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DNA-based identification and descriptions of immatures of *Kempnyia* Klapálek (Insecta:Plecoptera) from Macaé River Basin, Rio de Janeiro State, Brazil

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Abstract: Deoxyribonucleic acid (DNA) barcode (cytochrome *c* oxidase subunit I [COI]) sequences have been used to associate adult and immature stages of stoneflies as a rapid and effective alternative to rearing immatures. However, no molecular associations have been made for Neotropical species, which have very few described immature stages. *Kempnyia* Klapálek (Perlidae) is an endemic South American genus. Currently, 20 females have been associated with males of the 40 valid species, but only 5 nymphs have been described. We collected stonefly nymphs at Macaé River Basin, Rio de Janeiro State, Brazil, and *Kempnyia* nymphs were separated those in 6 morphotypes. These morphotypes were associated to nominal *Kempnyia* species using 38 COI sequences (471 base pairs [bp]) of immatures and identified adults of 10 species occurring in southern and southeastern Brazil. Based on a neighbor-joining analysis of Kimura-2-parameter (K2P) pairwise divergences, we found 6 genetic *Kempnyia* lineages at Macaé River Basin represented by all morphotypes of nymphs included in our study. These nymphs were associated with males of *Kempnyia alterosarum*, *K. colossica*, *K. gracilentata*, *K. petersorum*, *K. obtusa*, and *K. varipes*. Intraspecific divergences in *Kempnyia* varied from 0 to 15%, but $\sim\frac{1}{2}$ of comparisons had values $>3\%$. These data are in agreement with previous studies with stonefly populations, which tend to show high COI divergences. Based on our results, we described previously unknown immatures of *K. alterosarum*, *K. petersorum*, and *K. obtusa*, and the male genitalia of *K. varipes* for the first time. We also described a new adult color morph of *K. petersorum*. Our study increased the number of *Kempnyia* species recorded at Macaé River Basin from 5 to 11. We recorded for the first time *K. alterosarum* and *K. reticulata* from Rio de Janeiro State and *K. jatim* from Espírito Santo State.

Key words: stoneflies, taxonomy, COI, Atlantic Forest

Benthic macroinvertebrates are the most commonly used freshwater organisms for assessing water quality because: 1) they are ubiquitous and can be used to detect environmental disturbances in many types of aquatic systems and 2) their large number of species offers a spectrum of responses to environmental stressors (Rosenberg and Resh 1993, Bae et al. 2005). Macroinvertebrates have been used in freshwater monitoring programs in the Neotropics (Galdan et al. 2000, Callisto et al. 2001, Buss et al. 2002) and indicate that stonefly nymphs are very sensitive to environmental change (Junqueira and Campos 1998, Miserendino

and Pizzolón 1999). Most such studies in the Neotropics are based on identifications at the family level (e.g., Junqueira and Campos 1998, Miserendino and Pizzolón 1999) because of high diversity and the lack of appropriate taxonomic tools for identification at a lower level, even though some authors recommend identification at species level for greater sensitivity (Jones 2008, Sweeney et al. 2011).

Taxonomic descriptions of most Neotropical stoneflies are based only on adult males, and species identification of stonefly nymphs is almost impossible based on the published literature. Nymphal stages of few Neotropical spe-

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cies have been described because of the difficulty of associating Plecoptera nymphs with adults. For example, adults often are not found with nymphs because females tend to fly upriver to oviposit (Müller 1954). Rearing nymphs to adults can be difficult because nymphs often die before reaching the laboratory (Novaes et al. 2012). Some authors have reared last-instar nymphs to the adult stage in the field to avoid transporting live specimens to the laboratory (Bispo et al. 2013). Avelino-Capistrano (2010) had some success rearing *Kempnyia gracilentata* (Enderlein) and *K. reticulata* (Klapálek) in the laboratory because they are more resistant to transportation death than other species (e.g., *Anacroneuria debilis* (Pictet)), and Novaes et al. (2012) reared nymphs of *Anacroneuria ofaye* Froehlich in an apparatus that simulates river currents. Nevertheless, most nymphal species remain unassociated.

Molecular tools have been used to associate immature and adult stages of insects (Mynott et al. 2011), address phylogenetic questions (Fochetti et al. 2009, 2011, Boumans and Baumann 2012) and cryptic species problems (Tierno-de-Figueroa et al. 2011), and identify species based on the GenBank database (Hajibabaei et al. 2011, Sweeney et al. 2011). Use of molecular tools to associate life stages is rapid and effective (Miller et al. 2005, Caterino and Tishechkin 2006, Zhou et al. 2007, Hayashi and Sota 2010, Trivinho-Strixino et al. 2012) because genetic regions are identical in all life stages. Deoxyribonucleic acid (DNA) barcode sequences based on cytochrome *c* oxidase subunit I (COI) have been used to associate adult and nymphal stoneflies in Australia (Gray 2009, Mynott et al. 2011) to provide diagnostic characteristics and tools for identification of nymphs. To date, no similar study has been done in the Neotropics.

Currently, 168 species of Plecoptera are recorded from Brazil. Of those, 40 occur in Rio de Janeiro State (Nessimian et al. 2009, Froehlich 2010, 2011a, b, Avelino-Capistrano et al. 2013). Only 8 species are recorded for Macaé River Basin in Rio de Janeiro State: *Anacroneuria subcostalis* Klapálek, *A. petersi* Froehlich, *A. simulans* Froehlich, *Gripopteryx garbei* Navás, *Kempnyia obtusa* Klapálek, *K. puri* Avelino-Capistrano, Souza & Nessimian, *K. serrana* (Navás), and *K. taunayi* (Navás) (Nessimian et al. 2009, Avelino-Capistrano et al. 2013, Baldin et al. 2013). However, Baptista et al. (1998) found 13 morphotypes of stonefly nymphs in the Macaé River Basin while conducting an ecological study, a result highlighting the lack of taxonomic information for this region.

MATERIALS AND METHODS

Background on *Kempnyia* (Perlidae) stoneflies

Kempnyia is an endemic genus of Perlidae from South America and currently includes 40 species (Froehlich 2011a, b). The genus is distributed from central to southern Brazil, but may also occur in neighboring areas of Argentina, Paraguay, and Uruguay. Recently, nymphs were

found in Venezuela (Derka et al. 2009). The genus was erected by Klapálek based on *K. tenebrosa* Klapálek from Teresópolis, Rio de Janeiro State (Stark et al. 2009). According to Stark et al. (2009), these insects can be characterized by: 1) presence of 2 or 3 ocelli, 2) head with short ridge extending from posterior margin to junction of epicranial suture, which bends forward between the ocelli, and 3) male sternum IX with hammer greatly variable. Nymphs of *Kempnyia* live in headwater areas of preserved rivers where they are found among the litter. Adults can be found flying or resting on vegetation near the stream (Dorvillé and Froehlich 2001, Henriques-Oliveira and Nessimian 2010). *Kempnyia* nymphs are used in freshwater biomonitoring in Brazil because they and other stoneflies are considered sensitive to chemical and physical changes (Mugnai et al. 2008). However, only 5 nymphs of *Kempnyia* are described and associated to nominal species: *Kempnyia colossica* (Navás), *K. gracilentata* (Enderlein), *K. neotropica* (Jacobson & Bianchi), *K. reticulata*, and *K. tijucana* Dorvillé & Froehlich (Dorvillé and Froehlich 2001, Bispo and Froehlich 2008, Avelino-Capistrano et al. 2011, Bispo et al. 2013). We collected *Kempnyia* Klapálek nymphs in the Macaé River Basin and used DNA barcodes to associate them with identified males occurring in southeastern Brazil.

Study area

Macaé River is the largest river entirely within the State of Rio de Janeiro, Brazil. Its basin covers ~1765 km² and includes part of the cities of Nova Friburgo, Casimiro de Abreu, Rio das Ostras, Conceição de Macabu, Carapebus, and Macaé, where 82% of the basin occurs. Macaé River begins at Pico do Tinguá (Nova Friburgo) at 1560 m asl at Serra de Macaé in the Atlantic Rain Forest and empties into the Atlantic Ocean at Macaé (136 km). The river receives rural and urban domestic sewage, and only the headwaters are well preserved (SEMADS 2001). We sampled at 105 collecting stations throughout the basin (altitudes were 7–1103 m asl) along a gradient from well preserved to impacted (Nova Friburgo, Casimiro de Abreu, and Macaé municipalities).

Specimen sampling

We collected most of our study material between November 2008 and September 2010. We collected nymphs manually and adults with light traps, and fixed and preserved all specimens in 93% ethanol to maximize DNA preservation. We also included specimens from the Macaé River Basin previously deposited at Coleção Entomológica by José Alfredo Pinheiro Dutra (DZR); Universidade Federal do Rio de Janeiro). We included identified *Kempnyia* males collected at other localities in southern or southeastern Brazil in the DNA study (Appendix S1), when these species were not represented by males from Macaé River Basin.

Specimen imaging

We took optical photographs of the material with the aid of a stereomicroscope (MZ16; Leica, Wetzlar, Germany) and a coupled camera (Leica DFC420). We stacked images with the softwares Auto-Montage[®] (Synoptics, Frederick, Maryland) and Leica IM50. We took scanning electron micrographs (SEMs) of 2 nymphs of each species. We rehydrated nymphs in a series of 70, 50, 30, and 15% alcohol for 10 min each and washed them in pH 7.2 sodium cacodylate buffer for 10 min before fixation in 2.5% glutaraldehyde and postfixation in 1% osmium tetroxide for 1 h. After washing in the same buffer, we dehydrated nymphs in a series of increasing ethanol concentrations and submitted them to critical-point drying, using superdry CO₂ in Balzer's apparatus. We set the dried material on metallic stubs, covered it with a thin layer of Au (20–30 nm), and observed it via a JSM-6390LV scanning electron microscope (JEOL, Tokyo, Japan) at the Electron Microscopy Platform, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz.

DNA sequences

We extracted DNA from all identified adults and included males and females that occurred at the same site. We also extracted DNA from nymphs collected at sites where identified adults were present to increase the likelihood of association. In addition, we extracted DNA from 5 nymphs of each morphotype, when available, even if no adults had been collected. We used the DNeasy[®] Blood and Tissue Kit (Qiagen, Hilden, Germany) to extract DNA following a modified protocol from the manufacturer's instructions and amplified partial COI by polymerase chain reaction (PCR) using the primers LCO-1490 or C1-J-1718 in combination with HCO-2198 (Folmer et al. 1994, Simon et al. 1994). We used a 2-part PCR program with 5 initial cycles with annealing temperature of 45°C followed by 35 cycles with annealing temperature of 51°C. PCR amplifications were purified and sequenced by Macrogen (Seoul, Korea). Chromatograms were viewed and consensus sequences were adjusted manually with Sequencher 4.14 (Gene Codes, Ann Arbor, Michigan). Sequences were checked with Basic Local Alignment Search Tool (BLAST; Altschul et al. 1997) against the GenBank nucleotide database to check the amplified product. Alignment of sequences was done with ClustalW (Thompson et al. 1994) in MEGA (version 5.1; Tamura et al. 2011). For some species, bases obtained only at the start or end of the alignment were trimmed. The result was a 471-base pair (bp) alignment of 38 sequences for *Kempnyia* and 3 outgroup sequences (*Anacroneuria debilis*, *A. itatiaiensis* Balducci, Bispo et Novaes, and *Paragripopteryx* sp.).

Phylogenetic analyses

We calculated pairwise distances corrected using the Kimura-2-parameter (K2P) model of evolution in MEGA

and clustered sequences via neighbor-joining (NJ) with 500 bootstrap replicates. We conducted a Bayesian analysis (2 independent runs of 4 Monte Carlo–Markov Chains for 1 million generations, 25% generation burn-in) in MrBayes (version 3.2.2; Ronquist et al. 2013) to assess support and monophyly of the clades recovered. We used jModelTest (Posada 2008) to choose the evolutionary model Hasegawa-Kishino-Yano + invariant + gamma (HKY+I+G) on the basis of Akaike's Information Criterion as the best model for the COI sequences.

RESULTS

Nymphal and female associations

We identified 10 distinct lineages or well supported clusters of *Kempnyia* (bootstrap and Bayesian posterior probabilities) in the NJ analysis (Fig. 1; color version available online). Three of these clades included conspecific males identified through male genital characters. Distances between males were 4.6% for *K. petersorum* (Froehlich), 9.6% for *K. obtusa*, and up to 15.1% for *K. colossica*. We assumed the highest value as the cutoff for maximum intraspecific divergences and used these values to delimit specific lineages that included ≥ 1 specimen. We separated unidentified nymphs into 6 morphotypes and associated representatives of morphotypes to male adults of *K. alterosarum* Froehlich, *K. colossica*, *K. gracilentata* Enderlein, *K. obtusa*, *K. petersorum* Froehlich, and *K. varipes* Klapálek. We separated adult female specimens into 3 morphotypes and associated representative specimens of each morphotype to identified males of *K. gracilentata*, *K. jatim* Froehlich, and *K. petersorum*.

COI intra- and interspecific divergences

Pairwise K2P corrected *p*-distances between all sequences are given in Table S1. Within the 29 *Kempnyia* specimens studied, minimum intraspecific divergences were 0.0 to 10.7%, and maximum intraspecific divergences were 4.6 to 15.1% (*K. alterosarum*: 6.4%; *K. colossica*: 10.7 to 15.1%, mean = 13.0; *K. gracilentata*: 0.0 to 11.2%, mean = 3.1; *K. jatim*: 0.2 to 7.7%, mean = 5.1; *K. obtusa*: 0.4 to 9.6%, mean = 6.4; *K. petersorum*: 0.0 to 4.6%, mean = 1.7; and *K. varipes*: 5.5 to 9.6%, mean = 8.0). Interspecific intrageneric divergences ranged from 7.5 to 25.0% (mean = 19.1). Thus, *Kempnyia* specimens showed an overlapping barcode gap (Fig. 2).

Kempnyia occurring in Macaé River Basin

We found 11 *Kempnyia* species in the Macaé River Basin. Four of these species were previously recorded from this basin (*K. obtusa*, *K. serrana*, *K. taunayi*, and *K. puri*), but we did not collect the latter 3 species during our study. Thus, we recorded 7 new *Kempnyia* species from this basin based on our study of 240 specimens

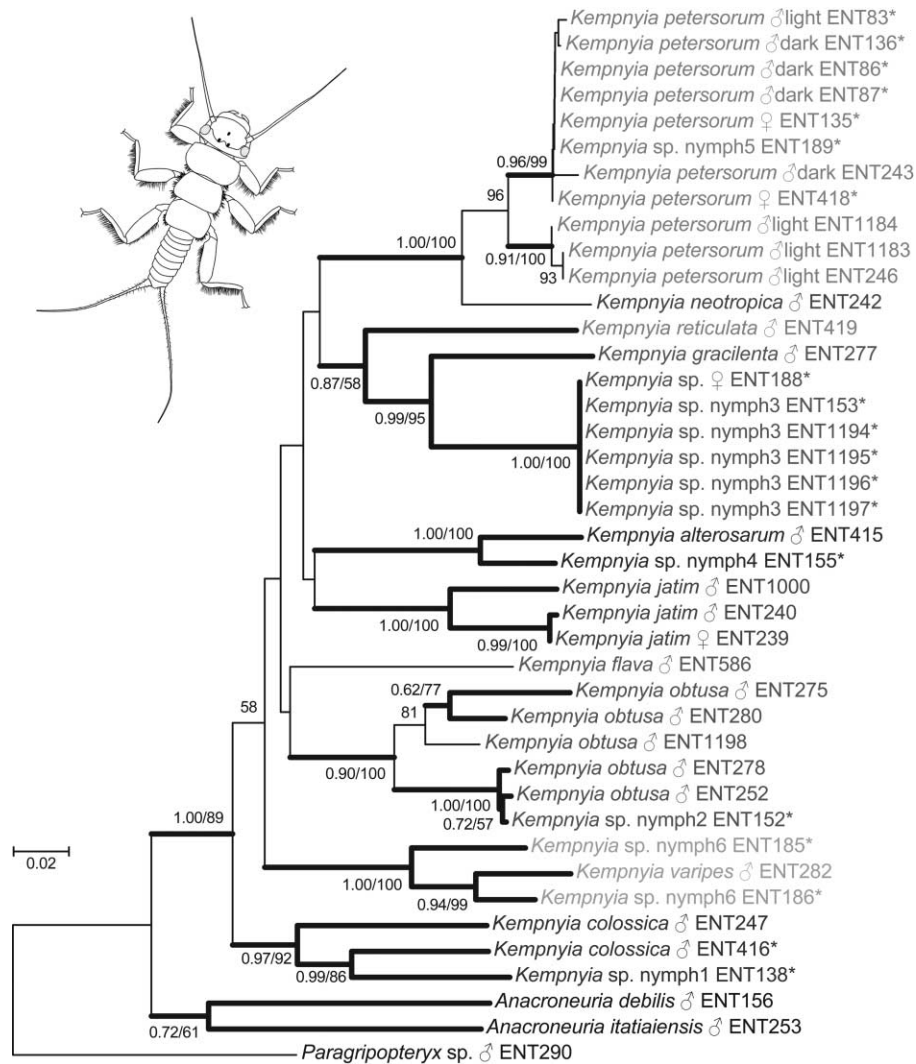


Figure 1. Neighbor-joining tree for cytochrome *c* oxidase subunit I (COI) sequences (471 base pairs) from *Kempnyia* and related stoneflies from southeastern and southern Brazil modeled by Kimura-2-parameter (K2P). The tree is rooted with a representative of Gripopterygidae. Clades shown with thick lines were recovered in the Bayesian analysis. Numbers are Bayesian posterior probabilities (when existing)/bootstrap percentages >50%. Colors correspond to *Kempnyia* species clades. * indicates specimens collected in Macaé River Basin.

(169 nymphs, 67 adults) collected at 23 of the 105 stations. Among the adults, 39 males represented 5 *Kempnyia* species: *K. colossica*, *K. gracilenta*, *K. jatim*, *K. petersorum*, and *K. obtusa*. We separated nymphs into 6 morphotypes and identified 1 morphotype as *K. reticulata* based on the nymphal morphological description given by Avelino-Capistrano et al. (2011). This record is the 1st for this species in Rio de Janeiro State. We used COI to associate 2 of these unidentified nymphal morphotypes with adult male *K. alterosarum* and *K. varipes* from other localities.

Morphological descriptions of previously unknown immatures, *K. alterosarum*, *K. obtusa*, and *K. petersorum* and a new color morph of the male adult of *K. petersorum* are given below. The species *K. varipes* was originally described based only on external morphology. Therefore, we have in-

cluded the adult male and associated nymphs. Short descriptions and notes on other studied species are given below. Head measurements are the maximum width of the head capsule. Forewing measurements are the distance from the base of the anal lobe to the wing apex. For nymphs, wing pad measurements refer to the distance from the anterior margin of the mesonotum to the tips of wing pads. Morphological terminology follows Stark et al. (2009).

DESCRIPTION OF SPECIES AND REMARKS

***Kempnyia alterosarum* Froehlich, 1988 (Fig. 3A–G; color version available online)**

Description. **Distribution Brazil.** Minas Gerais and Rio de Janeiro (new record) states.

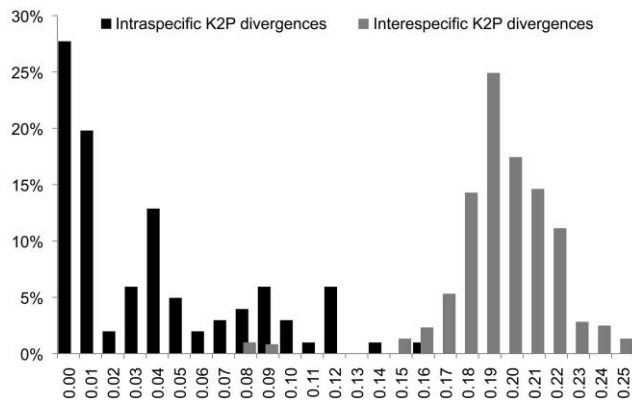


Figure 2. Percentages of pairwise intra- and interspecific comparisons by Kimura-2-parameter (K2P) divergences showing an overlapping barcode gap of *Kempnyia* specimens.

Material examined Brazil. MG: Itabirito, 2♂ [ENT415]; Itamonte, 1♂. RJ: Nova Friburgo, 1n [ENT155].

Measurements Nymph ($n = 1$). head: 3.36 mm; wing pads: 3.37 mm. Adult male ($n = 3$). head: 2.03–2.62 mm; forewing: 12.13–15.26 mm.

Nymph General color stramineous. Head with labrum and anterior frons with reddish brown band; posterior area of frons, clypeus, and parietalia, light brown (Fig. 3A). M-line light brown, but poorly delimited. Epicranial suture not exceeding the paired ocelli. Three ocelli partially

black. Eyes black. Spines covering $\frac{1}{3}$ of ocular sclerite. Antennae light brown (Fig. 3A, B). Pronotum rectangular, brown with a yellow area forming a characteristic pattern, covered by dark brown bristles (Fig. 3A); flange light brown, with margin brown; posterior flange area thicker than lateral. Meso- and metanotum stramineous with lighter spots (Fig. 3C), and with many spines laterally arranged. Pro-, meso-, and metanotum covered with many setae. Thoracic gills present: ASC [1,2,3], PSC [1,2,3], PT [3], and AT[1,2]. Legs stramineous, with longitudinal row of plumose setae on femur, tibia, and reduced on tarsi (Fig. 3D, E); numerous reddish-brown spines covering the femur in dorsal view; in ventral view, these spines are sparse and fewer in number; longitudinal row of reddish-brown spines along tibia (Fig. 3D, E). Abdomen, in dorsal view brown, becoming darker to the apex; in the each segment, posterior margin is darker; a row of spines is disposed on the base of each tergum (Fig. 3F); tergum X dark brown (Fig. 3G); general color yellow in ventral view, except by the last 5 segments of abdomen, which become brown to dark brown. Anal gills present (Fig. 3G). Cerci brown, bearing long bristles; brown spines are present in each article of the cerci. Moderate number of long fine bristles covering the base of cerci (Fig. 3G); each cercus article with a ring of 10 spines.

Remarks Froehlich (1988) described this species from Serra do Cipó (Minas Gerais State) based on many male

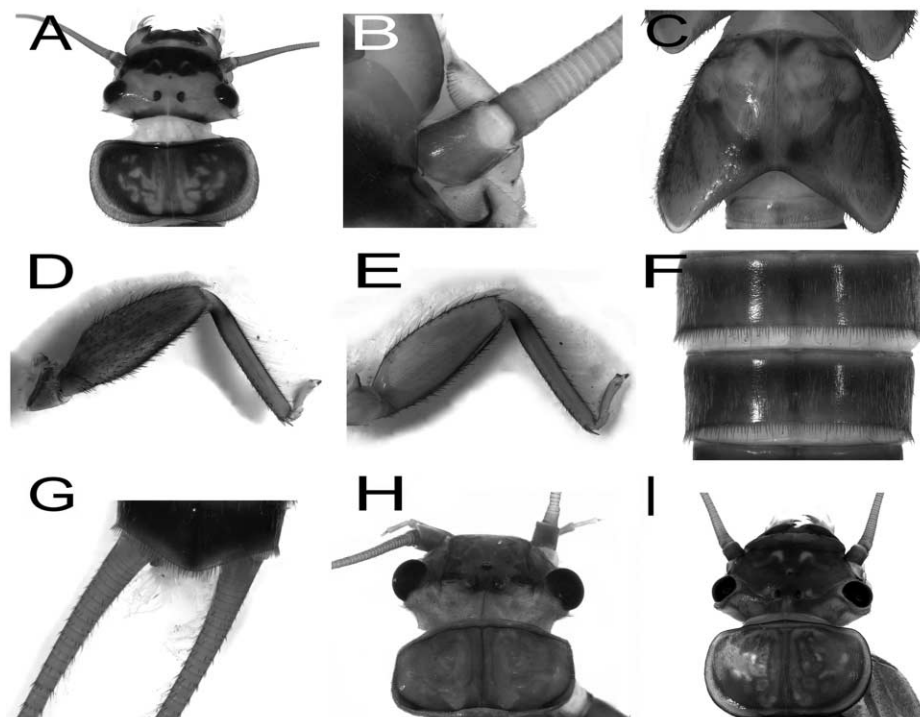


Figure 3. *Kempnyia alterosarum* Froehlich. Nymph: A.—Head and pronotum; B.—Flagellum of antennae, dorsal view; C.—Metanotum; D.—Foreleg, anterior view; E.—Foreleg, posterior view; F.—Tergum VI–VIII; G.—Tergum X and cerci. *Kempnyia colossica* (Navás). Adult male: H.—Head and pronotum. Nymph: I.—Head and pronotum.

and female specimens. No nymphs have been previously described. No additional records are available for this species from southeastern Brazil, and ours is the 1st record for Rio de Janeiro State. No adults were collected, and only 1 nymph from Nova Friburgo was associated with specimens from Itabirito and Itamonte. Males from Itabirito were smaller than the male from Itamonte, but both males were in the range of measurements given by Froehlich (1988). The nymph is larger than the adult males from Minas Gerais State and differs from other *Kempnyia* nymphs by the color pattern, a brown ring on anterior tibia, and few long bristles on cerci base (see Fig. 3A–G).

***Kempnyia colossica* (Navás, 1934) (Fig. 3H, I; color version available online)**

Description. *Distribution Brazil.* Minas Gerais, Paraná, Rio de Janeiro, Santa Catarina, and São Paulo states.

Material examined Brazil. MG: Itamonte, 6n. **RJ:** Nova Friburgo, 3♂ [ENT416], 1n [ENT138]; **SP:** São José do Barreiro, 3♂ [ENT247].

Measurements Nymph ($n = 7$). head: 3.57–6.50 mm; wing pads: 2.82–8.00 mm. Adult male ($n = 6$). head: 3.33–4.41 mm; forewing: 24.76–29.60 mm.

Nymph (additional characters) General color reddish-yellow dorsally and whitish yellow ventrally. Eyes black with white contour; 3 black ocelli, light comma-like paired spots beside ocelli (Fig. 3I). Pronotum oval, with many bristles; surface rugose forming a pattern of ornamentation; flange thin, with parts sometimes darker. Meso- and metanotum darker on anterior margin than posterior margin. Legs with femora and tibiae with a fringe of brown bristles. Abdomen reddish-brown with longitudinal line light brown.

Remarks Navás (1934) described this species as *Laeissa colossica* based on an exemplar from Nova Bremen (now Presidente Getúlio, Santa Catarina State). Froehlich (1988) transferred this species to *Kempnyia* based on characteristics shared with *Kempnyia brasiliensis* (Pictet) (large size and type of penial armature). He pointed out differences between males from Santa Catarina and Campos do Jordão, especially in the size and shape of penial armature. Specimens from Rio de Janeiro State (Fig. 3H, I) are similar to the specimen from Santa Catarina State with penial armature similar to that illustrated by Froehlich (1988; Fig. 3G–I). The nymph of this species was recently described by Bispo et al. (2013) based on material from Paranapiacaba Mountains (São Paulo State) and is similar to those analyzed from Nova Friburgo. Characters given in the nymphal description above were not cited in this description or are slightly different.

***Kempnyia gracilentia* (Enderlein, 1909) (Figs S1A–H, 4A–C; color version available online)**

Description. *Distribution Brazil.* Espírito Santo, Minas Gerais, Rio de Janeiro, and São Paulo states.

Material examined Brazil. ES: Castelo, 3♂ [ENT277]. Santa Teresa, 1♂ 1♀ **RJ:** Nova Friburgo, 1♀ [ENT188], 38n [ENT153, 1194, 1195, 1196, 1197].

Measurements Nymph ($n = 38$). head: 1.73–4.12 mm; wing pads: 1.11–4.01 mm. Adult female ($n = 2$). head: 3.25–3.27 mm; forewing: 19.6–21.50 mm. Adult male ($n = 4$). head: 2.32–2.46 mm; forewing: 14.17–15.68 mm; penial armature: 1.80 mm.

Nymph (additional characters) Spines covering ½ of ocular sclerite (Fig. S1A). Antennae bearing light brown bristles; each flagellomere bearing tufts of short digitiform setae (Fig. S1B). Pronotum rectangular (Fig. S1C). Femora covered by numerous spines and bristles (Fig. S1D, E). Thoracic gills present: ASC [1,2,3], PSC [1,2,3], PT[3], and AT[1,2]. Abdomen light brown; a row of spines on posterior margin of each tergum (Fig. S1F); numerous long fine bristles covering the base of cerci (Fig. S1G, H); long brown spines on basal articles of cerci (Fig. S1G, H). Anal gills present (Fig. S1G).

Remarks Enderlein (1909) described *K. gracilentia* from adult males and females from Espírito Santo State. The species is recorded from Rio de Janeiro (Petrópolis, Teresópolis; Jewett 1960), Minas Gerais (Serra do Caraça; Froehlich 1984), and São Paulo (Campos do Jordão; Froehlich 1984). The female subgenital plate of *K. gracilentia* was drawn by Froehlich (1984), but later this author attributed these figures (1–3) as belonging to *K. tamoya* (Froehlich 2011a). We did not examine the material studied by Froehlich, but specimens of *K. gracilentia* reared by Avelino-Capistrano et al. (2011) (Fig. 4A) and the female associated to this species from Macaé River Basin [ENT188] (Fig. 4B, C) agree with the original figures by Froehlich (1984).

***Kempnyia obtusa* Klapálek, 1916 (Figs 4D, S2A–H; color version available online)**

Description. *Distribution Brazil.* Minas Gerais, Rio de Janeiro, and São Paulo states.

Material examined Brazil. ES: Alto Caparaó, 1♂ [ENT275]. **MG:** Itabirito, 1♂ [ENT280]. **RJ:** Nova Friburgo, 3♂, 31n [ENT152]; Petrópolis, 4♂ [ENT252, 1198]. **SP:** São José do Barreiro, 4♂, 2♀ [ENT278].

Measurements Nymph ($n = 31$). head: 1.25–5.14 mm; wing pads: 0.73–5.23 mm. Adult male ($n = 13$). head: 2.40–3.24 mm; forewing: 15.49–20.60 mm. Adult female ($n = 2$). head: 2.19–2.81 mm; forewing: 18.84–19.42 mm.

Nymph General color stramineous, with dark bristles (Fig. 4D). Head with dark brown area, with arched stains, ranging from lateral margin of clypeus to area between the ocelli (Fig. 4D). Labrum brown. Clypeus with anterior margin brown. M-line barely evident, with glabrous surface. Epicranial suture does not exceed paired ocelli. Three ocelli completely black. Eyes black. Spines covering basal ⅓ of ocular sclerite (Fig. S2A). Antennae stramineous, with brown setae on scape base of pedicel (Fig. S2B) and in each

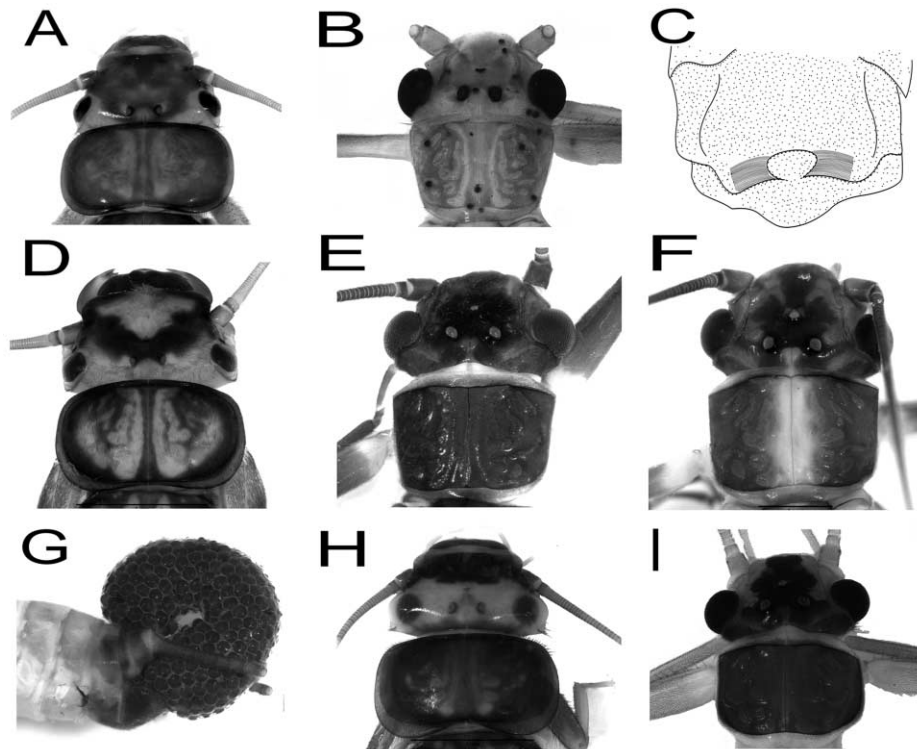


Figure 4. *Kempnyia gracilentata* (Enderlein). Nymph: A.—Head and pronotum. Adult female: B.—Head and pronotum; C.—Female subgenital plate. *Kempnyia obtusa* Klapálek. Nymph: D.—Head and pronotum. *Kempnyia petersorum* Froehlich. Adult male: E.—Dark brown morph, head and pronotum. Adult female: F.—Dark brown morph, head and pronotum; G.—Subgenital plate and egg mass. Nymph: H.—Head and pronotum. Adult male: I.—Light brown morph, head and pronotum.

flagellomere. Pronotum oval shape (Fig. S2C); anterior margin brown; lateral and posterior margins light brown; marginal groove of pronotal disc brown; 2 longitudinal pale lines parallel to median line suture, forming a characteristic pattern. Meso- and metanotum covered by numerous bristles (Fig. S2D); general color light brown with pale areas forming a characteristic pattern. Thoracic gills present: ASC [1,2,3], PSC [1,2,3], PT[3], and AT[1,2]. Legs yellow with brown stripe; a row of silver plumose setae on femur and tibia (Fig. S2E, F); numerous bristles covering the femur; spines present near on anterior and posterior margins of dorsal surface of femur. Abdomen light brown; posterior margin of each segment brown; a row of spines on posterior margin of each tergum (Fig. S2G); tergum X brown; in ventral side, all segments are pale brown, except segment X. Anal gills absent (Fig. S2H). Cerci with few long bristles on the base (Fig. S2H), reducing in number and in size to apex; brown spines are present on each article.

Remarks Klapálek described *Kempnyia obtusa* based on male specimens from Teresópolis (Rio de Janeiro State). Specimens from Rio de Janeiro, Minas Gerais, and São Paulo states agree with the original description, except the material from Serra do Caparaó, where specimens were smaller. Froehlich (1988) later recorded females from Serra de Macaé and Parque Nacional do Itatiaia, but immatures of this species were previously unknown. Nymphs are char-

acterized by the characteristic pattern of the pale area on the head (Fig. 4D), by dark brown stripes on the femur, absence of anal gills (Fig. S2H), and many long bristles at cerci base.

***Kempnyia petersorum* Froehlich, 1996 (Figs 4E–I, S3A–H; color version available online)**

Description. *Distribution Brazil.* Minas Gerais, Rio de Janeiro, Paraná, and São Paulo states.

Material examined Brazil. RJ: Nova Friburgo, 2♂ [ENT83], 6♀, 2n [ENT189]; Macaé, 11♂ [ENT86, 87, 136], 5♀ [ENT135, 418], 4n; Petrópolis, 2♂ [ENT243], 2♀. **PR:** Morretes, 3♂ [ENT246, 1183, 1184]. **SP:** Iporanga, 3♂, 2♀. **MG:** Itabirito, 1♂.

Measurements Nymph ($n = 6$). head: 2.42–3.99 mm; wing pads: 1.73–4.10 mm. Adult male ($n = 22$). head: 1.49–2.48 mm; forewing: 8.20–14.61 mm. Adult female ($n = 15$). head: 2.35–3.11 mm; forewing: 13.25–19.39 mm.

Adult male, dark brown morph General color brown (Fig. 4E). Eyes black. Three ocelli partially black. Central area of head dark brown; lateral side brown; yellow heart-shaped spot inside loop of epicranial suture. Antennae light brown; scape of antennae brown. M-line present, lighter than the rest of head. Epicranial suture does not exceed the paired ocelli. Pronotum rectangular and brown;

very rugose. Wings brown, with dark brown veins. Legs brown with fore coxae, fore trochanters, and base of fore tibiae yellow; other legs with same color, but brown area at tibiae starts at middle (2nd legs) and apical end (3rd legs). Abdomen light brown in dorsal and ventral views. Cerci brown, with many spines in the base reducing in number apically; many bristles covering all cerci. Tergum X with peg-like area, with few and sparse spines; paraprocts digitiform. Subgenital plate round; hammer subtriangular (similar to fig. 10 in Froehlich 1996). Penial armature as illustrated by Froehlich (1996; figs 13–15).

Adult female, dark brown morph Color pattern similar to male (Fig. 4F), but with distinct yellow longitudinal median band on pronotum. Subgenital plate rounded and brown. Egg mass violet (Fig. 4G).

Nymph General color brown. Head with brown area anterior to anterior ocellus extending to frons; clypeus brown (Fig. 4H). M-line not evident. Epicranial suture does not exceed paired ocelli. Three ocelli partially black. Eyes black. Spines are present only at base of the compound eyes (Fig. S3A). Antennae yellow; scape brown with setae (Fig. S3B). Pronotum subtrapezoidal brown (Figs 4H, S3C), with a pale area forming characteristic pattern; flange thin laterally, and thicker posteriorly. Meso- and metanotum light brown with lighter spots; with many spines laterally arranged (Fig. S3D). Thoracic gills present: ASC [1,2,3],

PSC [1,2,3], PT[3], and AT[1,2]. Posterior margin of each segment of abdomen brown with a row of spines on posterior margin of each tergum (Fig. S3E). Legs yellow, with sparse row of setae on femora (Fig. S3F) and tibiae, sparser on tarsi. Abdomen brown, darkening towards segment X; row of spines on posterior margin of each tergum (Fig. S3G); tergum X dark brown. Cerci with few bristles only at base (Fig. S3G, H); brown spines present in each article. Anal gills absent (Fig. S3G).

Remarks The nymph described herein differs from other *Kempnyia* nymphs by the dark brown color pattern of the head, similar to the nymph of *K. neotropica* described by Bispo and Froehlich (2008). However, these nymphs could be distinguished by the absence of anal gills in *K. petersorum*. Based on the molecular association, adult specimens described above as dark brown morphs are conspecific with individuals with the typical (originally described) light brown color pattern (Fig. 4I). Both color morphs occur at Macaé River Basin (see discussion).

***Kempnyia varipes* Klapálek, 1916 (Figs 5A–H, S4A–H; color version available online)**

Description. Distribution Brazil. Rio de Janeiro State.

Material examined Brazil. RJ: Nova Friburgo, 6n [ENT186]; Macaé, 1n [ENT185]; Teresópolis, 4♂ [ENT282].

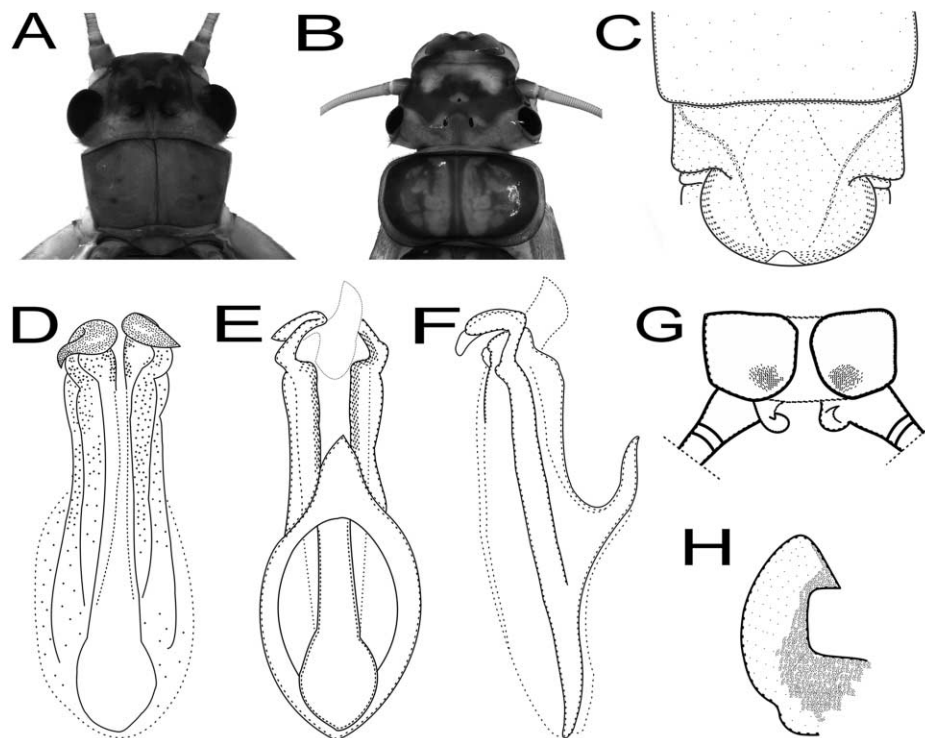


Figure 5. *Kempnyia varipes* Klapálek. Adult male: A.—Head and pronotum. Nymph: B.—Head and pronotum. Adult male: C.—Tergum X; D–F.—Penial armature, dorsal, ventral, and lateral views; G.—Subgenital plate and hammer; H.—Right paraproct, lateral view.

Measurements Nymph ($n = 7$). head: 2.87–4.22 mm; wing pads: 2.65–3.31 mm. Adult male ($n = 4$). head: 2.49–2.98 mm; forewing: 15.44–17.53 mm.

Male General color, in dorsal view brown, with light spots along the body (Fig. 5A). Ventral side pale. Head covered with many setae. M-line present. Three ocelli partially black. Eyes black. Antennae yellow and covered by many setae. Epicranial line does not exceed ocelli. Pronotum trapezoidal, brown laterally and lighter on disc. Wings light brown; veins of forewings light brown, excepting R, r, Cu, and A. Legs yellow, except by dark brown area at femoral base, and at base and apex of tarsus. Abdomen and cerci covered by numerous bristles. Cerci light brown. Subgenital plate rectangular and outlined by brown; hammer triangular (Fig. 5C). Penial armature dark brown. Hooks making a compact curve (comma-shaped) (Fig. 5D–F). Ring with median projection (Fig. 5E, F) with pointed apex. Tergum X with areas with peg-like bristles (Fig. 5G). Paraprocts directed dorsally with sharp point near apex (Fig. 5H).

Nymph General color stramineous (Fig. 5B). Head with labrum light brown; clypeus with lateral areas light brown; front with broad light-brown macula posteriorly, with small stramineous spot surrounding anterior ocellus. M-line not evident. Epicranial suture exceeding paired ocelli. Eyes black; with spines at base (Fig. S4A). Antennae yellow, with few setae on scape (Fig. S4B). Pronotum oval, borders of disc and median longitudinal band light brown; pronotal flange thicker posteriorly and wider laterally, with some spines at apex and base (Fig. S4C). Meso- (Fig. S4D) and metanotum light brown with lighter spots, with many spines laterally. Legs yellow; in dorsal view, femora covered by bristles and spines; longitudinal fringe of bristles on femora and tibiae (Fig. S4E). Abdomen light brown; a row of spines on posterior margin of each tergum (Fig. S4F). Anal gills present (Fig. S4G). Cerci moderately covered by long bristles at base, reducing in number and size towards the apex, but a tuft is present on final segments; each segment bearing brown spines (Fig. S4H).

Remarks This species has comma-shaped hooks similar to *K. alterosarum*, *K. vanini* Froehlich, and *K. tupinamba* Froehlich, but the median projection of the penial ring and the absence of spiny patches on the penial tube separate *K. varipes* from the others. This projection can be found in *K. reticulata* and *K. tijucana*, but these species have differently shaped hooks. The nymph is characterized by the epicranial suture that exceeds the paired ocelli, and the light-brown color delimited by pale areas, forming a characteristic pattern on the head and pronotum.

DISCUSSION

COI intra- and interspecific divergences

COI intraspecific and intraspecific divergences are commonly reported in DNA barcoding studies (Bergsten

et al. 2012). Both are used to calculate the barcode gap, which affects the accuracy of species identifications. Early studies of intraspecific variation indicated barcode gaps <3% divergence in insect species and >8% divergence in most animals studied (Hebert et al. 2003a, b). However, substitution rates are heterogeneous in mitochondrial DNA, so universal thresholds for divergences should not be assumed in species delimitations (Galtier et al. 2009).

We regarded unidentified nymphs and females as conspecific with identified males when they formed a monophyletic group and their maximum divergence was less than the maximum intraspecific divergence found between 2 males identified on the basis of morphological characters. Intraspecific divergences varied from 0 to 15.1% (Table S1), but $\sim\frac{1}{2}$ of comparisons had values >3%. The maximum divergence was found between 2 male specimens of *K. colossica* (ENT247 vs ENT416). These intraspecific divergence values seem high when compared to those for other insects (Zhou et al. 2009, Hayashi and Sota 2010, Park et al. 2011, Renaud et al. 2012), but the stoneflies studied so far tend to show high intraspecific divergences. For perlids, Fochetti et al. (2011) reported maximum intraspecific divergences of 8.3% in *Besdolus bicolor* (Navás) and 7.7% in *Perlodes microcephalus* (Pictet). For chloroperlids, Weiss et al. (2012) found maximum divergences of 6.2% in *Siphonoperla torrentium* (Pictet) and 5.1% in *S. hajastanica* (Zhiltzova). For gripopterygids, Mynott et al. (2011) reported an intraspecific divergence of 5.8% in *Riekoperla alpina* McLellan and 4.2% in *R. karki* McLellan. Mynott et al. (2011) pointed out that these high values could be related to geographic isolation among populations at high altitudes and might indicate the presence of cryptic species. Most species of *Kempnyia* in our study, e.g., *K. colossica*, *K. gracilentata*, and *K. obtusa*, show high intraspecific divergences, and these species have been collected in isolated mountain ranges in southeastern Brazil. High genetic divergences found in *Kempnyia* may be the result of the generally low vagility (Boumans and Baumann 2012) and conservative morphology of stoneflies, which makes it difficult to find reliable diagnostic characteristics for differentiating isolated populations or cryptic species.

In contrast, the populations of *K. petersorum* we studied were relatively homogeneous. Five individuals from Macaé River Basin shared the same haplotype, even though some were collected in localities separated by ~ 35 km and 800 m in altitude. The highest divergences among *K. petersorum* individuals were found between a specimen from Paraná State (Morretes) and all other specimens from Rio de Janeiro State (Petrópolis and Macaé) (2.6–4.6%; ENT418 vs ENT1184 and ENT243 vs ENT246). These localities were separated by ~ 700 km. Nevertheless, *K. petersorum* shares a pair of lateral spinose patches on the membranous penial tube (Froehlich 2011b)

with *K. neotropica* and a few other *Kempnyia* species, including *K. auberti* Froehlich, *K. ocellata* Froehlich, *K. sazimai* Froehlich, *K. tupinamba*, *K. umbrina* Froehlich, and *K. vanini*. Some of these species are very similar in genital morphology, and are distinguished only by color pattern and size (Froehlich 1996). The distinction of these nominal species based on color pattern may be problematic because we found 2 color morphs of *K. petersorum* male and female adults in Macaé River Basin. The light color morph (Fig. 4I) matches Froehlich's (1996, 2011b) description based on specimens from São Paulo State, which have a distinctly stramineous head with a longitudinal brown band and uniformly brown pronotum, and is represented in our study by male specimens from Macaé River Basin [ENT83] and Paraná State [ENT246, 1183, and 1184]. Other *K. petersorum* males from Macaé River Basin in our study were dark brown (Fig. 4E), occurred in the same collecting sites, and shared the same size and penial armature with the light morph. This is the first reported case of color dimorphism in *Kempnyia*. Given the high morphological similarity, color plasticity, and the low interspecific divergences (relative to other divergences in our study) among members of this group of species (7.5–8.9% interspecific divergences in Fig. 2; ENT242 vs ENT1183 and ENT242 vs ENT1184), it is possible that *K. petersorum*, *K. neotropica*, and perhaps other related nominal species belong to the same species.

Kempnyia from Macaé River Basin

Knowledge of species-level identification and geographic range is essential for ecosystem conservation. This level of understanding is nascent for most Neotropical aquatic insects. Among the 15 *Kempnyia* species recorded from Rio de Janeiro State, only 4 had been described from Macaé River Basin based on material collected by E. Garbe in the early 1900s (Froehlich 1988, 2010, Nessimian et al. 2009). A new species was described recently based on material collected in 1996 and deposited in DZRJ (Avelino-Capistrano et al. 2013). Thus, our study increases these numbers to 11 species from Macaé River Basin and 17 from Rio de Janeiro State.

Currently, stonefly species from Rio de Janeiro State make up 24% of the total number of species recorded from Brazil, a percentage that is similar to the 27% in Trichoptera and 23% in Ephemeroptera (Salles et al. 2013, Santos et al. 2013). The area of this state is <1% of the area of Brazil, so the high proportion of recorded species in the state probably is a reflection of the early establishment of research centers in the region. However, historical inventories were done in only a few areas and little is known about the insect fauna in the rest of the state, especially in the northern region (Couri et al. 2009). The information on Plecoptera is very sparse because of a lack of specialists

and focused diversity studies (Nessimian et al. 2009). *Kempnyia taunayi* and *K. serrana* were recorded in early collections (Froehlich 2010), but they did not occur in our samples.

Kempnyia taunayi was described by Navás (1936) based on 1 adult female, previously synonymized by Jewett (1960) with *K. klugii* (Pictet). Froehlich (2010) did not treat this species as a synonym, but as a *species inquirenda*, probably because Plecoptera females have a few diagnostic characters, and species diagnoses are based mostly on male genitalia. On the other hand, *K. serrana* has been recorded from Rio de Janeiro, Espírito Santo, and São Paulo states (Froehlich 2011b). We might have failed to collect it because it might not be attracted to light traps, as is the case for *K. tenebrosa* (Froehlich 2011b). Use of additional traps, like Malaise traps, might increase the efficiency of collecting events.

Our work contributes significantly to nymphal taxonomy and increases the number of *Kempnyia* nymphs described to species level from 5 to 9. Descriptions of nymphs are common from the Nearctic and Palearctic regions (Claassen 1931, Hynes 1940, Zhiltzova et al. 2012) and are becoming more common from the Neotropics (Dorvillé and Froehlich 2001, Bispo and Froehlich 2008, Avelino et al. 2011, Bispo et al. 2013). In general, reliable diagnostic characters for separating nymphs to species level are few, and authors do not agree on which characters to use. Many authors have used coloration and external morphology to describe nymphs (Claassen 1931, Dorvillé and Froehlich 2001, Bispo and Froehlich 2008, Avelino-Capistrano et al. 2011, Bispo et al. 2013). We attempted to find new diagnostic characters by using SEMs, but we found none other than those that can be observed with light microscopy. However, we did find 1 important character, the absence of anal gills for 2 species of *Kempnyia*, *K. obtusa*, and *K. petersorum*, not previously observed in other described nymphs, and it seems to be of diagnostic relevance.

Conclusions

We are the first to use molecular tools to associate life stages of Neotropical stoneflies. Molecular associations to identified adult male *Kempnyia*, a southern South American genus from Macaé River Basin, resulted in identification of 6 nymphal morphotypes. Of the 6 *Kempnyia* species identified, 4 of those lacked formal morphological descriptions of their immature stages. These descriptions will enable morphological identification of immatures occurring in this basin at the species level, which will help refine biotic indices for freshwater monitoring. During our 3-y inventory, adult males of 2 of these species were not collected. Had these nymphs not been associated using DNA barcodes to identified males from other regions in Brazil, these species would have been neglected as oc-

curing in this basin. Our study is far from being a comprehensive study that resolves species limits in *Kempnyia*, but it contributes to our knowledge of haplotype diversity in stonefly populations, which may be useful to future barcode identifications of *Kempnyia* individuals in south-eastern Brazil for a wide variety of ecological and monitoring studies. Our study also revealed interesting aspects of stonefly morphological taxonomy (e.g., color polymorphism) that should be better addressed. In our opinion DNA barcode associations provided an easy and effective means to identify immature stages of Neotropical stoneflies and should be a standard method for associating stages when rearing of immatures is not possible.

ACKNOWLEDGEMENTS

We are grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, procs. 470.316/2009 and 481.554/2012) and Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ, procs. E-26/171.281/2006 and E26/111.389/2010) for partially funding this study. Fellowships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and CNPq to FACS and JLN, respectively, are also appreciated. This manuscript highly benefited from comments and suggestions made by editors P. Silver and B. Sweeney and 2 anonymous referees on a previous version. We thank L. S. Barbosa for the suggestions on photographs. For sharing important literature, we thank L. Lecci, R. Fochetti, W. Graf, and C. Froehlich, who also shared his own illustrations of the genitalia of the type of *K. varipes*, so that a correct identification of our material could be made. S. Freitas helped with some SEMs at Rudolf Barth Electron Microscopy Platform of Instituto Oswaldo Cruz. Last, we thank all colleagues who helped with field work: A. L. Henriques-Oliveira, A. P. M. Santos, B. Clarkson, B. H. L. Sampaio, G. A. Jardim, I. C. Gonçalves, L. L. Dumas, L. F. L. Silveira, M. R. Souza, N. Ferreira Jr., R. A. Carvalho, and V. P. Alecrim.

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