

## A Mitochondrial DNA Perspective on the Molecular Systematics of the Sunfish Genus *Lepomis* (Actinopterygii: Centrarchidae)

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Complete mitochondrial DNA cytochrome *b* gene sequences from 56 specimens representing all 12 species of *Lepomis* were used to examine phylogenetic relationships within the genus. Results supported the monophyly of *Lepomis* and all previously recognized subgenera, except *Eupomotis*, but there was no support for previously proposed relationships among subgenera. Seven species were recovered as monophyletic lineages, while five species (*L. auritus*, *L. macrochirus*, *L. marginatus*, *L. miniatus*, and *L. symmetricus*) were recovered as either poly- or paraphyletic or were placed as parts of unresolved polytomies with other species. Parametric bootstrapping tests rejected monophyly for only two of the five species (*L. auritus* and *L. symmetricus*). Without additional data, including increased geographic sampling and a comparable nuclear gene phylogeny, it is not possible to determine whether the failure to support monophyly for these two species reflects the presence of cryptic species or results from hybridization.

*LEPOMIS* Rafinesque, 1819 is the largest genus within Centrarchidae (sunfishes and basses) with 12 species (Nelson et al., 2004). Because of their importance as game fishes, common occurrence, and widespread distribution, species of *Lepomis* have been the focus of many ecological and evolutionary studies (Birmingham and Avise, 1986; Wainwright, 1996; Schaefer et al., 1999).

Despite the popularity of *Lepomis* as model organisms, species-level relationships within the genus have received limited attention. Some species-level relationships within *Lepomis* have been consistently recovered (e.g., *L. marginatus* sister to *L. megalotis*; Bailey, 1938; Mabee, 1993). But most hypotheses of relationships either preceded or did not employ phylogenetic methods (Bailey, 1938; Branson and Moore, 1962; Avise and Smith, 1974) or produced conflicting results (Mabee, 1993). These conflicts probably result from the considerable morphological and color variation exhibited by *Lepomis* (Bailey, 1938), perhaps from the impact of hybridization (Hubbs, 1955; Hester, 1970; Scribner et al., 2000), and/or different methods of character analysis and phylogenetic reconstruction. We used mitochondrial DNA (mtDNA) cytochrome *b* gene sequences for phylogenetic inferences among species of *Lepomis* to provide an independent assessment of lineage evolution within sunfishes in order to reconcile previous conflicting hypotheses of relationships.

### MATERIALS AND METHODS

At least two individuals of each of the 12 species of *Lepomis* were collected, and every effort

was made to include samples from different localities. We avoided collecting specimens in ponds, lakes, and reservoirs where hybrids are more common (Hubbs, 1955; Scribner et al., 2000). Representatives from all other centrarchid taxa were used as outgroups (Roe et al., 2002) (see Material Examined). Taxonomy follows Nelson et al. (2004).

Mitochondrial DNA isolation methods, polymerase-chain-reaction amplification parameters, and DNA sequencing methods for cytochrome *b* are given in Roe et al. (2002). All sequences were aligned using BioEdit (Hall, 1999). No insertion-deletions were present. All sequences were translated into amino acid sequences as an additional check of the alignment using MacClade 4.05 (D. R. Maddison and W. P. Maddison, Sinauer Associates, Inc., Sunderland, MA, 2002).

Phylogenies were estimated by maximum parsimony (MP) and maximum likelihood (ML) analyses using PAUP\* (D. L. Swofford, Sinauer Associates, Inc., Sunderland, MA, 2002). The heuristic search option (100 random addition replications with tree bisection-reconstruction) was used to search for optimal trees in both MP and ML analyses. Non-parametric bootstrap analysis (Felsenstein, 1985) with 1000 pseudo-replicates and 10 random sequence additions was conducted for the MP analysis. Bootstrap analysis for the ML tree was conducted with 100 pseudo-replicates and “as-is” addition sequence. In both analyses, branches with bootstrap values of  $\leq 50\%$  were collapsed.

Modeltest (Posada and Crandall, 1998) was used to identify the optimal model of base substitution appropriate for analysis. The selected

model was the transversion model with variable nucleotide base frequencies, variable transversions, transitions equal, some sites assumed invariable and variable sites assumed to follow a discrete gamma distribution (i.e., TVM + I +  $\Gamma$ ). Maximum-likelihood model parameter values were as follows: nucleotide frequencies, A = 0.2622, C = 0.3569, G = 0.1553 and T = 0.2255; rate matrix, A-C = 0.9516, A-G = 8.1232, A-T = 1.0902, C-G = 0.5807, C-T = 8.1232, G-T = 1.0000; proportion of invariable sites (I) = 0.5092; discrete gamma distribution shape parameter ( $\Gamma$ ) = 1.0633.

Parametric bootstrapping (Swofford et al., 1996; Goldman et al., 2000) was used to test for the apparent non-monophyly observed in some taxa following the methods and parameters outlined by Shull et al. (2001) and D. R. Maddison (Testing monophyly of a group of beetles. Study 1 in Mesquite: a modular system for evolutionary analysis, version 1.01, [http://mesquiteproject.org/Mesquite\\_Folder/docs/mesquite/studies/study001/index.html](http://mesquiteproject.org/Mesquite_Folder/docs/mesquite/studies/study001/index.html)), with the exception that only 100 simulated matrices were used. This procedure was implemented in the Genesis module within the Mesquite system for phylogenetic computing (W. P. Maddison and D.R. Maddison. 2004. Mesquite: a modular system for evolutionary analysis, version 1.02, <http://mesquiteproject.org>).

#### RESULTS AND DISCUSSION

Analysis of the complete mtDNA cytochrome *b* gene (1140 bp) among 80 specimens of *Lepomis* yielded 504 (44.2%) variable sites, of which 466 (40.8%) were parsimony informative. Topologies recovered by MP and ML analyses were in good agreement regarding the major mtDNA clades within *Lepomis* (Fig. 1) and hypotheses of relationships among and within the major clades were consistent between the two analyses. Basal relationships were more highly resolved in the MP analysis (Fig. 1); otherwise, the only differences between results from the two analyses were in levels of resolution for within-species samples of the major clades.

Our analyses supported the monophyly of *Lepomis*, a result consistent with the hypotheses of Bailey (1938), Avise and Smith (1974), and Roe et al. (2002). Interestingly, however, monophyly was rejected in the results presented by Avise and Smith (1977), Wainwright and Lauder (1992), and Mabee (1993), three studies upon which most of the recent comparative evolutionary investigations have been based. The mtDNA trees depict *Enneacanthus* as a monophyletic taxon outside of *Lepomis* (data not shown), and

place the warmouth (*L. gulosus*) within *Lepomis*. These results agree with some hypotheses (Avise and Smith, 1974; Mabee, 1993; Roe et al., 2002), but contrast with others (Bailey, 1938; Wainwright and Lauder, 1992).

The mtDNA trees recovered three pairs of sister species indicated by Bailey's (1938) classification (*L. cyanellus* and *L. symmetricus*, *L. humilis* and *L. macrochirus*, and *L. megalotis* and *L. marginatus*) and were consistent with a fourth such pair (*L. miniatus* and *L. punctatus* in an unresolved trichotomy with *L. microlophus*). Both MP and ML hypotheses rejected Bailey's (1938) subgenus *Eupomotis* (= *L. gibbosus* and *L. microlophus*), although this grouping has received support from studies of functional morphology (Lauder, 1986) and morphological ontogeny (Mabee, 1993). In our analysis, trees constrained to include a monophyletic *Eupomotis* were markedly longer than the shortest MP trees (105 extra steps; parametric bootstrapping test,  $P < 0.001$ ).

Previously reported synapomorphies in support of *Eupomotis* may represent homoplasious traits. These include presence of secondary color bars and pointed pectoral fin shapes (Mabee, 1993) and a complex of traits involved in snail eating (enlarged pharyngeal muscles and a crushing motor pattern; Lauder, 1986). Regarding the latter trait, Lauder (1986) suggested that *L. cyanellus* was convergent on *Eupomotis*, but that *L. cyanellus* and *L. gibbosus* used the crushing motor pattern only when feeding on snails, whereas *L. microlophus* used it for all prey types. This observation, together with our independent test of phylogenetic relationships, supports homoplasy for the traits used to unite *L. microlophus* and *L. gibbosus*. Discrepancies between morphological and molecular phylogenetic hypotheses for *Lepomis* may reflect the confounding effects of phenotypic plasticity on recognition of intra- and inter-specific morphological variation and/or coding such morphological characters for phylogenetic analysis (Wiens, 2000), or homoplasy due to character convergence. Phenotypic plasticity in trophic characters has been well documented in *Lepomis* (Ehlinger, 1990; Hegrenes, 2001; Yonekura et al., 2002), including *L. gibbosus* (Robinson and Wilson, 1996).

Evolutionary developmental studies must be grounded with phylogenetic hypotheses of the taxa under consideration (Zauner et al., 2003). This point is emphasized by consideration of two examples from Mabee's (1993) study of ontogenetic transformations and their use in reconstructing a phylogeny of centrarchids. Mabee (1993) depicted *L. symmetricus* as sister to *L.*

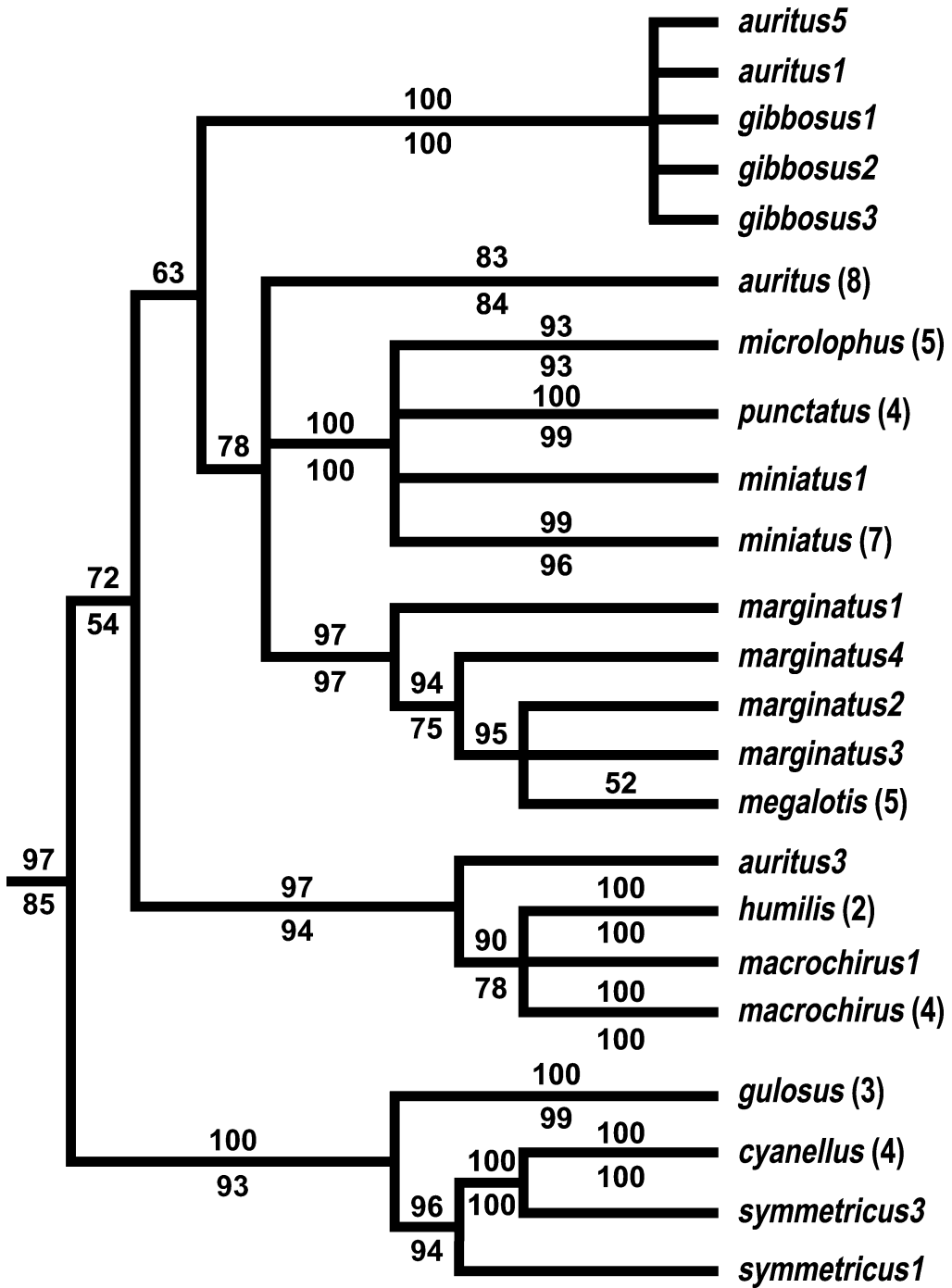


Fig. 1. Strict consensus of the 10 shortest maximum parsimony trees (length = 2419 steps; CI = 0.327, RI = 0.823, RC = 0.269). Numbers in parentheses are the number of haplotypes represented for that species. Numbers above and below branches indicate support based on, respectively, the maximum parsimony and maximum likelihood bootstrap analyses.

*macrochirus* in her hypothesis derived from using outgroup polarization. Two synapomorphies supported this relationship, both of which were considered terminal ontogenetic additions (character states 53B—dorsal fin spot; 18I—number of infraorbital bones 4 to 7). In contrast, both MP and ML hypotheses resolved *L. symmetricus* sister to *L. cyanellus* (53A—terminal addition) and *L. macrochirus* sister to *L. humilis* (18G—terminal deletion). Thus, both the interpretation of character evolution and frequency of terminal additions and deletions in the ontogenetic evolution of *Lepomis* differs from those that would be indicated by our phylogenetic reconstructions.

Mabee's (1993) results also indicated a sister relationship between *L. microlophus* and *L. gibbosus*, which was supported by two synapomorphies representing terminal ontogenetic additions (her 56B—secondary color bars and 41A—posttemporal margin). The mtDNA tree, however, places *L. microlophus* in a trichotomy with *L. miniatus* and *L. punctatus*, and *L. gibbosus* is depicted as sister to a clade of six other species (*L. auritus*, *L. microlophus*, *L. miniatus*, *L. punctatus*, *L. marginatus*, and *L. megalotis*). If our tree correctly represents the phylogeny of *Lepomis*, then Mabee's (1993) synapomorphies become homoplasious, and the overall number of terminal additions observed increases, a conclusion that alters one's interpretation of both the potential application of ontogeny in systematics and developmental evolution in these fishes.

Our analyses failed to support mtDNA monophyly for five species. Three species (*L. auritus*, *L. marginatus*, and *L. symmetricus*) were resolved as either poly- or paraphyletic and samples from two species (*L. macrochirus* and *L. miniatus*) were placed as parts of unresolved polytomies with other species. Constraining these five species to be "monophyletic," resulted in significantly increased tree lengths (parametric bootstrapping test,  $P < 0.05$ ) only for *L. auritus* (87 steps) and *L. symmetricus* (27 steps). In the absence of further information, including more thorough geographic sampling and knowledge of the phylogenetic pattern in the nuclear genome, it is not possible to determine whether the failure to support monophyly for these two species reflects the presence of cryptic species or results from hybridization.

*Lepomis auritus* was recovered in multiple locations in the mtDNA tree. Eight of the 11 specimens formed a monophyletic lineage, but two were weakly divergent (average = 0.2% under our ML substitution model) from *L. gibbosus*, and markedly divergent (average = 21.6%) from the remaining *L. auritus* specimens. On-

going hybridization has been documented between *L. auritus* and *L. gibbosus* (Schwartz, 1972; Scribner et al., 2000) and may account for the placement of these two specimens. A third specimen was sister to a clade comprising *L. humilis* and *L. macrochirus*, and it was less divergent from this clade (average = 14.8%, 7.2%, respectively) than from the clade of eight *L. auritus* haplotypes (average = 17.8%). The placement of this specimen is enigmatic, but it might reflect relatively ancient hybridization and genetic introgression.

Our results identify several systematic problems within traditionally recognized species of *Lepomis* that warrant both further investigation and concern in future comparative biological studies using these species as model organisms. More detailed intraspecific taxonomic and systematic studies are needed to clarify the ranking of subspecific taxa currently recognized within *Lepomis* (e.g., *L. megalotis*, with up to six recognized subspecies; Page and Burr, 1991). In addition, the recovery of '*L. symmetricus*' as paraphyletic suggests that unrecognized biodiversity possibly exists within this species. In both cases, current stocking or transplantation practices by fisheries managers may be obfuscating and/or disrupting either natural evolutionary lineages or evolutionary significant units.

#### MATERIAL EXAMINED

Institutional abbreviations follow Leviton et al. (1985). KJR tissue extraction numbers, collection localities, voucher specimen collection numbers, and GenBank accession numbers are as follows: *Acantharchus pomotis* 1 (KJR-56)—INHS 45049, AY115993; *A. pomotis* 2 (KJR-82)—UAIC 11844.02, AY115994; *Ambloplites cavifrons* 1 (KJR-7)—UAIC 12285.02, AY115979; *A. cavifrons* 2 (KJR-182)—UAIC 13074.02, AY115980; *A. rupestris* 1 (KJR-12)—UAIC 12253.06, AY115977; *A. rupestris* 2 (KJR-115)—SIUC 37911, AY115978; *Archoplites interruptus* 1, 2 (KJR-61, 62)—AY115995, AY115996; *Centrarchus macropterus* 1 (KJR-35)—UAIC 11865.02, AY115981; *C. macropterus* 2 (KJR-165)—UAIC 13070.07, AY115982; *Enneacanthus chaetodon* 1 (KJR-103)—UAIC 13139.04, AY115983; *E. chaetodon* 2 (KJR-84)—UAIC 11844.03, AY115984; *E. gloriosus* 1 (KJR-1)—UAIC 11704.14, AY115985; *E. gloriosus* 2 (KJR-63)—UAIC 12367.01, AY115986; *E. obesus* 1, 2 (KJR-85, 159)—UAIC 11844.04, AY115987, AY11598; *Lepomis auritus* 1 (KJR-18)—Town Creek at MD route 51, 3.7 mi ESE of Old Town, Allegany Co., MD, UAIC 12287.02, AY115969; *L. auritus* 2 (KJR-94)—Middle Fork of Broad River at GA

- Hwy 57, WNW of Franklin Springs, Franklin Co., GA, UAIC 12376.02, AY115970; *L. auritus* 3 (KJR-27)—Town Creek at CR 674 in Philpott, Henry Co., VA, UAIC 12285.03, AY828949; *L. auritus* 4 (KJR-106)—Blue Springs Creek at Lower Blue Springs Road, 10 mi WSW of Hamilton, Harris Co., GA, UAIC 12371.03, AY828950; *L. auritus* 5—Little River at Bagley boat ramp off of CR 2144, Johnston Co., NC, UAIC 12585.05, AY828951; *L. auritus* 6—Tar River at Hunter Hill Road, 2 km S of West Mount, Nash Co., NC, UAIC 12586.02, AY828952; *L. auritus* 7—South River at U.S. Hwy 701, ca. 2 km SSW of Garland, Sampson/Bladen Co. line, NC, UAIC 12587.05, AY828953; *L. auritus* 8—Lake Waccamaw and adjacent canal at public boat ramp, W side of lake, Columbus Co., NC, UAIC 12588.07, AY828954; *L. auritus* 9—Back Swamp at Back Swamp Road, 12 km WSW of Lumberton, Robeson Co., NC, UAIC 12589.08, AY828955; *L. auritus* 10—Jack's Creek at SC Hwy 26, Clarendon Co., SC, UAIC 12590.05, AY828956; *L. auritus* 11—unnamed creek to Savannah River at boat ramp, ca. 11 km S of Purnysburgh, Jasper Co., SC, UAIC 12591.01, AY828957; *L. cyanellus* 1 (KJR-8)—Horseshoe Run at CR 7, Tucker Co., WV, UAIC 12253.07, AY115973; *L. cyanellus* 2 (KJR-146)—Whiteside Creek at WI Hwy 78, 4 mi NE of Wiota, Lafayette Co., WI, UAIC 12528.01, AY115974; *L. cyanellus* 3 (KJR-9)—Potomac River at mouth of Wills Creek in Cumberland, Allegany Co., MD, UAIC 12288.02, AY828958; *L. cyanellus* 4 (KJR-10)—Town Creek at CR 674 in Philpott, Henry Co., VA, UAIC 12285.04, AY828959; *L. gibbosus* 1 (KJR-59)—Lake Andrusia, 5 mi WNW of Cass Lake, southeast shore, Beltrami Co., MN, INHS 39505, AY828960; *L. gibbosus* 2 (KJR-75)—North branch of Snowy Creek at CR 46, 1 km NNE of Hopemont, Preston Co., WV, UAIC 12254.08, AY828961; *L. gibbosus* 3 (KJR-86)—Poplar Lick at Spillway of New Germany Reservoir, New Germany State Park, Garrett Co., MD, UAIC 11834.01, AY828962; *L. gulosus* 1 (KJR-14)—Hatchet Creek at FL Hwy 222, Alachua Co., FL, UAIC 12286.06, AY115971; *L. gulosus* 2 (KJR-107)—Easley Creek at Clark/Hot Springs Co. line, E of Witherspoon, AR, UAIC 12420.01, AY115972; *L. gulosus* 3 (KJR-32)—Barnishee Bayou, Shelby Co., TN, UAIC 11865.03, AY828963; *L. humilis* 1 (KJR-58)—Mississippi River, 2.2 mi SSW of Fulton, U.S. Hwy 30, Whitestone Co., IL, INHS 40071, AY828964; *L. humilis* 2 (KJR-66)—Kansas River, below Bowersock Dam in Lawrence, Douglas Co., KS, KU 25192, AY828965; *L. macrochirus* 1 (KJR-19)—Little Orange Creek at Hwy 21, Putnam Co., FL, UAIC 12290.02, AY115975; *L. macrochirus* 2 (KJR-109)—Easley Creek at Clark/Hot Springs Co. line, E of Witherspoon, AR, UAIC 12420.03, AY115976; *L. macrochirus* 3 (KJR-17)—Deep Creek Lake at Glen Acres, Youghiogeheny River, Garrett Co., MD, UAIC 12289.01, AY828966; *L. macrochirus* 4 (KJR-26)—Town Creek at CR 674 in Philpott, Henry Co., VA, UAIC 12285.05, AY828967; *L. macrochirus* 5 (KJR-123)—Turkey Creek ca. 1 km N of Pinson at Tapwingo Road, Jefferson Co., AL, UAIC 12511.07, AY828968; *L. marginatus* 1 (KJR-16)—Little Orange Creek at Hwy 21, Putnam Co., FL, UAIC 12290.03, AY828969; *L. marginatus* 2 (KJR-88)—Cedar Creek at U.S. Hwy 43, Mobile Co., AL, UAIC 11704.15, AY828970; *L. marginatus* 3 (KJR-127)—Wilkes Creek, 0.5 mi W of Pleasant Ridge, Green Co., AL, UAIC 12508.07, AY828971; *L. marginatus* 4 (KJR-205)—Everglades Canal L-31W, S of FL Hwy 9336 bridge, Dade Co., FL, UAIC 12367.03, AY828972; *L. megalotis* 1 (KJR-20)—Town Creek at MD Rt. 51, 3.7 mi ESE of Old Town, Allegany Co., MD, UAIC 12287.02, AY828973; *L. megalotis* 2 (KJR-80)—Rock Creek at unnumbered county road 2.7 mi S of Minot, Colbert Co., AL, UAIC 12237.02, AY828974; *L. megalotis* 3 (KJR-92)—Rockcastle River, ca. 9 km SSW of Livingston on I-75, Laurel/Rockcastle Co. line, KY, UAIC 12354.03, AY828975; *L. megalotis* 4 (KJR-114)—Indian Creek at bridge on TN Hwy 64 near Olive Hill, Hardin Co., TN, SIUC 3707, AY828976; *L. megalotis* 5 (KJR-118)—Meramec River 2.75 mi S of Gray Summit, Franklin Co., MO, SIUC 37913, AY828977; *L. microlophus* 1 (KJR-73)—Shoal Creek at Pine Glen Recreation Area, 5.5 mi NW of Helflin, Cleburne Co., AL, UAIC 11897.01, AY828978; *L. microlophus* 2 (KJR-135)—Baron Fork of Illinois River at Camp Egan, Cherokee Co., OK, OSUS 27530, AY828979; *L. microlophus* 3 (KJR-213)—Orange Creek at FL Hwy 315, N of Orange Springs, Putnam/Marion Co. line, FL, UAIC 12601.05, AY828980; *L. microlophus* 4 (KJR-215)—Little River at Bagley Boat Ramp of CR 2144, Johnston Co., NC, UAIC 12585.07, AY828981; *L. microlophus* 5 (KJR-216)—Ocklokonee River at FL Hwy 153 SE of Havana, Gadsden Co., FL, UAIC 12650.11, AY828982; *L. miniatus* 1 (KJR-50)—Lightsey's Pond at AL Hwy 219, 2.5 mi S of Centreville, Bibb Co., AL, UAIC 10931.05, AY828983; *L. miniatus* 2 (KJR-72)—Poor House Branch at AL Hwy 34, 2.7 mi N of Shocco Springs, Talladega Co., AL, UAIC 12428.01, AY828984; *L. miniatus* 3 (KJR-111)—Easley Creek E of Witherspoon, Clark/Hot Springs Co. line, AR, UAIC 12420.06; *L. miniatus* 4 (KJR-129)—Wilkes Creek, 0.5 km W of Pleasant Ridge, Green Co., AL, UAIC 12508.13,

AY828986; *L. miniatus* 5 (KJR-125)—Deverell Spring along Union Road, 0.7 mi WNW of Union Church, Bradley Co., TN, UAIC 12522.01, AY828987; *L. miniatus* 6 (KJR-141)—Cypress Creek at Hwy 326 S of Kountze city limits, Hardin Co., TX, SIUC 37827, AY828988; *L. miniatus* 7 (KJR-218)—Saline River at AR Hwy 15, ca. 6 mi NNE of Warren, Bradley Co., AR, UAIC 12682.26, AY828989; *L. miniatus* 8 (KJR-224)—Bell Creek at AR Hwy 26, 2 mi W of Hollywood, Clark Co., AR, UAIC 12679.05, AY828990; *L. punctatus* 1 (KJR-214)—Rocky Creek at GA Hwy 56, ca. 2 km E of Johnson Co., Toomes Co., GA, UAIC 12592.06, AY828991; *L. punctatus* 2 (KJR-217)—Jacks Creek at SC Hwy 26, Clarendon Co., SC, UAIC 12590.08, AY828992; *L. punctatus* 3 (KJR-65)—Everglades Canal L-31W, S of FL Hwy 9336 bridge, Dade Co., FL, UAIC 12367.01, AY828993; *L. punctatus* 4 (KJR-21)—Santa Fe River at public boat ramp, downstream of U.S. Hwy 41, N of Highsprings, Alachua/Columbia Co. line, FL, UAIC 12291.01, AY828994; *L. symmetricus* 1 (KJR-120)—Bridge over Wolf Lake on road to chemical plant, Union Co., IL, SIUC 37912, AY828995; *L. symmetricus* 3 (KJR-139)—Oxbow near Little River at Dead Man's Point, 4 mi S of Broken Bow, McCurtain Co., OK, OSUS 27537, AY828996; *Micropterus dolomieu* 1 (KJR-15)—UAIC 12253.08, AY115997; *M. dolomieu* 2 (KJR-96)—UAIC 12354.05, AY11598; *M. salmoides* 1 (KJR-133)—OSUS 27528, AY115999; *M. salmoides* 2 (KJR-189)—UAIC 12590.09, AY116000; *Pomoxis annularis* 1 (KJR-157)—UAIC 11821.02, AY115989; *P. annularis* 2 (KJR-166)—UAIC 12610.08, AY115990; *P. nigromaculatus* 1 (KJR-6)—UAIC 12309.04, AY 115991; *P. nigromaculatus* 2 (KJR-138)—OSUS 27536, AY115992.

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