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DNA Barcoding and New Records of Odonates (Insecta: Odonata) from Paraíba State, Brazil

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Abstract: Odonates (Insecta: Odonata) are important insects in the food chains of freshwater environments around the world, being used as a model species for areas of behavior and analysis of environmental quality. In Brazil, especially in the Northeastern region, both knowledge about the distribution and molecular information of odonate species found in the two main biomes of the region is still limited. Aiming to improve these issues, here, we carried out an Odonata survey in two locations and built a DNA barcode database for species from the state of Paraíba. In total, 15 first records were reported for this Brazilian state and 142 specimens from 27 genera and 45 species had their 'Folmer' cytochrome c oxidase subunit I (COI) fragment evaluated. The database we generated includes data for 70% of the Odonata species found in Paraíba state. For 16 species, this is the first DNA barcode available in public sequence repositories. Our results demonstrate that using the COI in the regional scale can help identify and delimit those evaluated. Eight species (17%) showed a low percentage of differentiation (<2%) compared to other species currently deposited in the GenBank or BOLD System; nevertheless, we present morphological traits that reaffirm our identifications. Barcode data provide new insights into Neotropical diversity and deliver basic information for taxonomic analyses.

Keywords: dragonfly; damselflies; DNA barcode; Brazilian northeast

Citation: Koroiva, R.; Gomes, V.G.N.; Vilela, D.S. DNA Barcoding and New Records of Odonates (Insecta: Odonata) from Paraíba State, Brazil. *Diversity* **2022**, *14*, 203. https://doi.org/10.3390/d14030203

Academic Editor: Luc Legal

Received: 21 February 2022
Accepted: 8 March 2022
Published: 10 March 2022

1. Introduction

Odonates (Insecta: Odonata) are a group of fascinating insects that can be found all over the world (except for Antarctica) [1]. They have been used as model animals in several areas, such as behavioral studies and the analysis of environmental quality (e.g., [2–4]). This group plays an important role in the trophic network of freshwater environments, both as an efficient predator of invertebrates and as a prey for several vertebrates [5]. Over the last decade, scientists have increasingly discovered new species, which has improved the taxonomic knowledge of odonates in the Neotropical realm; however, information on their taxonomy ('Linnean' shortfall), distribution ('Wallacean' shortfall) and genetic diversity remains scarce (see [6,7]).

The traditional identification of odonates based on their phenotypic traits is often assumed to be difficult, considering that the older depictions are based on brief descriptions and rare illustrations (see examples in [8]). Even the most recent definitions present limited information to facilitate the identification of both larvae and adults. In general, in adults, anal appendages, wing venation and genitalia are often used to identify and classify odonates [9,10]. Despite the possibility of identifying some species at the larval level, the number of this type of descriptions is still scarce, especially in the Neotropical region [9].

Since Hebert and colleagues [11,12] suggested using cytochrome oxidase subunit I (COI) sequences as a global bioidentification system for animals in 2003, there has been great progress in using it for species identification and species discovery of odonates. This system, which is called DNA barcoding, has proven to be highly effective for delimiting and identifying different groups (e.g., [13,14]). As a result of an initiative by researchers at the University of Guelph (Ontario, Canada), a worldwide database has been maintained for the deposit and public identification of sequences; this database is called the BOLD system (the DNA Barcode of Life Data System) [15]. Among the advantages of this system compared with other public sequence repositories (e.g., GenBank) is that it is easy to access and download specimen data and sequences. For Neotropical odonates, the use of DNA barcoding is still limited, and there has been some discussion regarding its effectiveness by Koroiva et al. [16]. Moreover, Vilela et al. [17,18] have substantiated its direct application through integrative taxonomy, i.e., the framework to delimit and describe taxa by integrating information from multiple and complementary perspectives [19], using molecular and morphological data.

Currently, there are about 872 species of odonates in Brazil [20]. Several biomes can be found in this country, including the Atlantic Forest and 'Caatinga'. The former is considered a biodiversity hotspot, and it is one of the most threatened tropical forests in the world [21]. Meanwhile, the latter is the only biome exclusive to Brazil. It is characterized by a semi-arid climate and has been recognized as an affected biome as a result of anthropic actions, such as free-living livestock and fuelwood extraction [22,23]. These two biomes are present in the state of Paraíba, a small territory state localized in Northeastern Brazil. The region's list of Odonata species was recently published by Koroiva et al. [8]. In this work, the authors identified 49 species living in the region; this provides a good sampling of the diversity of the state, despite the few collections present in the Atlantic Forest. Nonetheless, it is essential to elaborate upon a broad and diverse species collection in order to realize representative genetic databases. In turn, using them to construct a reference database is the next step to enable the use of new molecular tools (e.g., DNA metabarcoding) in this region.

Keeping in mind the difficulties in the morphological identification of dragonflies, molecular tools present a promising way to solve this impediment in Neotropical species [16]. In this study, we present a DNA barcode library for the Odonata identification in the Paraíba state. This work aims (i) to improve the information on the species present in the Atlantic Forest of Paraíba; (ii) to establish DNA barcode libraries for the odonatofauna of Paraíba based on the COI gene and (iii) to evaluate the accuracy of the DNA barcodes in defining species in relation to both the regional and global databases.

2. Materials and Methods

2.1. Ethics Statement

This study was conducted with the appropriate permission (SISBIO license number 74324-5 and JBBM license number 002/2021/JBBM/SUDEMA).

2.2. Data Collection

Between May 2020 and November 2021, 740 specimens were collected from 10 municipalities in the state of Paraíba. Information about climate classification, precipitation, vegetation types and the geology of the sampling area are available in the work of Koroiva et al. [8]. In addition, two sites not considered by Koroiva et al. [8] were sampled during the 10 sampling campaigns between October 2020 and November 2021: João Pessoa Botanical Garden (JBBM), which is located in the municipality of João Pessoa (−7.135867, −34.860025; datum WGS84), and the "Banho do Jair" stream, found in the municipality of Santa Rita (−7.000965, −34.98836; datum WGS84). Notably, both areas are located in the Atlantic Forest fragments. The morphological identification of all specimens was done with the help of experts (see Acknowledgements) in Odonata taxonomy and by using the taxonomic keys of Lencioni [9,24,25] and Garrison et al. [10,26]. Our collection of specimens followed the

methodology presented in Vilela et al. [27]. In terms of the classification, we followed Paulson et al. [28]. Voucher specimens were deposited into the Entomological Collection of the Department of Systematics and Ecology at the Federal University of Paraíba (DSEC/UFPB).

2.3. Extracting, Amplifying and Sequencing

All the DNA from the samples was extracted using the Blood & Tissue DNA Mini Kit (Ludwig Biotec, Alvorada, Brazil) from one leg, and it was preserved in ethanol. We amplified the genetic material of 44 specimens of 21 species collected in the two sampled sites mentioned above. We also used another 70 specimens of the 27 species previously collected by Koroiva et al. [8] and deposited in DSEC/UFPB (see Figure 1). For most species, we used specimens from different municipalities in an effort to analyze different populations (see BOLD dataset on http://dx.doi.org/10.5883/DS-ODOPB, accessed on 11 February 2022). In total, 658 bp were amplified from the 5′ region of the Cox1 gene using the M13-tailed primers OdoF1_t1 (5′–TGTAAAACGACGGCCAGTATTCAACHAATCATAARGATATTG G-3′) and OdoR1_t1 (5′–CAGGAAACAGCTATGACTAAACTTCTGGATGYCCRAARA AYCA-3′) (Semotok, unpublished, BOLD Systems http://www.boldsystems.org/index.php/Public_Primer_PrimerSearch, accessed on 27 January 2022). When it was not possible to amplify them, an approximately 421 bp long fragment at the 3′ end of the barcoding region was amplified by using the forward primer BF2 (5′-GCHCCHGAYATRGCHTTYCC-3′) and the reverse primer BR2 (5′-TCDGGRTGNCCRAARAAYCA-3′) described by Elbrecht and Leese [29]. This procedure was performed with consideration that the primers commonly used in COI amplification (OdoF1_t1-OdoR1_t1, HCO2198-LCO1490 [30], HCO2198_t1-LCO1490_t1 [31], LepF1-LepR1 [32] and LepF1_t1-LepR1_t1 [33]) were not successful in the amplification for many species, especially in Zygoptera, with exceptions of *Telebasis corallina* (Selys, 1876) and *Hetaerina rosea* Selys, 1853.

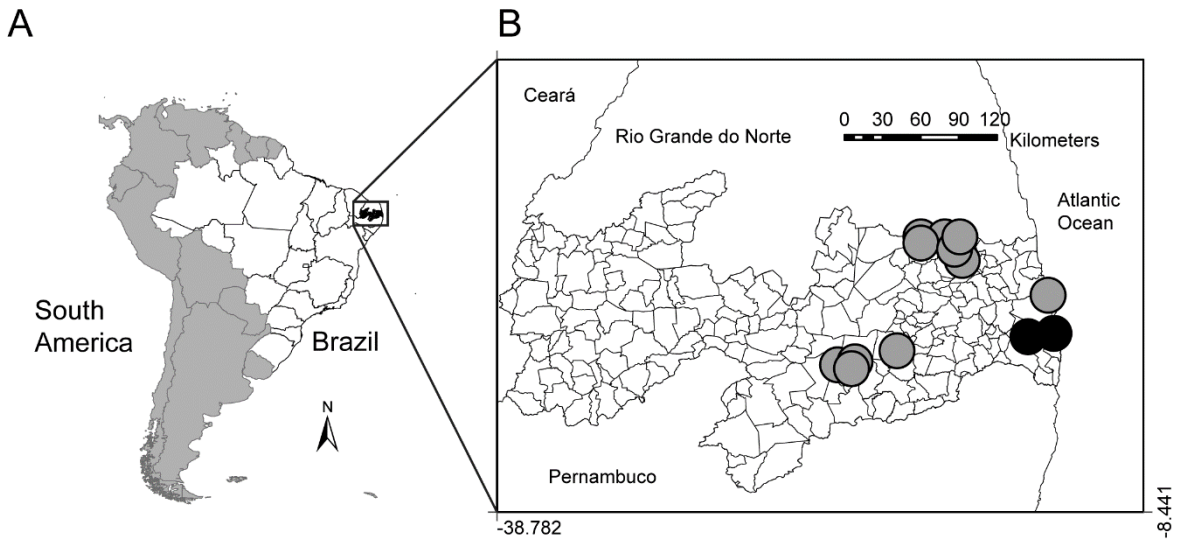

Figure 1. Geographical location of sampled specimens of odonates. (**A**) Map of South America (dark grey) highlighting the geopolitical division of Brazil (white); (**B**) municipality division of Paraíba with the locality of our two sampling sites (black dots) and the other localities for specimens obtained in DSEC/UFPB (grey dots).

The PCR conditions for amplification consisted of 1 × buffer (Colorless GoTaq® Flexi Buffer; Promega Corp., Madison, WI, USA), 0.2 mM dNTP mix, 0.2 µM of each primer, 2 mM $MgCl_2$, 1U Taq polymerase (GoTaq® G2 hot start polymerase, Promega Corp., Madison, WI, USA) and 2 µL of template DNA; these materials were placed in a total reaction volume of 25 µL. The PCR cycling program to OdoF1_t1 and OdoR1_t1 followed Vilela et al. [18]. For the BF2-BR2 primers, the PCR cycling program was run as follows: initial denaturation step with 3 min at 95 °C, 35 cycles of denaturation for 30 s at 95 °C, annealing for

45 s at 50 °C and extension for 1 min at 72 °C, and final extension for 5 min at 72 °C. The PCR products were purified with ethanol/sodium acetate and sequenced in an ABI 3130 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA). The OdoF1_t1 and OdoR1_t1 sequencing were performed using M13 Forward (5′-TGTAAAACGACGGCCAGT-3′) and M13 Reverse primers (5′-CAGGAAACAGCTATGAC-3′), respectively. The sequence data were uploaded to GenBank (accession numbers OL806732 to OL806735 and OL806621 to OL806730) and were made available on BOLD as a dataset (http://dx.doi.org/10.5883/DS-ODOPB, accessed on 11 February 2022).

2.4. Data Analysis

To check the sequence quality of both strands and to assemble and edit them if necessary, we used GENEIOUS v 9.0.5 [34]. Furthermore, we aligned the sequences for each gene loci using Muscle v3.8.425 [35] (module implemented in GENEIOUS v 9.0.5) at the default setting. Five species that had less than three individuals were found to have sequenced in our database (i.e., singletons and doubletons; see on http://dx.doi.org/10.5883/DS-ODOPB, accessed on 11 February 2022). Using the sequences from the Brazilian specimens deposited in the BOLD system (accessed on 27 January 2022), we determined that our database had the incorporation of all sequences of these species (28 specimens); in turn, this allowed the analysis of intraspecific variation, totaling 142 specimens. The genetic distances between and within species were estimated using the Kimura's two-parameter substitution model (K2P) (but see Srivathsan and Meier [36]); these were calculated using the MEGA X software [37]. To increase the robustness of the homology statement and to elevate the matrix occupancy, long sequences were truncated to cover only the 'Folmer' region of the COI gene. This is the most commonly used region for DNA barcoding, as it covers 658 nt of the 5′-end of the gene. For insects, the region can be amplified using the 'Folmer' primer pair (HCO2198 and LCO1490; [30]); subsequently, truncation was carried out following the positioning of these primers.

Next, we calculated the mean and maximum genetic divergence values and the lowest genetic distance on our (regional) database to the nearest neighbor in MEGA X [37]. We then plotted the empirical K2P values associated with intra- and interspecific comparisons against each other following the methods detailed by Koroiva and Kvist [38], in order to highlight and visualize any potential "barcode gap" (but see discussion on Wiemers and Fiedler [39]). To evaluate them in the global databases, we used the default settings of Web BLAST (Basic Local Alignment Search Tool; https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 11 February 2022) on GenBank [40] in order to identify the nearest matching sequences and the Ident and E(xpect) values. In addition, we used the Species Level Barcode Records option on BOLD (http://www.boldsystems.org/index.php/IDS_OpenIdEngine; accessed on 11 February 2022, [41]) to obtain a list of the sequences with the highest similarity. With consideration of previous works (e.g., [16]), we examined the species closest to those that showed less than 2% genetic divergence.

2.5. Species Delimitation

We used two methods to delimit the species: distance-based and tree-based methods. For a distance-based method, we performed ABGD (Automatic Barcode Gap Discovery analysis) online (http://wwwabi.snv.jussieu.fr/public/abgd/, accessed on 11 February 2022) [42] because the program is optimized for the COI gene; we used the values of Pmin = 0.001 and Pmax = 0.10, steps = 20, relative gap width (X) = 0.75 and Nb bins = 20 and K2P. The ABGD resulted in a stable genetic group count with a range of prior intraspecific values (P = 0.0113–0.0483) and the results of these grouping are presented. ASAP (Assemble Species by Automatic Partitioning) [43] analyses were performed on the website (https://bioinfo.mnhn.fr/abi/public/asap/, accessed on 11 February 2022) using K2P; we considered only the partition showing the lowest ASAP score.

For tree-based methods, we used the Poisson tree process (PTP) as implemented in the PTP (http://species.h-its.org/, accessed on 11 February 2022) using the default

settings [44,45] and the generalized mixed Yule coalescent (GMYC) method. To run the PTP analysis, we first built a tree with RAxML (v 8.2.12) [46] using a GTR GAMMA I model and 1000 bootstrap replicates. The resulting tree was used as the input tree to run on the web server.

A single-threshold GMYC analysis was conducted in the species delimitation service (https://species.h-its.org/gmyc/, accessed on 11 February 2022) [47]. An ultrametric single-locus gene tree was obtained using BEAST v.2.6.6 [48] with 1.5×10^8 MCMC generations under relaxed lognormal clock, the Yule process tree model, and a burn-in of the first 10% generations of the final consensus tree. The posterior distributions (ESS > 200) were examined in Tracer v. 1.6 [49]. The best fitting available model was identified through the jModelTest v. 2.1.7 (AIC & BIC, GTR + I + G) [50,51].

Finally, we created a neighbor-joining (NJ) dendrogram to provide a graphic representation of the divergence pattern between species. An NJ tree was inferred using MEGA X software [37]. In all the tree-based analysis, three species were used as the out group (HQ941355 *Baetis adonis*, HQ943539 *Baetis phoebus* and HQ987969 *Ephemerella mucronata*). It should be noted that the tree presented here is only intended to represent the distance matrix and it should not be interpreted as a phylogenetic hypothesis.

3. Results

3.1. Sampling

Our samples added 15 new species records to the odonatofauna of Paraíba: *Dasythemis venosa* (Burmeister, 1839), *Dythemis nigra* Martin, 1897, *Epipleoneura metallica* Rácenis, 1955, *Erythrodiplax* cf. *fervida* (Erichson in Schomburgk, 1848), *Idioneura ancilla* Selys, 1860, *Macrothemis imitans* Karsch, 1890, *Metaleptobasis bicornis* (Selys, 1877), *Micrathyria didyma* (Selys in Sagra, 1857), *Micrathyria mengeri* Ris, 1919, *Nephepeltia berlai* Santos, 1950, *Orthemis flavopicta* Kirby, 1889, *Perithemis lais* (Perty, 1834), *Tauriphila australis* (Hagen, 1867), *Telebasis griffinii* (Martin, 1896) and *Triacanthagyna septima* (Selys in Sagra, 1857).

After performing the morphological analysis, all specimens (including those deposited in the DSEC/UFPB) that had previously been identified as *Anatya guttata* (Erichson in Schomburgk, 1848) were now identified as *Anatya januaria* Ris, 1911.

3.2. Genetic Variation

A total of 142 mitochondrial COI barcode sequences were obtained from five families, 27 genera and 45 species (mean by species 3.15; max = 15, min = 1, Table 1, Figure 2). All of the analyzed sequences were larger than 353 bp. The average base pairs of the sequences were 524 bp (SD = 116.54) and the median was 592 bp. Thirteen singletons were registered in the database (Table 1). In the regional database (Table 1), the greatest intraspecific variation was the Erythrodiplax fusca (Rambur, 1842) with 1.85% and the smallest inter-specific variation occurred between the Erythrodiplax leticia Machado, 1996 and the Erythrodiplax cf. fervida, with 4.87%.

Table 1. Similarity match approach of the generated sequences of collected specimens with the regional database sequences and global database sequences of GenBank and BOLD (Species Level Barcode Records) for species identification. N, number of barcode sequences; Mean(%) = average of intraspecific genetic distance value (expressed as percent); Max(%) = maximum intraspecific genetic distance value (expressed as percent); Nearest neighbor = most closely related species retrieved; DNN = lowest genetic distance to the nearest neighbor (expressed as percent).

				Similarity Match Regional Database				Similarity Match Global Database		
Species	**N**	**Mean (%)**	**Max (%)**	**Nearest Neighbor**	**DNN (%)**	**GenBank id. (%)**	**GenBank E-Value**	**GenBank Species**	**Bold Similarity**	**Bold Species**
Acanthagrion gracile	1	-	-	*Lestes forficula*	19.01	92.17	1.00×10^{-149}	*Acanthagrion cuyabae*	no match	no match
Anatya januaria	3	0.36	0.57	*Erythrodiplax umbrata*	15.44	99.73	0	*Anatya* sp.	99.74	*Anatya guttata*
Argia reclusa	3	0.16	0.25	*Orthemis discolor*	18.88	98.74	0	*Argia reclusa*	98.73	*Argia reclusa*
Brachymesia furcata	2	0.85	0.85	*Brachymesia herbida*	10.55	99.67	0	*Brachymesia furcata*	99.67	*Brachymesia furcata*
Brachymesia herbida	1	-	-	*Brachymesia furcata*	10.55	90.05	0	*Brachymesia furcata*	99.33	*Brachymesia herbida*
Diastatops obscura	3	0.86	1.3	*Orthemis aequilibris*	12.2	99.83	0	*Diastatops obscura*	99.83	*Diastatops obscura*
Dythemis nigra	3	0.16	0.25	*Orthemis discolor*	13.87	100	0	*Dythemis multipunctata*	100	*Dythemis nigra*
Epipleoneura metallica	1	-	-	*Dythemis nigra*	20.59	85.4	0	*Argia fumipennis*	no match	no match
Erythemis plebeja	6	0	0	*Erythemis carmelita*	10.64	90.95	0	*Erythemis peruviana*	100	*Erythemis plebeja*
Erythemis vesiculosa	5	0.32	0.50	*Erythrodiplax umbrata*	14.62	87.50	0	*Acisoma attenboroughi*	99.85	*Erythemis vesiculosa*
Erythemis peruviana	2	0.33	0.33	*Erythemis plebeja*	13.02	88.39	0	*Agrionoptera insignis*	100	*Erythemis peruviana*
Erythemis carmelita	3	0.10	0.16	*Erythemis plebeja*	10.64	97.90	0	*Erythemis* sp.	98.67	*Erythemis mithroides*
Erythrodiplax avittata	2	0	0	*Erythrodiplax basalis*	11.04	86.93	0	*Erythrodiplax paraguayensis*	no match	no match
Erythrodiplax umbrata	3	1.14	1.37	*Orthemis discolor*	13.74	99.84	0	*Erythrodiplax umbrata*	99.83	*Erythrodiplax umbrata*
Erythrodiplax leticia	2	0.16	0.16	*Erythrodiplax cf fervida*	4.87	92.53	0	*Erythemis* sp.	no match	no match
Erythrodiplax fusca	15	0.89	1.85	*Erythrodiplax avittata*	15.75	94.53	0	*Erythrodiplax connata*	99.85	*Erythrodiplax fusca*
Erythrodiplax cf. *fervida*	2	0	0	*Erythrodiplax leticia*	4.87	91.71	0	*Erythemis* sp.	no match	no match
Erythrodiplax basalis	6	0.11	0.75	*Erythrodiplax avittata*	11.04	87.24	6.00×10^{-118}	*Erythrodiplax paraguayensis*	99.74	*Erythrodiplax paraguayensis*
Erythrodiplax cf. *unimaculata*	1	-	-	*Erythrodiplax leticia*	8.58	95.86	6.00×10^{-163}	*Libellulidae* sp.	97.11	*Erythrodiplax kimminsi*
Hetaerina rosea	3	0.52	0.78	*Ischnura capreolus*	19.6	98.96	0	*Hetaerina sanguinea*	98.95	*Hetaerina sanguinea*
Ischnura fluviatilis	4	0.12	0.26	*Telebasis filiola*	15.96	99.22	0	*Ischnura fluviatilis*	98.68	*Ischnura fluviatilis*
Ischnura capreolus	4	0.25	0.49	*Lestes forficula*	13.2	100	0	*Ischnura capreolus*	100	*Ischnura capreolus*
Lestes forficula	3	0.69	1.03	*Ischnura capreolus*	13.2	100	0	*Lestes forficula*	98.78	*Lestes forficula*
Macrothemis griseofrons	6	0.1	0.17	*Erythrodiplax umbrata*	15.39	88.07	0	*Agrionoptera insignis*	no match	no match
Mecistogaster kesselringi	1	-	-	*Lestes forficula*	14.76	95.92	6.00×10^{-178}	*Mecistogaster amalia*	no match	no match
Metaleptobasis bicornis	4	0.12	0.25	*Telebasis corallina*	13.75	93.42	6.00×10^{-163}	*Metaleptobasis selysi*	no match	no match
Miathyria marcella	6	0.53	0.92	*Perithemis lais*	13.19	87.84	0	*Agrionoptera insignis*	100	*Miathyria marcella*
Micrathyria hesperis	4	0.38	0.51	*Uracis imbuta*	14.37	87.98	4.00×10^{-125}	*Micrathyria stawiarskii*	no match	no match
Micrathyria ocellata	3	0.11	0.16	*Nephepeltia belai*	13.76	100	0	*Micrathyria ocellata*	100	*Micrathyria ocellata*
Nephepeltia berlai	1	-	-	*Micrathyria ocellata*	13.76	98.11	0	*Nephepeltia aequisetis*	98.1	*Nephepeltia aequisetis*
Orthemis schmidt	4	0.62	0.78	*Orthemis discolor*	5.00	99.48	0	*Orthemis schmidti*	99.48	*Orthemis schmidti*
Orthemis aequilibris	1	-	-	*Orthemis discolor*	4.92	95.25	0	*Orthemis discolor*	99.83	*Orthemis aequilibris*
Orthemis discolor	7	0.51	0.91	*Orthemis aequilibris*	4.92	99.03	0	*Orthemis* sp.	100	*Orthemis discolor*
Orthemis flavopicta	1	-	-	*Orthemis discolor*	9.69	93.79	4.00×10^{-176}	*Orthemis cultriformis*	no match	no match
Pantala flavescens	3	0.65	0.65	*Tramea cophysa*	14.9	99.84	0	*Pantala flavescens*	99.83	*Pantala flavescens*
Perithemis tenera	3	0.87	1.27	*Brachymesia furcata*	13.28	91.1	0	*Libellulidae* sp.	99.83	*Perithemis icteroptera*
Perithemis lais	1	-	-	*Brachymesia furcata*	13.14	88.21	0	*Libellulinae* sp.	99.25	*Perithemis lais*
Progomphus dorsopallidus	1	-	-	*Perithemis tenera*	18.93	86.22	4.00×10^{-171}	*Tanypteryx hageni*	no match	no match
Tauriphila australis	1	-	-	*Orthemis flavopicta*	14.64	100	0	*Tauriphila australis*	100	*Tauriphila australis*
Telebasis corallina	4	0.47	0.8	*Orthemis schmidt*	13.08	88.28	0	*Telebasis willinki*	97.9	*Telebasis corallina*
Telebasis filiola	4	0.26	0.51	*Telebasis corallina*	13.26	98.72	0	*Telebasis willinki*	98.96	*Telebasis willinki*
Telebasis griffinii	3	0.52	0.78	*Uracis imbuta*	17.12	88.89	8.00×10^{-127}	*Telebasis digiticollis*	no match	no match
Tramea cophysa	4	0.83	1.02	*Brachymesia furcata*	12.88	99.11	0	*Tramea cophysa*	98.34	*Tramea binotata*
Uracis imbuta	1	-	-	*Nephepeltia berlai*	14.35	94.08	1.00×10^{-179}	*Uracis imbuta*	99.04	*Uracis imbuta*
Zenithoptera lanei	1	-	-	*Telebasis corallina*	19.33	98.24	8.00×10^{-166}	*Zenithoptera* sp.	no match	no match

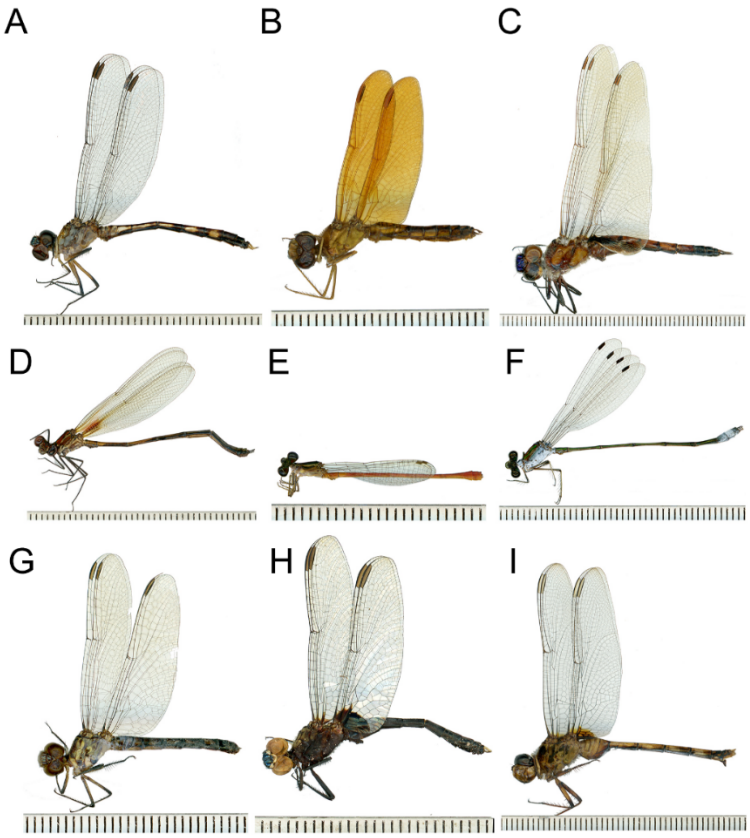

Figure 2. Examples of odonates (Insecta: Odonata) collected and sequenced from Paraíba State, Brazil. (**A**) *Anatya januaria*, (**B**) *Perithemis tenera*, (**C**) *Tramea cophysa*; (**D**) *Hetaerina rosea*, (**E**) *Telebasis filiola*, (**F**) *Lestes forficula*; (**G**) *Micrathyria hesperis*, (**H**) *Erythrodiplax basalis* and (**I**) *Erythemis plebeja*.

Figure 3 shows the clear separation of intraspecific and interspecific distances and the so-called "barcoding gap" on the regional DNA barcode library. Considering the global databases, eight species showed close proximity (<2%) to records of other deposited species (Table 1): *A. januaria*, *Erythemis carmelita* Williamson, 1923, *Erythrodiplax basalis* (Kirby, 1897), *H. rosea*, *N. berlai*, *Perithemis tenera* (Say, 1840), *Telebasis filiola* (Perty, 1834) and *Tramea cophysa* Hagen, 1867.

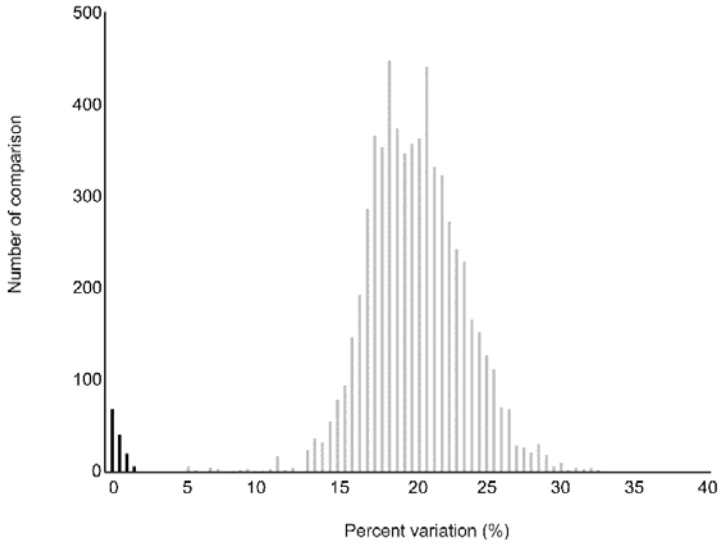

Figure 3. Frequency distribution of intraspecific (black) and interspecific (grey) genetic divergence in the sampled odonates (regional database).

3.3. Species Delimitation

Species delimitation analyses provided 45 species defined by morphological delimitation. Using molecular methods to delimit species, the results were 45 for both the ABGD-initial partition and ABGD-recursive partition, 39 for ASAP, 46 for PTP and GMYC (Figure 4).

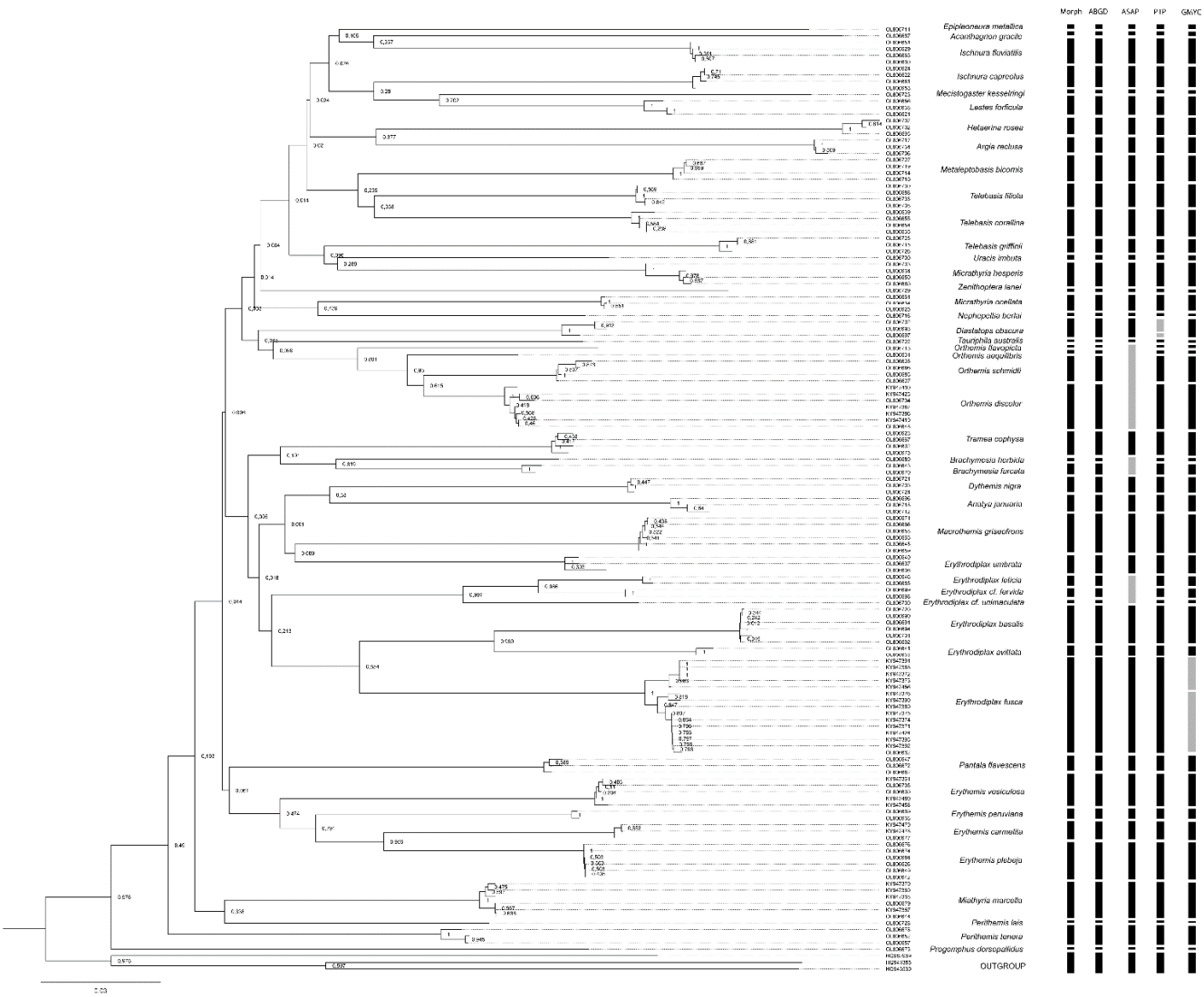

Figure 4. Molecular species delimitation of odonates from the Paraíba state, Brazil, based on DNA barcodes. Black bars indicate congruent results between the molecular and morphological identifications, and grey bars indicate divergence results from morphological identification. It should be noted that the neighbor-joining tree presented here is only intended to represent the distance matrix, and it should not be interpreted as a phylogenetic hypothesis.

4. Discussion

This study adds 15 Odonata species to the state of Paraíba. As a result, this state now has 64 species recorded; thus, it is third in number of species in the Northeast region of Brazil, behind only Bahia and Ceará (174 species [52] and 73 species [53], respectively). This library of DNA sequences is the first publication of DNA barcoding for the 16 Odonata species in public sequence repositories: *Acanthagrion gracile* (Rambur, 1842), *A. januaria*, *Ep. metallica*, *E. avittata* Borror, 1942, *E. leticia*, *E.* cf. *fervida*, *E. basalis*, *E.* cf. *unimaculata* (De Geer, 1773), *M. griseofrons* Calvert, 1909, *Mecistogaster kesselringi* Soldati and Machado, 2019, *Metaleptobasis bicornis* (Selys, 1877), *N. berlai*, *O. flavopicta*, *Progomphus dorsopallidus*

Byers, 1934, *T. filiola* and *Zenithoptera lanei* Santos, 1941. The database generated for the present study provides data for 70% (45 species) of the odonate species found in Paraíba state. Despite all these numbers, we recognize that this work is a starting point for the study of odonates in Paraíba state, considering that the likely diversity of the region must be far greater than our estimates.

It is important to highlight the methodological limitations that we faced for the amplification of Neotropical odonates. Although many studies indicate the effectiveness of the tested primers on several continents (e.g., [54,55]), they did not have the amplification capacity for all the species amplified in this study, most notably in Zygoptera. Problems with amplifying Neotropical species using primers commonly used in the world is not new, and it is not exclusive to odonates (see [13] for frogs and [14] for fishes). Jennings and collaborators [14] highlight the importance of this type of information considering that the trial and error nature for the choice of primers wastes labor and reagents.

In addition to demonstrating that the two pairs of primers used in this study are capable of amplifying the COI fragment in the two suborders present in the Neotropical region, this study shows that it is also important to consider the usefulness of this marker to discriminate between different species in terms of their DNA barcode and metabarcoding studies. The regional database does not present the overlapping of inter- and intraspecific genetic variation; however, in the global database analysis (using Genbank and the BOLD System), eight species showed close genetic proximity to the species we examined (<2%). Below, we discuss the most likely hypothesis for each taxon as well as the disagreements we noticed in the global dataset evaluation.

Our results showed that the analyzed specimens of *A. januaria* from Paraíba state are 99.74% similar to the available sequences for *A. guttata* (Erichson, 1848). Problems with determining the species of *Anatya* are largely recognized, mainly due to some specific variable characteristics being wrongly interpreted (see [10], p. 224). Our specimens had subtle differences among them, including the size of cerci and body length; however, based on the comparison of these structures drawn by Ris ([56], p. 424) and Garrison et al. [10], the shape of the posterior hamule left almost no doubt that our specimens belong to *A. januaria* (Figure 5A).

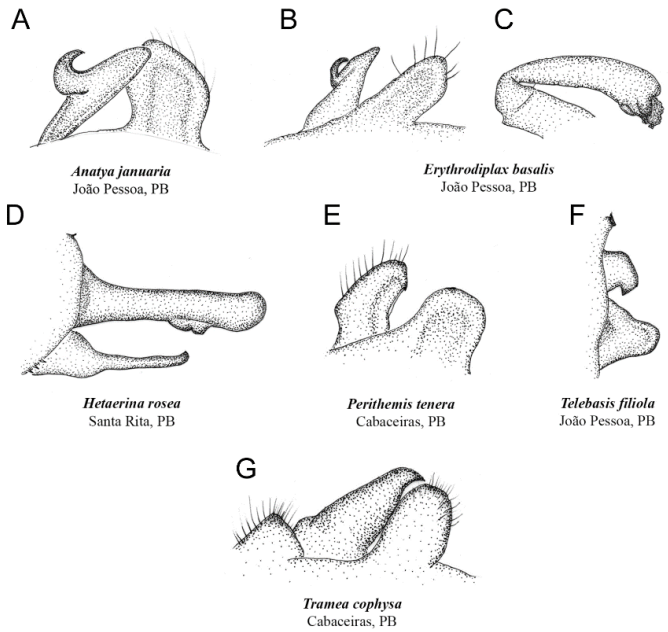

Figure 5. Morphology of male Odonata adults collected in Paraíba state, Brazil (lateral view of): (**A**) secondary genitalia of *Anatya januaria*; (**B**) secondary genitalia and (**C**) vesica spermalis of *Erythrodiplax basalis*; (**D**) caudal appendages of *Hetaerina rosea*; (**E**) secondary genitalia of *Perithemis tenera*; (**F**) caudal appendages of *Telebasis filiola*; (**G**) secondary genitalia of *Tramea cophysa*.

In turn, the *E. carmelita* we analyzed was 98.67% similar to a sequence identified as the species *E. mithroides*. We are confident with regard to our morphological identification because, despite their color resemblance, these two species are very easily distinguishable because of their abdominal characteristics. *Erythemis carmelita* belongs to the group of *Erythemis* species that possesses greatly swollen basal abdominal segments (which are slightly swollen in *E. mithroides*) and narrow remainder segments (which are broad in *E. mithroides*) ([10], p. 240–241).

In addition, we found a high similarity between the sequences of our *E. basalis* and the deposited sequences of *E. paraguayensis* (Förster, 1905). Borror [57] states that specimens of *E. paraguayensis* are "apt to be confused with small individuals of *basalis*" (p. 153); however, morphologically, these species are difficult to be confused with one another because of multiple characteristics: *E. basalis* belongs to Borror's basalis group, which consists of species that have hamules that are usually slender and have an outer branch a little longer than their inner branch (evident in lateral view, Figure 5B), a terminal segment of a slender penis, small and rounded lateral lobes (Figure 5C)—characteristics that we can easily observe in *E. basalis* specimens [57].

In contrast, *E. paraguayensis* belongs to the connata group, in which males present hamules that are moderately robust, their outer branch is almost equal in size to inner branch, and the median process of the penis is very prominent and slender, similar to what we can observe in the males of *E. paraguayensis*. In addition, *E. paraguayensis* is one of the smallest species of the genus and the extent of the small spots of the hind wings nearly trespasses upon the cubital space. In contrast, *E. basalis* is a much larger species, its hind wing spots reaching (or in some cases, trespassing upon) the first antenodal vein. Based on these characters, we argue that our morphological identification is correct.

Although our sequences of *H. rosea* are 98.95% similar to the ones assigned to *H. sanguinea* Selys, 1853, those two species are highly unlikely to be confused with one another. This is mainly because of the paraprocts (Figure 5D) are long and well developed in *H. rosea*, while they are vestigial in *H. sanguinea* (see [58] for a revision). Another character that can easily separate the two species is the median lobe of cercus. It is bilobed in *H. rosea* and entirely so in *H. sanguinea* (see [24], pp. 66–67).

Nephepeltia berlai shares several morphological characters with *N. aequisetis* Calvert, 1909, with the most prominent among them being the tubercle on the venter of thorax, the length and placement of the hind tibiae spurs, and also the vesica spermalis (see [59] for a revision). However, there are some characters on the vesica spermalis (medio-ectal distal process) and the cercus (level of the distal end of ventral toothed carina at about distal fourth of cercus length) that allow for a separation of the two species. Based on those characters, we believe that our specimens are *N. berlai*.

We based our identifications of the genus *Perithemis* largely on the study of von Ellenrieder and Muzón [60], which was the first study to separate the species of this genus using characteristics other than coloration and wing venation. The males we identified as *P. tenera* (Say, 1840) (regarded as *P. mooma* Kirby, 1889 in [60]) present wings that are uniformly colored (as in *P. icteroptera* Selys in Sagra, 1857), have a tip of hamuli (Figure 5E) at least 0.40 of the ventral margin (at the level of ventral margin in *P. icteroptera*), and a penis with a first segment trapezoidal (rounded in *P. icteroptera*). Our sequences were 99.83% similar to the sequences assigned to *P. icteroptera*; however, the characteristics described above led us to identify our specimens as *P. tenera*.

The sequences of our specimens identified as *T. filiola* were 98.96% identical to the specimens deposited assigned as *T. willinki* Fraser, 1948. These species are very similar morphologically, as it is stated in the revision of the genus [61]. However, despite the great resemblance of these taxa, Garrison [61] properly diagnosed the two species, showing that the cercus of *T. filiola* (Figure 5F) is distinctly shorter than the paraproct (it is subequal to the paraproct in *T. willinki*). In turn, we followed the same diagnosis to assign our specimens as *T. filiola*.

Lastly, we identified some males of *T. cophysa* showing agreement with the diagnosis presented in DeMarmels and Rácenis [62]. However, our sequences were 98.34% similar to the sequences assigned to *T. binotata* (Rambur, 1842) in the BOLD System. These two taxa, although they belong to the same genus, are quite different. Due to their color and morphological differences, they were placed in different groups within *Tramea*. The cophysa group, to which *T. cophysa* belongs, is composed of four species that share "only one constant characteristic common to all four species and, peculiar to the "cophysa-group", are two oblique pale lateral bands on the synthorax" [62]. Such a characteristic is present in our specimens while it is absent in *T. binotata*, as this is a species with an overall blue-grey coloration, contrasting with the reddish coloration of the species of the cophysa group. Additionally, as a result of our comparison of morphological features, such as the shape of the posterior hamule and the length and shape of the cercus (Figure 5G), we were able to make a safe distinction between our specimens to other taxa within *Tramea*.

The molecular species delimitation results were identical to the morphological results for most of the species we examined. The presence of divergent results is commonly used to indicate possible cryptic species (e.g., [63]); in turn our identical results in terms of morphological, ABGD, and the occasional divergences suggest that COI has the ability to delimit in the evaluated species.

In summary, our results demonstrate that DNA barcoding can be used to delimit and differentiate odonates on a regional scale. Of the 45 species evaluated in this study, only eight species (17%) showed any disagreement with the global databases and all species (100%) could be identified when we consider only our regional DNA barcode database. Considering the issues with the global databases, our results for the number of species that can be readily identified using their DNA barcoding (83%) are close to those found in other regions of the world. In a genetic database for the Central and North European odonates, the effectiveness was 88% in a set of 103 species [64]. Values between 79% and 89% were also found in datasets in countries such as the Philippines (in a set of 38 species; [54]), Italy (in a set of 88 species; [65]) and Malta (in a set of 10 species, [55]). In Brazil, the only evaluation (in a set of 38 species) performed indicated a success rate between 79% and 94% depending on the analysis criteria [16].

Subsequently, all these results indicate the importance of performing careful morphological analysis (see this kind of problem in [66]). Moreover, as indicated by Koroiva and Kvist [38], it reinforces that the use of the global database does not allow the correct establishment of all molecular identifications to be correct. Many reasons justify the discrepancy between the morphological and molecular results. Among in odonates, three main causes are the presence of cryptic species, rapid and/or recent radiation events and errors in identifying the deposited specimens [38]. Regarding this last issue, the description and presentation of the key structures for identification presented above aimed to facilitate future comparisons in this sense.

5. Conclusions

The establishment of an Odonata DNA barcode library for the Paraíba state is a milestone that will improve the taxonomy and biodiversity conservation for Neotropical species. Despite the difficulties of using traditional primers for amplifying the Neotropical species, our results demonstrate that using the COI in the regional scale can help identify and delimit those evaluated. Our results for the number of species that can be readily identified using their DNA barcoding (83%) are close to the results found in other regions of the world. Keeping in mind the problems of using public genetic databases for identification, here, we present morphological evidence for our identifications in the cases of disagreement. In turn, this facilitates comparisons and allows for new questions to arise about the genetic diversity of tropical species.

Author Contributions: Conceptualization, R.K.; methodology, R.K., V.G.N.G. and D.S.V.; formal analysis, R.K., V.G.N.G. and D.S.V.; data curation, R.K. and D.S.V.; writing—original draft preparation, R.K., V.G.N.G. and D.S.V.; writing—review and editing, R.K., V.G.N.G. and D.S.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partially funded by PROPESQ/PRPG/UFPB, grant number PVA13280-2020 and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES), finance Code 001. DSV thanks Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for a postdoctoral fellowship grant (Proc. 2019/26438-9).

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Data are contained within the article; specimens analyzed in this study are deposited in Entomological Collection of the Department of Systematics and Ecology of the Federal University of Paraíba (DSEC/UFPB) and are available on request to the collection managers. The sequences are available at GenBank (accession numbers OL806732 to OL806735 and OL806621 to OL806730) and BOLD system (http://dx.doi.org/10.5883/DS-ODOPB, accessed on 11 February 2022).

Acknowledgments: We are thankful to Jardim Botânico de João Pessoa (JBBM/SUDEMA) for the assistance in sampling. We are grateful to Frederico Augusto de Atayde Lencioni and Rosser W. Garrison for confirmation of species identifications. We are also grateful to the academic editor and the anonymous reviewers for their valuable comments.

Conflicts of Interest: The authors declare no conflict of interest.

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