DNA barcodes for species delimitation in Chironomidae (Diptera): a case study on the genus *Labrundinia*

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Abstract—In this study, we analysed the applicability of DNA barcodes for delimitation of 79 specimens of 13 species of nonbiting midges in the subfamily Tanypodinae (Diptera: Chironomidae) from São Paulo State, Brazil. Our results support DNA barcoding as an excellent tool for species identification and for solving taxonomic conflicts in genus *Labrundinia.* Molecular analysis of cytochrome c oxidase subunit I (COI) gene sequences yielded taxon identification trees, supporting 13 cohesive species clusters, of which three similar groups were subsequently linked to morphological variation at the larval and pupal stage. Additionally, another cluster previously described by means of morphology was linked to molecular markers. We found a distinct barcode gap, and in some species substantial interspecific pairwise divergences (up to 19.3%) were observed, which permitted identification of all analysed species. The results also indicated that barcodes can be used to associate life stages of chironomids since COI was easily amplified and sequenced from different life stages with universal barcode primers.

Résumé—Notre étude évalue l'utilité des codes à barres d'ADN pour délimiter 79 spécimens de 13 espèces de moucherons de la sous-famille des Tanypodinae (Diptera: Chironomidae) provenant de l'état de São Paulo, Brésil. Notre étude confirme l'utilisation des codes à barres d'ADN comme un excellent outil pour l'identification des espèces et la solution de problèmes taxonomiques dans genre *Labrundinia.* Une analyse moléculaire des séquences des gènes COI fournit des arbres d'identification des taxons, délimitant 13 groupes cohérents d'espèces, dont trois groupes similaires ont été reliés subséquemment à une variation morphologique des stades larvaires et nymphal. De plus, un autre groupe décrit antérieurement à partir de caractères morphologiques a été relié à des marqueurs moléculaires. Il existe un écart net entre les codes à barres et, chez certaines espèces, d'importantes divergences entre les espèces considérées deux par deux (jusqu'à 19,3%), ce qui a permis l'identification de toutes les espèces examinées. Nos résultats montrent aussi que les codes à barres peuvent servir à associer les différents stades de vie des chironomides, car il est facile d'amplifier et de séquencer le gène COI provenant des différents stades avec les amorces universelles des codes à barres.

Introduction

Nonbiting midges of the genus *Labrundinia* Fittkau (Diptera: Chironomidae: Tanypodinae) are minute Diptera (1.0–2.5 mm). Currently, this genus contains 14 species, all of which are found in the New World with the exception of *Labrundinia longipalpis* (Goetghebuer, 1921) (Ashe and O'Connor 2009; Silva *et al.* 2011). The genus was erected by Fittkau (1962) based on the Palearctic species *L. longipalpis* (original combination *Tanypus longipalpis*), which has immatures that live in a variety of unpolluted water bodies from small streams and ponds to lakes and bays (Silva *et al.* 2011). Morphology-based species identifications of *Labrundinia* are

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considerably difficult or even impossible, particularly for adults. The few diagnostic characters used to separate species demonstrate considerable intraspecific variation, making their application to one species difficult. For example, male abdominal coloration, an important diagnostic species character, has an extensive variation within the genus.

The descriptions of *Labrundinia* species currently available are based mainly on males and do not include diagnostic characters for larvae and pupae at the species level. Association of chironomid immature stages can be achieved by individual rearing of larvae and collecting cast larval and pupal skins to establish the associations between each life stage (*e.g.* Ekrem *et al.* 2007). This procedure is time-consuming, however, and is not always successful for species with special environmental requirements. Thus, life stage associations based on genetic similarity of short DNA fragments (so-called DNA barcodes) may be more effective for species identification. Several studies have recognised the benefit of DNA sequences for associating immature stages with adult stages (*e.g.* Hebert *et al.* 2004a; Thomas *et al.* 2005; Ekrem *et al.* 2010a; Stur and Ekrem 2011; Silva *et al.* 2012). DNA barcoding has previously been used to differentiate six Australian species within another genus of the subfamily Tanypodinae (*Procladius* Skuse, Carew *et al.* 2011).

Tanypodinae is the third most diverse subfamily after Chironominae and Orthocladiinae, respectively (Ashe and O'Connor 2009). Thienemann and Zavřel (1916) established this subfamily based on the immature stages and its monophyly is strongly supported, with Podonominae as its sister-group (Cranston *et al.* 2012). Larval *Labrundinia* have modified head capsules that are adapted for a predatory lifestyle, as is found in most members of Tanypodinae. Comparative morphological studies on larval head capsules (Gouin 1959; Bryce and Hobart 1972) indicate that the features distinguishing the Tanypodinae larvae from those of the other subfamilies were regarded as adaptations for predation.

DNA barcoding employs standardised genomic fragments to enable species identification and discovery of cryptic taxa (Hebert *et al.* 2003; Kress *et al.* 2005; Savolainen *et al.* 2005). Supporters of this technique argue that a short standardised fragment of DNA can be used to recognise taxa as well as increase the speed, objectivity, and efficiency of species identification (Meyer and Paulay 2005). Initial tests of genetic barcoding using mitochondrial markers on animals have shown that a 658-base-pair fragment of the mitochondrial gene, cytochrome c oxidase subunit I (COI) is usually effective as a barcode sequence, providing more than 95% species-level resolution (Hebert *et al.* 2003, 2004a; Hajibabaei *et al.* 2006; Smith *et al.* 2006). Nevertheless, despite these highly encouraging results, the success rate of this approach relies on the delineation between intraspecific and interspecific genetic divergence (Hebert *et al.* 2004b; Meyer and Paulay 2005). On the other hand, intraspecific and interspecific DNA sequence variation depends directly on the evolutionary and biogeographical relationships of the group in question, which might differ severely from one population or region to another (Kirkendale and Meyer 2004).

Regarding the identification of insects, DNA barcoding has a number of drawbacks (Virgilio *et al.* 2010). Recent speciation, incomplete lineage sorting, interspecific hybridisation, and infection by endosymbiotic bacteria such as *Wolbachia* Hertig (Rickettsiaceae) (Funk and Omland 2003; Whitworth *et al.* 2007; Foottit and Adler 2009) may all negatively affect the performance of DNA barcoding in insects (Virgilio *et al.* 2010). Undoubtedly, and perhaps more importantly, the reliability of DNA barcoding in insects might be challenging given their immense diversity (Foottit and Adler 2009), which affects the comprehensibility of DNA barcode sequence libraries to adequately represent the immense diversity in insects (Virgilio *et al.* 2010).

In this study, we evaluate the feasibility of using DNA barcodes for species delimitation in *Labrundinia* using the standard barcode fragment of COI amplified with the universal primers of Folmer *et al.* (1994), LCO1490 and HCO2198. We also investigate whether partial COI gene sequences can be employed to associate life stages of species within this genus. We choose to focus on species of *Labrundinia* because this genus is currently being revised by the first author and material of numerous species is available from different localities in São Paulo State, Brazil. All unnamed species referenced in

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this study will be described in a future taxonomic manuscript, and published elsewhere.

Materials and methods

The taxa included in this study were selected to represent as many of the known morphotypes in *Labrundinia* as possible. Field work was conducted in São Paulo State, Brazil, without any design to test sampling regime or spatial distribution. Larvae and pupae were collected from aquatic systems < 200 km apart, using a hand-net. Some larvae and pupae were isolated in small vials covered with nylon screen and reared in the laboratory to obtain emerged adults. Several samples of different aquatic macrophyte species were collected and placed in a plastic tray in order to rear specimens to adulthood. Immature chironomids were preserved in absolute ethanol while imagines were kept in slightly dilute ethanol (~ 80–85%) to avoid breakage. More than one life stage was sequenced from all the included species (Appendix). Different morphotypes were recognised based on variation in all observable morphological traits such as colouration, genital structures, shapes of pupal thoracic horn, and larval claws.

Ethanol-preserved specimens were dissected under a stereo microscope, and the wings, one pair of legs, and the antennae were mounted in Euparal on microscope slides. DNA was extracted from the remaining body parts in a buffered solution with the enzyme proteinase-K. DNA extraction, polymerase chain reaction (PCR), and bi-directional sequencing were performed at the Canadian Centre for DNA Barcoding (Guelph, Ontario, Canada), using standard protocols (http://ccdb.ca/pa/ge/research/protocols). Other specimens were analysed in the molecular lab at the Norwegian University of Science and Technology Museum in Trondheim (Norway), where DNA extraction followed the tissue protocol using a GeneMole robot (MoleGenetics, Lysaker, Norway). Each PCR was made in a total volume of 25 μL and contained 2 μL DNA template (concentration not measured), 1 × Qiagen PCR Buffer (Tris-Cl, KCl, $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$), 2.0 μM additional $MgCl_2$, 0.8 mM of dNTPs, 0.4 μM of each of the suggested standard barcode primers (Folmer *et al.* 1994) LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′), 1 unit of HotStarTaq DNA Polymerase (Qiagen, Oslo, Norway), and 14.3 μL of ddH2O. Amplifications for the COI region were performed in a thermocycler with an initial denaturation step of 95 °C for 15 minutes, followed by five cycles of 94 °C for 30 seconds, 45 °C for 30 seconds, 72 °C for 1 minute, followed by 35 cycles of 94 °C for 30 seconds, 51 °C for 30 seconds, 72 °C for 1 minute, and one cycle at 72 °C for 5 minutes, then held at 4 °C.

The PCR products were purified using ExoSAP-IT (USB Products, Affymetrix, Santa Clara, United States of America) following protocols recommended by the manufacturer. Purified products were sequenced in both directions using BigDye 3.1 (Applied Biosystems, Life-technologies, Oslo, Norway) termination reactions and analysed on ABI 3730 genetic analysers. Sequences were assembled and edited using DNA BASER Sequence Assembler 3.2.4 (Heracle BioSoft S.R.L., Pitesti, Romania), checked for stop-codons and aligned as translated amino acids using default ClustalW options (Thompson *et al.* 1999) as implemented in MEGA 5.03 (Tamura *et al.* 2011). The alignment was trivial as no indels were observed in the sequences.

Neighbour-joining (NJ) and maximum likelihood trees were produced in MEGA 5.03 (Tamura *et al.* 2011), using Kimura two-parameter (K2P) (Kimura 1980) and GTR + G + I (Lanave *et al.* 1984) models, respectively. Support for specific tree topologies was estimated by bootstrap analysis with 1000 pseudoreplicate data sets (Felsenstein 1985). Pairwise sequence divergences within and between genetic clusters were calculated under a K2P model in MEGA. The analysis of intraspecific and interspecific genetic distances were based on the K2P (Kimura 1980) and maximum composite likelihood Tamura *et al.* (2004) models and were as the best-fit models of nucleotide substitution and base frequencies also calculated with MEGA 5.03. Intraspecific and interspecific distances were plotted as a histogram using PAST version 2.14b (Hammer *et al.* 2001). A summary of species sequenced and their respective voucher and GenBank accession numbers is provided in the Appendix. All specimens and DNA barcodes are deposited in the Barcode of Life Data Systems (boldsystems.org, Ratnasingham and Hebert 2007) in the project Neotropical Tanypodinae (NEOTA).

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Results

Partial COI gene-sequences were obtained from 79 specimens of 13 species (Appendix). The Folmer-primers worked well on templates from all tested species and no difference was observed in amplification or sequencing success with regard to different life stages. The aligned sequences in the majority (98.7%) were 657 base pairs long with 209 variable sites (31.8%), of which 200 (95.6%) were informative. Most variable sites occurred in the third codon-position (Table 1). The sequences were heavily AT-biased, specifically in the third position, which exhibited a combined average AT-composition of 89.9% (Table 1). A hierarchical likelihood ratio test of aligned sequences in MEGA 5.03 returned the general time reversible model with a parameter for invariable sites and gamma correction for rate heterogeneity (GTR + G + I) as the best model ($-\ln L = 4110.404$, BIC = 9991.709, AIC = 8531.870).

Based on clustering of exemplars in the NJ analysis we were able to identify 12 new, undescribed *Labrundinia* species. In addition, *Labrundinia tenata*, previously described by means of morphology (Roback 1987; Silva and Fonseca-Gessner 2009) was successfully sequenced and DNA barcodes were produced from all life stages.

The pairwise distances for the analysed *Labrundinia* specimens, produced by both K2P and maximum composite likelihood models, showed distinctly larger interspecific than intraspecific divergences. Thus, there were distinct barcode gaps (Fig. 1). There were no identical gene sequences between species, and all species were distinguishable by genetic distances. Average intraspecific and interspecific K2P-distances for all analysed *Labrundinia* species were 0.91% and 14.53%, respectively. Maximum intraspecific divergence was observed in *Labrundinia* species 2 (4.78%), followed by *Labrundinia* species 25 (3.94%) and *Labrundinia* species 10 (3.46%) (Table 2). The lowest interspecific distances were found between *Labrundinia* species 10 and *Labrundinia* species 25 (average 10.8%), followed by *Labrundinia* species 10 and *Labrundinia* species 15 (average 11.23%). Intraspecific and interspecific distances produced by the maximum composite likelihood model provided similar results.

The specimens identified morphologically as *L. tenata* were divided into two separate barcode clusters. Nucleotide sequences of these specimens differed by a minimum of 2.5% and in up to 22 nucleotide sites, but there were no observable morphological characters that differentiated the specimens belonging to theses clusters. Specimens of *L. tenata* from the two clusters were collected from four ecosystems in São Paulo State, three in São Carlos municipality, and one in Luiz Antonio municipality (Appendix). All specimens from one cluster were collected at the São Carlos Ecological Park, so there was some geographical structure in the clustering even though the localities in São Carlos are not more than 8.5 km from each other. Similarly, the specimens identified as *Labrundinia* species 10 also formed two distinct groups, but no morphological differences were observed. Nucleotide sequences of these specimens diverged by a minimum of 2.7% and up to 25 nucleotide sites. In this case, all specimens were collected in the same geographical locality (Ecological Park/ Monjolinho stream).

DNA barcode sequences also indicated that three of the clusters were related species. This was subsequently confirmed by morphological analysis of the immature stages: *Labrundinia* species 8 resembled *Labrundinia* species 6 in the

Table 1. Variable and informative sites and average nucleotide composition in the analysed COI gene sequences

Nucleotide position	Variable sites (%)	Informative sites (%)	Adenine (%)	Cytosine (%)	Guanine (%)	Thymine (%)
1st	13.69	12.5	26.6	15.6	30.7	26.8
2nd	0.91	0.5	13.6	26.5	15.9	43.7
3rd	80.8	87	45.6	7.20	2.71	44.3
All	31.8	95.6	28.6	16.4	16.5	38.3

COI, cytochrome c oxidase subunit I.

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Fig. 1. Histogram of the calculated intraspecific and interspecific distances of partial COI sequences for the analysed *Labrundinia* specimens: (A) Kimura two-parameter. (B) Maximum composite likelihood.

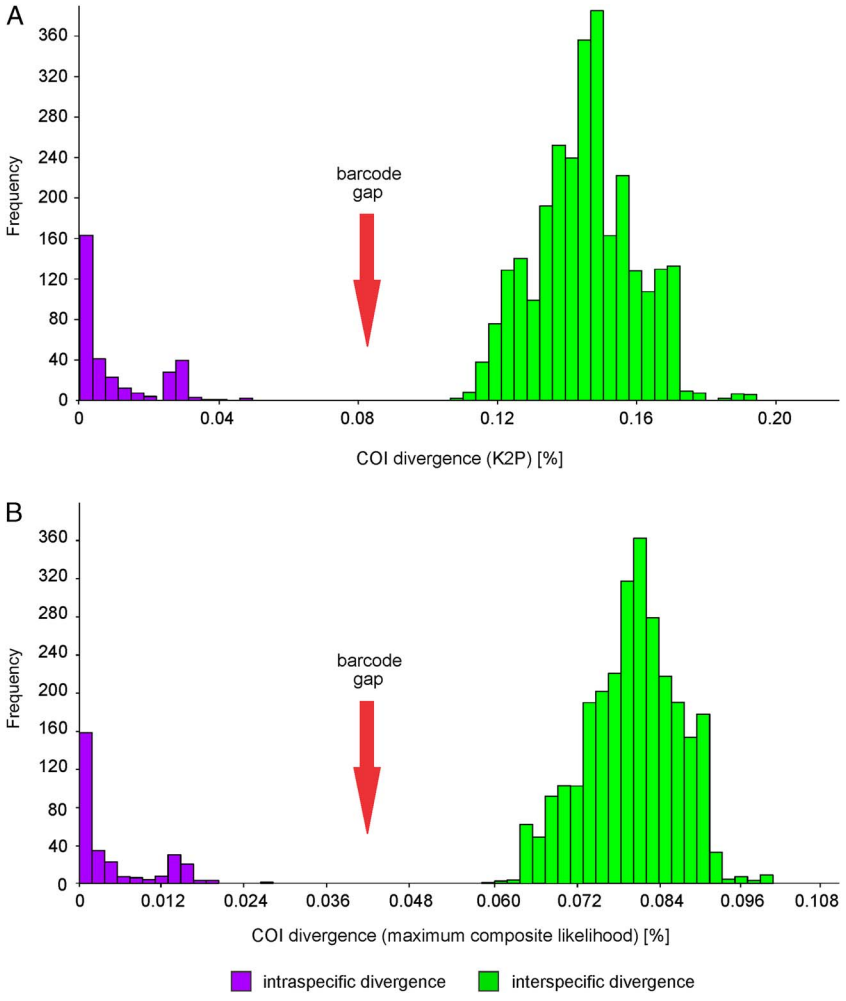

Table 2. Summary of intraspecific and interspecific Kimura two-parameter distances between morphological species of the genus *Labrundinia*

Species	Mean intraspecific K2P distance	Maximum intraspecific K2P distance	K2P distance to nearest neighbour	Mean interspecific K2P distance
Labrundinia species 2	3.48	4.78	11.64	14.52
Labrundinia species 3	0.13	0.31	10.86	14.58
Labrundinia species 6	0.67	0.93	11.92	15.44
Labrundinia species 7	0.31	0.8	11.22	14.68
Labrundinia species 8	0.31	0.31	11.22	14.56
Labrundinia species 10	1.94	3.46	10.15	13.68
Labrundinia species 15	0.91	1.55	10.50	14.81
Labrundinia species 20	0.10	0.15	12.10	16.02
Labrundinia species 21	0.15	0.31	11.55	14.61
Labrundinia species 23	0.54	1.08	10.84	13.56
Labrundinia species 24	1.37	2.02	11.42	14.52
Labrundinia species 25	3.94	3.94	10.15	14.34
Labrundinia tenata	1.46	2.98	11.37	14.35

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Fig. 2. Neighbour-joining tree based on partial COI sequences (DNA barcodes) and the Kimura two-parameter substitution model. Numbers on branches are bootstrap values >70%. DNA barcodes enabled association of different life stages, which are labeled on the terminals. Colours denote different species.

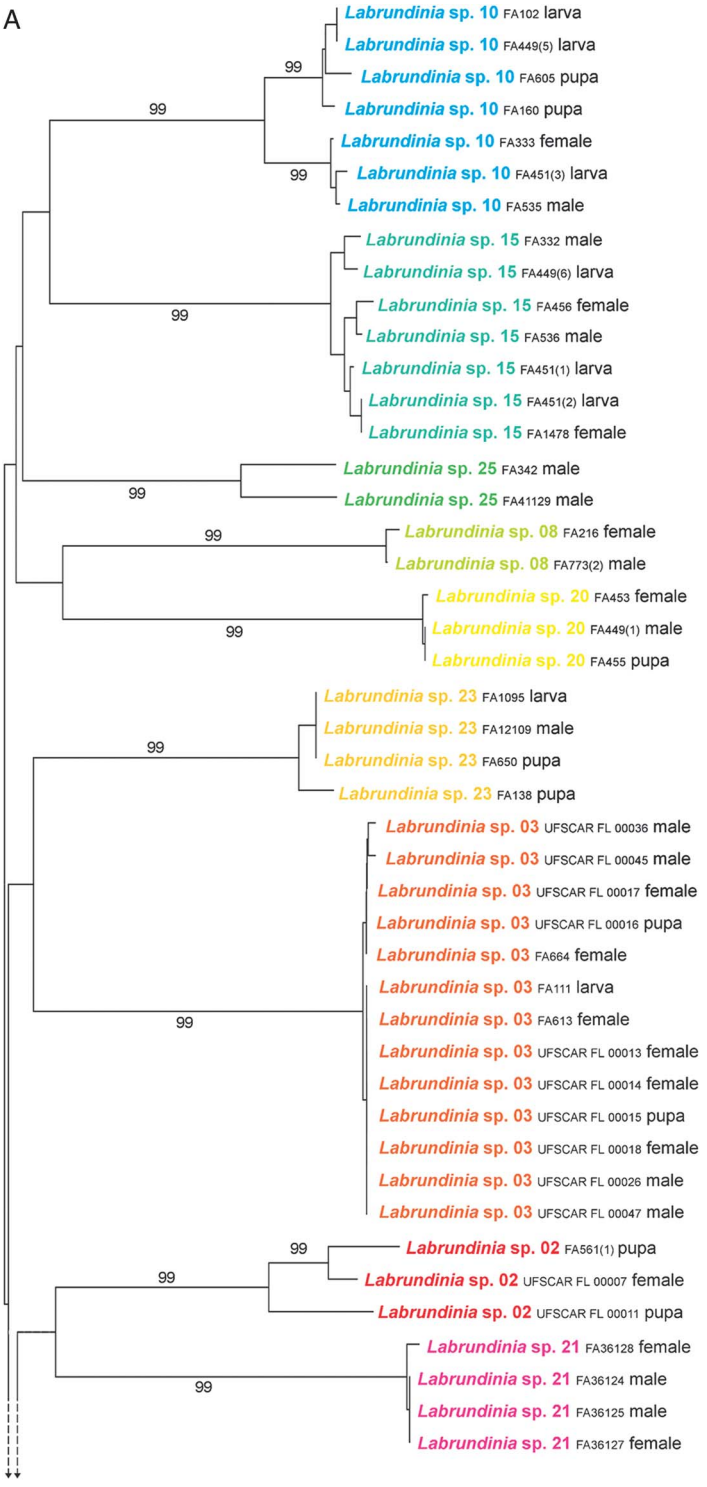

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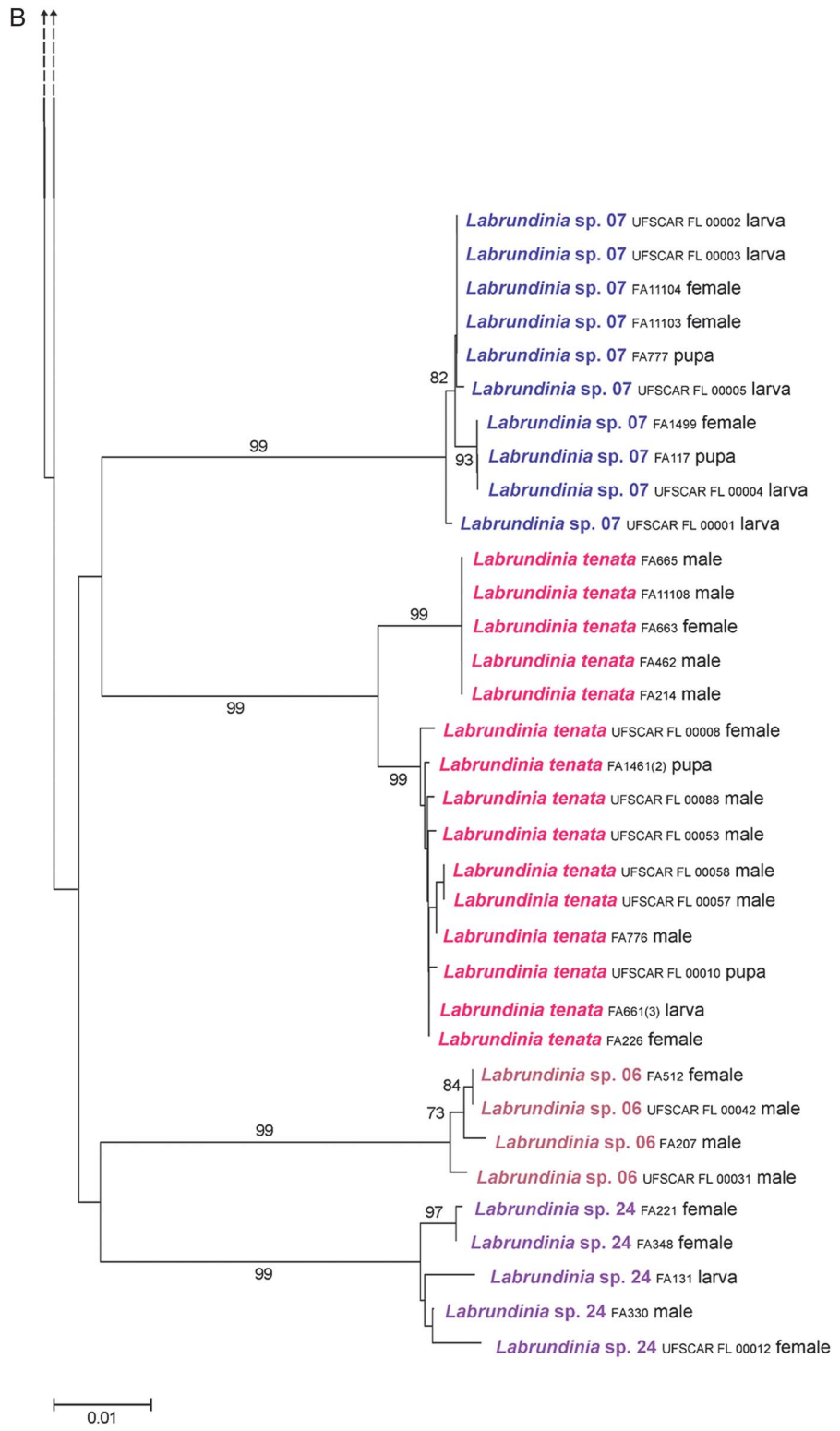
Fig. 2. Continued



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presence of a serrated claw in the larva and in the male abdominal colouration, but differed in the pupal thoracic horn ratio (length/width) that was higher in *Labrundinia* species 8. Nucleotide sequences of these specimens differed by minimum 15.6% and in up to 90 nucleotide sites. *Labrundinia* species 23 and *Labrundinia* species 25 were similar to *Labrundinia* species 2 in the male abdominal colouration, but diverged distinctly in the size and shape of the pupal thoracic horn, and in the number of spines that constitutes the lateroventral spine group (LVS) of the larval head. Nucleotide sequences of these specimens differed by minimum of 12.6% and in up to 133 nucleotide sites.

A NJ tree demonstrated that a substantial barcode divergence exists between all the analysed species of *Labrundinia*. For easier comparison with other DNA barcode studies, we here present the NJ trees produced by using the K2P model (Fig. 2). The NJ analysis, using maximum composite likelihood, yielded identical NJ trees, whereas trees resulting from maximum-likelihood analysis (using K2P and GTR + G + I models of substitution) only differ in the placement of *Labrundinia* species 25, which groups with *Labrundinia* species 10 as its closest cluster (data not shown). Boostrap support exhibited minor variation among all analyses, but were always 98% or higher for all species clusters.

Discussion

DNA barcoding focuses on species delineation and identification, and not on phylogenetic inference. Moreover, COI has been found to be too variable for reliable phylogenetic analysis in Chironomidae (Ekrem *et al.* 2007, 2010b). However, the NJ tree based on analysis of pairwise COI distances (Fig. 2) can be useful as a graphical representation of the genetic differences between sequences and clusters of sequences in the dataset. In this tree (which is not a representation of the most probable phylogeny of the included taxa), it is noticeable that there is a substantial barcode divergence between all the analysed species of *Labrundinia*.

The use of DNA barcodes also enables the association of different life stages based on genetic similarity of phenotypic characteristics (Hebert *et al.* 2003). In many chironomids, descriptions and diagnoses are largely or entirely based on a single life stage or even a single sex of a life stage. Consequently, incomplete knowledge of the life stages of a species precludes the use of morphological characters and natural history information that might be of particular interest for testing ecological, phylogenetic, and evolutionary hypotheses. The successful life stage associations made in this, as well as previous studies (Carew *et al.* 2005, 2007, 2011; Sinclair and Gresens 2008; Wiedenbrug *et al.* 2009; Ekrem *et al.* 2010a; Stur and Ekrem 2011; Silva *et al.* 2012), show that similar success may be expected in the association of adults and immatures of other chironomids (and other insects), which enables a complete taxonomic description of species where rearing is difficult or even impossible.

We observed low levels of intraspecific divergence within the species analysed. This result is perhaps not surprising given that most of the specimens from a certain species were sampled in the same locality. Nevertheless, our results clearly demonstrate the potential of using DNA barcodes in the identification of chironomids, at least in a local geographical scale. Moreover, the average intraspecific variation reported here is similar to other studies of insects. Average intraspecific variation of 0.9% (Ekrem *et al.* 2007) and 2.32% in Chironomidae (Sinclair and Gresens 2008); 2.76% (Hebert *et al.* 2004a) and 0.46% (Hajibabaei *et al.* 2006) in Lepidoptera; and 1.1% in mayflies (Ball *et al.* 2005) have been recorded. The aforementioned results indicate that a single threshold value for species delimitation is not appropriate. Even though recent efforts have been made to overcome this drawback (*e.g.* Automatic Barcode Gap Discovery) for primary species delimitation (Puillandre *et al.* 2011), character-based methodologies (DeSalle *et al.* 2005; DeSalle 2007), and Barcode Index Numbers (Ratnasingham & Hebert, 2013), additional studies will be required to determine the best method for species delineation using DNA barcodes.

According to Aliabadian *et al.* (2009), the barcode gap, *i.e.*, the difference between intraspecific and interspecific distances, allows for identification success in distance-based barcoding. Our data show that the distribution of intraspecific and interspecific divergences in *Labrundinia*

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exhibit a clear barcode gap which may be considered as a predictor of the identification success. Nonetheless, DNA barcodes were not enough for precise identification in all cases. For example, *L. tenata* specimens separated into two distinct groups, having nucleotide sequence divergences up to 2.5% and no observable morphological differences. Based on the suggested threshold of 2–3% applied to distinguish species (Hebert *et al.* 2003), the two clusters can be considered one species. *Labrundinia* species 10 exhibited similar results. Using DNA barcodes, Ekrem *et al.* (2007) also obtained two separate clusters of *Micropsectra notescens* (Walker) (Diptera: Chironomidae: Chironominae) specimens, even though they were unable to find any distinct morphological differences separating the clusters. In contrast, Sinclair and Gresens (2008), found one cluster represented by two species of *Cricotopus* van der Wulp (Diptera: Chironomidae: Orthocladiinae). The specimens had <3% COI sequence divergence, but presented consistent morphological differences. These results indicate that we need further studies using nuclear markers and morphological and ecological data in order to elucidate relationships between specimens that currently appear to belong to *L. tenata* and *Labrundinia* species 10.

DNA barcodes are argued to be valuable in species identification of taxonomic groups that are difficult to identify using morphology and Hebert *et al.* (2003, 2004a) stressed that DNA barcoding permits the assignment of unidentified specimens to known species as well as to identify species new to science. Our findings ratify this assertion as *Labrundinia* species 8, *Labrundinia* species 23, and *Labrundinia* species 25 were unveiled as new, undescribed species only after their significantly different and deeply divergent barcode clusters were discovered. These species differ mainly on the pupal thoracic horn, which seems to provide good diagnostic characters for many *Labrundinia* species, showing consistent patterns of interspecific variation. However, before the DNA barcode analyses, the pupal thoracic horn differences were treated as subtle morphological variation. Similarly, Carew *et al.* (2011) separated apparently cryptic species of *Procladius* Skuse (Diptera: Chironomidae: Tanypodinae) by means of morphological characters in the immature stages only after the analysis of the DNA barcode data. In a more general perspective, our results support DNA barcoding using COI as a promising approach for accurate interpretation of morphological variations within nonbiting midges in the subfamily Tanypodinae.

The DNA barcoding approach has been argued to be imprecise for consistent species delimitations by several authors (*e.g.* DeSalle *et al.* 2005; Will *et al.* 2005; Rubinoff *et al.* 2006; Ebach 2011), mostly due to general methodological reservations. The presence of mitochondrial pseudogenes (Bensasson *et al.* 2001) or incomplete lineage sorting may lead to species-level paraphyly and polyphyly (Funk and Omland 2003), blurring delineation of species by monophyletic barcode clusters criterion (Ekrem *et al.* 2010a). Nevertheless, none of these drawbacks seems to be an issue using COI sequences in species-level identification of Chironomidae (Carew *et al.* 2005, 2007; Sinclair and Gresens 2008; Ekrem *et al.* 2010b; and this study).

Conclusions

In our study of *Labrundinia* species, analysis of DNA barcode sequences using NJ trees supported 13 cohesive species clusters, of which three similar groups were subsequently linked to distinct morphological characters in the immature stages. DNA barcodes also assisted in associating different life stages. A distinct barcode gap and considerable interspecific pairwise divergences were observed, which allowed for unambiguous identification of all analysed species. Moreover, although DNA barcodes worked well for species delimitation in *Labrundinia*, inclusion of sequence data from extra nuclear markers is recommended in order to strengthen these results. Finally, efforts should be made to obtain specimens of *L. longipalpis* from the Palearctic as knowledge of their genetic distance to the New World species and to North American populations of *L. longipalpis* may be essential to understand the species limits within the genus.

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Appendix. List of analysed specimens with associated sample localities in São Paulo State, Brazil, voucher reference numbers and GenBank accessions. ''FA'' indicates samples processed by Fabio Laurindo da Silva; ''UFSCAR FL'' indicates samples processed by Mateus Pepinelli

Taxon	Locality	Voucher number	Accession number
Labrundinia tenata	São Carlos, Ecological Park, 19.iv.2010, F. L. Silva	FA214	JX887530
Labrundinia tenata	São Carlos, Valparaíso reservoir, 26.xi.2011, F. L. Silva	FA226	JX887529
Labrundinia tenata	São Carlos, Ecological Park, 19.iv.2010, F. L. Silva	FA462	JX887538
Labrundinia tenata	São Carlos, Fazzari reservoir, 27.iv.2010, F. L. Silva	FA661(3)	JX887537
Labrundinia tenata	São Carlos, Ecological Park, 19.iv.2010, F. L. Silva	FA663	JX887536
Labrundinia tenata	São Carlos, Ecological Park, 19.iv.2010, F. L. Silva	FA665	JX887535
Labrundinia tenata	São Carlos, Valaparaíso reservoir, 04.iv.2011, F. L. Silva	FA776	JX887534
Labrundinia tenata	São Carlos, Ecological Park, 19.iv.2010, F. L. Silva	FA11108	JX887533
Labrundinia tenata	São Carlos, Fazzari reservoir, 27.iv.2010, F. L. Silva	FA1461(2)	JX887532
Labrundinia tenata	São Carlos, Fazzari reservoir, 06.xii.2008, F. L. Silva	UFSCAR FL 00008	HM379516
Labrundinia tenata	São Carlos, Fazzari reservoir, 06.xii.2008, F. L. Silva	UFSCAR FL 00010	HM379517
Labrundinia tenata	Luis Antonio, Beija-Flor reservoir, 09.xii.2008, F. L. Silva	UFSCAR FL 00053	HM379556
Labrundinia tenata	Luis Antonio, Beija-Flor reservoir, 09.xii.2008, F. L. Silva	UFSCAR FL 00057	HM379559
Labrundinia tenata	Luis Antonio, Beija-Flor reservoir, 09.xii.2008, F. L. Silva	UFSCAR FL 00058	HM379560
Labrundinia tenata	São Carlos, Fazzari reservoir, 06.xii.2008, F. L. Silva	UFSCAR FL 00088	HM379573
Labrundinia species 2	São Carlos, Fazzari reservoir, 27.iv.2010, F. L. Silva	FA561(1)	JX887483
Labrundinia species 2	São Carlos, Fazzari reservoir, 06.xii.2008, F. L. Silva	UFSCAR FL 00007	HM379515
Labrundinia species 2	São Carlos, Fazzari reservoir, 06.xii.2008, F. L. Silva	UFSCAR FL 00011	HM379518
Labrundinia species 3	São Carlos, Fazzari stream, 27.iv.2010, F. L. Silva	FA111	JX887484
Labrundinia species 3	São Carlos, Fazzari stream, 10/09/2010, F. L. Silva	FA613	JX887486
Labrundinia species 3	São Carlos, Canchin reservoir, 02.ii.2009, F. L. Silva	FA664	JX887485
Labrundinia species 3	São Carlos, Fazzari reservoir, 06.xii.2008, F. L. Silva	UFSCAR FL 00013	HM379520
Labrundinia species 3	São Carlos, Fazzari reservoir, 06.xii.2008, F. L. Silva	UFSCAR FL 00014	HM379521
Labrundinia species 3	São Carlos, Fazzari reservoir, 06.xii.2008, F. L. Silva	UFSCAR FL 00015	HM379522
Labrundinia species 3	São Carlos, Canchin reservoir, 02.ii.2009, F. L. Silva	UFSCAR FL 00016	HM379523
Labrundinia species 3	São Carlos, Canchin reservoir, 02.ii.2009, F. L. Silva	UFSCAR FL 00017	HM379524
Labrundinia species 3	São Carlos, Canchin reservoir, 02.ii.2009, F. L. Silva	UFSCAR FL 00018	HM379525
Labrundinia species 3	São Carlos, Canchin reservoir, 02.ii.2009, F. L. Silva	UFSCAR FL 00026	HM379533
Labrundinia species 3	São Carlos, Canchin reservoir, 02.ii.2009, F. L. Silva	UFSCAR FL 00036	HM379540
Labrundinia species 3	São Carlos, Canchin reservoir, 02.ii.2009, F. L. Silva	UFSCAR FL 00045	HM379548
Labrundinia species 3	São Carlos, Canchin reservoir, 02.ii.2009, F. L. Silva	UFSCAR FL 00047	HM379550
Labrundinia species 6	São Carlos, Canchin reservoir, 02.ii.2009, F. L. Silva	FA207	JX887489

Appendix. *Continued*

Taxon	Locality	Voucher number	Accession number
Labrundinia species 6	São Carlos, Canchin reservoir, 02.ii.2009, F. L. Silva	FA512	JX887487
Labrundinia species 6	São Carlos, Canchin reservoir, 02.ii.2009, F. L. Silva	UFSCAR FL 00031	HM379535
Labrundinia species 6	São Carlos, Canchin reservoir, 02.ii.2009, F. L. Silva	UFSCAR FL 00042	HM379546
Labrundinia species 7	Gália, Caetetus reservoir, 08.07.2008, F. L. Silva & J. F. Nunes	FA117	JX887490
Labrundinia species 7	Gália, Caetetus reservoir, 08.07.2008, F. L. Silva & J. F. Nunes	FA777	JX887494
Labrundinia species 7	Gália, Caetetus reservoir, 08.07.2008, F. L. Silva & J. F. Nunes	FA11103	JX887493
Labrundinia species 7	Gália, Caetetus reservoir, 08.07.2008, F. L. Silva & J. F. Nunes	FA11104	JX887492
Labrundinia species 7	Gália, Caetetus reservoir, 08.07.2008, F. L. Silva & J. F. Nunes	FA1499	JX887491
Labrundinia species 7	Gália, Caetetus reservoir, 08.07.2008, F. L. Silva & J. F. Nunes	UFSCAR FL 00001	HM379510
Labrundinia species 7	Gália, Caetetus reservoir, 08.07.2008, F. L. Silva & J. F. Nunes	UFSCAR FL 00002	HM379511
Labrundinia species 7	Gália, Caetetus reservoir, 08.07.2008, F. L. Silva & J. F. Nunes	UFSCAR FL 00003	HM379512
Labrundinia species 7	Gália, Caetetus reservoir, 08.07.2008, F. L. Silva & J. F. Nunes	UFSCAR FL 00004	HM379513
Labrundinia species 7	Gália, Caetetus reservoir, 08.07.2008, F. L. Silva & J. F. Nunes	UFSCAR FL 00005	HM379514
Labrundinia species 8	São Carlos, Valparaíso reservoir, 26.xi.2011, F. L. Silva	FA216	JX887496
Labrundinia species 8	São Carlos, Valaparaíso reservoir, 04.iv.2011, F. L. Silva	FA773(2)	JX887495
Labrundinia species 10	São Carlos, Ecological Park, 19.iv.2010, F. L. Silva	FA102	JX887503
Labrundinia species 10	São Carlos, Ecological Park, 19.iv.2010, F. L. Silva	FA160	JX887501
Labrundinia species 10	São Carlos, Monjolinho stream, 11.iv.2011, F. L. Silva	FA333	JX887497
Labrundinia species 10	São Carlos, Monjolinho stream, 11.iv.2011, F. L. Silva	FA449(5)	JX887502
Labrundinia species 10	São Carlos, Monjolinho stream, 11.iv.2011, F. L. Silva	FA451(3)	JX887498
Labrundinia species 10	São Carlos, Monjolinho stream, 11.iv.2011, F. L. Silva	FA535	JX887499
Labrundinia species 10	São Carlos, Ecological Park, 19.iv.2010, F. L. Silva	FA605	JX887500
Labrundinia species 15	São Carlos, Monjolinho stream, 11.iv.2011, F. L. Silva	FA332	JX887504
Labrundinia species 15	São Carlos, Monjolinho stream, 11.iv.2011, F. L. Silva	FA449(6)	JX887507
Labrundinia species 15	São Carlos, Monjolinho stream, 11.iv.2011, F. L. Silva	FA451(1)	JX887506
Labrundinia species 15	São Carlos, Monjolinho stream, 11.iv.2011, F. L. Silva	FA451(2)	JX887505
Labrundinia species 15	São Carlos, Monjolinho stream, 11.iv.2011, F. L. Silva	FA456	JX887509
Labrundinia species 15	São Carlos, Monjolinho stream, 11.iv.2011, F. L. Silva	FA536	JX887510
Labrundinia species 15	São Carlos, Monjolinho stream, 11.iv.2011, F. L. Silva	FA1478	JX887508
Labrundinia species 20	São Carlos, Monjolinho stream, 11.iv.2011, F. L. Silva	FA449(1)	JX887511
Labrundinia species 20	São Carlos, Monjolinho stream, 11.iv.2011, F. L. Silva	FA453	JX887512
Labrundinia species 20	São Carlos, Monjolinho stream, 11.iv.2011, F. L. Silva	FA455	JX887513
Labrundinia species 21	São Carlos, Espraiado stream, 19.viii.2011, S. Wiedenbrug	FA36124	JX887517

Appendix. *Continued*

Taxon	Locality	Voucher number	Accession number
Labrundinia species 21	São Carlos, Espraiado stream, 19.viii.2011, S. Wiedenbrug	FA36125	JX887516
Labrundinia species 21	São Carlos, Espraiado stream, 19.viii.2011, S. Wiedenbrug	FA36127	JX887515
Labrundinia species 21	São Carlos, Espraiado stream, 19.viii.2011, S. Wiedenbrug	FA36128	JX887514
Labrundinia species 23	Luis Antonio, Óleo oxbow lake, 07.i.2011, S. T. Strixino	FA138	JX887521
Labrundinia species 23	Luis Antonio, Óleo oxbow lake, 07.i.2011, S. T. Strixino	FA650	JX887518
Labrundinia species 23	Luis Antonio, Óleo oxbow lake, 07.i.2011, S. T. Strixino	FA1095	JX887520
Labrundinia species 23	Luis Antonio, Óleo oxbow lake, 05.i.2011, S. Wiedenbrug	FA12109	JX887519
Labrundinia species 24	São Carlos, Mayaca reservoir, 11.iv.2011, F. L. Silva	FA131	JX887525
Labrundinia species 24	São Carlos, Mayaca reservoir, 20.iv.2011, F. L. Silva	FA221	JX887524
Labrundinia species 24	São Carlos, Mayaca reservoir, 11.iv.2011, F. L. Silva	FA330	JX887522
Labrundinia species 24	São Carlos, Mayaca reservoir, 11.iv.2011, F. L. Silva	FA348	JX887523
Labrundinia species 24	Luis Antonio, Beija-Flor reservoir, 09.xii.2008, F. L. Silva	UFSCAR FL 00012	HM379519
Labrundinia species 25	São Carlos, Mayaca reservoir, 11.iv.2011, F. L. Silva	FA342	JX887527
Labrundinia species 25	Luis Antonio, Beija-Flor reservoir, 05.i.2011, S. Wiedenbrug	FA41129	JX887526