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Copeia 107, No. 2, 2019, 208–218

Redescription and Recognition of *Etheostoma cyanorum* from Blue River, Oklahoma

William J. Matthews[1] and Thomas F. Turner[2]

***Etheostoma cyanorum*, endemic to the Blue River drainage of southern Oklahoma, is redescribed and recognized as a distinct species within the *Etheostoma whipplei–Etheostoma radiosum* complex, separating it from *E. radiosum*. Originally described as *Poecilichthys radiosus cyanorum*, it was one of three putative subspecies of *E. radiosum* (with *E. r. radiosum* and *E. r. paludosum*) considered valid until now, defined in part by drainage-specific allopatry. Two separate mtDNA gene trees show that *E. cyanorum* forms a distinct and strongly supported lineage. Ten meristic and 16 morphometric traits are reexamined and new information included, confirming traits separating *E. cyanorum* from *E. radiosum*, and clarifying ambiguities about "bluntness of the snout" as diagnostic for *P. r. cyanorum*. *Etheostoma cyanorum* differs from *E. radiosum* by lower counts of unpored lateral line scales, higher counts of pored lateral line scales, and greater interorbital width. Large adult *E. cyanorum* have a deep body and blunt snout per earlier studies, but those traits are not diagnostic due to allometry. Head depth and head width can separate *E. cyanorum* from most populations of *E. radiosum*, but they overlap with some populations of *E. radiosum* in southwest Arkansas. All evidence supports recognition of *E. cyanorum* as a valid species. A broad geographic, molecular assessment to supplement existing morphological information is needed to assess validity of the two remaining subspecies of *E. radiosum*.**

We redescribe and recognize *Etheostoma cyanorum* as a valid species, separate from *Etheostoma radiosum* (Orangebelly Darter; Moore and Rigney, 1952; Collette, 1965; Matthews and Gelwick, 1988), within the larger *Etheostoma whipplei* (Redfin Darter) species complex, or clade (Retzer et al., 1986; Lang and Mayden, 2006; Near et al., 2011). We review its taxonomic history and recent treatments, supplement and reevaluate previous morphological data, and add molecular genetic data supporting specific recognition. *Etheostoma cyanorum* is known only from the Blue River drainage in southcentral Oklahoma, USA, a tributary of Red River. The range of *E. radiosum*, minus *E. cyanorum*, includes tributaries of the Washita River west of Blue River, and all south-flowing drainages in Oklahoma or Arkansas east of Blue River from the Clear-Muddy Boggy drainage to the upper Ouachita River basin in Arkansas (Fig. 1). In the Saline River of the Ouachita River basin (note there are two "Saline" rivers in Arkansas) *Etheostoma artesiae* (Redspot Darter) represents the clade. *Etheostoma whipplei* occurs north of *E. cyanorum* and *E. radiosum*, limited in the region to the Arkansas River basin (Retzer et al., 1986; Piller et al., 2001; Robison and Buchanan, 1988, in press). Thus, the four species (including *E. cyanorum*) of the *E. whipplei* clade exhibit near complete allopatry, with a narrow zone of sympatry between *E. radiosum* and *E. artesiae* above the Fall Line in the Red and Ouachita river drainages (Piller et al., 2001). But in hundreds of samples from southwest Arkansas or southeast Oklahoma by WJM since 1976, no two of the species have been found together.

The Redfin Darter complex, and *Etheostoma radiosum cyanorum* (Moore and Rigney, 1952) in particular, have a complicated taxonomic history (details in Supplemental Text A; see Data Accessibility). The first species described in the group was *Boleichthys whipplii* (now = *E. whipplei*) from Coal Creek, Indian Territory (now Pittsburg County, Oklahoma; Girard, 1859). Jordan and Gilbert (1886) collected specimens within the range of the future *E. radiosum* in the upper Ouachita River basin in Arkansas, reporting them as *Etheostoma whipplei* (amended spelling) but noting a "lack of red spots" (unlike *E. whipplei*). The first collections of *E. r. paludosum*, as "*E. whipplei*," later described as *Poecilichthys radiosus paludosus* (Moore and Rigney, 1952), were in the Kiamichi River drainage by Seth E. Meek in 1894 (Meek, 1896), and by H. A. Pilsbry in 1903 in the Muddy Boggy drainage (Fowler, 1904). Thus by 1904, *Etheostoma whipplei* (or *whipplii*) was viewed as one species in Oklahoma (then Indian Territory), Arkansas, and extreme north Texas, but with considerable variation noted. Ortenburger and Hubbs (1926) reported *Poecilichthys whipplii* (spelled with two "ii"s) from a collection on 19 June 1925 in Yanubbe Creek, McCurtain County, Oklahoma, comprising the first collection of *E. radiosum* from the Little River basin. The Blue River drainage was not sampled by ichthyologists until the 1940s, thus *P. r. cyanorum* (Moore and Rigney, 1952), now *E. cyanorum*, remained undetected.

Hubbs and Black (1941) recognized *Poecilichthys whipplei radiosus*, from the Caddo River, Arkansas, as a new subspecies. However, Hubbs and Black (their map, p. 3) included specimens from the (eastern) Saline River in Arkansas and rivers in Texas, now known to have *Etheostoma artesiae* (Piller et al., 2001) instead of *E. radiosum*. Moore and Rigney (1952) elevated *Poecilichthys radiosus* to species and provided diagnoses for three new subspecies: *P. r. cyanorum* from Blue River, *P. r. paludosus* from the Clear Boggy and Kiamichi systems, and *P. r. radiosus* throughout the rest of the range to the east. Moore and Rigney (1952) and Scalet (1971) diagnosed the Blue River subspecies as having a blunter and more "decurved" snout, deeper head, a "larger, heavier body" (but see Results about validity of these traits), fewer unpored scales, and more pored scales than other subspecies. Bailey et al. (1954) placed all darters in three genera, moving *Poecilichthys* into the genus *Etheostoma*.

Echelle et al. (1975) assessed lactate dehydrogenase LDH-1 and esterase ES-3 enzymes at multiple sites within each drainage in the range of *E. radiosum* as then known. For both LDH-1 and ES-1 Blue River fish differed substantially from

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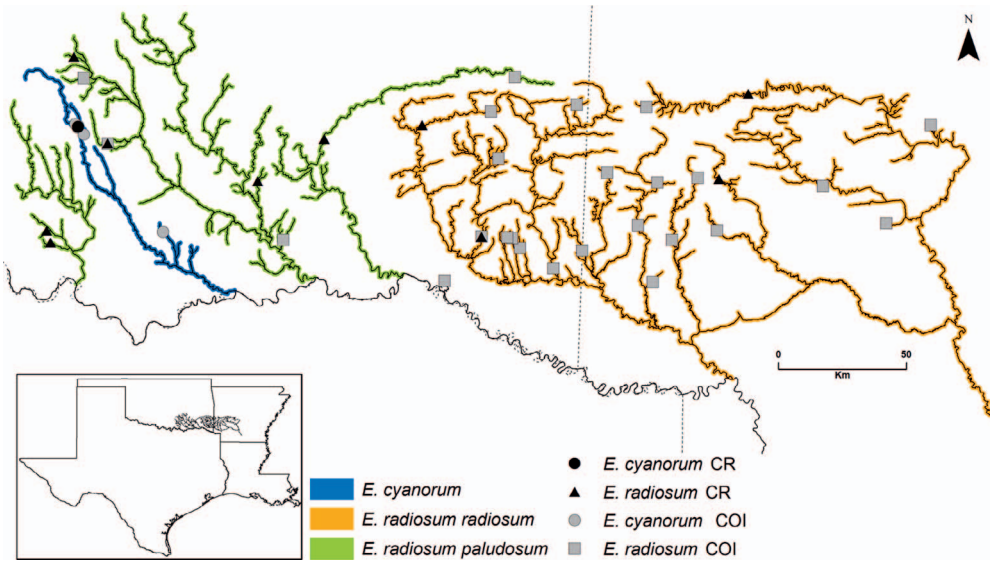

Fig. 1. Range map of *E. cyanorum* and *E. radiosum* in the south-central United States. *Etheostoma cyanorum* is found only in the Blue River drainage, Oklahoma, USA. Nominal subspecies *E. r. radiosum* is restricted to the Ouachita and Little River drainages to the east, and *E. r. paludosum* is restricted to the Kiamichi, Muddy Boggy, Clear Boggy systems, and tributaries of the Red River to the west. Drainages were identified by layering georeferenced collection data (http://www.fishnet2.net) into GIS software. MtDNA CR (black) and COI (gray) sampling localities are superimposed on drainages.

those in the nearby Muddy Boggy and Kiamichi drainages, but not from populations in southwest Arkansas (figures 1 and 2 of Echelle et al., 1975).

Retzer et al. (1986) compared *E. radiosum* (as then understood) to *E. whipplei*, reiterating color characteristics (red spots on flanks of *E. whipplei*) separating the two species. However, they noted a lack of any overall morphometric differences between the two species in a principal components analysis. Piller et al. (2001) recognized *E. artesiae* (Hay, 1881) as distinct from *E. whipplei* due to meristic differences and allopatry and noted the close taxonomic relationship of both to *E. radiosum*.

Matthews and Gelwick (1988) assessed the subspecies of Moore and Rigney (1952) with geographically dense sampling, including newly discovered *E. radiosum* in tributaries of the Washita River (Matthews et al., 1986). They recognized substantial morphological differences between specimens from Blue River (then considered *E. radiosum)* and all other populations including head depth and pored and unpored lateral line scale counts. Matthews and Gelwick (1988) recommended continued recognition of the three subspecies of Moore and Rigney (1952) pending further investigation of variation, and they considered the new populations from the Washita River drainage to align with *E. r. paludosum*.

Two papers from 2011 took very different approaches, but each has important implications for systematics of *E. radiosum* and *E. cyanorum*. Near et al. (2011) compared 245 North American darter (Percidae) species (*a priori* including as species several recognized subspecies) with one mitochondrial and two nuclear genes for a comprehensive darter phylogeny. Near et al. (2011 and their supplementary material) included as full species one individual each of *Etheostoma cyanorum* (from lower Blue River near Milburn, Oklahoma); "*Etheostoma paludosum*" (from Kiamichi River headwaters west of the Oklahoma–Arkansas border; and *Etheostoma radiosum* (from Caddo River south of Caddo Gap, Arkansas). Near et al. (2011, appendix) erected "*Vexillapinna*" as a "new clade name" that included *E. whipplei*, *E. artesiae*, *E. radiosum*, *E. cyanorum*, and *E. paludosum*. A tree (their figure 3) including all 245 darters, showed *E. artesiae* sister to a group with all other species in the *Vexillapinna* clade, within which *E. paludosum* and *E. cyanorum* were sister, and *E. radiosum* and *E. whipplei* (in part) were sister. However, the analyses of Near et al. (2011) were based on a single individual of each of the three nominal subspecies of *E. radiosum*, thus did not address geographic variation within those taxa. Nevertheless, Near et al. (2011) represents the first time in the literature that species level names were applied to the recognized subspecies of *E. radiosum* (Page, 1983; Matthews and Gelwick, 1988).

April et al. (2011) used "DNA barcoding," following mtDNA protocols of the Fish Barcode of Life Campaign, for 752 species of North American fishes, to seek evidence of unrecognized "cryptic diversity." Their study included a large number of "*E. radiosum*" across its range including fish from Blue River (now = *E. cyanorum*), but not populations in the Washita River system (Matthews et al., 1986). Even with those western populations excluded, April et al. (2011, and their supporting information) detected a high degree of cryptic diversity within *E. radiosum*, including five separate clusters representing genetically dissimilar taxa. They noted (p. 10606) that such clusters might represent new species but called them "unconfirmed candidate species."

The results of Near et al. (2011) and April et al. (2011) provide contrasting views of possible systematics within the *E. radiosum* complex. Near et al. (2011: p. 569) accepted *a priori* that previously named subspecies of *E. radiosum* were full species, because those "allopatrically distributed subspecies exhibit morphological differences on the order of those observed between described species." In contrast, April et al. (2011; specimen data available at http://www.barcodinglife.com) used broad geographic sampling across most of the known range of *E. radiosum*. Five clusters (=their "BIN"s) from their analyses showed patterns important for taxonomy of the complex. Their most important finding relative to the current study was that their BIN (BOLD.AAA3140) was restricted to 15 specimens from multiple locations in Blue River, representing *E. cyanorum*, and no Blue River material was in any other BINs. Their BIN (BOLD.ABZ2860) contained 49 individuals from a geographically broad region, from the Clear and Muddy Boggy system in south Oklahoma to the Ouachita drainage near Hot Springs, Arkansas. A fourth BIN (BOLD.ABZ2859) included 46 individuals from a geographically limited area comprising the Little River basin in southeast Oklahoma and southwest Arkansas, but excluding the Cossatot River, from which all individuals were in a separate, fifth BIN (BOLD.AA3138). In summary, April et al. (2011) found all material from Blue River uniquely contained in one BIN, supporting designation of *E. cyanorum* as a distinct species.

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In the second edition of the *Peterson Field Guide to Freshwater Fishes of North America North of Mexico*, Page and Burr (2011) included *E. artesiae*, *E. radiosum*, and *E. whipplei* as valid species, noting *E. radiosum* had the three named subspecies from Moore and Rigney (1952). Finally, given all available information at the time of deliberation by the Committee on Common and Scientific Names of Fishes, Page et al. (2013) recognized *E. artesiae*, *E. radiosum*, and *E. whipplei* as distinct species, but not *E. r. cyanorum* or *E. r. paludosum*. We now recommend recognition of *E. cyanorum* as a valid species but withhold comment on *E. r. paludosum* or the nominal subspecies until a more detailed geographic molecular study of *E. radiosum* is completed.

MATERIALS AND METHODS

Morphology.—Meristic counts and measurements for specimens in all drainages having *E. radiosum* or *E. cyanorum* were taken from original, paper datasheets on which counts and measurements were recorded by hand for Matthews and Gelwick (1988). We checked a computerized database against those original datasheets, made a few minor corrections detected as typos, and re-examined results from Matthews and Gelwick (1988). Ten counts and 16 measurements (Supplemental Table A; see Data Accessibility) followed Hubbs and Lagler (1964) as modified by Retzer et al. (1986). MedCalc Version 18.9.1 (MedCalc for Windows Version 18.9.1, MedCalc Software, Ostend, Belgium) was used on 331 specimens for meristics and 320 for measurements to calculate mean, standard deviation, and 95% confidence intervals for the mean (equivalent to mean$\pm$2 standard errors) for *E. cyanorum* in the Blue River drainage and for 13 populations of *E. radiosum* across its entire range. Supplemental Table A (see Data Accessibility) and MedCalc calculations included 40 *E. cyanorum* for which all meristic counts were complete and 39 *E. cyanorum* for which all measurements were complete.

Additional morphological data.—In addition to counts from Matthews and Gelwick (1988), pored and unpored lateral line scales were counted for another 40 adult males in nuptial color collected in Blue River in August 2017 (BRTP1 and BRTP2) to compare to the Matthews and Gelwick (1988) data for these putative diagnostic characters (per Moore and Rigney, 1952).

Another 11 small to large (38–54 mm SL) adult male (determined by dissection) *E. cyanorum* collected in Blue River in 1994 by E. Marsh (ECM 94-8) were used to assess shape of the head relative to a "blunt snout," considered diagnostic of *E. r. cyanorum* by Moore and Rigney (1952). We measured "angle of the muzzle" (per Hubbs and Black, 1941), which we now call "angle of anterior profile" (AAP) as a better descriptor, emphasizing the angle between the horizontal axis of the body and the predominant head profile. The AAP was measured by placing each fish on a horizontal line level with the anteriormost point of the snout and parallel to the midline of the body, then, viewing from directly above, drawing a straight line at the angle of the dominant slope of the anterior profile or "muzzle" of Hubbs and Black (1941), intersecting the horizontal line to determine the angle (AAP; Supplemental Figure A; see Data Accessibility).

Coloration.—Descriptions of coloration for specimens in all drainages having *E. radiosum* or *E. cyanorum* were taken from original color slides of freshly caught material made in the field by WJM, standardized field sheets on which color for fresh specimens was recorded on schematic outlines of fish by F. Gelwick, and comments in field notes supporting Matthews and Gelwick (1988). Another 40 adult males in nuptial color collected in Blue River in August 2017 (BRTP1 and BRTP2, OMNH 86858) were examined for details of the color pattern in the spinous dorsal fin to compare to descriptions of subspecies of *E. radiosum* in Moore and Rigney (1952) and Matthews and Gelwick (1988). Twelve additional adult *E. cyanorum* in peak nuptial color and one adult female were collected from Blue River upstream of Connerville, Oklahoma, on 30 March 2018 (WJM 3645), with the largest male (OMNH 86859) and the female (OMNH 86860) photographed in color by N. Lang, within 30 minutes of being narcotized, for the species description (below). The other 11 (OMNH 86861) were used by WJM to further assess the color pattern in the spinous dorsal fin with fresh material.

Genetic methods.—Fifty-two specimens were collected from tributaries of the Red River in southeastern Oklahoma and southwestern Arkansas, USA (Fig. 1). Sampling was focused on the Blue River and geographically adjacent drainages including the Clear Boggy River to the east, and tributaries of the Washita River to the west. Collecting was mainly in upland portions of drainages where *E. radiosum* is most abundant. Fishes were collected with a seine or backpack electrofisher, anesthetized in MS-222 on site, and either frozen in liquid nitrogen or preserved in 95% ethanol in the field. Quantity and quality of genomic DNA did not differ between sample preservation methods, and all samples were readily amplified by polymerase chain reaction (PCR).

Nucleic acids were isolated from white muscle or fin clips using proteinase-K digestion and phenol/chloroform extractions followed by ethanol precipitation and resuspension in distilled water (Turner, 1997). PCR was conducted with oligonucleotide primers L15926 and H16498 that target a $\sim$450 bp fragment of the mitochondrial control region (CR) flanked by the mtDNA proline tRNA gene (Kocher et al., 1989; Shields and Kocher, 1991). Amplification occurred in 50 µl reaction volumes containing 5 µl sample DNA template, 5 µl 10X reaction buffer, 200 µM dNTPs, 2 µM $MgCl_2$, 0.5 units *Taq* DNA polymerase, and primers added at a final concentration of 0.8 µM. Thermocycling consisted of an initial denaturation step at 94°C for 2 min, followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing at 48°C for 30 seconds, and extension at 72°C for 1 minute. PCR products were sequenced directly in both directions using the BigDye™ version 3.1 cycle sequencing kit and an ABI Prism 3130 capillary sequencer, in accordance with manufacturer's instructions. Fragments were concatenated using Sequencher version 4.9 (Gene Codes Corp., Ann Arbor, MI) and aligned manually.

In addition to CR data, we examined nucleotide sequence variation in the mitochondrial cytochrome oxidase I (COI) gene obtained from the Barcode of Life Database (BOLD) public data portal (http://www.boldsystems.org). COI data for *E. radiosum* were distributed in four BINs (AAA3138, $n = 5$; AAA3140, $n = 15$; ABZ2859, $n = 46$; ABZ2860, $n = 49$), and we excluded BIN AAA3139 ($n = 1$). Sequences for 115 individuals were downloaded into the MEGA version 7.0.26 software environment (Kumar et al., 2016). Sequence alignments were checked and adjusted by eye and translated to corresponding amino acid sequences using the vertebrate mitochondrial DNA code in MEGA.

CR and COI datasets could not be combined into a single phylogenetic analysis because sequences were not obtained

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Fig. 2. Holotype of *Etheostoma cyanorum*, as *P. radiosus cyanorum* (Moore and Rigney, 1952). Photograph courtesy of the Fish Division, University of Michigan Museum of Zoology.

from the same individuals and the geographic coverage of the datasets differed (Fig. 1). The CR dataset included Washita River drainage samples from Glasses and Little Glasses creeks that drain into Lake Texoma in the western-most portion of the range of *E. radiosum*. The COI dataset had denser coverage of eastern localities including Little River tributaries in southeastern Oklahoma and southwestern Arkansas (Fig. 1) but did not include the Washita drainage.

Etheostoma whipplei was selected as outgroup based on evolutionary affinity with *E. radiosum* (Piller et al., 2001; Near et al., 2011). A single CR sequence (GenBank accession no. U77029) sampled from Lee Creek in western Arkansas was included in alignments of *E. radiosum* and *E. cyanorum*. A total of 58 COI sequences from *Etheostoma whipplei* was downloaded (BIN AAA3316, $n = 58$) from the BOLD data portal, aligned with sequences of *E. radiosum* and *E. cyanorum*, then translated and checked in MEGA as above. In all cases, data matrices included only unique DNA haplotypes.

Phylogenetic analysis.—Initial phylogenetic analysis of nucleotide sequence variation in the CR was conducted using the maximum-likelihood (ML) search algorithm implemented in MEGA. We selected the best-fit model of nucleotide substitution using MODELTEST (Posada and Crandall, 1998) as implemented in MEGA. Once a substitution model was selected, searches for the most likely tree were conducted relative to an initial tree obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances, and then selecting the topology with highest log-likelihood value. Support for nodes in the CR gene tree was assessed by bootstrap resampling with replacement with 1000 replicates. Nucleotide sequence variation in the control region was visualized in a median-joining haplotype network (Bandelt et al., 1999) rendered in POPART version 1.7 (Leigh and Bryant, 2015). For COI data, we used similar ML methods, model-testing, and search strategies, with node support assessed via bootstrap resampling (1000 replicates).

In addition to analyses in MEGA, we estimated CR and COI gene trees in separate runs using MRBAYES v. 3.2.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) run in the CIPRES Portal v. 3.3 cluster environment at the San Diego Supercomputer Center. Best-fit models of nucleotide substitution (identified from MEGA) were used, and analyses were run for $1x10^7$ generations with sampling the of Markov chain every 1000 generations. This procedure resulted in 100,000 trees for each analysis, of which 10,000 were discarded as burn-in. Support for nodes was determined by posterior probabilities obtained from the majority-rule consensus tree that was visualized using the program FIGTREE version 1.4.2 (Rambaut, 2016). Three replicate runs were conducted with different starting trees to ensure consistency.

RESULTS

Etheostoma cyanorum (Moore and Rigney, 1952), elevated to species

Blue River Orangebelly Darter
Figures 2, 3; Tables 1, 2; Supplemental Table A

Holotype.—UMMZ 161366, holotype of *Poecilichthys whipplii cyanorum*, adult breeding male, 68 mm SL, designated by Moore and Rigney (1952), collected by Moore in 1949 in Blue

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Fig. 3. (A) Male *E. cyanorum,* 63 mm SL, in nuptial color, from mainstem Blue River, 1.7 km west-northwest of Connerville, OK, 30 March 2018 (WJM 3645; OMNH 86859); (B) female *E. cyanorum,* 41 mm SL, same collection (OMNH 86860). Location: 34°27.067′N, 96°39.327′W. Photographs by N. Lang. (C) Detail of distal color band in nuptial male from same collection, same date (OMNH 86861). Photograph by WJM.

River at State Hwy 99 north of Connerville, Johnston County, Oklahoma (Fig. 2).

Paratypes.—Paratypes include 329 individuals collected by Moore or students from the Blue River drainage (museum acronyms and catalog numbers, Moore and Rigney, 1952: p. 10).

Diagnosis.—*Etheostoma cyanorum* differs from *E. radiosum* by their allopatric distribution, with *E. cyanorum* known only from Blue River and tributaries, versus *E. radiosum* occupying tributaries of Washita River west of Blue River and all drainages eastward from the Clear Boggy to the upper Ouachita and Little Missouri in southwest Arkansas. *Etheostoma cyanorum* differs from *E. radiosum* in having all assayed mtDNA haplotypes not shared with *E. radiosum*, lower counts of unpored lateral line scales, higher counts of pored lateral line scales, and a wider interorbital distance. *Etheostoma cyanorum* can be distinguished by a deeper and wider head from *E. radiosum* in all Oklahoma drainages, and in most but not all drainages in Arkansas. Nuptial males of *Etheostoma cyanorum* differ from nuptial *E. radiosum* in the geographically closest drainages (Washita, Clear Boggy, Muddy Boggy, and Kiamichi) by a solid blue distal band in the spinous dorsal (Fig. 3C), compared to the distal blue band in those populations of *E. radiosum* (=*E. r. paludosum* of Moore and Rigney) having an appearance of blue "dots" or "spots" bordered in white, between clear or orange tips of the fin spines.

Description.—*Etheostoma cyanorum* is a moderately large darter of the clade *Vexillapinna* (Near et al., 2011) and subgenus *Oligocephalus* (Lang and Mayden, 2006), averaging about 45 mm standard length; largest specimens about 70 mm SL. Holotype meristics, morphometrics, and colors in Moore and Rigney (1952). Meristics and morphometrics from Moore and Rigney (1952) and Gelwick and Matthews (1988) compared in Supplemental Table A (see Data Accessibility). Snout decurved and blunt in large adults, less so in smaller individuals. Moore and Rigney (1952) noted, for unusually large holotype, "back is little elevated, sloping in almost a straight line to the caudal peduncle." In smaller individuals,

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Table 1. Meristic characters for *Etheostoma cyanorum* (Blue drainage), including minimum, maximum, mean, and standard deviation, compared to 13 populations of *Etheostoma radiosum*.

River drainage	Blue	Washita	Clear Boggy	Muddy Boggy	Kiamichi	Little	Glover	Mountain Fork	Rolling Fork	Cossatot	Saline	Ouachita	Caddo	Little Missouri
n	40	71	32	15	32	15	15	15	15	15	18	17	16	15
Unpored lateral line scales														
Minimum	0	3	3	7	4	6	9	9	8	7	6	8	9	5
Maximum	10	24	15	14	16	18	18	15	15	10	13	18	16	15
Mean	5.1	9.7	9.8	10.7	10.5	13.5	11.9	12.3	12.3	8.6	9.6	12.6	12.6	10.0
SD	2.0	3.1	2.7	1.7	2.8	3.2	2.4	1.8	2.2	1.2	2.0	3.2	2.2	2.6
Pored lateral line scales														
Minimum	43	33	39	39	39	42	39	43	36	40	38	36	33	34
Maximum	58	54	52	50	48	52	56	49	49	50	47	45	48	44
Mean	49.5	44.8	45.3	43.6	43.7	45.9	46.4	45.9	44.0	45.6	41.9	40.2	39.2	38.9
SD	4.0	3.5	2.9	2.9	2.4	2.7	3.7	2.1	3.3	2.4	2.6	3.0	3.4	2.9

head slopes upward at about a 20° angle from eye to occiput with pronounced hump at the occiput, dorsum then elevated about 15° from horizontal posteriorly to origin of dorsal fin, beyond which body is relatively uniform depth to origin of soft dorsal and anal fins, beyond which dorsum and caudal peduncle taper slightly to caudal base. Lateral line scales average 55; unpored lateral line scales average 5.9; pored lateral line scales average 49. Dorsal spines average 10.4; dorsal soft rays average 13.6; anal-fin soft rays 7 to 8; pectoral-fin rays typically 12; pelvic-fin rays invariant at 6. Gill rakers average 10. Average measurements (in thousandths of standard length): predorsal length 345; body depth 205; caudal peduncle depth 116; head length 269; head depth 170; interorbital width 54; eye diameter 67; gape width 73; snout length 69. Distribution of scales on head and body in Moore and Rigney (1952); tuberculation in Collette (1965); natural history and ecology in Scalet (1971).

Coloration.—Coloration as in Moore and Rigney (1952) and Scalet (1971), except nuptial male *E. cyanorum* have more yellow or yellowish-orange on cheeks and lower sides of head and body (Fig. 3A) than does *E. radiosum*, particularly *E. radiosum* in Ouachita River drainage where coloration is more reddish-orange (Matthews and Gelwick, 1988). Reproductive females drab in comparison to males; light yellow on cheeks and pectoral, anal, and caudal fins, and light orange below the opercle (Fig. 3B). Distal blue bar in spinous dorsal of nuptial male *E. cyanorum* solid band passing across the membranes and including the spine tips, except the two posterior spines (Fig. 3C).

Snout shape not diagnostic.—Moore and Rigney (1952) considered blunt snout diagnostic of *E. r. cyanorum*, but this is strongly influenced by body size. Table 1 of Moore and Rigney showed snout length of *E. cyanorum* went fewest times into head length compared to other subspecies,

Table 2. Morphometric characters for *Etheostoma cyanorum* (Blue drainage), including minimum, maximum, mean, and standard deviation, compared to 13 populations of *Etheostoma radiosum*.

River drainage	Blue	Washita	Clear Boggy	Muddy Boggy	Kiamichi	Little	Glover	Mountain Fork	Rolling Fork	Cossatot	Saline	Ouachita	Caddo	Little Missouri
Percents of standard length														
n	39	62	32	15	33	15	15	14	15	15	18	17	15	15
Interorbital width														
Minimum	4.5	2.9	3.4	3.9	3.6	4.1	4.0	4.1	4.1	4.0	3.9	4.7	4.4	4.2
Maximum	6.5	5.6	5.7	5.2	5.7	6.5	5.3	5.2	5.7	5.4	5.3	5.6	5.8	5.9
Mean	5.5	4.5	4.6	4.8	4.6	4.9	4.6	4.8	5.0	4.8	4.6	5.1	5.0	4.8
SD	0.5	0.4	0.5	0.3	0.5	0.6	0.3	0.3	0.4	0.4	0.4	0.3	0.5	0.4
Head depth														
Minimum	16.3	14.2	15.4	15.1	14.4	15.0	15.0	15.7	15.2	15.2	15.0	14.8	15.7	15.5
Maximum	19.3	17.5	18.7	17.6	17.5	17.0	17.8	17.8	17.7	17.5	19.1	18.7	18.2	18.5
Mean	17.9	15.8	16.9	16.3	15.9	15.8	16.8	16.3	16.4	16.5	17.4	17.1	17.1	17.3
SD	0.8	0.7	0.8	0.8	0.8	0.6	0.8	0.6	0.7	0.7	1.1	0.9	0.7	0.8
Head width														
Minimum	13.3	11.2	12.2	12.7	11.8	12.2	12.0	12.1	10.9	12.5	12.9	13.1	12.2	13.3
Maximum	16.4	15.3	15.5	14.3	14.6	14.0	14.1	14.8	14.5	15.0	16.2	15.7	16.2	16.6
Mean	14.7	13.6	14.0	13.5	13.0	13.2	13.4	13.2	13.4	13.9	14.7	14.6	13.8	14.8
SD	0.8	0.9	0.9	0.5	0.7	0.6	0.6	0.8	0.9	0.7	1.0	0.9	1.1	1.0
Body depth														
Minimum	18.0	16.0	18.5	18.4	17.0	19.1	17.6	19.1	17.9	19.2	18.6	18.9	18.1	18.8
Maximum	24.0	20.2	22.1	20.0	21.9	21.5	23.9	22.1	20.8	22.3	23.8	22.5	21.3	22.3
Mean	20.5	18.2	20.2	19.2	19.6	20.1	20.3	20.3	19.5	20.7	20.7	20.6	19.7	20.8
SD	1.2	0.9	1.0	0.4	1.4	0.8	1.7	0.8	0.7	1.0	1.4	0.9	1.1	0.9

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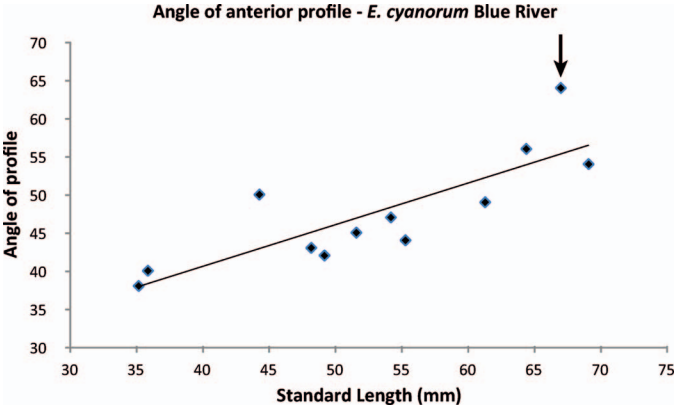

Fig. 4. Angle of the anterior profile for 11 male *E. cyanorum*, from Blue River in September 1994, with significant trendline, and the holotype (indicated by arrow; not included in regression or in trendline).

suggesting a longer, not shorter, snout length. Matthews and Gelwick (1988, their table 5) also found that specimens from Blue River had longest mean snout length, pointing out that a "blunt" snout is not necessarily equivalent to a "short" snout if the snout is greatly curved downward and is properly measured from eye to snout tip (on an angle) per Hubbs and Lagler (1964). Indication that a "blunt" snout was diagnostic (Moore and Rigney, 1952) was based on the large holotype (68 mm SL) described as "very robust," with a head "quite blunt" and "sharply decurved from the eyes to the snout tip." The holotype (Fig. 2) is larger than any of 300+ typically sized individuals evaluated by Matthews and Gelwick (1988; mean = 45, maximum = 64 mm SL). The holotype, although faded (Fig. 2), is well preserved, with extremely downturned snout, blunt face, and deep body per Moore and Rigney, but their description only applies to very large specimens. Of six nuptial males from Blue River August 2017, ranging 47 to 60 mm SL, only the largest (upper right in Supplemental Figure B; see Data Accessibility) approaches bluntness of snout described by Moore and Rigney (1952). For 11 males ranging 35 to 69 mm SL collected in the upper Blue River on 24 September 1994, larger individuals had a significantly more obtuse angle (regression of AAP on SL, $P = 0.0015$) between the horizontal axis of the body and the snout (Fig. 4). The holotype had a wider AAP (64°) between axis of the body than any specimens we examined (Fig. 4), confirming that the holotype was an individual with an unusually blunt snout.

Allometry.—Three other head or body traits have strong to moderate allometric trends. In Supplemental Figure C (see Data Accessibility), 26 male *E. cyanorum* used in Matthews and Gelwick (1988) from three locations in Blue River, traits (parts per thousand of standard length) are plotted against standard length with a trendline added by the Excel linear trendline function for significant regressions. Larger males had relatively shorter snouts (regression $P = 0.068$), consistent with the results in the section above, deeper bodies (regression $P = 0.011$), and a weak, but non-significant trend for a deeper caudal peduncle (Supplemental Figure C; see Data Accessibility).

Distribution.—*Etheostoma cyanorum* is known only from the Blue River drainage in southcentral Oklahoma, a tributary of Red River. It is most abundant in the upper, spring-fed, rocky portions of the drainage, and in some small tributary creeks, but is scarce or absent in lower, muddy portions of the drainage closer to Red River.

Etymology.—The specific name "*cyanorum*" (="of the Blues" referring to Blue River) was suggested for the subspecies, on advice from R. M. Bailey (Moore and Rigney, 1952), to reflect restriction of this form to Blue River and its tributaries. Linder (1955) and Echelle et al. (2015) referred to it as the "Blue River Orangebelly Darter," but Near et al. (2011) called it "Blue Darter." We follow Linder (1955) and Echelle et al. (2015) and recommend the common name "Blue River Orangebelly Darter," because *E. cyanorum* is not predominantly blue in coloration. This common name aligns with the practice of referring to other fishes in Oklahoma in a drainage-specific manner, including Red River Pupfish (*Cyprinodon rubrofluviatilis*), Red River Shiner (*Notropis bairdi*), and Arkansas River Shiner (*Notropis girardi*).

***Morphological comparisons with* Etheostoma radiosum.**—*Etheostoma cyanorum* differs from all populations of *E. radiosum* by fewer unpored lateral line scales and more pored lateral line scales (Table 1). Of 40 *E. cyanorum* and 291 *E. radiosum* from the original Matthews and Gelwick data, 80% of *E. cyanorum* had six or fewer unpored lateral line scales, compared to 95% of *E. radiosum* across all drainages having seven or more, often 8 to 16. For the additional 40 *E. cyanorum* collected in Blue River in 2017, 32 (=80%) had six or fewer unpored lateral line scales, in agreement with the earlier data. A mean of 5.1 unpored lateral line scales for *E. cyanorum* is fewer than for any population of *E. radiosum* (Table 1), and Supplemental Figure D-a (see Data Accessibility) shows that 95% confidence intervals around means were non-overlapping (=significantly different) between *E. cyanorum* and any population of *E. radiosum*. Pored lateral line scales with a mean of 49.5 also were significantly greater for *E. cyanorum* than for any population of *E. radiosum* (Table 1), separated by non-overlapping bars representing 95% confidence intervals (Supplemental Figure D-b; see Data Accessibility). Gill raker counts (not shown) were lowest for *E. cyanorum* but overlapped substantially with several populations of *E. radiosum* and are not diagnostic. No other meristic trait from Matthews and Gelwick (1988) separated *E. cyanorum* from *E. radiosum*.

The only morphometric trait statistically separating *E. cyanorum* from all *E. radiosum* is a wider interorbital width (Table 2), non-overlapping with any populations of *E. radiosum* at 95% confidence intervals (Supplemental Figure E-a; see Data Accessibility). Head depth of *Etheostoma cyanorum* was greater (Table 2) and non-overlapping at 95% confidence intervals (Supplemental Figure E-b; see Data Accessibility) for *E. radiosum* in any drainages except the Saline and Little Missouri drainages in Arkansas. Head width in *E. cyanorum* (Table 2 and Supplemental Figure E-c; see Data Accessibility) is greater than for any *E. radiosum* except in the Saline, Ouachita, and Little Missouri drainages. No other individual measurement traits separate *E. cyanorum* and *E. radiosum*, and Matthews and Gelwick (1988, their figure 4) found no separation of Blue River (now *E. cyanorum*) from *E. radiosum* in a sheared principal components analysis of 16 morphological measurements.

Moore and Rigney (1952) made a general statement (possibly based on the large holotype) that *P. r. cyanorum* has a "larger, heavier body" but did not document any supporting measurement such as body depth. The present analysis shows mean body depth of *E. cyanorum* (Table 2)

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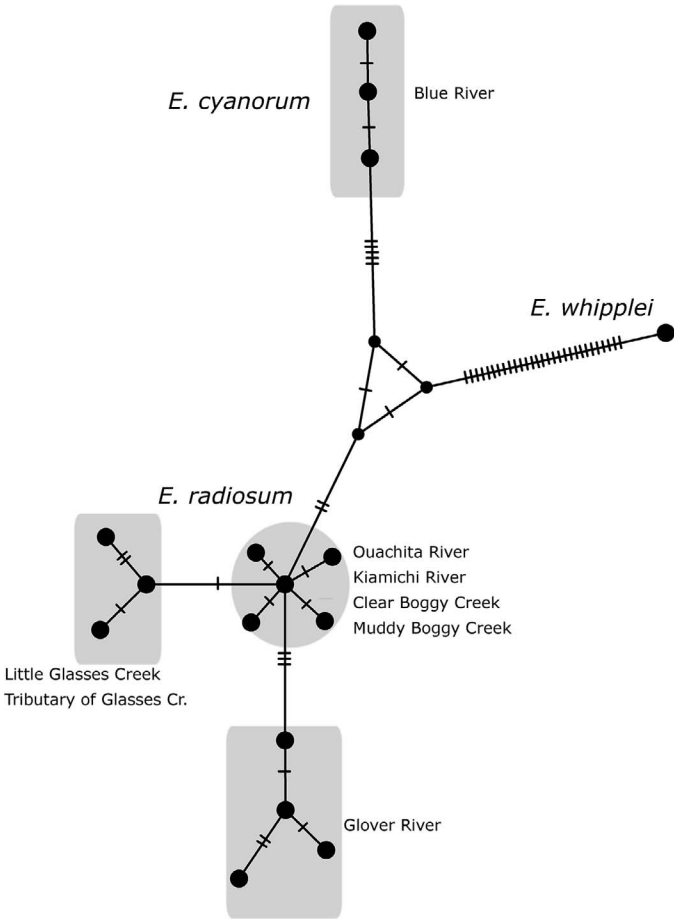

Fig. 5. A median-joining haplotype network depicting nucleotide sequence variation in the mtDNA control region (CR). Circles represent unique CR haplotypes and hash marks represent the number of nucleotide differences separating distinct haplotypes. Shaded polygons represent haplotypes grouped by drainage. Haplotypes of *E. cyanorum* are found only in the Blue River drainage. All haplotypes observed in the Caddo and Little Missouri rivers (not shown in figure) were also observed in the Ouachita River (shown in figure). *Etheostoma whipplei* is the outgroup taxon (GenBank accession no. U77029).

overlapping at a 95% confidence interval for nine of the 13 populations of *E. radiosum* (Supplemental Figure E-d; see Data Accessibility), so body depth does not separate *E. cyanorum* from *E. radiosum*.

Phylogenetic comparisons.—For the CR dataset, gene tree topology and branch lengths were inferred using the Tamura 3-parameter model (Tamura, 1992) that fit the data best as indicated by likelihood-ratio testing. A median-joining haplotype network illustrated nucleotide sequence differences of CR lineages (Fig. 5). For the COI dataset, the Kimura 2-parameter model fit best, and the gene tree with the highest log likelihood is shown in Figure 6. No stop codons or missing data were observed when COI sequences were translated, but there were a few samples where base pairs were missing at extreme 5′ or 3′ ends. These sites were deleted from the data matrix prior to phylogenetic analysis.

Maximum-likelihood and Bayesian gene tree topologies were identical for CR and COI datasets and support for recovered nodes was consistent, although Bayesian posterior probabilities offered slightly stronger support than bootstrap proportions. Likewise, considerable topological similarities were observed between CR and COI gene trees. Samples from the Blue River were identified as monophyletic and deeply divergent in all trees with strong support (99% and 100% bootstrap proportions, and 1.0 posterior probabilities for CR and COI trees, respectively). The CR and COI trees also indicated monophyly of haplotypes observed in the Little River Basin, but the extent of geographic sampling and the level of support differed across datasets. High bootstrap support (95% bootstrap support, 1.0 posterior probability) was observed in the CR tree (not shown) for the node joining lineages in the Little and Glover rivers. An unresolved node (66% bootstrap support, 0.74 posterior probability) was observed for samples from the Little, Mountain Fork, and Glover rivers in the COI tree. In all analyses, closely related haplotypes from the Ouachita, Caddo, and Little Missouri rivers in southeastern Arkansas and the Kiamichi and Clear-Muddy Boggy rivers in southeastern Oklahoma formed a large and geographically widespread lineage.

Topological differences that were observed across CR and COI datasets reflected differences in geographic sampling rather than analytical discordance. For example, the CR dataset included two distinct locations from the Clear Boggy drainage to the north and east, and Washita River tributaries to the west of the Blue River watershed (see Material Examined). Neither drainage shared haplotypes in common with the Blue River despite close geographic proximity. Rather, a distinct haplotype common to upper portions of the Clear Boggy River was shared with the Washita. Two Washita haplotypes were unique, but closely related to the common, shared haplotype (Fig. 5).

The COI dataset (Fig. 6) had more extensive representation of the Little River drainage than the control region dataset. A well-supported finding was monophyly (bootstrap support 99%, Bayesian posterior probability 1.0) and relatively deep divergence of samples obtained from the Cossatot River in Arkansas. This finding suggests a complex evolutionary history of populations in the Little River drainage that warrants more detailed investigation in future studies.

Habitat and ecological notes.—*Etheostoma cyanorum* is most common in rocky riffles and rapids of the Blue River mainstem and in riffles of larger tributaries to Blue River. Larger adults are generally in deeper and swifter parts of riffles with larger cobble, whereas smaller adults or juveniles are more abundant in slower currents with smaller substrate. There is noteworthy complementarity in microhabitat between *E. cyanorum* and the local form of Orangethroat Darter (*Etheostoma pulchellum*), which tends to occur in slower currents near shore, and more in spring runs than in the river mainstem (Echelle et al., 1974). See Scalet (1971) for comprehensive review of habitat, ecology, and behavior.

Since the 1950s, *E. cyanorum* has been known to hybridize with *E. pulchellum* in the Blue River mainstem (Linder, 1955; Branson and Campbell, 1969; Echelle et al., 1974). Collections by WJM in 2016 (Matthews et al., 2016), 2017, and 2018 confirmed that hybrids (with a range of anal fin coloration suggesting possible backcrossing) still exist. Most hybrids appear to be of the *E. pulchellum* body form, with orange in the anal fin ranging from a small spot to a larger, more suffuse orange blotch (in contrast to a blue anal fin in most Orangethroat Darters), and no individuals of apparent *E. cyanorum* body form observed in recent years (WJM) show physical evidence of traits from *E. pulchellum*, so hybridization or introgression may be mostly from *E. cyanorum* into *E. pulchellum*. However, this impression needs assessment using genetic tools.

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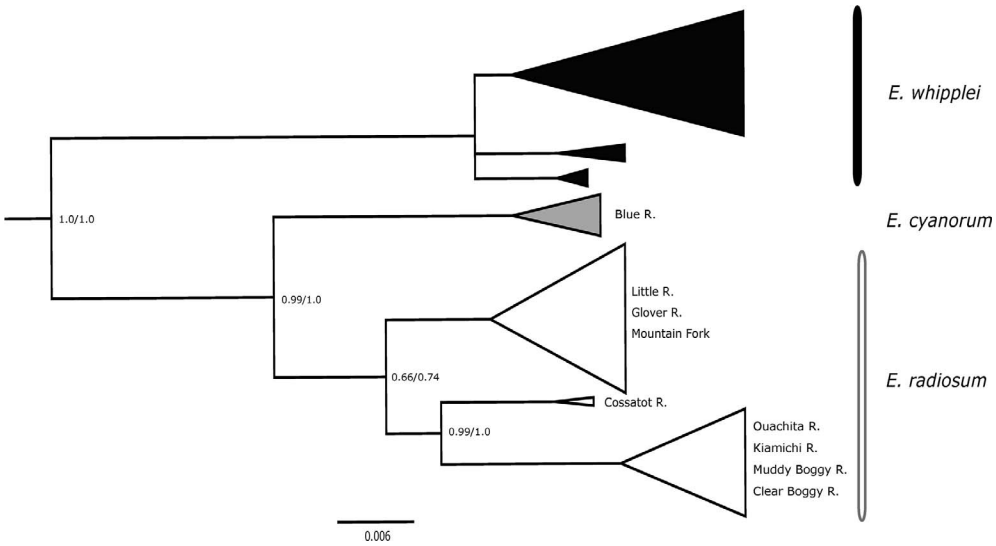

Fig. 6. A majority-rule consensus gene tree reconstructed from mtDNA cytochrome oxidase 1 (CO1) sequence data obtained from the Barcode of Life Database (BOLD). Maximum-likelihood (ML) and Bayesian trees had identical topologies. Branch lengths are proportional to inferred mutations. Shading of lineages represents samples from *E. whipplei* (outgroup) in black, *E. cyanorum* in gray, and *E. radiosum* in white. ML bootstrap proportions/Bayesian posterior probabilities are reported. See Data Accessibility for tree file.

DISCUSSION

Etheostoma cyanorum and *E. radiosum* have a long and complicated taxonomic history. The complex has been placed in several genera, confused with similar species (*E. artesiae*) in collecting accounts, and has had three subspecies described. Now, its allopatry with *E. radiosum*, and its distinctive morphology and unique genetics, warrant recognition of *E. cyanorum* as a full species. Evidence has been building for years that *E. cyanorum* is a species distinct from *E. radiosum*. Moore and Rigney (1952) recognized that it was morphologically the most divergent of their three putative subspecies, with extreme values for 13 of 15 meristic and morphometric traits. Echelle et al. (1975) showed in analyses of two enzymes that populations in the Blue River drainage differed markedly from those in nearby drainages. Morphological findings of Matthews and Gelwick (1988) could have justified recognition of *E. cyanorum*, but they chose to withhold recognition pending more evidence. That evidence now exists in the form of increased scrutiny about details of head shape, examination herein of additional morphological or color information since Matthews and Gelwick (1988), and new genetic information. Specifically, the mtDNA sequence assessments described here, including data from April et al. (2011), indicate that *E. radiosum* (including *E. cyanorum*) could include at least four genetically distinctive and unique lineages. Similar results were recovered in a mtDNA ND2 gene tree (not shown; N. Lang, pers. comm.).

It is worth noting that mitochondrial genes are linked and effectively represent a single genetic locus. While thorough phylogeographic assessment of nuclear DNA loci would have been ideal, existing data on RAG1 and S7 gene regions showed exceptionally low nucleotide sequence divergence between *E. cyanorum* and *E. radiosum* (Near et al., 2011), suggesting that these loci would be largely uninformative at the evolutionary timescale studied here. It is also clear that the mtDNA genome in darters is subject to deep introgression, a term that reflects a mtDNA transfer event prior to diversification of species within lineage *Vexillapinna*, including the *E. radiosum* complex (Near et al. 2011). Nonetheless, both mtDNA datasets in the present study showed nearly identical patterns of evolution across the geographic range of *E. radiosum* and indicate that *E. cyanorum* is distinct and the most deeply diverged lineage in the species complex. This result is robust to deep introgression and ongoing hybridization (Matthews et al., 2016) with the congener *E. pulchellum*.

All available evidence supports the recognition of *E. cyanorum* but suggests that the named subspecies *E. r. paludosum* or *E. r. radiosum* require additional studies of geographic variation in molecular and morphological characters if validity of those subspecies is to be determined. The mtDNA gene trees indicated recent and substantive gene flow across drainages previously thought to harbor different subspecies (e.g., Ouachita and Kiamichi basins share identical haplotypes). Closely related haplotypes from the Ouachita, Caddo, and Little Missouri rivers in southeastern Arkansas and the Kiamichi and Clear-Muddy Boggy rivers in southeastern Oklahoma formed a large and geographically widespread lineage with haplotypes shared across drainages, also suggesting relatively recent gene flow. These distinct watersheds span the putative geographic ranges of *E. r. radiosum* and *E. r. paludosum*. Moreover, earlier allozyme data indicated that the Kiamichi, Muddy, and Clear Boggy River samples were genetically differentiated (Echelle et al., 1975).

Etheostoma cyanorum is endemic to one drainage, distributed allopatrically from all other populations now considered to be *E. radiosum*. It is recognized morphologically on the basis of scale counts and a wider interorbital width. Some measurement traits are allometric such that large adults have a shorter snout and deeper body, with a blunter and more downturned snout, as noted by Moore and Rigney (1952) and evaluated in detail by Matthews and Gelwick (1988) and herein. But because these head and body shape traits are allometric, they are not useful for separating smaller individual *E. cyanorum* from *E. radiosum*, in spite of being considered diagnostic of the subspecies *E. r. cyanorum* (Moore and Rigney, 1952). Finally, there is clear evidence from mtDNA analysis that *E. cyanorum* markedly differs from all other populations considered *E. radiosum*. We conclude that *E. cyanorum* should be recognized as a distinct species, with the recommended common name of Blue River Orangebelly Darter.

MATERIAL EXAMINED

Genetic material

Etheostoma cyanorum: Blue River Drainage: Blue River: MSB 76601, 5.

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Etheostoma radiosum: Washita River Drainage: Little Glasses Creek: OMNH 74856, 5; Tributary of Little Glasses Creek: WJM-2970 (uncatalogued), 5. Clear Boggy Drainage: Delaware Creek: MSB 76561, 5; Jack Fork Creek: MSB 76574, 5. Muddy Boggy Drainage: Sandy Creek: MSB 76552, 5. Little River Drainage: Glover River: MSB 45447, 5; Little River: PU05-18 (uncatalogued), 5. Kiamichi River Drainage: Kiamichi River MSB 95950, 5. Ouachita River Drainage: Ouachita River: JCT1991 (uncatalogued), 5; Little Missouri River: TFT-92-32 (uncatalogued), 5.

Etheostoma whipplei: Arkansas River Drainage: Lee Creek: NLU 73112, 1.

Morphological material

Original morphological material examined by Matthews and Gelwick (1988) is in that paper, not repeated here. Newly examined morphological materials are:

Etheostoma cyanorum: Blue River Drainage: Blue River at Hwy 99, 0.7 km N of Connerville, OK, ECM 94-8 (OMNH 86857), 11; Blue River 1.7 km WNW of Connerville, OK, WJM3645 (male photographed, OMNH 86859, 1; female photographed, OMNH 86860, 1; additional material examined OMNH 86861, 9); Blue River 1.3 km SE of Connerville, OK, BRTP1 and BRTP2 (OMNH 86858), 40.

DATA ACCESSIBILITY

Supplemental information is available at https://www.copeiajournal.org/ci-18-054. Mitochondrial control region sequence data are available on GenBank under accession numbers: *E. cyanorum*, MK490957–MK490959; *E. radiosum*, MK481992–MK482003.

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