/Sanger sequenced for DNA2831 are identical to the sequences obtained from Illumina sequencing. Although we report here only results of eight genes, many other genes are present in the

**Table 2.** Comparison of the results of PCR/Sanger sequencing of ethanol-preserved Bembidion to Illumina sequencing of the single pinned B. orion (DNA2831) collected in 1968. Consider, for example, the sequences obtained for ArgK: the Illumina sequencing of the pinned B. orion yielded a sequence (contig) of length 1450 bases; 625 of those bases overlapped with the 638 bases sequenced using PCR/Sanger from ethanol-preserved B. orion; thus, only 13 bases or about 2% of the sequence was missing (although Illumina sequencing yielded 825 (= 1450–625) bases beyond those obtained by PCR/Sanger sequencing)

| Gene | PCR/Sanger sequencing results from ethanol-preserved material | Illumina results from pinned B. orion |
|------|---------------------------------------------------------------|-------------------------------------|
|      | Length of analyzed region, all species | Length of analyzed region, B. orion | Length of contig over analyzed region | % Illumina length relative to PCR/Sanger B. orion | Full length of contig |
| 28S  | 920–967 | 948–949 | 949 | 100 | 10114 |
| COI  | 658–766 | 766 | 766 | 100 | 13869 |
| CAD  | 749–798 | 765–798 | 798 | 100 | 2420 |
| Topo | 687–742 | 713–735 | 742 | 101 | 1625 |
| MSP  | 835–876 | 847–876 | 835 | 95 | 960 |
| wg   | 384–456 | 456 | 456 | 100 | 2157 |
| ArgK | 606–638 | 638 | 625 | 98 | 1450 |
| 18S  | 1373–1917 | 1916 | 1916 | 100 | 10114 |

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assembly, and comparison with assemblies from fresh specimens of other species of *Bembidion* suggest that the assembly from pinned specimen DNA2831 is of comparable quality. A more in-depth study of the genomic data obtained from the pinned specimen is beyond the scope of this paper, and will be published separately.

The maximum likelihood trees for each gene show monophyly for many of the inferred species in most genes (Figs 4 and 5, Table 3). 28S and COI in particular show monophyly of each of the six species, all supported with bootstrap percentages of 76 or greater (Table 3). Each species is monophyletic in three (*B. horni*) or more (all other species) of the gene trees.

There is slight geographic variation in DNA sequences within three species. Arizona and New Mexico populations of *B. cooperi* differ at two nucleotide sites in COI (the variants in the two populations code for the same amino acids). The Colorado specimen of *B. horni* is unusually distinct in COI, differing at five sites from all other *B. horni*; these five differences are synonymous. The two subspecies of *B. festivum* show no consistent differences in 28S, COI, or CAD, but the two specimens of *B. festivum festivum* sequenced do differ by two synonymous third-position differences in Topo from the six specimens of *B. festivum hilare* sequenced. Additional sampling will be needed to determine if the geographic distributions of those alleles match those of the two subspecies as defined with morphological data. No geographic substructuring is evident in the other three species.

The inferred species tree suggests that *B. orion* is the sister group of remaining *Liocosmius*, with *B. darlingtonielum* and *B. horni* as sisters, and with *B. cooperi* and *B. festivum* as sisters, although only the latter relationship is strongly supported (Fig. 6). The species tree inferred in the three *BEAST* analyses were very similar one to another, with all showing the same branching relationship among clades with posterior probability greater than 50% (Fig. 6A). The analyses only differed in the placement of *B. mundum*. The first and second analyses, both with an exponential prior for the standard deviation of the clock rate, showed *B. mundum* as sister to a clade consisting of the quartet *B. horni* + *B. darlingtonielum* + *B. festivum* + *B. cooperi* (Fig. 6B), although the support for this was low (posterior probability 0.40–0.45); the posterior probability in these analyses of *B. mundum* as sister to *B. festivum* + *B. cooperi* was 0.32–0.36. The third analyses showed *B. mundum* as sister to *B. festivum* + *B. cooperi* (Fig. 6C, posterior probability 0.41), with a less probable (0.35) placement of *B. mundum* as sister to the quartet.

**DISCUSSION**

The evidence that the genetic lineages of subgenus *Liocosmius* are organized into at least five species comes from multiple sources: monophyly patterns in multiple gene trees (Figs 4 and 5, Table 3), similarity of sequences within each species but distinctiveness between species in 28S and COI (Fig. 4), consistent differences in morphological structures, including genitalia (Figs 12–15), that are congruent with the DNA results, and the microsympatry of several species pairs (Table 4).

The only species pair whose distinctiveness might be in doubt is *B. festivum* and *B. cooperi*. In contrast to other species pairs, there are no observed differences between male genitalia of these two species, and externally the species are extremely similar, with only a slight difference noted in the hind angles of the pronotum (Fig. 8). They are morphologically no more different than are the two subspecies of *B. festivum*. *B. festivum* and *B. cooperi* are also allopatric. Our decision to treat them as distinct is thus based almost entirely on differences in DNA sequences. In that regard
Figure 6. Species relationships as inferred by *BEAST, using data from 28S, CAD, Topo, and COI. A, Majority-rule consensus tree of the post-burn-in trees; each of the three analyses showed the same tree topology, and differed only in the posterior probability estimates for the clades. These estimates are shown (as percentages) on each internal branch, with variation in values across analyses shown. B, Maximum clade credibility tree for the second analysis (Gamma prior for mean clock rate, exponential for standard deviation of clock rate); the first analysis (with uniform prior for mean clock rate) shows the same branching pattern; C, Maximum clade credibility tree for the third analysis (Gamma prior for mean clock rate and for standard deviation of clock rate). Scale bar on B and C 0.005, as reconstructed by *BEAST.

they are quite distinctive (Figs 4–5), as much as some other Liocosmius species pairs, and much more so than are the subspecies of B. festivum. Consistent differences between the two species include: (1) in CAD, two synonymous and one non-synonymous differences; (2) in Topo, one synonymous difference; (3) in 28S, seven base substitutions and three indels; (4) in COI, 23 synonymous base differences (i.e., 3% of the 766 bases total). The differences in 28S and COI in particular are consistent with those found between other pairs of similar-but-distinct, sympatric Bembidion (e.g., Maddison, 2008).

There has been increasing interest in sequencing DNA from museum specimens or ancient remains. Vertebrates have been subjects of much of this work, with over 100 mitochondrial genomes sequenced from museum specimens (Pajmans, Gilbert & Hofreiter, 2013), and thousands of exons sequenced from mammal museum skins (Bi et al., 2013). This work has been enabled by next-generation Illumina sequencing methods that are designed to sequence short pieces of DNA, such as those naturally created as DNA degrades within museum specimens (Dillon et al., 1996; Knapp & Hofreiter, 2010; Mason et al., 2011; Nachman, 2013). Within the much smaller insects, the first sequencing of DNA from pinned material was performed using standard PCR/Sanger sequencing (Dillon et al., 1996; Junqueira, Lessinger & Azeredo-Espin, 2002; Goldstein & DeSalle, 2003; Mandrioli, Borsatti & Mola, 2006; Gilbert et al., 2007; Thomsen et al., 2009; Andersen & Mills, 2012; Vuataz et al., 2013), or PCR followed by pyrosequencing (Shokralla et al., 2011). Studies of DNA based on next-generation sequencing methods from museum specimens of insects are only beginning to be explored for mitochondrial genomes (Staats et al., 2013). Our study is the first of which we are aware that uses next-generation methods to sequence general elements of the nuclear genome of a pinned insect.

**DESCRIPTIONS AND IDENTIFICATION OF TAXA**

**SUBGENUS LIOCOSMIUS CASEY, 1918**

Type species *Ochthedomus mundus* LeConte, 1852, designated by Lindroth (1963).

Beetles of the subgenus *Liocosmius* have the general appearance of typical members of the *Bembidion* Series (that is, small, thin, delicate, and spotted), to which they do not belong. *Liocosmius* instead is in a clade with the *Ocydromus* and *Odontium* Series, the *Plataphus* Complex, and related groups (Maddison, 2012), many of which are larger, darker, and more robust.

**Morphological Diagnosis**

Small to medium (2.9–4.5 mm), shiny *Bembidion*, with four pale spots on the elytra. Frontal furrows single, broad, shallow, well-marked, not convergent or extended onto clypeus. Eyes not reduced. Posterior angle of pronotum with a basolateral carina. Posterior margin of pronotum not strongly sinuate laterally, its basal
transverse furrow not conspicuously or coarsely punctate. Elytron with lateral bead ending at humerus, not prolonged onto base; with a humeral and subapical pale spot (occasionally infuscate); one to four or more incomplete, faint discal striae (punctostriate for the most part, in many specimens barely visible), outer striae increasingly evanescent; intervals flat; discal setae on third interval, on or close to the third stria. Elytra not iridescent (occasionally possessing a bluish sheen). Mesosternal process without subapical setae. Metasternal process not margined in anterior half. Apices of parameres normally with two setae. Spermatheca not inflated basally.

There are two other groups of Bembidion with small, delicate, four-spotted adults in the geographic range of Liocosmius. Members of subgenus Lindrochthus Maddison, including B. wickhami Hayward, differ in many other features, including having the hind margin of the pronotum distinctly sinuate (‘notched’, as in Fig. 7B in Maddison (2012)); the hind, transverse furrow of the pronotum coarsely, deeply punctate; the elytra brightly iridescent; and the metasternal process narrowly margined laterally near its anterior point. At the eastern edge of the range of Liocosmius some specimens of Bembidion (Bembidion) quadriracematum might be encountered with four spots (as opposed to the more typical two-spotted western forms); these differ from Liocosmius in having a more notably constricted pronotum whose hind margin is also distinctly sinuate, with a small notch just behind and medial to the seta at the hind angle, and in having larger punctures in the striae in the anterior half of the elytra.

Habitat
Adults of Liocosmius are most often encountered on steep sand banks, often partly shaded, along bodies of water (Fig. 3). The banks need not be immediately adjacent to open water (a suitable bank of damp sand 5 m or more from shore can have abundant beetles).

Although river and creek banks are the typical habitat, the beetles can be common around stagnant backwaters of creeks (e.g., B. cooperi at Oak Creek south of Sedona, Arizona; B. festivum, B. darlingtoniellum, and B. mundum at North Branch Cache Creek, California), or small inlets at the edge of lakes (e.g., B. orion at Lily Lake, California). The only exception to this typical habitat appears to be B. orion, which occurs both in this habitat and on lake shores with dark, damp, organic soil (e.g., Lily Lake near Lake Tahoe).

Composition
The known species of subgenus Liocosmius are:
- Bembidion horni Hayward, 1897
- Bembidion orion Cooper and Maddison, sp. nov.
- Bembidion mundum (LeConte, 1852)
- Bembidion darlingtoniellum Cooper and Maddison, sp. nov.
- Bembidion festivum Casey, 1918
- Bembidion festivum festivum Casey, 1918
- Bembidion festivum hilare Casey, 1918
- Bembidion cooperi Maddison, sp. nov.

IDENTIFICATION OF SPECIES USING MORPHOLOGICAL DATA

Many specimens of subgenus Liocosmius can be identified to species using pronotal shape, microsculpture, and colour. The pronotum varies from wide and transverse (Fig. 7A, B) to relatively narrow (Fig. 7C–F). The extent of microsculpture on the elytra can be definitive. When present, microsculpture is finely engraved, and is only visible under higher magnification (at least 50×) with appropriate illumination (a ring light is ideal, although diffuse light can suffice at higher magnifications). The most obvious differences between some species are in colours of their elytra. Two species have dark elytral margins and epipleura, whereas the remainder are pale. The four spots (maculae) on the
elytra vary in extent. The subhumeral maculae, behind the shoulder, vary from small and isolated (e.g., Fig. 9B) to large and confluent (Fig. 10B). The subapical maculae, toward the apex of the elytra, also vary in extent and shape, with many (but not all) species having the medial end of each macula tapered, and pointing posteriorly; the spot thus appears comma-shaped (e.g., Fig. 9A). However, some of these differences can be subtle, and not evident in all specimens; geographic variation in colour also complicates identification. For example, southern specimens of *B. festivum*, *B. mundum*, and *B. darlingtonielum* are all paler than northern specimens, such that southern *B. mundum* from the Los Angeles area are as pale as northern *B. darlingtonielum* from around San Francisco. For this reason, specimens need to be identified considering their geographic context, and ideally with well-identified, comparative material. When in doubt, examination of the flagellar complex of the male genitalia will be definitive, except for distinguishing *B. festivum* from *B. cooperi*.

Three dichotomous keys are given: a full key containing all taxa, which will be needed for most of California and regions near the eastern and southern California borders, and two simpler keys for limited faunas north and east of California.

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FULL KEY FOR CALIFORNIA AND ADJACENT TERRITORIES TO THE EAST AND SOUTH

1. Pronotum broader (PW/PL = 1.34–1.44; Figs 1A,B, 7A,B). Lateral gutter of elytra dark. Epipleura of elytra piceous or dark brunneous. Antennae dark, with most articles piceous, at most three basal articles partly rufous. Femora piceous or dark ferrugineous in most specimens..............................2
   – Pronotum narrower (PW/PL = 1.17–1.32, most specimens < 1.29; Figs 1C, D, 2A–C, 7C–F). Elytral margins pale. If antennae darkened, not piceous, and with two or more basal articles entirely pale. Epipleura of elytra flavous or testaceous. Legs pale..........................3

2. Subhumeral macula reaches medially well onto third interval in most individuals; subapical macula comma-shaped in most individuals, with medial end tapered, wrapping around seta ed5 and onto third or second interval (Fig. 9A). Flagellar complex of aedeagus longer (Fig. 13A,B). Widespread (Baja Cal. Norte, CA, AZ, UT, NM, CO). Commonly ..........B. horni Hayward
   – Subhumeral macula reaches medially to at most the third stria, but not onto the third interval; subapical macula oblique, more posterior on outer elytral intervals than on inner ones, with medial end blunt, not tapered and not prolonged posteriorly around ed5, extending medial of ed5 in very few individuals (Fig. 9B). Flagellar complex of aedeagus shorter (Fig. 13C,D). Sierra Nevada of California ..........B. orion Cooper and Maddison, sp. nov.

3. Head, pronotum and abdomen brunneous or castaneous. Antennae testaceous or pale rufous. Flagellar complex of aedeagus very large, sinuate (Fig. 15) Larger (3.5–4.5 mm, most ≥ 3.7 mm)..................................................4
   – Head, pronotum and abdomen black or piceous. Antennae testaceous or pale rufous, or apical articles infuscated. [Some specimens from Santa Barbara and Los Angeles counties and southward have dark castaneous heads and pronotum, and with pale antennae; smaller pale specimens from that area should follow this path if they have only two discal setae on each elytron.] Flagellar complex of aedeagus smaller, more or less straight (Fig. 13E–F) Smaller (3.1–4.1 mm)............................................................................6

4. Hind angles of prothorax forming a right angle or are slightly acute, with lateral edge just in front of hind angle parallel to main body axis (Fig. 8E–H). No microsculpture on disc of elytra. Pale areas of elytra less extensive (Fig 2C). AZ, NM ..........................................................B. cooperi Maddison, sp. nov.
   – Hind angles of prothorax obtuse, with in most specimens slightly rounded and with lateral edge of pronotum just in front of hind angle diverging anteriorly (Fig. 8A–D). Either with faint microsculpture on disc of elytra (Fig. 2D) or with three discal setae on each elytron (Fig. 10B). CA, OR (B. festivum Casey). ..................5

5. Three or more dorsal setae on each elytron. Elytral microsculpture lacking on disc. Most specimens paler (Figs 2B, 9B), although some specimens from north of Los Angeles County are darker. Southern California ..........B. festivum festivum Casey
   – Most specimens with two dorsal setae on each elytron. Elytra with fine, transverse microsculpture on disc. Darker (Figs 2A, 9A). Northern California, Oregon ..........B. festivum hilare Casey

6. Humeral macula in most specimens does not extend medially onto third elytral interval, and does not extend anteriorly on intervals 3 and 4. Antennae generally infuscated distally from article 4 or 5 outward. Specimens from the Los Angeles basin and surrounding mountains are paler, with the humeral macula extending onto the third interval in some specimens, but there is never an isolated dark patch on intervals 5 and 6. Microsculpture absent from elytral discs, but well-engraved (although very finely so) from near hind margin of subapical macula to elytral apex, with distinct sculpticells. Flagellar complex of aedeagus longer (Fig. 13E, F) ..........B. mundum LeConte
   – Humeral macula extends medially at least onto third elytral interval; macula on third and fourth intervals extended anteriorly in many specimens, thus isolating a dark patch on intervals 5 and 6 (Fig. 9D); northern specimens (e.g., north of San Francisco) are darker, and some do not have an isolated dark patch. At least antennal articles 4–6 testaceous or pale rufous. Microsculpture absent from elytral disc, at most feebly and irregularly engraved close to elytral apex. Flagellar complex of aedeagus shorter (Fig. 13G, H) .........................................................B. darlingtoniellum Cooper and Maddison, sp. nov.

KEY FOR NORTHWESTERN FAUNA (CA NORTH OF 41°N, OR, WA, ID, BC)

1. Head and pronotum brunneous or castaneous. Antennae testaceous or pale rufous. Elytra with fine, transverse microsculpture on disc. Flagellar complex of aedeagus very large, sinuate (Fig. 15B). Larger (3.7–4.2 mm)............................B. festivum hilare Casey
   – Head and pronotum black or piceous. Antennae with apical articles infuscated. Elytra without microsculpture on disc. Flagellar complex of aedeagus smaller, more or less straight (Fig. 13E–F). Smaller (3.1–3.9 mm, most ≤ 3.6 mm)..................................................B. mundum LeConte

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B. orion

Diagnosis: A broad, dark species (Figs 1A, 7A, 9A). Length 3.2–4.1 mm. Ground colour of head, thorax, and elytra (including side margins) black or piceous. Antennae dark, with most articles piceous, at most three basal articles partly rufous. Femora piceous or dark ferrugineous in most specimens; tibia dark rufous to testaceous. Subhumeral macula of elytra reaches medially onto third interval in most individuals; subapical macula comma-shaped in most individuals; with medial end tapered, wrapping around seta ed5 and onto third or second interval (Fig. 9A). Sutural stria finely but distinctly punctostriate anteriorly (at 25 × magnification), engraved posteriorly, traceable from at least basal fifth to elytral apex; parascutellar stria punctostriate, punctures sharply defined (Fig. 11A). Microsculpture of elytra variable, with some specimens having evident although finely engraved transverse sculpticells on disc, others lacking such microsculpture. Flagellar complex of aedeagus very large, sinuate (Fig. 15C). Geographic distribution: From Southern California and Baja California east to Arizona, Utah, Colorado and New Mexico (Fig. 16).

SP. NOV.

Bembidion orion Cooper + Maddison, sp. nov.

Holotype male (in CAS), here designated, labelled 'USA: California: El Dorado Co., Strawberry Creek at Sciots Camp, 38.7835°N 120.1463°W, 1760 m, 30.v.2012. DRM 12.049. Maddison, Kavanaugh, & Moore', 'David R. Maddison DNA3104 DNA Voucher' [printed on pale green paper], and 'HOLOTYPE Bembidion orion Cooper + Maddison' [partly handwritten on red paper]. Genitalia mounted in Euparal on small card (with DNA3104 written on it) beneath specimen; extracted DNA stored separately in CAS and OSAC. GenBank accession numbers for DNA sequences of the holotype are KJ624197 (28S), KJ624354 (COI), KJ624241 (CAD), KJ624307 (Tope), and KJ624263 (ArgK). Type locality: Strawberry Creek at Sciots Camp, 38.7835°N 120.1463°W, El Dorado County, California.

Geographic variation: Specimens from the eastern portions of the range (AZ, NM, UT, CO) are somewhat larger (3.4–4.1 mm, with most ≥ 3.5 mm, as opposed to 3.2–3.7 mm in the Los Angeles basin and surrounding mountains, most ≤ 3.5 mm), and many have paler tibiae than specimens from Southern California and Baja California.

Paratypes: 89 specimens from the following localities, deposited in OSAC, CAS, CSCA, and USNM: USA: California: El Dorado Co., Strawberry Creek at Sciots Camp, 1760 m, 38.8736°N 120.0817°W (1); USA: California: El Dorado Co., Lily Lake, 2000 m, 38.874°N 120.0817°W (1); USA: California: El Dorado Co., Lily Lake, 2020 m, 38.874°N 120.0817°W (1); USA: California: El Dorado Co., Lily Lake, 2030 m, 38.874°N 120.0817°W (1); USA: California: El Dorado Co., Lily Lake, 2040 m, 38.874°N 120.0817°W (1); USA: California: El Dorado Co., Lily Lake, 2050 m, 38.874°N 120.0817°W (1); USA: California: El Dorado Co., Lily Lake, 2060 m, 38.874°N 120.0817°W (1); USA: California: El Dorado Co., Lily Lake, 2070 m, 38.874°N 120.0817°W (1); USA: California: El Dorado Co., Lily Lake, 2080 m, 38.874°N 120.0817°W (1); USA: California: El Dorado Co., Lily Lake, 2090 m, 38.874°N 120.0817°W (1); USA: California: El Dorado Co., Lily Lake, 2100 m, 38.874°N 120.0817°W (1); USA: California: El Dorado Co., Lily Lake, 2110 m, 38.874°N 120.0817°W (1); USA: California: El Dorado Co., Lily Lake, 2120 m, 38.874°N 120.0817°W (1); USA: California: El Dorado Co., Lily Lake, 2130 m, 38.874°N 120.0817°W (1); USA: California: El Dorado Co.,
Derivation of specific epithet: Named after the Greek mythological hunter Orion and the constellation named after him. These hunting beetles, whose spots appear as sparkling pinpoints of light as they scamper on shorelines in the Sierra Nevadas, are reminiscent of twinkling stars.

Nomenclatorial note: ‘Bembidion toltichi’ is a manuscript name used for this species by KWC, and there are many specimens so labelled in collections, including at least two specimens labelled as holotypes. The name has appeared in various online databases (e.g.,...
Figure 9. Elytra of species of *Liocosmius*. A, *B. horni*; B, *B. orion*; C, *B. mundum*; D, *B. darlingtonielum*. 
Figure 10. Elytra of species of Liocosmius. A, B. festivum hilare; B, B. festivum festivum; C, B. cooperi.
Diagnosis: A small, dark, broad *Liocosmius* (Figs 1B, 7B, 9B). Length 2.9–3.6 mm. Broader, less convex than other species of *Liocosmius*. Head, pronotum and ventral surface deep black to piceous, weakly aeneous in some

in the Global Names Index, http://gni.globalnames.org/name_indices/59925152/name_index_records, accessed 15 January 2014). It should be considered a nomen nudum.

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fresh specimens. Pronotum and elytral epipleura black to brown, ground colour of elytra black. Maculae bright yellow, generally sharply delimited, not reaching boundaries of elytron, and smaller than in *B. horni*; humeral macula irregularly rhomboidal, infrequently surpassing 3rd interval, rarely enclosing anterior dorsal pore; subapical macula elongate, roughly rectangular, directed obliquely anteromedially, with medial end blunt, uncommonly surpassing the third stria or slightly comma-shaped. Antenna black to fusco-piceous, first (and occasionally second) article paler below; palpi piceous or at least infuscated; legs piceous to dark reddish brown, pro- and mesotrochanters and apical 0.8 of tibiae paler in some, with tarsi darker. Pronotum broader and more transverse than other *Liocosmius* (Fig. 7B): PW/PL = 1.37 to 1.44, with most specimens being ≥ 1.40; all other *Liocosmius* measured have PW/PL < 1.40. Microsculpture sculpticells (at 50× magnification) as in *B. horni*, but in most specimens more weakly developed: transverse closed or open mesh, generally obsolete on frons and pronotal disc, in most specimens absent from the disc of the elytra. Parascutellar striae absent, or mere trace of 2–3 weak, fine punctae (Fig. 11B); sutural stria generally not traceable anterior to anterior third; 2 to 3 (rarely to 5) weak discal striae represented by lines of very fine punctae. The aedeagus has the smallest flagellar complex within *Liocosmius* (Fig. 13C,D), and the ventral margin is more or less straight (Fig. 12C,D).

This species is most similar to *B. horni*, from which it can be distinguished externally by the subapical macula which is blunt-ended medially, the smaller subhumeral macula, darker appendages, and the more finely punctate parascutellar stria.

About 200 specimens examined.

**Geographic distribution:** Restricted to the Sierra Nevada of California (Fig. 16).

**Spatial relationships to other Liocosmius:** *B. orion* has been found at the same locality as *B. mundum*, and
within 52 km of B. horni. It is allopatric with other species of Liocosmius (Table 4).

Geographic variation: None noted.

**Bembidion mundum** LeConte

*Ochtedromus mundus* LeConte, 1852: 190. Lectotype male in MCZ, here designated, labelled: [gold dot], ‘O. mundus S. Jose Lec’ [handwritten], ‘B bifasciatum Mots.’ [handwritten], ‘MCZ TYPE 35335’ [partly handwritten, on red paper], ‘Aug–Dec 2004 MCZ Image Database’, ‘MCZ-ENT 00035335’ [printed with matrix code], ‘LECTOTYPE *Ochtedromus mundus* LeConte designated D.R. Maddison’ [partly handwritten, with red border]. Type locality: San Jose, Santa Clara County, California.

**Diagnosis:** A relatively small, narrow species of intermediate darkness (Figs 1C, 7C, 9C). Length 3.1–3.9 mm (most specimens 3.4–3.6 mm). Head, pronotum and abdomen black or piceous, although some southern specimens (in the Los Angeles basin and adjacent mountains) have dark castaneous pronota. Antennae with at least articles 5 through 11 infuscated, with dis-engraved (although very finely so) from near hind basin area, however, the humeral macula is more extensive, and extends onto the third elytral interval, and does not extend anteriorly on intervals 3 and 4. In some specimens from the Los Angeles basin area, however, the humeral macula is more extensive, and extends onto the third elytral interval. However, in contrast to *B. darlingtonielum*, there is never an isolated dark patch on intervals 5 and 6. The pronotum is narrower than in *B. horni* and *B. orion*, and is similar in form to *B. darlingtonielum*. Microsculpture absent from elytral discs, but well-engraved (although very finely so) from near hind margin of subapical macula to elytral apex, with distinct sculpticells. In this way *B. mundum* is distinct from *B. darlingtonielum* as in the latter the elytral apex is very shiny, and lacks well-engraved microsculpture. Flagellar complex of aedeagus moderately long and straight (Fig. 13E, F), much longer than in *B. darlingtonielum*, second in length only to *B. festivum* and *B. cooperi*. About 600 specimens examined.

**Geographic distribution:** One of the most widespread *Liocosmius*, occurring from southern California north to southern British Columbia, east to Idaho and Nevada (Fig. 17). The record in Bousquet (2012) from Coconino County, Arizona (CMNH) is based upon *Bembidion cooperi*, and the same is likely true of the Snow (1906) record. The record in Bousquet (2012) from Grand County, UT, is based upon specimens of *B. horni*. The record in Tanner (1928) is also likely based upon specimens of *B. horni*.

**Spatial relationships to other Liocosmius:** We have found *B. mundum* on the same shorelines as *B. horni*, *B. darlingtonielum*, *B. f. festivum*, and *B. f. hilare*, and it is found at the same locality as *B. orion* (Table 4). It is allopatric only with *B. cooperi*.

**Geographic variation:** As noted in the diagnosis, the specimens from the Los Angeles basin and adjacent mountains are paler than specimens north of this region.

**Bembidion darlingtonielum** Cooper and Maddison, sp. nov.

*Ochtedromus* (Figs 1D, 7D, 9D, 12G–H, 13G–H, 18)

**Holotype** male (in OSAC), here designated, labelled: USA: California: Lake Co., North Branch Cache Creek at hwy 20, 305 m, 38.9881°N 122.5400°W, 5.viii.2010. DRM 10.089. D. R. Maddison & K. W. Will’, ‘David R. Maddison DNA2617 DNA Voucher’ [printed on pale green paper], and ‘HOLOTYPE *Bembidion darlingtonielum* Cooper + Maddison’ [partly handwritten on red paper]. Genitalia mounted in Euparal on small card (with DNA2617 written on it) beneath specimen; extracted DNA stored separately. GenBank accession numbers for DNA sequences of the holotype are KJ624165 (28S), KJ624325 (COI), KJ624214 (CAD), KJ624280 (Topo), KJ624248 (ug), and KJ624258 (ArgK). Type locality: USA: California: Lake Co., North Branch Cache Creek at highway 20, 38.9881°N 122.5400°W.

**Paratypes:** 44 specimens from the following localities, deposited in OSAC, CAS, CSCA, EMEC, USNM, BMNH, MNHN, and CNC: USA: California: Yolo Co., Putah Creek, 38.5067°N 122.0427°W (6), USA: California: Yolo Co., Cache Creek at road 57, 129 m, 38.8238°N 122.184°W (1), USA: California: Lake Co., North Branch Cache Creek at hwy 20, 305 m, 38.9881°N 122.544°W (37).

**Derivation of specific epithet:** It gives us great pleasure to name this species after the late Philip J. Darlington, Jr., carabid systematist and biogeographer, and friend of KWC.

**Diagnosis:** Intermediate between *B. mundum* and *B. festivum* in size and colour (Figs 1D, 7D, 9D). Similar in body colour to *B. mundum*, but with pale antennae typical of *B. festivum*. Length 3.4–4.1 (most specimens 3.7–4.1 mm). Head, pronotum and abdomen black or piceous, although some southern specimens (in the Los Angeles area) have dark castaneous pronota. Antennae testaceous or pale rufous. Elytra with lateral
margins and epipleura pale. Humeral macula extends medially at least onto third elytral interval; macula on third and fourth intervals extended anteriorly in many specimens, thus isolating a dark patch on intervals 5 and 6 (Fig. 9D); northern specimens (e.g., north of San Francisco) are darker, and some do not have an isolated dark patch. Pronotum narrow, similar to that of B. mundum. Microsculpture absent from elytral disc, at most feebly and irregularly engraved close to elytral apex. Flagellar complex of aedeagus shorter than in B. mundum (Fig. 13G, H).

This species is most likely to be confused with B. mundum or B. festivum. In the southern end of the range (Los Angeles basin and surrounding mountains), where all three species have relatively pale members, B. darlingtonielum has only two discal setae on each elytron (in contrast to three in B. festivum festivum), and has very shiny elytral apices with no or extremely weak microsculpture (in contrast to B. mundum), and has the isolated subhumeral dark patch (Fig. 9D, in contrast to B. mundum). In the centre of its range (north of Los Angeles County but south of San Francisco Bay area), the two discal setae and shiny elytral apices are still distinctive. From San Francisco north the paler antennae and shinier elytral apices distinguish B. darlingtonielum from B. mundum, and the lack of discal microsculpture and darker pronotum distinguishes it from B. festivum hilare. The flagellar complex of the aedeagus should be examined if in doubt.

About 80 specimens examined.

Geographic distribution: Known only from the western half of California, from southern California north to near Clear Lake in Lake County (Fig. 18).

Spatial relationships to other Liocosmius: B. darlingtonielum has been found on the same shorelines as B. horni, B. mundum, B. f. festivum, and B. f. hilare (Table 4). It is allopatric with B. orion and B. cooperi.

Geographic variation: As noted in the diagnosis, northern specimens are darker than specimens from the south.

Bembidion festivum Casey, 1918: 45. Lectotype female, designated by Erwin (1984: 172), in USNM [# 36874], labelled ‘Cal.’ [underlined, with a stroke through the C], ‘festivus Csy’ [handwritten], ‘TYPE USNM 36874’ [partly handwritten, on red paper], ‘LECTOTYPE Bembidion festivum Csy By Erwin ’77’ [partly handwritten, with a male symbol]. Genitalia mounted in Euparal on small card (with ‘hilare type’ written on it) beneath specimen. Type locality: Santa Barbara, Santa Barbara County, California.

Bembidion hilare Casey, 1918: 44. Lectotype male, designated by Erwin (1984: 173), in USNM [# 36873], labelled ‘Cal.’ [with a red dot to the left of the C], ‘CASEY bequest 1925’, ‘hilaris Csy’ [handwritten], ‘TYPE USNM 36873’ [partly handwritten, on red paper], ‘LECTOTYPE Bembidion hilare Csy By Erwin ’77’ [partly handwritten, with a male symbol]. Genitalia mounted in Euparal on small card (with ‘hilare type’ written on it) beneath specimen. Type locality: Cloverdale, Sonoma County, California.

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Contrary to Lindroth (1963: 344), the type specimen of *Bembidion hilare* Casey does not belong to *B. mundum*, but rather to the subspecies here called *B. festivum hilare*. The type is teneral, with most of the left elytron missing, and thus it is more difficult to determine its characteristics. However, the shoulder macula is too extensive for *B. mundum*, and there is clear microsculpture on the elytral disc around ed3.

Figure 15. Male genitalia, right side. All to same scale (but different scale than Fig. 13); scale bar 0.1 mm. The flagellar complex is emphasized by an overlay of thin black lines showing its most prominent edges. *B. festivum festivum* (A: DNA2303), *B. festivum hilare* (B: DNA2688), *B.cooperi* (C: DNA2116, D: DNA2171). Photographs reprinted with permission, copyright David Maddison, released under a Creative Commons CC-BY 3.0 licence.

Figure 16. Distributions of *Bembidion horni* and *B. orion*. Black symbols: localities of specimens with sequenced DNA; grey symbols: all other specimens. Larger symbols surrounded by red or grey indicate type localities. The ‘?’ indicates the location of the Piute Mountains, a doubtful record of both *B. horni* and *B. orion* (see text).
More definitively, the aedeagus matches that of *B. festivum* and *B. cooperi* rather than *B. mundum*: the brush sclerite is of the same shape as in *B. festivum*, and the flagellum, while difficult to see because of the lack of sclerotization, is large and curved and the visible region matches that of *B. festivum*. While still in glycerine, the shape of the flagellum, and its match to that of *B. festivum*, was more evident.

Contrary to Lindroth (1963: 344) and Bousquet (1997: 331), the type specimen of *Lopha bifasciata* Motschulsky also belongs to *B. festivum hilare*. Motschulsky’s type has the transverse elytral

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**Figure 17.** Distribution of *Bembidion mundum*. Black symbols: localities of specimens with sequenced DNA; grey symbols: all other specimens. Larger symbols surrounded by red or grey indicate type localities.
microsculpture and colour pattern typical of B. f. hilare.

There are specimens in collections (at least CAS and CSCA) of B. festivum hilare labelled as ‘Bembidion (Liocosmius) festivum ssp. insculptum Cooper’, including one labelled as a holotype, and others as paratypes; this name should be considered a nomen nudum.

**Diagnosis:** A large, pale species (Figs 2A, 2B, 7E, 10A, B). Length 3.5–4.5 mm (most specimens 3.8–4.2 mm). Head, pronotum and abdomen brunneous or castaneous. Antennae testaceous or pale rufous. Elytra with lateral margins and epipleura pale. Elytral maculae variable, but with a distinctly comma-shaped subapical macula. Hind angles of prothorax obtuse, in most specimens slightly rounded and with lateral edge of pronotum just in front of hind angle diverging anteriorly (Fig. 8A–D). With faint but well-engraved microsculpture on disc of elytra (Fig. 2D) in B. festivum hilare, lacking in B. festivum festivum. With two discal setae on each elytron in B. festivum hilare, or three in B. festivum festivum (Fig. 10B). Flagellar complex of aedeagus very large, sinuate (Fig. 15). Most similar to B. cooperi, from which it differs most distinctly by DNA sequences (see under B. cooperi); the only consistent morphological difference observed is the more obtuse hind angle of the prothorax in B. festivum. About 300 specimens examined.

**Geographic distribution:** From southern California north to Corvallis, Oregon (Fig. 19). In central California known only from west of the Central Valley, except for the possible exception of the Highway 180 locality (see below, under ‘Geographic variation’).

**Spatial relationships to other Liocosmius:** B. festivum has been found on the same river or creek bank as B. horni, B. mundum, and B. darlingtonielum (Table 4). It is allopatric with B. orion and B. cooperi.

**Geographic variation:** This species occurs in two geographically distinct forms, which we are treating as two subspecies. Specimens of the southern form (B. festivum festivum, Fig. 19, squares) are very shiny, lacking discal microsculpture on the elytra, and they generally possess three or more discal setae on each elytron. The extra discal setae are of remarkably variable position, on some elytra occurring near the anterior discal setae, in others near the posterior. The number of setae is also variable. In the 57 specimens examined from El Casco along San Timoteo Canyon, Riverside County, California (CSCA), 48 specimens have three setae on each elytron (abbreviated as ‘3/3’), five specimens are 3/4, one is 4/4, one is 4/5, and only two specimens have less than three setae on one side, but they have three on the other side (both specimens are 2/3). In the north, B. festivum hilare adults have fine but evident microsculpture on the elytral disc (Fig. 2D), and have two discal setae on each elytron. Specimens of B. festivum festivum from the Los Angeles basin area are much paler than those of B. f. hilare (compare Fig. 10B to Fig. 10A), but the geographic pattern of colour does not match those of the other traits, as the colour of B. f. festivum specimens from the northern end of its range are similar in colour to those of B. f. hilare. The specimens for these two forms show no consistent differences in 28S, COI, and CAD sequences, but do have two third-position sites in Topo at which they show consistent synonymous differences.

Almost all known specimens of B. festivum hilare are from north of San Francisco, and all specimens of B. festivum festivum from south of the Bay Area. The exception is a series of seven specimens labelled ‘Hwy 180, Bridge 96-16’, collected by KWC in 1975. These
are evidently from Fresno County, as highway 180 does not run through Madera County; the labels state 'Madera County', but KWC’s original notes in his notebook lists these as from Fresno County. Other notes by KWC mention this locality as being ‘to Kings Canyon’, which suggests that the locality is on highway 180 east of Fresno. However, there is no bridge in California whose number is ‘96-16’, nor was there one in 1975 (Tim Sandoval, California Department of Transportation, and Wendy Nakagawa, Public Works and Planning, Fresno County, pers. comm.). ‘96-16’ might refer to postmile position, but there is no bridge near postmile 96 along highway 180, nor was there likely a bridge at postmile 96 in 1975 (Tim Sandoval, pers. comm.). Of the bridges that cross water along highway 180, the site that appears to have the most typical Liocosmius habitat is at Kings River just east of Centerville, CA. These seven specimens are almost typical \( B. f. hilare \), with clear discal microsculpture (although less engraved than most other \( B. f. festivum \) specimens), and with all but one of the specimens having two discal setae on each elytron.

Figure 19. Distributions of \( Bembidion festivum \) and \( B. cooperi \). Black symbols: localities of specimens with sequenced DNA; grey symbols: all other specimens. Larger symbols surrounded by red or grey indicate type localities. The open circle represents a possible location for the ‘Highway 80 Bridge 96-16’ specimens of \( B. festivum hilare \) (see text). The ‘?’ indicates the location of the Piute Mountains, a doubtful record of \( B. festivum \) (see text).
(the seventh specimen is 2/3). Wherever along highway 180 these are from, the locality is east of other *B. festivum festivum*, and well south of other known *B. festivum hilare*. In Figure 19 this uncertain locality is marked with an open circle.

**Bembidion cooperi Maddison, sp. nov.**

(Figs 2C, 7F, 8E–H, 10C, 14C–D, 15C–D, 19)

**HOLOTYPE** male (in OSAC), here designated, labelled ‘USA: New Mexico: Grant Co., Gila River at route 211, Gila, 1370 m, 32.9691°N 108.5872°W, 12.viii.2005. DRM 05.046. D. R. & J. H. Maddison, A. E. Arnold’, ‘David R. Maddison DNA2116 DNA Voucher’ [printed on pale green paper], ‘VOUCHER 05.046-2 David R. Maddison’ [partly handwritten on pale pink paper], and ‘HOLOTYPE Bembidion cooperi Maddison’ [partly handwritten on red paper]. Genitalia mounted in Euparal on small card (with DNA2116 written on it) beneath specimen; extracted DNA stored separately. GenBank accession numbers for DNA sequences of the holotype are KJ624155 (28S), KJ624315 (COI), KJ624204 (CAD), KJ624270 (Topo), and KJ624362 (MSP). Type locality: USA: New Mexico: Grant Co., Gila River at route 211, Gila, 1370 m, 32.9691°N 108.5872°W.

**Paratypes:** 68 specimens from the following localities, deposited in OSAC, USNM, BMNH, MNHN, CAS, CSCA, CMNH, and UAIC: USA: New Mexico: Catron Co., San Francisco River, 5.7 mi N of Alma, 1525 m 33.4519°N 108.9253°W (1); USA: New Mexico: Catron Co., Reserve, San Francisco River, 1750 m, 33.7167°N 108.7571°W (3); USA: New Mexico: Grant Co., Gila River near Gila, 1370 m, 32.9692°N 108.5868°W (9); USA: New Mexico: Grant Co., Gila River near Cliff, 1350 m, 32.9124°N 108.5897°W (2); USA: Arizona: Yavapai Co., Oak Creek nr Baldwins Crossing, 1215 m, 34.8233°N 111.799°W (20); USA: Arizona: Coconino Co., Sedona (23); USA: Arizona: Coconino Co., Oak Creek Canyon, 6000 ft. (1).

**Derivation of specific epithet:** It gives DRM great pleasure to name this species after his late co-author, Kenneth W. Cooper, who loved *Bembidion*, especially subgenus *Liocosmius*. Kenneth spent decades collecting and examining *Liocosmius*, and had filled many notebooks with drawings and other observations about variation within the group. He had seen specimens of this species from Sedona, Arizona, and thought they were likely *B. festivum*, but had entertained the possibility that they might be distinct. He was very excited about the new insights to be gained with molecular data; it is fitting to name a species after him that was discovered from differences in DNA sequences.

**Diagnosis:** A large, pale species (Figs 2C, 7F, 10C), very similar in appearance to *B. festivum hilare* or dark *B. festivum festivum*. Length 3.7–4.5 mm. Head, pronotum and abdomen brunnceous or castaneous. Antennae testaceous or pale rufous. Elytra with lateral margins and epipleura pale. Pale areas of elytra less extensive (Fig. 2C) than *B. festivum festivum*, but similar to *B. festivum hilare*. Hind angles of prothorax forming a right angle or are slightly acute, with lateral edge just in front of hind angle parallel to main body axis (Fig. 8E–H). In contrast to *B. festivum hilare*, there is no microsculpture on disc of elytra. In contrast to *B. festivum festivum*, with only two discal setae on each elytron. Flagellar complex of aedeagus very large, sinuate, similar to *B. festivum* (Fig. 15). 68 specimens examined.

In contrast to the slight morphological differences, *B. cooperi* is rather distinct from *B. festivum* in the sequenced genes. For example, within CAD, *B. cooperi* differs from *B. festivum* at three sites (sites 6, 105, and 350 in the aligned matrix). The difference at site 350 (*B. cooperi* has an A and *B. festivum* has a G) codes for a difference in amino acids: *B. cooperi* has a lysine at this site, whereas *B. festivum* has an arginine. Within 28S, there are seven sites at which the two species show a base substitution between them, as well as three insertion-deletion differences. The most notable of these is an extra six bases (ATTTAC) in *B. festivum* at site 655 in the alignment. *B. cooperi* shows a unique signature in COI as well, differing from *B. festivum* at 23 sites.

**Geographic distribution:** Known from upper Gila River watershed, from Oak Creek around Sedona, Arizona, and western New Mexico along the Gila and San Francisco Rivers, from 1200 to 1750 m in elevation.

**Spatial relationships to other Liocosmius:** *B. cooperi* has been found on the same river bank as *B. horni*, but is allopatric with the remaining species of *Liocosmius* (Table 4).

**Geographic variation:** No morphological variation was noted within this species. In the genes sequenced, COI shows a consistent difference at two sites between Arizona and New Mexico specimens. The different variants code for the same amino acid in the two populations.

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**SHARED DATA**

Data available from the Dryad Digital Repository (Maddison & Cooper, 2014).