UNIVERSIDADE FEDERAL DO PARANÁ

LEÓN ANDRÉS PÉREZ GUTIÉRREZ

AN INTEGRATIVE TAXONOMIC REVISION AND MOLECULAR PHYLOGENY OF THE NORTHERN ANDEAN DAMSELFLIES OF THE COMPLEX OF GENERA *HETEROPODAGRION* SELYS, 1885 AND *MESAGRION* SELYS, 1885 (INSECTA: ODONATA: HETERAGRIONIDAE AND MESAGRIONIDAE)

> CURITIBA 2024

## LEÓN ANDRÉS PÉREZ GUTIÉRREZ

# AN INTEGRATIVE TAXONOMIC REVISION AND MOLECULAR PHYLOGENY OF THE NORTHERN ANDEAN DAMSELFLIES OF THE COMPLEX OF GENERA *HETEROPODAGRION* SELYS, 1885 AND *MESAGRION* SELYS, 1885 (INSECTA: ODONATA: HETERAGRIONIDAE AND MESAGRIONIDAE)

Tese apresentada ao Programa de Pós-Graduação em Entomologia desenvolvida no Laboratório de Sistemática de Insetos Aquáticos (LABSIA), Departamento de Zoologia, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Entomologia.

Orientador: Prof. Dr. Ângelo Parisse Pinto (DZoo/UFPR) Coorientadora: Profa. Dra. Daniela Maeda Takiya (DZoo/UFRJ)

CURITIBA 2024

#### DADOS INTERNACIONAIS DE CATALOGAÇÃO NA PUBLICAÇÃO (CIP) UNIVERSIDADE FEDERAL DO PARANÁ SISTEMA DE BIBLIOTECAS – BIBLIOTECA DE CIÊNCIAS BIOLÓGICAS

Pérez Gutiérrez, León Andrés

An integrative taxonomic revision and molecular phylogeny of the northern andean damselflies of the complex of genera *Heteropodagrion* Selys, 1885 and *Mesagrion* Selys, 1885 (Insecta : Odonata : Heteragrionidae and Mesagrionidae) / León Andrés Pérez Gutiérrez. – Curitiba, 2024. 1 recurso on-line : PDF.

Tese (Doutorado) – Universidade Federal do Paraná, Setor de Ciências Biológicas, Programa de Pós-graduação em Ciências Biológicas (Entomologia).

Orientador: Prof. Dr. Ângelo Parisse Pinto (DZoo/UFPR). Coorientadora: Profa. Dra. Daniela Maeda Takiya (DZoo/UFRJ).

1. Libélula. 2. Taxonomia numérica. 3. Análise cladística. I. Pinto, Ângelo Parise, 1980-. II. Takiya, Daniela Maeda. III. Universidade Federal do Paraná. Setor de Ciências Biológicas. Programa de Pósgraduação em Ciências Biológicas (Entomologia). IV. Título.

Bibliotecária: Giana Mara Seniski Silva CRB-9/1406

## APPROVAL TERM

# LEÓN ANDRÉS PÉREZ GUTIÉRREZ

AN INTEGRATIVE TAXONOMIC REVISION AND MOLECULAR PHYLOGENY OF THE NORTHERN ANDEAN DAMSELFLIES OF THE COMPLEX OF GENERA *HETEROPODAGRION* SELYS, 1885 AND *MESAGRION* SELYS, 1885 (INSECTA: ODONATA: HETERAGRIONIDAE AND MESAGRIONIDAE)

Dissertation submitted to the Programa de Pós-Graduação em Ciências Biológicas (Entomologia), Setor de Ciências Biológicas, Universidade Federal do Paraná, as a partial requirement for obtaining the Doctor of Science (D.Sc.) degree in Entomology.

Evaluation Committee:

Prof. Dr. Ângelo Parise Pinto Advisor (president) – Departamento de Zoologia, UFPR

Dra. Yesenia Margarita Vega Sánchez Instituto de Investigaciones en Ecosistemas y Sustentabilidad, UNAM

Dr. Federico Lozano Universidad Nacional de Avellaneda, UNDAV

Dr. Fabrícius Maia Chaves Bicalho Domingos Departamento Zoologia, UFPR

Curitiba, May 22th, 2024



MINISTÉRIO DA EDUCAÇÃO SETOR DE CIÊNCIAS BIOLÓGICAS UNIVERSIDADE FEDERAL DO PARANÁ PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO PROGRAMA DE PÓS-GRADUAÇÃO CIÊNCIAS BIOLÓGICAS (ENTOMOLOGIA) - 40001016005P5

#### TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação CIÊNCIAS BIOLÓGICAS (ENTOMOLOGIA) da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de LEON ANDRES PEREZ GUTIERREZ intitulada: An integrative taxonomic revision and molecular phylogeny of the northern andean damselflies of the complex of genera Heteropodagrion Selys, 1885 and Mesagrion Selys, 1885 (Insecta: Odonata: Heteragrionidae and Mesagrionidae), sob orientação do Prof. Dr. ANGELO PARISE PINTO, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 22 de Maio de 2024.

Assinatura Eletrônica 24/05/2024 16:36:49.0 ANGELO PARISE PINTO Presidente da Banca Examinadora

Assinatura Eletrônica 23/05/2024 18:09:05.0 YESENIA MARGARITA VEGA SÁNCHEZ Avaliador Externo (UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO) Assinatura Eletrônica 13/06/2024 11:38:20.0 FEDERICO LOZANO Avaliador Externo (UNIVERSIDAD NACIONAL DE AVELLANEDA)

Assinatura Eletrônica 23/05/2024 18:08:48.0 FABRICIUS MAIA CHAVES BICALHO DOMINGOS Avaliador Externo (UNIVERSIDADE FEDERAL DO PARANÁ)

Departamento de Zoologia - Centro Politécnico - CURITIBA - Paraná - Brasil CEP 81531-980 - Tel: (41) 3361-1763 - E-mail: pgento@ufpr.br Documento assinado eletronicamente de acordo com o disposto na legislação federal Decreto 8539 de 08 de outubro de 2015. Gerado e autenticado pelo SIGA-UFPR, com a seguinte identificação única: 367926 Para autenticar este documento/assinatura, acesse https://siga.ufpr.br/siga/visitante/autenticacaoassinaturas.jsp e insira o codigo 367926

#### DEDICATION

I dedicate this work to teacher Jürg De Marmels, for the support provided at crucial stages of my career and for inspiring me in the study of megapodagrionids. To León Abel Pérez and Luzmila Gutiérrez, my parents and first teachers, to whom I owe everything. To Samuel León, my son, the most important of all my teachers. To students, to future generations of entomologists.

#### ACKNOWLEDGEMENTS

Especially to the residents of each of the towns visited during the expeditions that allowed access to their territories during collect campaigns.

To the director of the thesis, my advisor Professor Dr. Ângelo Parise Pinto for sharing your knowledge and for the support and guidance to make the completion of this work possible.

To the thesis co-director, my co- advisor Professor Dr. Daniela Maeda Takiya for her commitment and crucial teachings.

To the Department of Entomology of the Federal University of Paraná.

To the postgraduate in Entomology and administrative staff for instructions throughout the doctoral process.

To Leonardo Polizelli, Vinícius Silva and coffee room staff: Joao, Amelia, Gabriela, for offering good moments of conversation and company during my stay in UFPR, Curitiba.

To each of the teachers from whom I had the opportunity to learn in the courses attended in UFPR.

To my friend Luis Carlos Gutiérrez (Q.E.P.D.) and Universidad del Atlántico for their support during my doctoral study commission.

#### DISCLAIMER

This work is not issued for the purpose of zoological nomenclature, so that the names and acts are clearly not available and do not enter zoological nomenclature unintentionally according to recommendations 8E of the Article 8.2.

Please contact one of the authors with the following e-mail address to request access the matrices and unpublished data used in the analyses: leonperez@mail.uniatlantico.edu.co; appinto@ufpr.br; takiya@gmail.com

#### RESUMO

As relações filogenéticas entre as libélulas de Zygoptera (Odonata) representam uma questão de intenso debate e os resultados de diferentes análises não são conclusivos. Para resolver essas questões é pertinente abordar diferentes níveis hierárquicos do relacionamento filogenético da subordem para propor classificações baseadas no consenso de análises morfológicas, moleculares e filogenômicas. O objetivo desta tese é revisar a taxonomia de dois gêneros proximamente relacionados de Megapodagrionidae s.l., os megápodes da subordem Zygoptera. Esses gêneros estão hoje em famílias distintas, Heteropodagrion Selys, 1865 em Heteragrionidae e Mesagrion Selys, 1885 em Mesagrionidae, mas tradicionalmente têm sido considerados próximos morfologicamente e nas propostas de classificação, mas seu próximo relacionamento filogenético é questionado. Com um conjunto de abordagens metodológicas que integram exame morfológico detalhado com microscopia tradicional e eletrônica, somado a sequências de regiões de quatro marcadores moleculares, dois mitocondriais (citocromo c oxidase subunidade I - COI e subunidade 16S ribossomal - 16S) e dois nucleares ribossomais (espaçador interno transcrito 2 - ITS2 e subunidade 28S) objetivou-se inferir os limites entre espécies e propor entidades taxonômicas com critérios integrativos. Por fim, são propostas as relações filogenéticas desses dois táxons com os membros do clado Heteragrionidae e testado seu monofiletismo com a adição de dados abrangendo nove das dez espécies conhecidas até a execução deste trabalho. A investigação filogenética revela que Heteropodagrion e Mesagrion são taxa irmãos. As relações entre os gêneros de Heteragrionidae revelam que eles não são um táxon monofilético e que Heteropodagrion e Dimeragrion deveriam ser excluídos deste táxon. A adição de Heteropodagrion a Mesagrionidae é apoiada pelas evidências apresentadas aqui, enquanto o status de Dimeragrion requer mais estudos. Heteragrionidae não é monofilético e conclui-se que a circunscrição da família deve incluir apenas Heteragrion e Oxystigma, com a possível inclusão de Allopodagrion que parece relacionado em todas as análises efetuadas neste trabalho.

**Palavras-chave**: delimitação de espécies; filogenia; sistemática; taxonomia molecular; taxonomia integrativa.

#### ABSTRACT

The phylogenetic relationships among zygopteran odonates (Odonata) are a matter of intense debate and results of different approaches are not conclusive. To solve these issues, it is pertinent to address different levels of phylogeny within the order to reconcile classifications based on morphological, molecular, and phylogenomic analyses. The purpose of this thesis is to review the taxonomy of two genera with uncertain phylogenetic position (*incertae sedis*) within Zygoptera, *Heteropodagrion* of the family Heteragrionidae and *Mesagrion* of the family Mesagrionidae. They have been traditionally considered close in proposals of classification, but their close relationship was recently questioned. With a set of methodological approaches that integrate detailed morphological examination with optical and electron microscopy, added to sequences of regions of four molecular markers, two mitochondrial (cytochrome c oxidase subunit I - COI and ribosomal subunit 16S) and two nuclear ribosomal (internal transcribed spacer 2 - ITS2 and 28S), we seek to resolve limits between species and establish taxonomic entities with integrative criteria. Finally, phylogenetic relationships of these two taxa with the members of the Heteragrionidae clade are proposed and their monophyly tested with the addition of data covering 9 of the 10 species known up to the present of this work. Integrative analyses allowed the recognition of species limits in the *Heteropodagrion-Mesagrion* complex, revealing patterns of genetic divergence that allowed the assignment of 5 new species and clarification of the statuses of the 5 species known so far, particularly resolving that H. superbum exhibits color polymorphism within the phenotypic variation. A phylogenetic investigation reveals that Heteropodagrion and Mesagrion are sister taxa. The relationships between the genera of Heteragrionidae reveal that they are not a monophyletic taxon and that *Heteropodagrion* and *Dimeragrion* should be excluded from this taxon. The addition of *Heteropodagrion* to Mesagrionidae is supported by the evidence presented here, as the status of *Dimeragrion* requires further studies. Heteragrionidae is not monophyletic, and it is concluded that the family circumscription should include only *Heteragrion* and *Oxystigma*, with the possibility of including Allopodagrion which seems related in all the analyzes carried out in this work.

**Key words**: integrative taxonomy; molecular taxonomy; phylogeny; species delimitation; systematics.

#### **FIGURES LIST**

#### **CHAPTER 1**

- FIGURE 1 Fig. 1. Maximum likelihood gene trees based on COI sequences of Neotropical megapods with *Heliocharis* as outgroup. Node values refer to SH-like aLRT and ultrabootstrap percentages. Taxa colors refer to Bybee et al. (2021) classification: Blue: Megapodagrionidae s.e; olive green: Hypolestidae; Red: Heteragrionidae; purple – Thaumatoneuridae; Pink – Philogenidae
- FIGURE 2 Fig. 2. Maximum likelihood gene trees based on COI sequences of Neotropical megapods with *Heliocharis* as outgroup. Node values refer to SH-like aLRT and ultrabootstrap percentages. Taxa colors refer to Bybee et al. (2021) classification: Blue: Megapodagrionidae s.e; olive green: Hypolestidae; Red: Heteragrionidae; purple – Thaumatoneuridae; Pink – Philogenidae

#### **CHAPTER 2**

 FIGURE 4 – Fig. 1. Representatives of *Heteropodagrion and Mesagrion* genera. (a) *Heteropodagrion santuario* sp. nov (Colombia, Risaralda: Santuario. Rio San Rafael) (b) *Mesagrion leucorrhinum* mainting pair (Chirajara, Guayabetal Colombia. (c) Larvae of *Heteropodagrion santuario* sp. nov. (Colombia, Risaralda: Río San Rafael, Santuario). (d) Larvae of *Mesagrion leucorrhinum* (Colombia, Cundinamarca: Guayabetal, Quebrada Chirajara)

- FIGURE 8 Fig. 5. Male, head: frontal and dorsal view. (a) Heteropodagrion paramillo
   sp. nov. (b) Heteropodagrion santuario sp. nov. (c)
   Heteropodagrion superbum. (d) Heteropodagrion varipes Daigle,
   2014. (e) Mesagrion leucorrhinum. Photos by L.P.......93
- FIGURE 9 Fig. 6. Male, thorax: dorsal, ventral, and lateral view. (a) Heteropodagrion croizati. (b) Heteropodagrion cuyabri sp. nov. Photos by L.P. ....
  94

FIGURE 12 – Fig. 9. Male, thorax: dorsal, ventral, and lateral view. (*a*) *Heteropodagrion superbum*. (*b*) *Heteropodagrion varipes*. Photos by L.P. ...... **97** 

- FIGURE 15 Fig. 12. Male, wings: dorsal view. (*a*) *Heteropodagrion diabolum* sp. nov. (*b*) *Heteropodagrion donnellyi* sp. nov. Photos by L.P. ...... **100**
- FIGURE 16 Fig. 13. Male, wings: dorsal view. (*a*) *Heteropodagrion paramillo* sp. nov. (*b*) *Heteropodagrion santuario* sp. nov. Photos by L.P. ...... **101**
- FIGURE 17 Fig. 14. Male, wings: dorsal view of *Heteropodagrion superbum (a)* (Colombia, Cauca: San Antonio, SAIA681) (b) (Colombia, Cauca: Anchicayá, CEUA94670). Photos by L.P. ...... **102**
- FIGURE 19 Fig. 16. Male, cerci: dorsal view and tip detail. (a) Heteropodagrion croizati. (b) Mesagrion leucorrhinum. Photos by L.P. ...... 104
- FIGURE 20 Fig. 17. Male, cerci: dorsal view and tip detail. (a) Heteropodagrion cuyabri sp. nov. (b) Heteropodagrion diabolum sp. nov. (c) Heteropodagrion nigripes Daigle, 2014. Photos by L.P. ..... 105
- FIGURE 21 Fig. 18. Male, cerci: dorsal view and tip detail. (a) Heteropodagrion donnellyi sp. nov. Photos by L.P (b) Heteropodagrion paramillo sp. nov. Photos by L.P (c) H. sanguinipes (extracted from Daigle, 2014).
- FIGURE 23 Fig. 20. Male, cerci: dorsal view and tip detail. (a) *Heteropodagrion* superbum. (a) Type Locality in San Antonio, Cauca. (b) *Heteropodagrion superbum*. Rio Bravo (c) *Heteropodagrion* varipes. Photos by L.P.

- FIGURE 29 Fig. 26. Female, head: frontal view. (a) Heteropodagrion croizati. (b) Heteropodagrion cuyabri sp. nov. (c) Heteropodagrion donnellyi sp. nov. (d) Heteropodagrion paramillo sp. nov. (e) Heteropodagrion santuario sp. nov. (f) Heteropodagrion superbum. (g) Mesagrion leucorrhinum. Photos by L.P. ..... 114
- FIGURE 30 Fig. 27. Female, left pair wings: dorsal view. (*a*) *Heteropodagrion croizati*. (*b*). *Heteropodagrion cuyabri* sp. nov. Photos by L.P. ...... **115**

FIGURE 34 – Fig. 31. Female, ovipositor: lateral view. (a) Heteropodagrion croizati.
(b) Heteropodagrion cuyabri sp. nov. (c) Heteropodagrion donnellyi sp. nov. (d) Heteropodagrion paramillo sp. nov. (e) Heteropodagrion santuario sp. nov. (f) Heteropodagrion superbum. (g) Mesagrion leucorrhinum. Photos by L.P. ..... 119

- FIGURE 37 Fig. 34. Habitus of live specimens. Male. (a) Heteropodagrion paramillo sp. nov. (Colombia, Antioquia: Via Juntas de Uramita- Peque).
   Photo by L.P. (b) Heteropodagrion sanguinipes (Ecuador, Quito: San Francisco). Photo by K. Tennessen and Mauffray) ...... 122
- FIGURE 38 Fig. 35. Habitus of live specimens. Male. (a) Heteropodagrion santuario sp. nov. (Colombia: Risaralda, Santuario). (b) Heteropodagrion superbum (Colombia, Cauca: Cali, San Antonio, Km 18 via Dagua). Photos by L.P.
- FIGURE 39 Fig. 36. Habitus of live specimens. (a) Heteropodagrion varipes male in hand (Ecuador, Morona Santiago: Abanico stream LABSIA319).
   (b) Mesagrion leucorrhinum. (Colombia: Rio Claro, Antioquia) Photos by L.P.
- FIGURE 40 Fig. 37. Habitus of live specimens. (a) Heteropodagrion croizati (Colombia, Putumayo: Serrania de los Churumbelos Aika Wasi).
  (b) Heteropodagrion cuyabri sp. nov. (Colombia, Quindio: Filandia, Hacienda Brehmen, Barvas River). Photos by L.P. .....
  125

FIGURE 41 – Fig. 38. Habitus of live specimens. (a) Heteropodagrion diabolum sp. nov. (Colombia, Chocó: Tutunendo, El Diablo Stream). (b) *Heteropodagrion donnellyi* sp. nov. (Panamá, Ciudad de Panamá: Cerro Azul, Agua Fria waterfalls). Photos by L.P. **126** 

- FIGURE 44 Fig. 41. Habitus in lateral view of males of Heteropodagrion. (a) holotype of Heteropodagrion cuyabri sp. nov. (b) holotype of Heteropodagrion diabolum sp. nov. (c) holotype of Heteropodagrion (d) of donnellyi sp. nov. holotype Heteropodagrion paramillo sp. nov. (e) holotype of Heteropodagrion sp.nov. (f) Heteropodagrion santuario superbum. Photos by L.P. ..... 129

#### **CHAPTER 3**

FIGURE 47 – Fig. 1. Maximum likelihood gene tree of taxa studied included Heteropodagrion and Mesagrion based on COI data set. Node values refer to SH-like aLRT and ultrabootstrap percentages. Taxa colors refer to Bybee et al. (2021) classification: Blue: Megapodagrionidae s.e; olive green – Hypolestidae; Red –

#### LIST OF TABLES

#### **CHAPTER 1**

- TABLE 1 Table 1. Neotropical genera of megapod damselflies (Megapodagrionidaes.l.) and their classification according to different authors20
- TABLE 2 Table 2. Data set used and analysis executed in phylogenetic reconstruction towards Odonata with megapodagrionid representatives included .. 25

#### **CHAPTER 2**

#### **CHAPTER 3**

### SUMMARY

1 INTRODUCTION	21
1.2 REFERENCES	23
2 OBJECTIVES	27
3 CHADTER 1 & CRITICAL REVIEW OF THE DHVI OGENY AND TAXONOMY OF THE NEOTRODICAL ME	GADOD
DAMASELETES (ODONATA), MEGADODAGDIONIDAE S. L. ); AN ATTEMPT TO SHEDDING LIGHT INTO A	GAFOD
POLYPHYLETIC HISTORY	
3.1 INTRODUCTION	28
3.1.1 A background in taxonomy of Megapodagrionidae s.l.	
3.1.2 Biology and natural history of Neotropical megapods	
3.1.3 Morphological and molecular data in Megapodagrionidae s.l. systematics	
3.1.4 Larval morphology in Neotropical Megapodagrionidae systematics	
3.1.5 A review of the proposed phylogenetic relationships in Megapodagrionidae s.l.	
3.1.6 Distributional patterns of the "Neotropical" Megapodagrionidae s.l.	40
<b>3.2</b> Methods	42
3.3 RESULTS	44
3.3.1 Relationships between Neotropical clades of Megapodagrionidae s.l.	
3.4 Discussion	46
3.5 References	48
4 CHAPTER 2 – THE DAMSELFLIES OF HETEROPODAGRION SELYS, 1885 AND MESAGRION SELYS, 18	85
(ODONATA: HETERAGRIONIDAE AND MESAGRIONIDAE): AN INTEGRATIVE TAXONOMIC REVISION	FOR THIS
PANAMANIAN AND NORTHERN ANDEAN COMPLEX OF GENERA	58
4.1 INTRODUCTION	59
4.2 MATERIAL AND METHODS	65
4.3 RESULTS	70
4.4 Taxonomy	73
4.4.1 Family Heteragrionidae Rácenis, 1959	74
4.4.1.1 Genus Heteropodagrion Selys, 1885	74
Heteropodagrion croizati Pérez-Gutiérrez & Montes-Fontalvo, 2011	75
Heteropodagrion cuyabri sp. nov	76
Heteropodagrion diabolum sp. nov.	79
Heteropodagrion donnellyi sp. nov	81
Heteropodagrion nigripes Daigle, 2014	84
Heteropodagrion paramillo sp. nov.	85
Heteropodagrion sanguinipes Selys, 1885	87

Heteropodagrion santuario sp. nov.	88
Heteropodagrion superbum Ris, 1918	91
Heteropodagrion varipes Daigle, 2014	94
4.4.2 Family Mesagrionidae	
4.4.2.1 Genus Mesagrion Selys, 1885	95
Mesagrion leucorrhinum Selys, 1885	96
4.5 Discussion	
4.5.1 MORPHOLOGICAL DATA	
4.5.2 SPECIES DELIMITATION	
4.6 Conclusions	
4.7 References	145
5 CHAPTER 3 - A MOLECULAR PHYLOGENY OF <i>HETEROPODAGRION</i> SELYS, 1885 AND <i>MESAG</i> 1885: A COMPLEX OF NEOTROPICAL DAMSELFLIES (ODONATA: ZYGOPTERA: HETERAGRIONIE	R <i>ION</i> SELYS, DAE AND
MESAGRIONIDAE)	154
MESAGRIONIDAE)	<b>154</b> 155
MESAGRIONIDAE)	<b>154</b> 
MESAGRIONIDAE)	<b>154</b> 
MESAGRIONIDAE)	
MESAGRIONIDAE)	
MESAGRIONIDAE)	
MESAGRIONIDAE)	
MESAGRIONIDAE) 5.1 INTRODUCTION 5.2 METHODS 5.2.1 Specimen and sample acquisition 5.2.2 DNA extraction and amplification and alignment 5.2.3 Taxon sampling 5.3 RESULTS 5.4 DISCUSSION 5.5 CONCLUSIONS	
MESAGRIONIDAE) 5.1 INTRODUCTION 5.2 METHODS 5.2.1 Specimen and sample acquisition 5.2.2 DNA extraction and amplification and alignment 5.2.3 Taxon sampling 5.3 RESULTS 5.4 DISCUSSION 5.5 CONCLUSIONS 5.6 REFERENCES	
MESAGRIONIDAE) 5.1 INTRODUCTION 5.2 METHODS 5.2.1 Specimen and sample acquisition 5.2.2 DNA extraction and amplification and alignment 5.2.3 Taxon sampling 5.3 RESULTS 5.4 DISCUSSION 5.5 CONCLUSIONS 6 GENERAL CONCLUSIONS	

#### **1 INTRODUCTION**

Insects popularly known as dragonflies and damselflies (Odonata) are extant members belonging to several lineages of pterygotes, among the oldest inhabitants of the earth, currently it corresponds to a mixed remnant of other already extinct groups, but most of these groups did not survive into the Mesozoic (Misof, 2014; Archibald et al., 2021). They exhibit a set of unique and special traits that are appreciated by people in general, as they are hemimetabolous and also amphibiotic, active predators as adults and larvae (Kalkman et al., 2008, Newton et al., 2023), flight performance (Silsby, 2001; May, 2019; Paulson & Marinov, 2021) and secondary genitalia and direct flight musculature (Maggioni et al., 2021).

Therefore, fossil register indicates than the diversity of the current Odonata dates back to Permian (Jarzembowski & Nel, 2002) from lineages with different cladogenetic ages (Grimaldi & Engel, 2005; Suvorov et al., 2021).

The Odonata are just over 6,400 living species, grouped into three major suborders, Anisoptera (true dragonflies, 3,100 spp.), Zygoptera (damselflies, 3,200 spp.), and Anisozygoptera. The last suborder is represented only by three relictual living species in a single genus *Epiophlebia* Calvert, 1903, the rest of members are fossil taxa (Busse, 2016). In respect to phylogenetic relationships, Anisoptera + Anisozygoptera (Epiophlebiidae) are sister groups named Epiprocta and Zygoptera form respective monophyletic groups (Bybee et al., 2021; Büsse & Ware, 2022).

The Neotropics is the largest center of diversity for odonates (Pinto, 2016; Pinto et al., 2023), only comparable to the eastern pantropical region and reasonable estimates predict that between 1000 and 1500 (10% of total) species are still unknown only in South America (Pinto, 2016). Much of this undiscovered diversity is unique to such regions (Pinto, 2024). For this reason, it is also the center of much of the current taxonomic research on Odonata (Paulson & Marinov, 2021).

Dragonflies and damselflies are a fundamental element of the biotic component used in environmental monitoring strategies due to their singularities as a model taxon (Clautsnitzer et al., 2009; May, 2019; Grigoropoulou et al., 2023), because they respond very distinctly and have different degrees of sensitivity to disturbances. These qualities have positioned them as indicators of habitat impacts of homogenization caused by urbanization (Deacon & Samways, 2021) Taxonomic practice in Odonata turns on integrative framework that achieves greater rigor by treating species as hypotheses (Pinto et al., 2023) in stages that become corroborative and refutational, this is the new face of realm of integrative taxonomy with different criteria for species delimitation based on multiple data sets, theoretical background, and criteria of inference (e.g., Dayrat, 2005; De Queiroz, 2007).

The ultimate decision of the taxonomist on the specific status will be subject to the plausibility or implausibility of hypotheses supported by the disciplines used (Schlick-Steiner et al., 2010). So, species are treated as hypothesized entities within an existing framework with the aim of proposing classifications based on evolutionary history, or better on the phylogenetic system inferred from a hypothesis of common ancestry (Pinto et al., 2023).

The general workflow for species delimitation consists of steps that can include use of tools, such as morphological examination, morphospecies statement, use of a single marker (i.e., barcode) for establishing a primary species hypothesis, and combined analysis of different markers and coalescent theory (bPTP) seeking to understand the evolution of populations (Padial et al., 2010). In a conclusive phase, when new taxa and names are established, we have secondary species hypotheses (Pante et al., 2015).

Nucleotide sequence analyzes based on few markers using Sanger platform or more recently phylogenomic data via Illumina or other Next-generation sequencing (NGS) platforms, have addressed phylogenetic relationships in high hierarchical levels of Odonata (Dijkstra et al., 2014; Kohli et al., 2021; Bybee et al., 2021), but are quite discordant and there are major questions to be investigated. The same has been observed in lower ranks such species, which have remained dynamic in the systematic field research of Odonata. New techniques do not stop us from advancing and methods of phylogenetic inference promise to be increasingly more integrative (Padial et al., 2010; Orr et al., 2021).

This study was carried out on a group of damselflies endemic to the north of South America and south of Central America by means of a critical review of its taxonomic history and an investigation of its specific and generic limits, as well as their internal relationships and position among higher taxonomic levels. These inferences were based on integrative taxonomy and phylogenetic methods and are organized in three chapters which work as stages of treatment of the problem involving these groups of damselflies. These stages are intended to provide answers based on all evidence available and represent contributions at three different levels to the systematics of the group.

The first chapter focuses on a review with the exploration of the groups among the linages of Megapodagrionidae *s.l.* with Neotropical distribution, using the data available in GenBank of two markers, one mitochondrial (COI) and another nuclear (28S ribosomal RNA). Based on separate and combined analyzes with maximum likelihood criteria, hypotheses of relationships among Neotropical megapods have been explored, offering an initial overview for what is expected to be answered in more depth in chapters 2 and 3.

The second chapter is dedicated to a taxonomic revision of the complex of genera *Heteropodagrion* Selys, 1885 (6 species) and *Mesagrion* Selys, 1885 (monotypic) using an integrative approach that includes a review of the diagnostic characters for the genus and included species. New diagnoses and fully illustrated descriptions for all 11 species, being five newly introduced here, are provided. Traditional morphological delimitation criteria are allied with molecular methods based on distance criteria (ABGD and ASAP), Bayesian (bPTP) and Maximum Likelihood. Thus, species delimitation was based on combined data of morphology and fragments of the mitochondrial cytochrome oxidase I (COI) gene and the second transcribed internal spacer (ITS2), which sought to resolve various taxonomic uncertainties that exist in each genus, as well as a mandatory review of the status of all species, including the intriguing *Heteropodagrion superbum* Ris, 1918.

The third and last chapter offers a molecular phylogenetic hypothesis envisioned to investigate the systematic position of the genera *Heteropodagrion* and *Mesagrion* with other megapods especially within Heteragrionidae. Sequences of the nuclear ribosomal 28S, and mitochondrial ribosomal 16S and COI gene were used to recover phylogenetic relationships among these taxa. The focus of this chapter was to offer a phylogeny that included for the first time 9 of 10 known species of *Heteropodagrion* and the single *Mesagrion*, as members of two most closely related clades and consequently to test the monophyly of Heteragrionidae.

#### **1.2 REFERENCES**

- Archibald SB, Cannings RA, Erickson RJ, Bybee SM, Mathewes RW 2021 The Cephalozygoptera, a new, extinct suborder of Odonata with new taxa from the early Eocene Okanagan Highlands, western North America. *Zootaxa*, 4934 (1), 1–133. <u>https://doi.org/10.11646/zootaxa.4934.1.1</u>
- Büsse S. 2016 Morphological re-examination of *Epiophlebia laidlawi* (Insecta: Odonata) including remarks on taxonomy. International Journal of Odonatology 19(4): 221–238. https://doi.org/10.1080/13887890.2016.1257442
- Büsse S, Ware, J. 2022 Taxonomic note on the species status of *Epiophlebia diana* (Insecta, Odonata, Epiophlebiidae), including remarks on biogeography and possible species distribution. **ZooKeys**. 1127. 79-90. 10.3897/zookeys.1127.83240.
- Bybee S, Kalkman V, Erickson J, Frandsen PB, Breinholt JW, Suvorov A, Dijkstra, KDB, Cordero-Rivera A, Skevington JH, Abbott JC, Sanchez-Herrera M, Lemmon AR, Lemmon E, Ware JL. 2021 Phylogeny and classification of Odonata using targeted genomics, *Molecular Phylogenetics and Evolution*, 160, 107115, https://doi.org/10.1016/j.ympev.2021.107115.Kalkman V, Clausnitzer V, Dijkstra KD, Orr A, Paulson D, Tol J. 2008 Global diversity of dragonflies (Odonata) in freshwater. 10.1007/978-1-4020-8259-7\_38.
- Calvert PP. 1903 Odonata, pp. 129-144. In: *Biologia Centrali-Americana*: *Insecta Neuroptera*. R.H. Porter and Dulau Co., London
- Clausnitzer V, Kalkman V, Ram M, Collen B, Baillie J, Bedjanic M, Darwall W, Dijkstra KD, Dow R, Hawking J, Karube H, Malikova E, Paulson D, Schütte K, Suhling F, Villanueva RJ, Ellenrieder N, Wilson K. 2009 Odonata enter the biodiversity crisis debate: The first global assessment of an insect group. *Biological Conservation*. 142. 1864–1869. 10.1016/j.biocon.2009.03.028.
- Deacon C, Samways MJ. 2021 A Review of the Impacts and Opportunities for African Urban Dragonflies. Insects. 12(3):190. doi: 10.3390/insects12030190.
- De Queiroz K. 2007 Species concepts and Species Delimitation, **Syst. Biol**. 56 (6): 879–886.
- Dijkstra K-DB, Kalkman VJ, Dow RA, Stokvis FR, Van Tol JAN. 2014 Redefining the damselfly families: a comprehensive molecular phylogeny of Zygoptera (Odonata). *Syst. Entomol*. 39: 68–96.
- Grimaldi D. Engel, MS. 2005 Evolution of the Insects. Cambridge University Press, New York, New York.

- Grigoropoulou A, Ab Hamid S, Acosta R, Akindele, EO, Al-Shami S, Altermatt F, Amatulli G, Angeler D, Arimoro F, Aroviita J, Astorga-Roine A, Bastos R, Bonada N, Boukas N, Brand C, Bremerich V, Bush A, Cai Q, Domisch S. 2023. The global EPTO database: Worldwide occurrences of aquatic insects. *Global Ecology and Biogeography.* 32. 10.1111/geb.13648.
- Kalkman V, Clausnitzer V, Dijkstra KD, Orr A, Paulson D, Tol J. 2008 Global diversity of dragonflies (Odonata) in freshwater. 10.1007/978-1-4020-8259-7\_38.
- Kohli M, Letsch H, Greve C, Béthoux O, Deregnaucourt I, Liu S, Zhou X, Donath A, Mayer C, Podsiadlowski L, Gunkel S, Machida R, Niehuis O, Rust J, Wappler T, Yu X, Misof B, Ware J. 2021 Evolutionary history and divergence times of Odonata (dragonflies and damselflies) revealed through transcriptomics. *iScience*. 24(11):103324. doi: 10.1016/j.isci.2021.103324.
- May, ML. 2019 Odonata: Who They Are and What They Have Done for Us Lately: Classification and Ecosystem Services of Dragonflies. *Insects* 10, no. 3: 62. <u>https://doi.org/10.3390/insects10030062</u>
- Misof B, Liu S, Meusemann K, Peters RS, Donath A, Mayer C, Frandsen PB, Ware J, Flouri T, Beutel RG, Niehuis O, Petersen M, Izquierdo-Carrasco F, Wappler T, Rust J, Aberer AJ, Aspo" ck U, Aspo" ck H, Bartel D, Blanke A, Berger S, Bo" hm A, Buckley TR, Calcott B, Chen J, Friedrich F, Fukui M, Fujita M, Greve C, Grobe P, Gu S, Huang Y, Jermiin LS, Kawahara AY, Krogmann L, Kubiak M, Lanfear R, Letsch H, Li Y, Li Z, Li J, Lu H, Machida R, Mashimo Y, Kapli P, McKenna DD, Meng G, Nakagaki Y, Navarrete-Heredia JL, Ott M, Ou Y, Pass G, Podsiadlowski L, Pohl H, von Reumont BM, Schutte K, Sekiya K, Shimizu S, Slipinski A, Stamatakis A, Song W, Su X, Szucsich NU, Tan M, Tan X, Tang M, Tang J, Timelthaler G, Tomizuka S, Trautwein M, Tong X, Uchifune T, Walzl MG, Wiegmann BM, Wilbrandt J, Wipfler B, Wong TK, Wu Q, Wu G, Xie Y, Yang S, Yang Q, Yeates DK, Yoshizawa K, Zhang Q, Zhang R, Zhang W, Zhang Y, Zhao J, Zhou C, Zhou L, Ziesmann T, Zou S, Li Y, Xu X, Zhang Y, Yang H, Wang J, Wang J, Kjer KM, Zhou X. 2014 Phylogenomics resolves the timing and pattern of insect evolution. *Science*, 346:763-767.

Newton L, Tolman E, Kohli M, Ware JL. 2023 Evolution of Odonata: genomic insights.

CurrentOpinioninInsectScience,58https://doi.org/10.1016/j.cois.2023.101073

- Orr M, Ferrari R, Hughes A, Chen J, Ascher J, Yan Y-H, Williams P, Zhou X, Bai M, Rudoy A, Zhang F, Ma K, Zhu, Chao-Don. 2021 Taxonomy must engage with new technologies and evolve to face future challenges. *Nature Ecology & Evolution.* 5, 3-4
- Padial JM, Miralles A, De la Riva I, Vences M. 2010 The integrative future of taxonomy. *Frontiers in Zoology* 7:16.
- Pante E, Puillandre N, Viricel A, Arnaud-Haond S, Aurelle D, Castelin M, Chenuil A, Destombe C, Forcioli D, Valero M, Viard F & Samadi S. 2015 Species are hypotheses: Avoid connectivity assessments based on pillars of sand. *Molecular Ecology*. 24. 525–544. 10.1111/mec.13048.
- Paulson DR, Marinov M. 2021 Zootaxa 20th Anniversary Celebration: Odonata section. *Zootaxa*, 4979(1), 218–221. https://doi.org/10.11646/zootaxa.4979.1.21
- Pinto, AP. 2016 A fauna de libélulas da América do Sul: a última fronteira a ser desvendada. *Informativo Sociedade Brasileira de Zoologia*. 117. 7-9.
- Pinto AP. 2024 Cap. 15, Odonata Fabricius, 1793, pp. 187-233. In: Rafael, J.A.; Melo, G.A.R.; Carvalho, C.J.B. de; Casari, S. & Constantino, R. (eds). Insetos do Brasil:
  Diversidade e Taxonomia. 2<sup>a</sup> ed. Instituto Nacional de Pesquisas da Amazônia, Manaus. 880 pp. https://doi.org/10.61818/56330464c15
- Pinto AP, Bota-Sierra CA, Marinov M. 2023 Species identification and description, in: Cordoba-Aguilar A, Beatty Ch, Bried J (eds), Dragonflies and Damselflies: Model Organisms for Ecological and Evolutionary Research, 2nd edn. Oxford, Oxford Academic press, <u>https://doi.org/10.1093/oso/9780192898623.003.0019</u>.
- Ris F (1918) Libellulinen (Odonata) aus der Region der amerikanischen Kordilleren von Costarica bis Catamarca. *Archiv für Naturgeschichte (Abteilung A*) **82**, 1–197.
- Schlick-Steiner B, Steiner F, Seifert B, Stauffer C, Christian E, Crozier R. 2010 Integrative taxonomy: a multisource approach to exploring biodiversity. Annual Review of Entomology 55: 421–38.

Selys-Longchamps E De (1885) Programme d'une revision des Agrionines. *Comptes Rendus de la Societe Entomologique de Belgique* **29**, cxli– cxlvi (1–8 separate).

Silsby J. 2001 Dragonflies of the World. CSIRO Publishing, Collingwood.

#### **2 OBJECTIVES**

The main goal of this dissertation was to contribute to the taxonomic and phylogenetic knowledge of Neotropical damselflies of Megapodagrionidae *s.l.* investigating the systematics of the complex of genera *Heteropodagrion* Selys, 1885 (Heteragrionidae) and *Mesagrion* Selys, 1885 (Mesagrionidae).

Specific goals:

(1) Provide a historical overview on the systematics of Neotropical Megapodagrionidae s.l., including a reanalysis of the available molecular data;

(2) Provide an integrative taxonomic revision of the species of the generic complex *Heteropodagrion-Mesagrion*, based on morphological and molecular criteria for species delimitation;

(3) Test the monophyly and investigate the phylogenetic placement of the complex of genera *Heteropodagrion* and *Mesagrion* based on a molecular phylogenetic approach;

(4) Inferring the phylogenetic relationships among species of the complex *Heteropodagrion-Mesagrion*.

# 3 Chapter 1. A CRITICAL REVIEW OF THE PHYLOGENY AND TAXONOMY OF THE NEOTROPICAL MEGAPOD DAMSELFIES (ODONATA: MEGAPODAGRIONIDAE S.L.): AN ATTEMPT TO SHEDDING LIGHT INTO A POLYPHYLETIC HISTORY<sup>1</sup>

León Andrés Pérez-Gutiérrez<sup>A, B</sup>

<sup>A</sup> Semillero de Investigación Sistemática y Autoecologia de Insectos Acuáticos SAIA, Research Group "Biodiversidad del Caribe Colombiano. Programa de Ciências Biológicas, Universidad del Atlántico, Barranquilla, Colombia.

<sup>B</sup> Laboratory of Systematics on Aquatic Insects (LABSIA), Departamento de Zoologia, Universidade Federal do Paraná, P. O. Box 19020, 81531-980, Curitiba, PR, Brazil. Corresponding author: E-mail: leonperez@mail.uniatlantico.edu.co LPG: https://orcid.org/0000-0002-2775-3040

**Key words**, Zygoptera, monophyly, *Incertae sedis*, Stem group, crown group phylogeny.

#### 3.1 Introduction

The insects of the order Odonata, dragonflies and damselflies, correspond to extant lineages pertaining to the ancient winged insects clade Odonatoptera (Bybee et al, 2016; Archibald et al., 2021). Living odonates currently consists of three major lineages recognized as suborders, Anisoptera and Zygoptera which together represent 99% of the extant dragonflies, and Anisozygoptera, an old and relictual lineage named Epiophlebiidae represented by three species of the single genus *Epiophlebia* Calvert, 1903 (Büsse, 2016; Bybee et al., 2021). At the subordinal level, Anisoptera and Epiophlebiidae are sister groups, being this clade sister to Zygoptera (Bybee et al., 2016; Rehn, 2003; Dijkstra et al., 2013; Nel et al., 2012).

With more of 6,400 living species of odonates (Paulson et al., 2024), the group of zygopterans genera known traditionally as megapodagrionids today consists of 51 genera and 300 species allocated up to 15 family-level taxa, of which about of 14

<sup>&</sup>lt;sup>1</sup> To be submitted to the *International Journal of Odonatology* (Percentile Scopus 50%, Qualis: A4) author guidelines available at: <u>https://worlddragonfly.org/ijo/instructions-for-authors/</u>

genera of 7 families and 180 species are endemic to the neotropics (Table 1). Many other traditional megapodagrionids or "megapods" most likely are distantly related to the Neotropical lineages (Rácenis, 1959; Dijkstra et al., 2013; Kalkman & Theischinger, 2013).

It is a group with a complex evolutionary history, which is presumed to be very ancient and probably with highly reticulated radiations even before the Carboniferous period (Rehn, 2003; Nel et al., 2012; Bybee, et al., 2016; Kohli et al., 2016; Suvorov et al., 2021). This is true particularly in the humid tropics of the globe at different times and geotectonic scales as supported by fossil evidence (Petrulevicius et al., 2008; Huang et al., 2018; Petrulevicius, 2020; So & Won, 2021).

Internal relationships among zygopterans have been the subject of intense research since the early beginnings of Odonatology with Selys-Longchamps (see Trueman, 2007) to recent years, of which representatives of Megapodagrionidae s.l. have been one the most challenging for taxonomists (Dijkstra et al., 2013; Kalkman & Theischinger, 2013; Bybee et al, 2021).

Significant changes in the classification of the order have taken advantage of the results of more recent studies with a clear tendency to splitting groups that had traditionally been challenging (*incertae sedis*) (Dijkstra et al., 2014; May, 2019; Bybee et al., 2021).

The recalcitrant polytomies and spurious support values of arrangements of subsampled megapod taxa (see Rehn, 2003; Bybee et al., 2008; Dumont et al., 2010; Kalkman & Theischinger, 2013; Dijkstra et al., 2013; Bybee et al., 2021) remain as the common tendency in analyses hitherto executed with combined morphological data and Sanger sequences. In the present context, next generation sequencing emerges with genomics as a most powerful tool (Kjer et al., 2016; Bybee et al., 2021), although incongruences among hypotheses due to largely subsampled taxa persist.

Analyses based only on wing venation data to clarify relationships among groups could have been biased and inductive due to ad hoc considerations (see Trueman, 2007); but with the accelerated advancement of inference methods, other theorical and methodological problems emerged as confusing factors (see Young & Gillung, 2020).

•	•	-		-					•	
GENERA	Selys (1862)	Kirby (1890)	Calvert (1913)	Kennedy (1920)	Fraser (1957)	Rácenis (1959)	Davies & Tobin (1985)	Bridges (1994)	Dijkstra et al. (2014)	Bybee et al. (2021)
Archaeopodagrion Kennedy (1939 (9 spp.)		I	1	Megapodagrion inae		Tatocnemidina e	Argiolestinae	Argiolestinae	Philogeniidae	Philogeniidae
Philogenia Selys, 1862 (40 spp.)	Legion <i>Podagrion</i>	Legion Podagrion	Legion Podagrion	Megapodagrion inae	Argiolestinae	Philogenini	Argiolestinae	Argiolestinae	Philogeniidae	Philogeniidae
<b>Dimeragrion</b> Calvert, 1913 <b>(5 spp.)</b>	I	I	I	Megapodagrion inae	I	Philogenini	Argiolestinae	Argiolestinae	incertae sedis	Heteragrionidae
Heteragrion Selys, 1862 (62 spp.)	Legion Podagrion	Legion Podagrion	Legion Podagrion	Megapodagrion inae	I	Heteragrionini	Argiolestinae	Argiolestinae	Heteragrionidae	Heteragrionidae
Heteropodagrion Selys, 1885 (5 spp.)	Legion <i>Podagrion</i>	Legion Podagrion	Legion Podagrion	Megapodagrion inae	Argiolestinae	Philogenini	Argiolestinae	Argiolestinae	incertae sedis	Heteragrionidae
Oxystigma Selys, 1862 (3 spp.)	Legion <i>Podagrion</i>	Legion Podagrion	Legion Podagrion	Megapodagrion inae		Philogenini	Argiolestinae	Argiolestinae	Heteragrionidae	Heteragrionidae
<b>Hypolestes</b> Gundlach, 1888 <b>(3 spp.)</b>	I	I	I	Amphipterygina e	Pseudolestinae	I	Hypolestinae	Hypolestinae	Hypolestidae	Hypolestidae
Mesagrion Selys, 1885 (1 sp.)	Legion <i>Podagrion</i>	Legion Podagrion	Legion Podagrion	Megapodagrion inae		Philogenini	Argiolestinae	Argiolestinae	incertae sedis	Mesagrionidae
<b>Paraphlebia</b> Selys in Hagen, 1861 <b>(15 spp.)</b>	Legion <i>Podagrion</i>	Legion Podagrion	Legion <i>Podagrion</i>	Megapodagrion inae	Argiolestinae	Argiolestinae: Argiolestini	Argiolestinae	Argiolestinae	Thaumatoneurid ae	Thaumatoneurid ae
<b>Thaumatoneura</b> McLachlan, 1897 <b>(1 sp.)</b>	I	I	I	Megapodagrion inae	Thaumatoneuri nae	Dysagrionidae	Thaumatoneuri nae	Thaumatone urinae	Thaumatoneurid ae	Thaumatoneurid ae
<b>Sciotropis</b> Rácenis, 1959 <b>(1 sp.)</b>	I	I	I	I	I	Philogenini	Argiolestinae	Argiolestinae	incertae sedis	Incertae sedis
Heliocharis Selys, 1853 (1 sp.)	I	I	I	Agrioninae	I		Dicteriastidae	Dicteriadidae	Dicteriadidae	Dicteriadidae
Allopodagrion Forster, 1910 (3 spp.)	Legion <i>Podagrion</i>	Legion Podagrion	Legion <i>Podagrion</i>	Megapodagrion inae	Megapodagriin ae	Megapodagrion ini		Megapodagri oninae	Megapodagrioni dae	Megapodagrioni dae
<b>Megapodagrion</b> Selys, 1885 <b>(1 sp.)</b>	Legion Podagrion (Podagrion Mecalonus)	Legion Podagrion (Podagrion	Legion <i>Podagrion</i>	Megapodagrion inae	Megapodagriin ae	Megapodagrion ini	Megapodagrion inae	Megapodagri oninae	Megapodagrioni dae	Megapodagrioni dae
<b>Teinopodagrion</b> Demarmels, 2001 <b>(24</b> <b>spp.)</b>	Legion Podagrion	Legion Podagrion	Legion <i>Podagrion</i>	I	I	I	I	I	Megapodagrioni dae	Megapodagrioni dae

Table 1. Neotropical genera of megapod damselflies (Megapodagrionidae s.I.) and their classification according to different authors.

In the last 20 years we have seen changes in analytical paradigms, with a transition from phylogenetic hypotheses based on few morphological and molecular data to the current scenario of bigdata with genomic approach at different taxonomic levels of the dragonfly's evolutionary history (Bybee et al., 2008, 2016, 2021; Kjer et al., 2013; Suvorov et al., 2021; Kohli et al., 2021; Newton et al., 2023).

The Neotropical region is currently the most dynamic field of research for Odonatology (Pinto, 2016; 2024) because of studies with diversity inventories and taxonomy of Odonata with a growing number of new taxa that include genera and species, driven by the interest to fill taxonomic gaps in key groups, such as, the Heteragrionidae, Philogeniidae, and Thaumatoneuridae (see Ortega-Salas et al., 2021; Stand Perez et al., 2019; Bybee et al., 2016; Bota-Sierra, 2017; Mauffray & Tennessen, 2019; Amaya-Vallejo et al., 2021; Mendoza-Penagos et al., 2023; Vilela et al., 2024).

The monophyly between lineages that define the Megapodagrionidae s.l. in the Neotropics are questionable in the last hypotheses on the phylogenetic relationships of the suborder Zygoptera (e.g., Bybee et al., 2021), thus internal relationships among these groups remain unresolved.

At present, relationship hypotheses among the Megapodagrionidae s.l. recovered them as a polyphyletic group composed of 15 lineages with different degrees of relationship (Dumont et al., 2010; Dijkstra et al., 2014; Bybee et al., 2021). The monophyly of families such as Heteragrionidae remains untested (Pinto, 2024), the systematic position of genera is still enigmatic and, in general, the relative relationship between families is also unknown.

Questions about relationships among groups that now arise because of genomic data are still intriguing and require the inclusion of new data sets to satisfy the understanding the relationships with taxa that are not yet included, for example *Allopodagrion* Förster, 1910, *Mesagrion* Selys, 1885, *Sciotropis* Rácenis, 1859, and *Megapodagrion* Selys, 1885.

#### 3.1.1 A background in taxonomy of Megapodagrionidae s.l.

The first mention of some name referring to Megapodagrionidae was of Selys (1862) when he formally established the Légion Podagrion, which included 22 species in 8 genera: from the Neotropical Region (*Heteragrion* Selys, 1862; *Paraphlebia* Selys

in Hagen, 1861; *Perilestes* Hagen in Selys, 1862; *Philogenia* Selys, 1862; *Podagrion* Selys, 1862); from the Afrotropical Region *Chlorolestes* Selys, 1862; from Oceania *Argiolestes* Selys, 1862; and from Malaysia *Podolestes* Selys, 1862 and *Amphilestes* Selys, 1862. Later, Selys (1885) included other taxa to the group, the Neotropical genera *Megapodagrion* Selys, 1885, *Heteropodagrion* Selys 1885, and *Mesagrion* Selys, 1885 (see Table 1). Selys' Legion system was adopted by Kirby (1890) and Calvert (1913), then Kennedy (1920) using penis morphology and wing venation stablished Megapodagrioninae as a subfamily of Agrionidae.

Subsequently, Fraser (1957) divided the megapodagrionines into Argiolestinae (all in Pantropical region) and Megapodagriinae (all Neotropical). Shortly after, Rácenis (1959) grouped in Megapodagrioninae the tribes Philogenini, Heteragrionini, and Megapodagrionini (Table 1).

Finally, based on the results of phylogenetic analyses with criteria of maximum likelihood and Bayesian inference Dijkstra et al (2013), proposed a reclassification of Zygoptera (Dijkstra et al., 2014) which is complemented by Bybee et al (2021) with genomic data.

The supraspecific taxonomic categories that were traditionally proposed were intuitive and with the passage of time they consolidated by tradition as "miscellaneous" groups with obscure internal relationships that were not the object of investigation in those pre-Hennigian times (Rehn, 2003; Trueman, 2007; Pilgrim & von Dohlen, 2008).

The criteria for these classifications were based mainly on similarity assumptions of the reticulation patterns of wing venation as evidence of closely relationship between these groups, due to the complementary sectors or supplementary veins between IRP1 and RP2 (Tillyard, 1939; Fraser, 1957; Rácenis, 1959; Trueman 2007). This dense venation pattern has been suggested as support that its evolutionary history dates back to the oldest lineages of Zygoptera with very intricate evolutionary histories (Rácenis, 1959; Petrolevicius et al., 2008; Nel et al., 2012).

Moreover, many of the venation characters so far encoded in cladistic analyses have been highly homoplastic (Pilgrim & von Dohlen, 2008) due the wide spectrum of modification in vein disposition, that include fusion and reduction in vein number (May, 2019). Kennedy (1920) later claimed for scarce venation as ancestral condition in Zygoptera suborder and possible reversal process for actual configuration of venational patterns.

#### 3.1.2 Biology and natural history of Neotropical megapods

In general terms, the Megapodagrionidae s.l. are a heterogeneous group of Zygoptera inhabiting mostly lotic ecosystems in dense forested areas (Rácenis, 1959; De Marmels, 1994; Kalkman et al., 2008; Kalkman & Theischinger, 2013; Pinto, 2024). They are usually found associated to small mountain seepages, streamlets to large lowland rivers, and can be more common in median altitude due to strict mountain rocky habitats requirements reflecting gradients (De Marmels, 2001; Kalkman et al., 2008), besides many species are highly specialized in specific type of habitats, genera as *Dimeragrion* Calvert, *Heteropodagrion* Selys, *Mesagrion* Selys, *Sciotropis* Rácenis, *Thaumatoneura* McLachlan are found in different types of rocky walls in streams, rivers, besides waterfalls (as *Paraphlebia*) where they occupy higropetric or madicolous habits (Fraser, 1957; De Marmels, 1994; Novelo-Gutierrez, 2008; Tenessen 2010; Pérez-Gutiérrez & Montes-Fontalvo, 2011).

In the neotropics, tropical forests harbor the greatest diversity at the generic level (Kalkman et al, 2008), with a marked tendency for distribution patterns restricted to basins and micro-basins due to its low vagility capacity. It has also been found that their populations are usually small (Rácenis, 1959; De Marmels, 1999; 2001; 2004) and highly fragmented through altitudinal gradients, as is the case of Central American genera such as *Paraphlebia* Selys in Hagen, 1861 and *Thaumatoneura* McLachlan, 1897, exclusive Andean genera such as *Teinopodagrion* De Marmels, 2001, *Archaeopodagrion* Kennedy, 1939, *Heteropodagrion* Selys, 1885, *Mesagrion* Selys, 1885, *Sciotropis* Rácenis,1959, *Philogenia* Selys, 1862, and *Dimeragrion* Calvert, 1913 in the Guyana shield of Venezuela and Brazil.

*Heteragrion* Selys, 1862 with 62 described species is the genus with the greatest diversity (Vilella et al., 2023; Pinto et al., 2024) only followed by *Phillogenia* Selys, 1862 (41 spp.) and *Teinopodagrion* De Marmels, 2001 (25 spp.) (Paulson et al., 2024). These groups of species are most abundant in rocky and sandy stream beds, with good retention of allochthonous material from gallery forests in tropical forests (Kalkman et al., 2008).

# 3.1.3 Morphological and molecular data in Megapodagrionidae s.l. systematics

As it is general in Odonata, the earliest approximations to elucidate phylogenetic relationships of Megapodagrionidae were largely based on assumptions about similarities of wing venation (Fraser, 1957; Rácenis, 1959) and penis morphology (Kennedy, 1920); only Kalkman et al. (2010) proposed groupings based on larval morphology; all these approaches can be considered as phenetics, since they were never intended to evaluate relationship hypotheses.

Old systems had the sole goal of grouping for classification purposes and were often based on the intuition that similar characteristics suggested evolutionary and / or phylogenetic relationship, but were not tested under a formal investigation framework, such as, cladistic inference, with congruence tests of characters based on homology statements (see Rehn, 2003 and Trueman, 2007 for an overview).

Wing venation patterns had interpretation difficulties for resolving the evolutionary history of Odonata (Pilgrim & von Dohlen, 2008), although hypotheses of wing venation of the Odonata was strengthened and better understood with the proposal of ancestral configuration of venation pattern (Riek & Kukalova-Peck, 1984) and more recently with contributions such as that of Trueman & Rowe (2019a, 2019b) and Jaquelin et al. (2017).

Reference	Dataset used	Analysis executed
Rehn (2003)	Adult skeletal morphology and wing venation	MP
	(122 characters analyzed)	
Hasegawa & Kasuya (2006)	16S rDNA (507 bp) and 28S rDNA (747 bp)	NJ, MP, ML
Carle et al. (2008)	Portions of the large and small subunit nuclear	BI
	ribosomal RNA's (28S and 18S, EF-1 $\alpha$ ,	
	mitochondrial rDNA's (12S and 16S)	
Bybee et al. (2008)	12S rDNA, 16S rDNA, mitochondrial COII,	MP, IB
	Histone 3, and nuclear ribosomal 18S and 28S	
	rDNA)	
Dumont <i>et al</i> . (2010)	Morphology and nuclear ribosomal 5.8 S, 18S,	MP, BI)
	and ITS1 and 2	
Yu and Bu (2011)	External morphology of cercus, genitalia (adults)	MP
Dijkstra et al. (2014)	Mitochondrial genes (16S, COI and nuclear 28S)	NJ, MP, ML, IB
Bybee et al. (2021)	Anchored hybrid enrichment	ML, IB

Table 2. Data set used in phylogenetic analysis of Odonata with representatives of megapodagrionids included. P = Parsimony, NJ = Neighbor-Joining, ML = Maximum Likelihood, BI = Bayesian Analysis.

With a clear trend towards the use of larger molecular data sets (Table 2), the integration of morphological data with molecular data is not the common denominator among phylogenetic analyses (Lipscomb et al., 2020). For example, Bybee et al. (2008), used morphological characters based on Rehn (2003), which is the most inclusive sampling analysis that included morphological data with significant taxonomic representativeness of extant and fossil taxa as well, however, the taxa sampling was insufficient in this study to solve megapod questions, mainly, only the Neotropical genera *Sciotropis* Selys, 1859, *Heteragrion* Selys, 1862, *Teinopodagrion* De Marmels, 2001, *Hypolestes* Gundlach, 1888, and *Philogenia* Selys, 1862 were included in the analyses by Bybee et al (2008) which is less than half of the Neotropical taxa.

The congruence between phylogenetic relationships using wing venation traits and molecular data have been and still remain highly discordant and incongruent (Bybee et al., 2008; Dijkstra et al., 2014; Trueman, 2017; May, 2019).

The reticulated history of Megapodagrionidae s.l. lineages deserves a much more rigorous integration to give a conclusive character to the phylogenomic hypotheses, the future challenge really consists of using tools and interpreting not only the correspondences and congruences between topologies and data sets (Hasegawa & Kasuya, 2006; Young & Gillung, 2020), but also the discordance have informative value (Suvorov et al., 2021) in the revealed patterns since behind the phylogenetic homoplasy are mechanisms that explain them and there are rigorous tests to verify them (Wake et al., 2011). These approaches must also be at hand from an integrative perspective in phylogenetic inference (Gomez-Daglio & Dawson, 2019; Vences, 2020).

In this context it is worth highlighting the inclusion of data from fossil which is becoming more and more necessary and is recommended to reconstruct the modifications of the wing mechanisms (Bybee et al., 2016) and probably understand the homoplastic trend of the features that have so far been included in the morphological analyses (Jaquelin et al., 2018; May, 2019). Although frequently the exclusion of fossil data has not affected the congruence with respect to topologies that do include them, clearly the reason is the very small representativeness of terminal fossil in phylogenetic hypotheses (Bybee et al., 2008).

#### 3.1.4 Larval morphology in Neotropical Megapodagrionidae systematics

The description of the larval stages of Neotropical megapodagrionids has been particularly slow and with few enthusiasts dedicated to the study of larval morphology and habitats of these Odonata (Tenessen, 2010). These damselflies have marked mesohabitats preferences (see Calvert, 1915; De Marmels 1982, 1994; Novelo-Gutierrez, 2008; Ramirez & Gutierrez, 2013). Inefficiency of conventional collecting methods for aquatic insects, make difficult to access them and collect larvae of many species of the megapods (see De Marmels, 2004; Kalkman et al., 2008; Lieftnick, 1976).

The distribution of morphological characters in the larvae shows a mosaic pattern that suggests a high homoplastic degree due to adaptive convergences to occupy similar habitats (Vick, 1998; Tennessen, 2010), although to date this has not been evaluated in a context of character evolution that allows it to be concluded (Kalkman et al., 2010).

Although an attempt has been made to classify megapods based on the similarity of gill morphology (Kalkman et al., 2010), until now, a strict evaluation of such groups has not been made, such as for the adults, interpretations of some larval traits has been considerably superficial, simplistic and, largely in a mistrustful overall similarity.

Tennessen (2010) mentioned the possible convergent characteristics among larvae of different damselfly families such as *Argia talamanca* Calvert, 1907 and *A. variegata* Forster, 1914 (Coenagrionidae) and *Stenocnemis pachystigma* Selys, 1886 (Platycnemididae) due to same selective pressures deriving in convergent traits as can be found in the higropetric habitat.

The morphology of the larvae reveals a variety of adaptations important for inhabiting stream environments, such as, waterfalls (Vick, 1998; De Marmels, 2004, Novelo-Gutierrez, 2008), splash zones, and hygropetric habits, especially developed in the larvae of Neotropical genera as *Heteropodagrion* Selys, 1885, *Mesagrion* Selys, 1885, *Paraphlebia* Selys in Hagen, 1861 and *Sciotropis* Rácenis, 1859, these habitat requirements are so strict as to impose selective pressures and brought about their evolutionary modification. The horizontal plane of the gills in *Dimeragrion* Calvert (named fan gills) (De Marmels, 1999; Kalkman & Theischinger, 2013) appears as the common ground-plan articulation in Argiolestidae and not in the rest Neotropical megapodes (Kalkman et al., 2010).

Definitely the notorious resemblance between the morphology of *Mesagrion* and *Heteropodagrion* include the row of denticles on the ventral surface of the prementum,
sacoid gills with grooves, and articulated terminal filament like in *Paraphlebia*, *Dimeragrion*, and *Heteragrion*, and not flat as in Argiolestidae (Tennessen, 2010; Pérez-Gutiérrez & Montes-Fontalvo, 2011), suggest convergence rather than simplesiomorphy, as the gills of *Paraphlebia* and *Mesagrion- Heteropodagrion* might suggest, too, this is a deduction because due their non monophyly, although this remain debatable.

The larval morphology in Megapodagrionidae s.s. (*Teinopodagrion* De Marmels, 2001, *Allopodagrion* Forster, 1910 and *Megapodagrion* Selys, 1885) are consistent in (Neiss et al., 2011; De Marmels, 2001) and confirm the close relationship of these three genera in a solid monophyletic group (Bybee et al., 2021); likewise, genomic data suggest a close relationship with Argiolestids in group 4 of Bybee et al., (2021). Recently descriptions of larvae of *Archaeopodagrion* Kennedy, 1939 reinforced morphologically the plausible Philogeniidae monophyly (Novelo-Gutierrez et al., 2020; Amaya-Vallejo et al., 2021).

# 3.1.5 A review of the proposed phylogenetic relationships in Megapodagrionidae s.l.

The position of the Megapodagrionidae was never well-supported among the Zygoptera phylogenetic hypotheses (Rehn, 2003; Bybee et al., 2008; Dumont et al., 2010; Dijkstra et al., 2014; Bybee et al., 2016).

The multiple lineages that make up the suborders of Odonata validate the analyses of different morphological scales together, with the incorporation of molecular data (partial sequences or genomic approaches), this is surely the most consistent way to hypothesize the internal relationships of the great clade Odonatoptera, thus as well as solving the ancestries between extinct lineages and more recent lineages, in other words, clarifying the relationships between crown and stem groups (Nel et al., 2012; May, 2019; Archibald et al., 2021).

In the present, use of fossil taxa data is especially useful in the divergence times calculated with molecular clocks (Kjer et al., 2016; Kohli et al., 2016) with the discovery of new fossil taxa it becomes more evident that within the suborders there are different stem clades (Petrulevicius, 2020) and in this regard the wing venation pattern still has much to clarify about the modification and the consequent diversification of the plan

observed in Odonatoptera lineages (Bybee et al., 2008; Nel et al., 2012; Jaquelin et al., 2017; Trueman & Rowe, 2019a).

Since Rehn (2003), the only study that includes morphological data of Megapodagrionidae s.l. was Yu & Bu, 2011 with emphasis on the systematic position of *Priscagrion* Zhou & Wilson, 2001, again the misrepresentation of taxa sample was notable. Analyses based on molecular data all failed to demonstrate its monophyly, and phylogenomic analyses have recently suggested that the megapodagrionids are composed of about 15 lineages (Bybee et al., 2021).

The interpretation of the also very heterogenous palette of variation observed among the *bauplan* of Megapodagrionidae s.l. is necessary and goes in a way much more coherent with integrative taxonomy and the hypothesis of multiple stem lineages among Megapodagrionidae s.l. is now more real than ever, also involving pantropical lineages (Petrulevicius et al., 2008).

Dijkstra et al. (2013) quoted: "further splits are considered more likely than the remerging of groups", but this argument without a real and exhaustive integrative analytical support can generate taxonomic inflation (Vences, 2020) and this is not a major problem at the species-level because the species concepts offer methodological options that avoid taxonomic inflation (De Queiroz, 2007; Dayrat, 2005; Padial et al., 2010), but at suprageneric levels instability is practically unavoidable since, as long as the Linnaean system is followed, the species will be associated with their higher taxonomic entities and the successive changes of taxonomic location make this cumbersome location for taxonomists or in other words for the practice of taxonomy (Gómez-Daglio & Dawson, 2019; Christenhusz, 2020).

Although relationships have not been satisfactorily resolved, a few clustering patterns are constant between the phylogenies, such as the sister-group relationship of *Paraphlebia* and *Thaumathoneura*, *Oxystigma* and *Heteragrion*, *Archaeopodagrion* and *Philogenia* (Bybee et al., 2008; Dijkstra et al., 2014; Bybee et al., 2021). However, the instability of the groups has also been repeatedly noted through the different hypotheses that tried to clarify the relationships between Neotropical and pantropical megapods (e.g., *Allopodagrion* and *Megapodagrion* with Argiolestids) (De Marmels, 2002).

Dijkstra et al.'s (2014) study was the first of a contemporary new generation wave of molecular taxonomical studies that elevated Heteragrionidae, Hypolestidae, Philogeniidae, Philosinidae, and Thaumatoneuridae to family status. They did "*ad hoc*" morphological analyses after the obtained molecular results. Dijkstra et al. (2013), largely based on published studies of paper of 2014 and Bybee et al. (2021), proposed a new classification where Megapodagrionidae s.s. are limited to the genera *Allopodagrion, Megapodagrion, and Teinopodagrion* and the rest of megapods have been elevated or revalidated to the rank of family, many of them monotypic due to been scattered through the Zygoptera phylogeny (see Table 1).

The phylogenomic analysis of Bybee et al. (2021), supported the close relationship of *Heteragrion* + *Dimeragrion*, in a new status of Heteragrionidae, however, nor *Oxystigma* nor *Heteropodagrion* were included in their data. In contrast, the phylogenetic tree by Dijkstra et al. (2014), *Mesagrion* was not sampled in such a way that the relationship between *Heteropodagrion* and *Mesagrion* cannot be addressed. In addition, in Bybee et al. (2021) there is low support for a new family status for the monotypic genera *Mesagrion*, Mesagrionidae (QC <0) suggesting that other relationships between taxa may be possible, and the relationship with Dicteriadidae (*Heliocharis amazona* Selys, 1853) and Hypolestidae (*Hypolestes* Gundlach, 1888) can be largely artifactual to the analysis as claimed by the authors of the cited article.

A matter such as the phylogeny of the Megapodagrionidae s.l. is far from being resolved, as Dijkstra et al. (2013) stated a few years ago, the reticulated relationships that exist between the miscellaneous lineages that made up the so-called megapods, are not entirely clarified, even with the vast dataset of those that are currently available.

Today the questions revolve around the lines of diversification and times of divergence that explain the complicated affiliations that hide behind the trees of the scarce phylogenetic studies. From the morphological point of view, the main question continues to be to clarify the evolution of the characters that seem scattered throughout the group, sometimes aberrant and highly autopomorphic or homoplastic (Pilgrim & von Dohlen, 2008).

The panorama research on cladogenesis in Odonata is open to both hypothesize on patterns of modification and special interest on speciation in Neotropical groups. However, there is a fact that is problematic and challenging: the oldest Odonatoptera lineages are extinct, we only have parts of what was their history as the stem groups of modern Odonata, and they are morphological parts, not DNA. That is the reality and for this reason the advance in techniques for morphological studies in extinct and extant taxa will continue to be valid and necessary (Wheeler 2004; Gomez-Daglio and Dawson, 2019).

# 3.1.6 Distributional patterns of the "Neotropical" Megapodagrionidae s.l.

The different lineages that megapods represent are distributed in pantropical zones of Africa, Asia, Australia, and in Neotropical America (Rácenis, 1959; Kalkman & Teischinger, 2013; Kohli et al., 2021).

The affiliations among genera that had been suggested from the morphological or molecular evidence, with the estimation of phylogenies also support biogeography, although the complex events that exist in the configuration of the distribution patterns of Megapodagrionidae s.l. involved a mixture of processes occurred *in situ* in the Neotropical region as the Andean and Amazonian orogeny (De Marmels, 2001).

The configuration of the Pacific Ocean and the fusion of North America with South America due to the establishment of the Isthmus of Panama among other geological/ tectonic features typical of the complex Neotropical region suggest that geographical range of ancient lineages today in the neotropics were broadly distributed in paleotropics and some regions of Palearctic and this suggests a very fragmented ancestral odonate fauna (Petrolevicius et al., 2008; Huang et al., 2018; Petrolevicius, 2020).

A robust phylogenetic hypothesis of Neotropical clades is needed to test hypotheses on the influence of orogenic events that have taken place in the complex area that comprises the union of North America, Central, and South America in the current distribution of taxa, such as the great American exchange (Coates & Stallard, 2013).

In phylogenetic hypotheses based in morphological, genetics, and genomics, close relationships have been suggested between Thaumatoneuridae (Central America) and Riphidolestidae (Pacific Paleotropical), as well as Megapodagrionidae *s.s.* and Argiolestidae (De Marmels, 2002; Kalkman & Theischinger, 2013; Bybee et al., 2021) supported by fossil evidence as well (Petrulevicius et al., 2008; Huang et al., 2018; Petrulevicius, 2020).

In the area comprised by the Isthmus of Panama, the island arc model (Grehan 2001; Coates et al., 2004) could have played a decisive role in the marked endemism of taxa with low dispersal capacity such as *Thaumatoneura* (South of Mexico-

Nicaragua), *Paraphlebia*, and *Heteropodagrion*. This set of evidence that correspond to taxa with a distribution restricted to lotic environments casts doubt on the existence of "a great American exchange" as an explanation for the occurrence of these taxa throughout Central and South America. On the contrary, the existence of Paleo-basins related to ancient island arches with potential diversification of metapopulations is a more congruent model with these patterns (Coates et al., 2004; Coates & Stallard, 2013, Grehan, 2001; Heads & Grehan, 2021), testing this hypothesis is, therefore, a topic of primary interest in biogeographical affinities of the Neotropical lineages with other taxa outside of presently known as neotropics.

The most diverse and widely distributed genera in the Neotropics are *Heteragrion* Selys, 1862 (62 spp.), *Philogenia* Selys, 1862 (40 spp.), and *Teinopodagrion* De Marmels, 2001 (29 spp.) and there are also genera that are markedly less diverse, with a high incidence of monospecific such as *Thaumatoneura* McLachlan, 1897 and the incertae sedis *Sciotropis* Rácenis, 1859, this fact is a possible sign of divergence patterns probably very recent in comparison to others that appear to be ancient, the speciation dynamism in these groups is a topic that requires special attention.

Dichotomous patterns in phylogenetic hypotheses are masked by deep ancestral introgression, which explains the phylogenetic discordance between ancient (stem) and (crown) lineages of Calopterygoidea, including Megapodagrionidae s.l., therefore, monophyly among focal groups is not supported or conversely, internal relationships emerge due to ancestral introgression (Suvorov et al., 2021).

The dynamic endemism concept (Nihei, 2006) offers an integrative perspective for a comprehensive explanation of manner than mixture of process, major tectonic, displacement of plates, continental accretion, and the junction of terrains modeled the present distribution of megapods in the Neotropics, undoubtedly a history independent of ancient areas in the paleotropics is not possible for understand the intricate megapodagrionidae s.l. diversification history, the disjunct connections within megapodagrionids genera imply ancestral lineages than were distributed out of present tropics around the globe.

If there are no close phylogenetic relationships between the Neotropical and paleotropical clades then, questions about the mechanisms of speciation of smaller and more manageable clusters of species could be addressed. In conclusion, with all the important advances in the clarification of relationships among Neotropical megapods, information is still far from being satisfactory and as a general pattern reticulated, intricated and puzzling.

In this review, available DNA sequence data was reanalyzed with the aim of conducting a phylogenetic exploration and testing relationships among strictly Neotropical clades and test of monophyly of the diverse lineages that traditionally make up the group Megapodagrionidae s.l., including representatives of genera and families, and have provided one comprehensive estimates of the phylogenetic relationships of the major lineages within Megapodagrionidae s.l. Sequences available in GenBank were from nuclear 28S rDNA and mitochondrial COI encompassing representative species from 12 of the 14 Neotropical genera.

#### 3.2 Methods

Nucleotide sequences of the Neotropical taxa available in GenBank were downloaded for the mitochondrial gene Cytochrome Oxidase I (COI) and partial nuclear subunit 28S rDNA (Table 3). Alignments were made with MUSCLE algorithm in MEGA X (Kumar et al., 2018) for COI matrix and for 28S the Q-INS-i algorithm in MAFFT (Katoh et al., 2019), then genetic distance matrices were made with Kimura-2-parameter and 250 replicates for bootstrap for a grouping exploration in MEGA X (see Suppl. mat.).

The matrix for COI with 19 sequences was downloaded including the *Heliocharis amazona* Selys, 1853 as outgroup, the length of the COI matrix was 658 sites, variable sites= 279, parsimoniously informative=237, and 384 conserved sites. For the 28S matrix, 18 sequences were aligned with 610 sites, conserved sites= 565, variable sites= 53, parsimony informative= 32 and conserved sites= 565. Finally, the concatenated alignment with 19 sequences with 1274 sites, variable sites= 338, parsimony-informative= 269 and 955 constant sites. The model for COI was GTR+F, the model for 28S was GTR+F and the best model for concantenated data GTR+I+G.

The workflow consisted of calculating the Neighbor-Joining (NJ) and Maximum Likelihood (ML) trees separately searching relationship patterns among Neotropical megapodagrionids with the data sets. Concatenated analysis including the two markers (COI and 28S) were performed only under Maximum Likelihood criteria.

Taxon	Voucher code	Collecting data	COI	28S
Dicteriadidae				
Heliocharis amazona	RMNH.INS.505205	SURINAME	KF369392	KF370124
Heteragrionidae				
Heteropodagrion sanguinipes	RMNH.INS.501970	ECUADOR: Quito	KF369399	KF370133
Heteragrion inca	RMNH.INS.501859	PERU	KF369398	KF370132
Heteragrion bickorum	RMNH.INS.502100	ECUADOR: Orellana	KF369396	KF370130
Heteragrion chrysops	RMNH.INS.502036	VENEZUELA: Aragua	KF369397	KF370131
Heteragrion triangulare	BNBTO_0132	BRAZIL	KY947445	-
Dimeragrion percubitale	RMNH.INS.502032	VENEZUELA	KF369361	KF370087
<i>Oxystigma</i> sp.	RMNH.INS.228855	SURINAME	KF369470	KF370216
Thaumatoneuridae				
Thaumatoneura inopinata	RMNH.INS.501982	COSTA RICA	KF369572	KF370332
Paraphlebia zoe	RMNH.INS.504309	MEXICO	KF369481	KF370229
Paraphlebia quinta	RMNH.INS.504317	MEXICO	KF369480	KF370228
Hypolestidae				
<i>Hypolestes</i> sp.	RMNH.INS.503671	DOMINICAN REPUBLIC	KF369402	KF370136
Philogeniidae				
Philogenia ferox	RMNH.INS.502040	VENEZUELA	KF369492	KF370241
Philogenia cassandra	RMNH.INS.502038	VENEZUELA	KF369491	KF370240
Philogenia iquita	RMNH.INS.501714	PERÚ	KF369493	KF370242
Archaeopodagrion armatum	RMNH.INS.501971	ECUADOR	KF369302	KF370020
Megapodagrionidae				
Teinopodagrion meridionale	RMNH.INS.502087	VENEZUELA	KF369564	KF370323
Teinopodagrion venale	RMNH.INS.502035	VENEZUELA	KF369565	KF370324
Incertae sedis				
Sciotropis cyclanthorum	RMNH.INS.502029	VENEZUELA	-	KF370297

Table 3. Species included for the analysis of the Neotropical Megapodagrionidae s.l. and GenBank accession numbers for COI and 28S sequences.

The NJ tree was calculated for a preliminary exploration in MAFFT online version, ML analyses were performed on the portal server CIPRES GATEWAY using IQ-TREE multicore version 1.6.12 (Miller et al., 2015; Nguyen et al., 2014). For COI the parameters used were iqtree2 -nt 6 -bnni -s infile.txt -st DNA -m TESTNEWMERGE - alrt 1000 --sprrad 6 -B 1000 --prefix output using SPRNG - Scalable Parallel Random Number Generator), 1000 samples for ultrafast bootstrap (UFBoot), 1000 replicates for Shimoidara-Hasegawa-like approximate likelihood ratio test (SH-aLRT), and with value of initial seed 959965. For the 28S matrix the parameters used were iqtree2 -nt 6 -bnni -s infile.txt -st DNA -m TESTNEWMERGE -alrt 1000 --prefix output, branch support was tested using 1000 replicates of ultrafast bootstrap and 1000 replicates of SH-aLRT, finally, for the concatenated analysis the parameters used iqtree2 -nt 6 -bnni -s infile.txt -st meters used analysis the parameters used iqtree2 -nt 6 -bnni -s infile.txt -bsam GENE -keep\_empty\_seq -p partition\_file.txt -st

DNA -rclusterf 50 -m TESTMERGE -alrt 1000 --sprrad 6 -B 1000 --prefix output – symtest with initial seed value 845303 using SPRNG.

# 3.3 Results

# 3.3.1 Relationships between Neotropical clades of Megapodagrionidae s.l.

The maximum likelihood gene trees (Fig 1-2) and the concatenated tree (Fig 3) reveal several stable groups with high support values, *Teinopodagrion* as member of Megapodagrionidae s.s. (UFBoot = 100) that is not directly related to any of the other Neotropical clades (Figs 1-3).



0.09

Fig. 1. Maximum likelihood gene trees based on COI sequences of Neotropical megapods with *Heliocharis* as outgroup. Node values refer to SH-like aLRT and ultrabootstrap percentages. Taxa colors refer to Bybee et al. (2021) classification: Blue: Megapodagrionidae s.s; olive green: Hypolestidae; Red: Heteragrionidae; purple – Thaumatoneuridae; Pink – Philogeniidae

A second clade with high support (100% UFBoot) is the one that relates *Hypolestes* + *Sciotropis*, although only 28S and not COI data are available for *Sciotropis*, this group emerges with high support (100).

All analyses show a split between the representatives of Heteragrionidae, in two large groups, one grouping *Oxystigma* and *Heteragrion* with 74% of BS support and on the other hand, a mixed group gathering *Dimeragrion+ Heteropodagrion + Paraphlebia*, although excluding *Thaumatoneura*, which appears closer to the Philogeniidae (*Archaepodagrion* and *Philogenia*) supported with 74% BS.



0.005

Fig. 2. Maximum likelihood gene trees based on 28S sequences of Neotropical megapods with *Heliocharis* as outgroup. Node values refer to SH-like aLRT and ultrabootstrap percentages. Taxa colors refer to Bybee et al. (2021) classification: Blue: Megapodagrionidae s.e; olive green: Hypolestidae; Red: Heteragrionidae; purple – Thaumatoneuridae; Pink – Philogeniidae



0.03

Fig. 3. Maximum likelihood tree based on the concatenated dataset (COI and 28S) of Neotropical Megapodagrionidae. Node values refer to SH-like aLRT and ultrabootstrap percentages. Taxa colors refer to Bybee et al. (2021) classification: Blue: Megapodagrionidae s.s; olive green: Hypolestidae; Red: Heteragrionidae; purple – Thaumatoneuridae; Pink – Philogeniidae

#### 3.4 Discussion

The gene trees showed unstable groups than not explain the internal relationships separately (Figs 1-2). Regarding Megapodagrionidae, we can only refer to its position based on sequences from *Teinopodagrion* because of the absence of sequences from *Allopodagrion* and *Megapodagrion*. It remains in question the internal relationships of this clade claimed as monophyletic (De Marmels, 2001; Dijkstra., 2014; Bybee et al., 2021). In any case, the analyses carried out so far show *Teinopodagrion* with phylogenetics relationships distant to Neotropical clades except in 28S gene tree.

The separate analyses as well as the concatenated one do not recover the monophyly of clades such as Heteragrionidae. It is important to note that

Heteragrionidae according to this reanalysis is only composed of *Oxystigma* Selys, 1862 and *Heteragrion* Selys, 1862 *(sensu* Dijkstra et al., 2013), phylogenetics relationships with *Dimeragrion* Calvert, 1913 and *Heteropodagrion* Selys, 1885 as suggested by Bybee et al., 2021 are not recovered in our analysis.

In general, it can be observed that the COI data set recovers relationships with better resolution compared to 28S (Figs. 1-2). When reexamining a resampling of the matrix of Dijkstra et al. (2014) resulting trees with all megapods including the taxa outside the Neotropics, it is remarkable that a monophyletic Neotropical group as such appears highly fragmented, the relationships between endemic taxa in the Neotropics in the trees include taxa of the pantropical region.

For 28S and COI matrixes, the analysis shows Neotropical representatives of Megapodagrionidae s.l., as polyphyletic group, with split of the various lineages than are confirmed in this reanalysis.

The true Megapodagrionidae (sensu Dijkstra et al., 2014) is a lineage apart from the rest of the other traditional Neotropical megapods, the same can be said of Hypolestidae that, being endemic to the Greater Antilles in the Caribbean, is postulated as the closest group to Megapodagrionidae s.s. (*Allopodagrion, Megapodagrion* and *Teinopodagrion*) although the first two are not included in the study of Bybee et al. (2021), nor Dijkstra et al. (2014), also with distant relationships with the Central American and South American megapodagrionids.

In the analyses conducted herein, Thaumatoneuridae (*Thaumatoneura* + *Paraphlebia*) is not recovered, since our results phylogenetically bring *Paraphlebia* closer to *Heteropodagrion* and *Dimeragrion* (Fig. 3).

Philogeniidae is another partially retained group with reasonable support in the analysis performed (74%), but the relationships among Philogeniidae and Thaumatoneuridae (only *Thaumatoneura*) obtained in the concatenated analyzes was until now a hypothesis not considered therefore further research should confirm the relationship between these Neotropical taxa.

The arrangement of the genera in Heteragrionidae, Philogeniidae and Thaumatoneuridae and their relationships are not very clear, and patterns emerge that differ among the datasets, as well as low supports that do not allow to fully conclude which is the sister group of Philogeniidae.

The phylogenetic closeness between *Heteropodagrion* and *Dimeragrion* is another topic that requires further research to be clarified, and the low support values

so far obtained leave open the possibility of other relationship arrangements for example *Paraphlebia*.

#### 3.5 References

- Amaya V, Bota-Sierra C, Novelo-Gutiérrez R, Herrera M. 2021 Two new species of Archaeopodagrion (Odonata, Philogeniidae) from the western foothills of the Tropical Andes, with biological observations and distributional records. **ZooKeys**. 1036. 21-38. 10.3897/zookeys.1036.64230.
- Archibald SB, Cannings RA, Erickson RJ, Bybee SM, Mathewes RW 2021 The Cephalozygoptera, a new, extinct suborder of Odonata with new taxa from the early Eocene Okanagan Highlands, western North America. **Zootaxa**, 4934 (1), 1–133. <u>https://doi.org/10.11646/zootaxa.4934.1.1</u>
- Bota-Sierra CA Novelo-Gutiérrez R. 2017 The genus *Heteragrion* (Odonata: Zygoptera) in Northwestern Colombia, with the description of *Heteragrion tatama* sp. nov. *Zootaxa*, 4347 (3), 553–571. <u>https://doi.org/10.11646/zootaxa.4347.3.8</u>
- Bybee S, Kalkman V, Erickson J, Frandsen PB, Breinholt JW, Suvorov A, Dijkstra, KDB, Cordero-Rivera A, Skevington JH, Abbott JC, Sanchez-Herrera M, Lemmon AR, Lemmon E, Ware JL. 2021 Phylogeny and classification of Odonata using targeted genomics, *Molecular Phylogenetics and Evolution*, 160, 107115, https://doi.org/10.1016/j.ympev.2021.107115.
- Bybee S, Cordoba-Aguilar A, Duryea, MC, Futahashi R, Hansson, B, Lorenzo-Carballa, MO, Schilder R, Stoks R, Suvorov A, Svensson EI, Swaegers J, Takahashi Y, Watts PC, Wellenreuther M. 2016 Odonata (dragonflies and damselflies) as a bridge between ecology and evolutionary genomics. *Front. Zool*. 13, 46.
- Bybee SM, Ogden TH, Branham, MA, Whiting MF. 2008 Molecules, morphology and fossils: a comprehensive approach to odonate phylogeny and the evolution of the odonate wing. *Cladistics* 24: 477–514.
- Calvert PP. 1903 Odonata, pp. 129-144. In: *Biologia Centrali-Americana*: *Insecta Neuroptera*. R.H. Porter and Dulau Co., London
- Carle FL. 2012 A new Epiophlebia (Odonata: Epiophlebioidea) from China with a review of epiophlebian taxonomy, life history, and biogeography. *Arthropod. Systemat*. Phylogeny 70: 75–83.

- Carle F, Kjer K, May, M. 2008 Evolution of Odonata, with Special Reference to Coenagrionoidea (Zygoptera). *Arthropod Systematics & Phylogeny*. 66. 37– 44. 10.3897/asp.66.e31679.
- Coates AG, Stallard RF. 2013 How old is the Isthmus of Panama? *Bulletin of marine science*. 89 (4): 801–813. <u>Http://dx.doi.org/10.5343/bms.2012.1076</u>
- Coates A, Collins L, Aubry M-P, Berggren W. 2004 The Geology of the Darien, Panama, and the late Miocene-Pliocene collision of the Panama arc with northwestern South America. Geological Society of America Bulletin, *Geol. Soc. Amer. Bull*. 116. 10.1130/B25275.1.
- Büsse S. 2016 Morphological re-examination of *Epiophlebia laidlawi* (Insecta: Odonata) including remarks on taxonomy. International Journal of Odonatology 19(4): 221–238. https://doi.org/10.1080/13887890.2016.1257442
- Calvert P. 1913 The fossil Odonate *Phenacolestes*, with a discussion of the venation of the legion Podagrion Selys. Proceedings of the Academy of Natural Sciences of Philadelphia 65: 225-272.
- Carle FL, Kjer KM, May ML. 2008 Evolution of Odonata, with special reference to Coenagrionoidea (Zygoptera). *Arthropod Syst. Phylogeny* 66: 37–44.
- Calvert PP. 1915 Studies on Costa Rican Odonata. VI. The waterfall-dwellers: the transformation, external features and attached diatoms of *Thaumatoneura* larva. *Entomological News*, 26, 295-305.
- Christenhusz MJM. 2020 On species concepts, phylogenetics and the science of natural history—three current issues facing taxonomy. *Megataxa* 001 (1): 067–072.
- Clausnitzer V, Kalkman V, Ram M, Collen B, Baillie J, Bedjanic M, Darwall W, Dijkstra KD, Dow R, Hawking J, Karube H, Malikova E, Paulson D, Schütte K, Suhling F, Villanueva RJ, Ellenrieder N, Wilson K. 2009 Odonata enter the biodiversity crisis debate: The first global assessment of an insect group. *Biological Conservation*. 142. 1864–1869. 10.1016/j.biocon.2009.03.028.
- Dayrat B. 2005 Towards integrative taxonomy. *Biological Journal of the Linnean Society* 85: 407-415.
- Deacon C, Samways MJ. 2021 A Review of the Impacts and Opportunities for African Urban Dragonflies. Insects. 12(3):190. doi: 10.3390/insects12030190. PMID: 33668371; PMCID: PMC7996324.

- De Marmels J. 2004 Heteragrion makiritare sp. nov with descriptions of hitherto unknown females and larvae of other species from Venezuela (Odonata: Megapodagrionidae, Lestidae). International Journal of Odonatology, 7. 439-458. 10.1080/13887890.2004.9748230.
- De Marmels J. 2002 Phylologenetic relationships of *Priscagrion* Zhou and Wilson, 2001, with a description of *Teinopodagrion croizati* spec. nov. from Ecuador (Zygoptera: Megapodagrionidae). *Odonatologica*. 31: 389-394.
- De Marmels J. 2001 Revision of *Megapodagrion* Selys, 1886 (Insecta, Odonata: Megapodagrionidae). Unpublished Ph.D dissertation, Mathematischnaturwissenschaftliche Fakultat der Universitat Zurich, 230 p.
- De Marmels J. 1999 A new species of *Dimeragrion* Calvert 1913 from Pantepui, Venezuela (Odonata: Megapodagrionidae). *Bol. Entomol. Venez*. 14(1): 27-36.
- De Marmels J. 1994 *Sciotropis lattkei* sp. n., eine neue Kleinlibelle aus Venezuela (Odonata: Megapodagrionidae). Mitt. Entom. Ges. Basel 44(1): 30-35.
- De Marmels J. 1982 Dos nayades nuevas de la familia Megapodagrionidae (Odonata Zygoptera). *Boletfn de Entomologia Venezolana*, Nueva Serie 2: 89-93.
- De Queiroz K. 2007 Species concepts and Species Delimitation, *Syst. Biol*. 56 (6): 879–886.
- Dijkstra K-DB, Kalkman VJ, Dow RA, Stokvis FR, Van Tol J. 2014 Redefining the damselfly families: a comprehensive molecular phylogeny of Zygoptera (Odonata). *Syst. Entomol*. 39: 68–96.
- Dijkstra K-DB, Bechly G, Bybee SM, Dow RA, Dumont HJ, Fleck G, Garrison RW, Hämäläinen M, Kalkman VJ, Karube H. 2013 The classification and diversity of dragonflies and damselflies (Odonata) In: Zhang, Z.-Q. (Ed.) Animal Biodiversity: An Outline of Higher-level Classification and Survey of Taxonomic Richness (Addenda 2013). *Zootaxa*; 3703: 36–45.
- Dumont HJ, Vierstraete A, Vanfleteren JR. 2010 A molecular phylogeny of the Odonata (Insecta). *Systematic Entomology*, 35: 6–18.
- Fraser FC. 1957 A reclassification of the order Odonata, based on some new interpretations of the venation of the dragonfly wing by the late R. J. Tillyard and F. C. Fraser, revision. *Royal Zoological Society of New South Wales*, Sydney, 133 pp.

- Gómez-Daglio L, Dawson MN. 2019 Integrative taxonomy: ghosts of past, present and future. *Journal of the Marine Biological Association of the United Kingdom* 99: 1237–1246. https://doi.org/10.1017/ S0025315419000201
- Grehan J. 2001 Biogeography and evolution of the Galapagos: integration of the biological and geological evidence, *Biological Journal of the Linnean Society*, 74: 267-287.
- Grimaldi D. Engel, MS. 2005 Evolution of the Insects. Cambridge University Press, New York, New York.
- Grigoropoulou A, Ab Hamid S, Acosta R, Akindele, EO, Al-Shami S, Altermatt F, Amatulli G, Angeler D, Arimoro F, Aroviita J, Astorga-Roine A, Bastos R, Bonada N, Boukas N, Brand C, Bremerich V, Bush A, Cai Q, Domisch S. 2023 The global EPTO database: Worldwide occurrences of aquatic insects. *Global Ecology and Biogeography.* 32. 10.1111/geb.13648.
- Hasegawa E, Kasuya E. 2006 Phylogenetic analysis of the insect order Odonata using 28S and 16S rDNA sequences: a comparison between data sets with different evolutionary rates. *Entomol. Sci*. 9: 55–66.
- Heads M, Grehan JR. 2021 The Galápagos Islands: biogeographic patterns and geology. *Biol Rev*, 96: 1160-1185. <u>https://doi.org/10.1111/brv.12696</u>
- Huang D, Azar D, Nel A. 2018 The oldest 'Megapodagrionidae' (Odonata, Zygoptera) discovered in the Lower Cretaceous Yixian Formation, China, *Cretaceous Research*, 84: 426-430, https://doi.org/10.1016/j.cretres.2017.12.003.
- Jacquelin L, Desutter-Grandcolas L, Chintauan-Marquier IC, Boistel R, Zheng D, Prokop J, Nel A. 2017 New insights on basivenal sclerites using 3D tools and homology of wing veins in Odonatoptera (Insecta). *Scientific Reports*, 8.
- Kalkman V, Clausnitzer V, Dijkstra KD, Orr A, Paulson D, Tol J. 2008 Global diversity of dragonflies (Odonata) in freshwater. 10.1007/978-1-4020-8259-7\_38.
- Kalkman VJ, Choong CY, Orr AG, Schütte K. 2010 Remarks on the taxonomy of Megapodagrionidae with emphasis on the larval gills (Odonata). *Int. J. Odonatol*. 13: 119–135. https://doi.org/10.1080/13887890.2010.9748366.
- Kalkman VJ, Theischinger G. 2013 Generic revision of Argiolestidae (Odonata), with four new genera. *International Journal of Odonatology*, 16: 1–52.
- Katoh K, Rozewicki J, Yamada KD. 2019 MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization, *Briefings in Bioinformatics*, 20 (4): 1160–1166, <u>https://doi.org/10.1093/bib/bbx108</u>

- Kennedy CH. 1920 The phylogeny of the zygopterous dragonflies as based on the evidence of the penes. *Ohio journ. sci.*, 21, p. 19-29.
- Kirby WF. 1890 A Synonymic Catalogue of Neuroptera Odonata, or Dragonflies, with an Appendix of Fossil Species. Gurney and Jackson, London, ix + 202 pp.
- Kjer K, Borowieck ML, Frandsen PB, Ware J, Wiegmann BM. 2016 Advances using molecular data in insect systematics, *Current Opinion in Insect Science*, 18: 40-47.
- Kohli M, Ware J, Bechly G. 2016 How to date a dragonfly: Fossil calibrations for odonates. *Palaeontologia electronica*. 19. 10.26879/576.
- Kohli M, Letsch H, Greve C, Béthoux O, Deregnaucourt I, Liu S, Zhou X, Donath A, Mayer C, Podsiadlowski L, Gunkel S, Machida R, Niehuis O, Rust J, Wappler T, Yu X, Misof B, Ware J. 2021 Evolutionary history and divergence times of Odonata (dragonflies and damselflies) revealed through transcriptomics. *iScience*. 24(11):103324. doi: 10.1016/j.isci.2021.103324. PMID: 34805787; PMCID: PMC8586788.
- Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018 MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution* 35:1547-1549.
- Lieftinck, M.A., 1976 The dragonflies (Odonata) of New Caledonia and the Loyalty Islands. Part 2. Immature stages. Cahiers O.R.S.T.O.M., Hydrobiologie 10: 165-200. Lipscomb D, Platnick N, Wheeler Q. 2003 The intellectual content of taxonomy: a comment on DNA taxonomy. *Trends in Ecology and Evolution*. 18, 2.
- Mauffray W, Tennessen K. 2019 A Catalogue and Historical Study of the Odonata of Ecuador. Zootaxa. 4628. 1-265. 10.11646/zootaxa.4628.1.1.
- May, ML. 2019 Odonata: Who They Are and What They Have Done for Us Lately: Classification and Ecosystem Services of Dragonflies. *Insects* 10, no. 3: 62. <u>https://doi.org/10.3390/insects10030062</u>
- Mendoza C, Gonçalves M, Vilela D. 2023 A new species of *Dimeragrion* Calvert, 1913 (Odonata: Zygoptera: Heteragrionidae) from Northwestern Brazil. Zootaxa. 5318.
  411-420. 10.11646/zootaxa.5318.3.6.
- Miller MA, Schwartz T, Pickett, BE. 2015 A RESTful API for Access to Phylogenetic Tools via the CIPRES Science Gateway. Evolutionary Bioinformatics 11, 43-48.DOI: 10.4137/EBO.S21501.

- Misof B, Liu S, Meusemann K, Peters RS, Donath A, Mayer C, Frandsen PB, Ware J, Flouri T, Beutel RG, Niehuis O, Petersen M, Izquierdo-Carrasco F, Wappler T, Rust J, Aberer AJ, Aspo" ck U, Aspo" ck H, Bartel D, Blanke A, Berger S, Bo" hm A, Buckley TR, Calcott B, Chen J, Friedrich F, Fukui M, Fujita M, Greve C, Grobe P, Gu S, Huang Y, Jermiin LS, Kawahara AY, Krogmann L, Kubiak M, Lanfear R, Letsch H, Li Y, Li Z, Li J, Lu H, Machida R, Mashimo Y, Kapli P, McKenna DD, Meng G, Nakagaki Y, Navarrete-Heredia JL, Ott M, Ou Y, Pass G, Podsiadlowski L, Pohl H, von Reumont BM, Schutte K, Sekiya K, Shimizu S, Slipinski A, Stamatakis A, Song W, Su X, Szucsich NU, Tan M, Tan X, Tang M, Tang J, Timelthaler G, Tomizuka S, Trautwein M, Tong X, Uchifune T, Walzl MG, Wiegmann BM, Wilbrandt J, Wipfler B, Wong TK, Wu Q, Wu G, Xie Y, Yang S, Yang Q, Yeates DK, Yoshizawa K, Zhang Q, Zhang R, Zhang W, Zhang Y, Zhao J, Zhou C, Zhou L, Ziesmann T, Zou S, Li Y, Xu X, Zhang Y, Yang H, Wang J, Wang J, Kjer KM, Zhou X. 2014 Phylogenomics resolves the timing and pattern of insect evolution. *Science*, 346:763-767.
- Neiss UG, Fiorentin GL, De Marmels J. 2011 The larva of Allopodagrion brachyurum De Marmels, 2001 (Odonata: Zygoptera: Megapodagrionidae) from Southern Brazil. **Zootaxa**, 2836: 44-50.
- Nel A, Bechly G, Prokop J, Bethoux O, Fleck G. 2012 Systematics and evolution of paleozoic and mesozoic damselfly-like odonatoptera of the 'protozygopteran' grade. *Journal of Paleontology*, 86(1): 81–104
- Newton L, Tolman E, Kohli M, Ware JL. 2023 Evolution of Odonata: genomic insights.CurrentOpinioninInsectScience,58<a href="https://doi.org/10.1016/j.cois.2023.101073">https://doi.org/10.1016/j.cois.2023.101073</a>
- Nihei, SS. 2008 Dynamic endemism and 'general' biogeographic patterns. Biogeografía: Bulletin of the Systematic and Evolutionary Biogeographical Association 3:2-6
- Nguyen L-T, Schmidt H, von Haeseler A, Minh B. 2014 IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. *Molecular biology and evolution*. 32. 10.1093/molbev/msu300.
- Novelo-Gutiérrez R, Bota-Sierra C, Amaya V. 2020 Description of the larva of the genus Archaeopodagrion Kennedy, 1939 (Zygoptera: Philogeniidae). Zootaxa. 4816. 325-332. 10.11646/zootaxa.4816.3.3.

- Novelo-Gutiérrez R. 2008 Description of the larva of *Paraphlebia zoe* Selys In Hagen, 1861 (Odonata: Megapodagrionidae). *Zootaxa.* 29-34. 10.5281/zenodo.184109.
- Orr M, Ferrari R, Hughes A, Chen J, Ascher J, Yan Y-H, Williams P, Zhou X, Bai M, Rudoy A, Zhang F, Ma K, Zhu, Chao-Don 2020 Taxonomy must engage with new technologies and evolve to face future challenges. *Nature Ecology & Evolution*. 5(1)
- Ortega-Salas H, Gonzalez-Soriano, E Jocqué M. 2022 Untangling the waterfall damsels, a review of the Mesoamerican genus *Paraphlebia* Selys in Hagen, 1861 (Odonata: Thaumatoneuridae) with descriptions of 11 new species. Zootaxa. 5089. 1-66. 10.11646/zootaxa.5089.1.1.
- Padial JM, Miralles A, De la Riva I, Vences M. 2010 The integrative future of taxonomy. *Frontiers in Zoology* 7:16.
- Paulson DR, Marinov M. 2021 Zootaxa 20th Anniversary Celebration: Odonata section. *Zootaxa*, 4979(1), 218–221. https://doi.org/10.11646/zootaxa.4979.1.21
- Paulson, DR, Schorr, M, Deliry, C. 2024 World Odonata list: https:// www2.pugetsound.edu/academics/academic-resources/slater-museum/ biodiversity-resources/dragonflies/world-odonata-list2/ Acess: Marzo/2024
- Pérez-Gutiérrez LA, Montes-Fontalvo JM. 2011 Rediscovery of *Mesagrion leucorrhinum* (Zygoptera: Megapodagrionidae): a "formal" description of female and ultimate stadium of larva with notes on habits. *Int. J. Odonatol*. 14, 91–100.
- Petrulevicius JF, Wappler T, Wedmann S, Rust J, Nel A. 2008 New Megapodagrionid Damselflies (Odonata: Zygoptera) from the Paleogene of Europe. *Journal of Paleontology*, 82, 1173–1181. https://doi.org/10.1666/07-091
- Petrulevicius JF. 2020 First argiolestid damselfly (Odonata: Zygoptera) from the late Palaeocene of Northwest Argentina, *Palaeoentomology* 003 (6): 541–545 <u>https://doi.org/10.11646/palaeoentomology.3.6.1</u>
- Pilgrim EM, von Dohlen CD. 2008 Phylogeny of the Sympetrinae (Odonata: Libellulidae): further evidence of the homoplasious nature of wing venation. *Systematic Entomology*, 33(1), 159–174
- Pinto ÂP. 2024 Cap. 15, Odonata Fabricius, 1793, pp. 187-233. In: Rafael, J.A.; Melo, G.A.R.; Carvalho, C.J.B. de; Casari, S. & Constantino, R. (eds). Insetos do Brasil: Diversidade e Taxonomia. 2<sup>a</sup> ed. Instituto Nacional de Pesquisas da Amazônia, Manaus. 880 pp. https://doi.org/10.61818/56330464c15

- Pinto ÂP, Bota-Sierra CA, Marinov M. 2022 Species identification and description, in: Cordoba-Aguilar A, Beatty Ch, Bried J (eds), Dragonflies and Damselflies: Model Organisms for Ecological and Evolutionary Research, 2nd edn (Oxford, 2022; online edn, Oxford Academic, 15 Dec. 2022) https://doi.org/10.1093/oso/9780192898623.003.0019, accessed 13 Nov. 2023.
- Pinto, ÂP. 2016 A fauna de libélulas da América do Sul: a última fronteira a ser desvendada. *Informativo Sociedade Brasileira de Zoologia*. 117. 7-9.
- Rácenis J. 1959 Notas taxonómicas sobre la familia Megapodagrionidae (Odonata: Zygoptera) con la sinopsis de las especies venezolanas. *Acta Biologia Venezuelica*, 2 (30): 335–367.
- Ramirez A, Gutiérrez-Fonseca P. 2013 The larvae of *Heteragrion majus* Selys and *H. atrolineatum* Donnelly, with a key to known species from Costa Rica (Odonata: Megapodagrionidae). *Zootaxa*. 3609. 96-100. 10.11646/zootaxa.3609.1.8.
- Rehn AC. 2003 Phylogenetic analysis of higher-level relationships of Odonata, 28. *Syst, Entomol*, pp. 181–240.
- Riek EF, Kukalova–Peck J. 1984 A new interpretation of dragonfly wing venation based upon early Carboniferous fossils from Argentina (Insecta: Odonatoidea) and basic characters states in pterygote wings. *Canadian Journal of Zoology*, 62:1150–1166.
- Selys-Longchamps E De. 1862 Synopsis des *Agrionines*. 3e legion: *Podagrion*. *Bulletin Academie Royale Belgique* Serie **2**, 5–44.
- Selys-Longchamps E De. 1885 Programme d'une revision des Agrionines. *Comptes Rendus de la Societe Entomologique de Belgique* **29**, cxli– cxlvi (1–8 separate).
- Selys-Longchamps E De. 1886 Revision du Synopsis des Agrionines. Première partie comprenant les légions *Pseudostigma–Podagrion–Platycnemis* et *Protonevra*. *Memoires couronnes et autres memoires, l'Academie royale de medecine de Belgique* 38, 1–233.
- Silsby J. 2001 Dragonflies of the World. CSIRO Publishing, Collingwood.
- Simmons MP, Kessenich J. 2020 Divergence and support among slightly suboptimal likelihood gene trees. **Cladistics**, 36: 322-340. <u>https://doi.org/10.1111/cla.12404</u>
- So KS, Won CG. 2021 The first 'Megapodagrionidae' (Odonata, Zygoptera) from the Lower Cretaceous of Democratic People's Republic of Korea, *Cretaceous Research*, 130, <u>https://doi.org/10.1016/j.cretres.2021.105054</u>.

- Stand-Pérez M, Bota-Sierra C, Pérez-Gutiérrez L. 2019 Heteragrion demarmelsi sp. nov., with taxonomic notes on Colombian Heteragrion species (Odonata: Heteragrionidae). Zootaxa. 4623. 090-112. 10.11646/zootaxa.4623.1.6.
- Suvorov A, Scornavacca C, Fujimoto MS, Bodily P, Clement M, Crandall KA, Whiting MF, Schrider DR, Bybee SM. 2021 Deep ancestral introgression shapes evolutionary history of dragonflies and damselflies, *Systematic Biology*, syab063, <u>https://doi.org/10.1093/sysbio/syab063</u>
- Tennessen KJ. 2024 *Polythore vexilla* sp. nov. from southwestern Ecuador (Odonata: Polythoridae). *Zootaxa*. 5424. 467-475. 10.11646/zootaxa.5424.4.5.
- Tennessen KJ. 2010 The madicolous nymph of *Heteropodagrion sanguinipes* Selys (Odonata: Megapodagrionidae). **Zootaxa**, 2531: 29–38. https://doi.org/10.5281/ZENODO.196541.
- Tillyard, RJ. 1939 A reclassification of the order Odonata. Based on some new interpretations of the venation of the dragonfiy wing. Part 11. Australian Zoologist 9(3): 195-22 I. Trautwein MD, Wiegmann BM, Beutel R, Kjer KM, Yeates DK. 2012 Advances in insect phylogeny at the dawn of the postgenomic era. *Annu. Rev. Entomol.* 57:449–68.
- Trueman JWH. 2007 A brief history of the classification and nomenclature of Odonata.
   **Zootaxa**, 1668: 381–394 In: Zhang, Z.-Q. & Shear, W.A. (Eds) Linnaeus
   Tercentenary: Progress in Invertebrate Taxonomy. **Zootaxa**, 1668: 1–766.
- Trueman JWH, Rowe RJ. 2019a The wing venation of Odonata. *Int. J. Odonatol*, 22 (1): 73-88. DOI: 10.1080/13887890.2019.1570876
- Trueman JWH, Rowe RJ. 2019b Reply to Nel, Garrouste, and Schubnel (2019) "The wing venation of Odonata. *Int. J Odonatol*, 22 (3–4): 167–172, https://doi.org/10.1080/13887890.2019.1654932
- Vences M. 2020 The promise of next-generation taxonomy. *Megataxa* 001 (1): 035– 038 doi.org/10.11646/megataxa.1.1.6
- Vick GS. 1998 Notes on some damselfly larvae from Cameroon (Zygoptera: Perilestidae, Amphipterygidae, Playcnemididae). *Odonatologica* 27(1): 87-98.
- Vilela D, Lencioni F, Bota-Sierra C, Ware J, Bispo P. 2023 Taxonomic revision of the Neotropical genus *Heteragrion* Selys, 1862 (Zygoptera: Heteragrionidae): male morphology, new species and illustrated key. *Zootaxa*. 5356. 1-96. 10.11646/zootaxa.5356.1.1.

- Wake D, Wake M, Specht C. 2011 Homoplasy: From detecting pattern to determining process and mechanism of evolution. *Science* (New York, N.Y.). 331. 1032-5. 10.1126/science.1188545.
- Ware J, Barden P. 2016 Incorporating fossils into hypotheses of insect phylogeny. *Current Opinion in Insect Science*. 18. 10.1016/j.cois.2016.10.003.
- Wheeler Q. 2004 Taxonomic triage and the poverty of phylogeny. *Philosophical Transactions of The Royal Society B Biological Sciences*. 359. 571-583. 10.1097/rstb.2003.1452.
- Wright AM. 2019 A systematist's guide to estimating bayesian phylogenies from morphological data, *Insect Systematics and Diversity*, 3(3), <u>https://doi.org/10.1093/isd/ixz006</u>
- Young AD, Gillung JP. 2020 Phylogenomics principles, opportunities, and pitfalls of big-data phylogenetics. **Systematic Entomology**, 45: 225-247. <u>https://doi.org/10.1111/syen.12406</u>
- Yu X, Bu W. 2011 A preliminary phylogenetic study of Megapodagrionidae with focus on the Chinese genera *Sinocnemis* Wilson & Zhou and *Priscagrion* Zhou & Wilson (Odonata: Zygptera). *Hydrobiologia* 665:195–203 DOI 10.1007/s10750-011-0622-7

# 4 Chapter 2 – THE DAMSELFLIES OF *HETEROPODAGRION* SELYS, 1885 AND *MESAGRION* SELYS, 1885 (ODONATA: HETERAGRIONIDAE AND MESAGRIONIDAE): AN INTEGRATIVE TAXONOMIC REVISION FOR THIS PANAMANIAN AND NORTHERN ANDEAN COMPLEX OF GENERA

León Andrés Pérez-Gutiérrez<sup>, A,B,C</sup>, Daniela Maeda Takiya<sup>D</sup>, Maria Paula Rozo<sup>D</sup>, and Ângelo Parise Pinto<sup>B</sup>

<sup>A</sup> Laboratorio de Entomología, Research Group "Biodiversidad del Caribe Colombiano. Programa de Ciencias Biológicas, Universidad del Atlántico, Barranquilla, Colombia. E-mail: <u>leonperez@mail.uniatlantico.edu.co</u>

<sup>B</sup> Laboratório de Sistemática de Insetos Aquáticos (LABSIA), Departamento de Zoologia, Universidade Federal do Paraná, P. O. Box 19020, 81531-980, Curitiba, PR, Brazil.

<sup>c</sup> Programa de Pós-graduação em Entomologia, Departamento de Zoologia, Universidade Federal do Paraná.

<sup>D</sup> Laboratório de Entomologia, Instituto de Biologia, Universidade Federal do Rio de Janeiro, Caixa Postal 68044, Cidade Universitária, 21941-971, Rio de Janeiro, RJ, Brazil.

LPG: <u>https://orcid.org/0000-0002-2775-3040</u> DMT: <u>https://orcid.org/0000-0002-6233-3615</u> MPR: <u>https://orcid.org/0000-0003-2792-2091</u> APP: <u>https://orcid.org/0000-0002-1650-5666</u>

**ABSTRACT.** *Heteropodagrion* Selys 1885 and *Mesagrion* Selys 1885 are genera of Neotropical damselflies that together have an intricated taxonomic history pending of revision. Both genera include 6 described species restricted and distributed from the northern mountain pacific ranges at the Isthmus of Panamá to south at northern Andes in Colombia and Ecuador. In the present revision, species delimitations were proposed based on comparative morphological analyses, genetic distances, and phylogenetic criteria using multi-locus mtDNA (COI) and nDNA (ITS2) sequences. Specimens from populations of uncertain identity were included to associate them to the available names, as well as new taxa are described, and their variation fully illustrated. ABGD, ASAP, and bPTP analyses allow us to support into integrative framework that *Heteropodagrion croizati* Pérez-Gutiérrez and Montes-Fontalvo, 2011, *H. sanquinipes* Selys, 1885, *H. superbum* Ris, 1918, and *H. varipes* Daigle, 2014 correspond to valid

species names, in addition to five new species introduced herein: *H. cuyabri* **sp. nov**., *H. diabolum* **sp. nov**., *donnellyi* **sp. nov**., *H. paramillo* **sp. nov**., and *H. santuario* **sp. nov**. Our study also reveals that *H. superbum* show geographic variation but belong to the same species. Higher genetic diversity is evident in the Mesagrionidae lineage with intraspecific distances considerably greater, but more evidence is necessary to suggest cryptic diversity or possible processes of incipient speciation. The inter- and intra-generic genetic distances detected in *Heteropodagrion* agree with those found in other studies with odonates at generic and species level. Finally, a crucial achievement for studies in Odonata, is the useful concatenation of the nuclear marker ITS2 with mitochondrial COI for consistent species delimitations aiding to diagnose species in groups which are difficult do based on morphology alone.

**Key words.** cryptic species, DNA-barcoding, Megapodagrionidae, phylogeny, taxonomy, Zygoptera,

# 4.1 Introduction

The taxonomy of Western Hemisphere damselflies (suborder Zygoptera) remains poorly resolved (Pinto, 2023). Recent revisions of genera reveal deep Wallacean and Linnean shortfalls and the underestimation of diversity in groups endemic to this region, which harbors the greatest diversity of Odonata, dragonflies and damselflies, on the planet (Clausnitzer et al., 2009; Pinto, 2016).

The recent "split" of the non-monophyletic family Megapodagrionidae s.l. (Dijkstra et al., 2014; Bybee et al., 2021), with reinstatement of old taxa and introduction of new families opened an uncertain scenario for the intergeneric relationships of the Neotropical clades traditionally included into a large concept of megapod damselflies recognized as Megapodagrionidae s.l.

Since the erection of *Heteropodagrion* Selys, 1885 and *Mesagrion* Selys, 1885 by Selys (1885), these genera were treated as members of the "Légion *Podagrion*" (Selys, 1862) (Fig. 1a-b). Selys (1885) proposed both *Mesagrion* —type species *Mesagrion leucorrhinum* Selys, 1885— and *Heteropodagrion* —type species *Heteropodagrion sanguinipes* Selys, 1885— as monotypic within the "2<sup>me</sup> Légion: Podagrion", interpreting the similarity in characters of the wing venation and caudal appendages. Their similarity is primarily based on characteristics of immature stages, and it is noteworthy that larvae of *Heteropodagrion* and *Mesagrion* are virtually indistinguishable morphologically (see Tennessen, 2010; Kalkman et al., 2010; Perez-Gutierrez & Montes-Fontalvo, 2011 and Fig. 1c-d).

Traditionally, such as for all odonates, the classification of these genera has been deeply based on wing venation characters (e.g., Selys, 1886; Tillyard, 1917; Fraser, 1957; Bechly, 1995). Characters supporting them as distinct genera are in *Mesagrion* the RP3-4 raising at level of the subnodus, while in *Heteropodagrion* the RP3 raises proximal to subnodus level, and in *Mesagrion* the origin of IRP2 is distal to subnodus, at level of first postnodal crossvein, while in *Heteropodagrion* originates at subnodus level. In addition, *Heteropodagrion* has supernumerary longitudinal veins (the supplementary sectors) between RP2 and IRP2, which are lacking in *Mesagrion* (Fig. 2a–b).

The importance of these wing venation characters as putative apomorphies have not been evaluated under a phylogenetic framework. Characters of ligula were not investigated and consequently underestimated in species diagnoses due to little variation observed (see Garrison et al., 2010; Pérez-Gutiérrez & Montes-Fontalvo, 2011b).

The genera *Heteropodagrion* and *Mesagrion* are referred as a complex (Pérez-Gutiérrez & Montes-Fontalvo, 2001), because at least interspecifically the general appearance in some species and even several morphological shared traits, make traditionally hard and biased recognize species limits due unknown intraspecific variation (Pinto, 2024).



Fig. 1. Representatives of *Heteropodagrion* and *Mesagrion* genera. (a) *Heteropodagrion santuario* sp. nov (Colombia, Risaralda: Santuario. San Rafael stream) (b) Mesagrion *leucorrhinum*, manting pair (Chirajara, Guayabetal Colombia. (c) Larva of *Heteropodagrion santuario* sp. nov. (Colombia, Risaralda: Río San Rafael, Santuario). (d) Larva of *Mesagrion leucorrhinum* (Colombia, Cundinamarca: Guayabetal, Quebrada Chirajara). Photo: LP.

They are specialized in occupying hygropetric or madicolous biotypes as rocky drains in lower-order lotic ecosystems, small streams, headwaters of first-order lotic

systems, streams of water that run over rocks, and areas of ridges in waterfalls where adult males and females can be seen perching on lianas and surrounding vegetation (Rácenis, 1959; Tennessen, 2010; Mauffray & Tennessen, 2019). Differently from other Heteragrionidae, adults of *Heteropodagrion and Mesagrion* perch with wings closed, and especially males, exhibit vivid colors that include commonly red, black, yellow, and white in patterns with unknown taxonomic relevance (Selys, 1885; 1886; Donnelly, 1992).

Females are more secretive in terms of coloration with mixture of pale brown, making them particularly difficult to detect in the field (Mauffray & Tennessen, 2019). Besides, larvae can be seen covered by debris accumulated due to exposure to the drag of the current of water and females oviposit directly on humid rocks in these biotypes (Tennessen, 2010; Pérez-Gutiérrez & Montes-Fontalvo, 2011a).

The strict microhabitat requirement by these damselflies suggests that isolation mechanisms at the level of micro-watersheds in the Andes may occur in species of this group, additionally in this context evidence of sympatric populations of *Heteropodagrion* and *Mesagrion* has been found on the Colombian massif, in Putumayo, these observations reveal a scenario where several hypotheses of speciation can be tested. In some cases, the possibility of hybrids (Zhang et al., 2021; Schneider et al., 2021) and polymorphisms (Cordero & Andres, 1996; Sanchez et al., 2010; Sanchez- Guillen et al., 2020) have been argued, although so far there is scarce evidence of conspecificity that showed this pattern common and homogeneous in the order Odonata (Mitchell & Samways, 2005)

*Mesagrion* is still a monotypic genus with its type species, *M. leucorrhinum*, distributed from the eastern to central Colombian Andes. Since the milestone study by Ris (1918) on odonates from Costa Rica along all Andean Mountain Chain to Argentina, when he described *H. superbum*, a century elapsed to see the third species described in *Heteropodagrion*, probably explained by scarce collected specimens.





– Mesagrion leucorrhinum —

Fig. 2. Venational characters in *Heteropodagrion* and *Mesagrion*. (a) Right anterior and posterior wings of *Heteropodagrion superbum* ♂ (Colombia, Cauca: San Antonio Km8 via Dagua). (b) Left pair wings of *Mesagrion leucorrhinum* ♂. *Abbreviations: MA* (Medial anterior); RP3-4; IR2 (Interradial 2); IR1 (Interradial 1); CUP (Cubital posterior); CUA (Cubital anterior). Arrows and dots indicate generic venational diagnostic characters between *Heteropodagrion* and *Mesagrion*. Photos: LP.

*Heteropodagrion* currently includes five recognized species, *H. croizati* Pérez & Montes, 2011, *H. nigripes* Daigle, 2014, *H. sanguinipes* Selys, 1885, *H. superbum* Ris, 1918, and *H. varipes* Daigle, 2014, distributed along the northern mountain pacific ranges of the Panamá Isthmus southwards to the northern Andes of Colombia and Ecuador (Tenessen, 2010; Daigle, 2014; Pinto, 2024).

Modern taxonomic revisions are based on as many lines of evidence as possible, for this, the addition of molecular data unequivocally not only assists the

taxonomist in decision-making (Marinov et al., 2016; Pimenta et al., 2019; Jones et al., 2020; Zhang et al., 2020; Yu & Xue, 2020; Schneider et al., 2021; Pinto et al., 2022), but perhaps more importantly, reveal insights into lineage evolution history (Osozawa et al., 2017; Bourguignon et al., 2013). In special to odonates, such integrative approach was strongly supported advocating the interaction among three stages of the inference: (1) problem discovery, (2) data investigation, and (3) data analysis, adopting as many sources as possible of data available to evaluate species-level hypotheses (Pinto et al., 2022). A taxonomic revision is needed when traditional diagnostic characters overlap between taxa, especially when new taxa emerge in the chronologic time, when classifications was based on phenetic criteria that do not reflect relationships between taxa, the opposite of classifications in the current context, phylogenetically framed.

The distributional range of *Heteropodagrion* and *Mesagrion*, as well as in many other Neotropical megapod damselflies (e.g., *Dimeragrion* Calvert, 1913; *Heteragrion* Selys, 1862; *Philogenia* Selys, 1862; *Teinopodagrion* De Marmels, 2001, and *Thaumatoneura* McLachlan 1897), indicates that speciation in these groups is high in very small geographic ranges and, therefore, distributional patterns are keys to solving interspecific limits.

Several studies show the plausibility of obtaining robust hypotheses on the evolution of lineages at genus level, as well as to delimit species (see Pons *et al.* 2006; Rach et al., 2008; Yu & Xue, 2020; Djan et al., 2020) with the incorporation of molecular markers from different regions of genomes being either mitochondrial or nuclear. This aspect is deeply related to the possibility of detecting processes that underlie speciation, such as incomplete lineage sorting, hybridization, or introgression among data sets (Schneider et al., 2021; Zhang et al., 2021).

Long-time taxonomic problems involving intricated taxa have been solved using integrative methodologies with molecular species delimitation approaches (Mitchell & Samways, 2005; Yu & Xue, 2020; Jones et al., 2021). Although, in many studies results of distinct molecular markers or methods are incongruent in proposing species hypotheses (Marinov et al., 2016), in such cases the decision ultimately rests on the taxonomist (Pimenta et al., 2019; Vilela et al., 2019; Zhang et al., 2021; Schneider et al., 2021).

Nevertheless, in an integrative framework, the taxonomist can to confront these incongruences in favor or against a hypothesis of relationship at different levels of taxa

hierarchy, this is especially evident in the topologies of individual molecular partitions obtained with mitochondrial data (e.g. COI and COII) when compared with those obtained with nuclear markers (e.g., ITS and 28S region) (Yu et al., 2015), such incongruences may represent different or complementary levels of ancestry (Hillis et al., 2021) and it is, therefore, fundamental to avoid delimitation with a single marker and with a single methodological approach (Dupuis et al., 2012; Meier et al., 2021) or overestimate results derived of single marker (Yu & Xue, 2020).

When species are observable and discernible, but difficult to diagnose using traditional taxonomic tools, an integrative framework with different sources of molecular markers is highly recommended (Cordero-Rivera & Lorenzo-Carballa, 2010; Lorenzo-Carballa et al., 2022; Guo & Kong, 2022). The recognition of cryptic species is possible indirectly through molecular evidence making use of different speciation scenarios where the species hypotheses are tested (Bourguignon et al., 2013).

Due to uncertain taxonomic studies on character variation and bias for an accurate species identification, this integrative taxonomic revision was focused in establishing limits of the known species of genera *Heteropodagrion* and *Mesagrion* with molecular and morphological evidence as to recognize inter- e intra specific variation of species as evolutionary lineages. Furthermore, previous species were corroborated, and new taxa are described based on multiple species delimitation approaches.

#### 4.2 Material and Methods

#### Taxon sampling

Specimens were collected in the field using entomological aerial nets fixed in acetone and preserved dried or fixed and preserved in 98% ethanol, with replacement after 24h, and then stored in the refrigerator at -20°C for DNA studies. Tissue samples were obtained from specimens collected between 2006–2023, thus 17 years old was the oldest sample. DNA and morphological vouchers were the same in all analyses. Morphotypes (i.e., primary species inference) were tentatively identified largely based on cerci morphology.

Specimens from the following collections were studied:

- DZUP Entomological Collection "Pe Jesus Santiago Moure", Department of Zoology, Federal University of Paraná, Curitiba, Brazil.
- **MNRJ** Entomological collection, Department of Entomology, Museu Nacional, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil.
- **UARC** Universidad del Atlántico Región Caribe Barranquilla, Atlántico, Colombia.
- **UDEA –** Entomological Collection of the University of Antioquia (CEUA), Universidad de Antioquia, Medellin, Colombia.

Holotypes of *H. croizati*, specimens collected in type localities of *H. varipes*, *H. superbum*, and *H. cf. superbum* Donnelly, the holotype of *M. leucorrhinum* and syntypes of *H. superbum* and *H. sanguinipes* were examined by photos or illustrations.

For the phylogenetic analyses, except for *H. nigripes*, specimens of all valid names included in *Heteropodagrion* and *Mesagrion*, as well as specimens from populations representing five putative new species were studied (Table 1). Outgroup species including representatives of Calopterygidae, Coenagrionidae, Heteragrionidae and Lestidae were selected according to the phylogenies of Dijkstra et al. (2014) and Bybee et al. (2021).

#### **DNA sequencing and alignment**

DNA was extracted using the DNeasy® Blood and Tissue kit (Qiagen Inc., Hilden, Germany) optimizing the original protocol by incubating the tissue for lysis for 48h and generating two separate elutions of 50ul of DNA extract, instead of one of 100ul. DNA was extracted from thoracic muscle samples of adults by dissection with forceps and through detachment the right metacoxa, with the remainder of the specimen kept as voucher.

Fragments of the mitochondrial gene cytochrome c oxidase subunit I (COI) and the nuclear Internal transcribed spacer II (ITS2) were amplified by PCR using the following primers: LCO1490 (5'-GGTCA ACAAA TCATA AAGAT ATTGG) and C1-J-1718 (5'-GGAGGATTTGGAAATTGATTAGTTCC) and CAS5p8sFc (5'-TGAACATCGACATTTYGAACGCACAT) and CAS28sB1d (5'-TTCTTTTCCTCCSCTTAYTAATATGCTTAA) (Folmer et al., 1994; Simon *et al.* 1994; Jie *et al.* 2003). All PCR reactions had a total volume of 25ul, containing 5µl of 5x Taq buffer (Promega), 3.5µl MgCl2 (50nM, Promega), 2µl BSA (Promega), 1µl dNTP mix (10uM, Promega), 0.5µl of each 10µM primer (Invitrogen), 0.2µl of the enzyme Go®Taq DNA polymerase (Promega), and 1.0 to 2.0µl of genomic DNA. The thermocycler profile consisted of 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C, with a final step of 7 min at 72°C.

PCR products were stained with GelRedTM (Biotium), subjected to agarose gel electrophoresis in 1.0% TBE and visualized under UV light. Amplicons were purified using ExoSAP-IT® (USB Affymetrix) and sequenced in forward and reverse direction using the same PCR primers by Macrogen (Seoul, South Korea). Electropherograms were assembled in Geneious 10 (2021.0.3) to generate consensus sequences (Kearse et al., 2012). The identity of all sequences was checked using BLAST on Genbank.

In addition to few COI sequences available at GenBank, consensus sequences of COI were aligned in MEGA 11.0.13 (Tamura et al., 2021) using the MUSCLE algorithm, while the ITS2 sequences were aligned in MAFFT online version with the L-INS-i algorithm (Katoh et al., 2019). Alignments were further checked visually in MEGA11 (Tamura et al., 2021) (Supplementary material 1).

#### Genetic distances and phylogenetic inference

Pairwise distances were calculated based on K2P for COI and P- Corrected for the ITS2 alignment, for each alignment a Neighbor-Joining dendrogram with 100 replicates of non-parametric bootstrap was conducted in MEGA 11 (Tamura et al., 2021) (Supplementary Figures 2-3, and Tables 2-3), Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair.

Substitution models and maximum likelihood (ML) trees were calculated in IQ-TREE multicore version 2.1.2 in Cipres Science Gateway (Miller et al., 2016) for both matrices separately and for a concatenated matrix. Sequence matrix1.7.8 (Vaidya et al., 2011) was used for concatenate matrices, ModelFinder (Kalyaanamoorthy et al., 2017) selected using BIC the models GTR+F for COI, TPM2+F+I+G4 for the ITS2, and SYM+I+G4 for the concatenated matrices. These selected models were used to calculate ML trees and 1000 pseudoreplicates of ultrafast bootstrap (UFBoot) and branch support with SH-like aLRT. Table 1. Species included for the analysis of the Mesagrion-Heteropodagrion complex and outgroups with respective voucher specimen code, adult gender (G), collecting data, and GenBank accession numbers for COI and ITS2 sequences. SEQ: stands for sequence generates herein, which will be substituted later from GenBank code after paper revision.

Taxon	Voucher code	G	Collecting data	COI	ITS2
Caloptervoidae		-			
Hetaerina brightwelli	ODO-LABSIA 676	2	BRAZII : Rio de Janeiro	SEQ	SEO
Hetaerina brightwelli	MNR.I 0117	2	BRAZII : Rio de Janeiro	SEQ	SEO
Coenagrionidae		U			
Acanthagrion gracile		2	BRAZII : Paraná, Curitiba	SEO	SEO
Metalentohasis selvsi		2	BRAZII · Paraná Antonina	SEO	-
Heteragrionidae		0	DIVALIE. Falana, AntUllilla	SLQ	-
Dimeragrian percubitala	ACX05000				
Difference on the second secon		.7	VLINLZUELA RRAZII : Daraná Direguera	AG 1 90090	-
Heterogrion ourontio		Ċ.	DRAZIL. Parana, Piraquara	SEQ	SEQ
		?	( DDAZIL - Derené Merrete -	SEQ	-
Heteragrion aurantiacum	DNA EBI-001	ď,	BRAZIL: Parana, Morretes	SEQ	-
Heteragrion ct. ovatum	DNA 673	ď	BRAZIL: Rio de Janeiro, Municipio?	-	SEQ
Heteragrion cf. tiradentense	DNA 674 CV05	ð	BRAZIL: Rio de Janeiro, Italiaia	-	SEQ
Heteragrion bariai	DNA 675	6	BRAZIL: Rondônia, Porto Velho	SEQ	SEQ
Heteragrion sp.	ODO-LABSIA 158	3	BRAZIL: Paraná, Piraquara	SEQ	SEQ
Heteragrion triangulare	ASM58149		BRAZIL	ASM58149	-
Heteragrion inca	AGY95127		PERU	AGY95127	-
Oxistigma sp.	AGY95199		SURINAME	AGY95199	-
Lestidae					
Lestes forficula	ENT-2789	8	BRAZIL: Rio de Janeiro, Nova Friburgo	SEQ	-
Megapodagrionidae		-			
Allopodagrion contortum	DNA BA-017	3	BRAZIL: Bahia, Una	SEQ	-
Protoneurinae		~			
Forcepsioneura sancta	MNRJ 2369	2	BRAZIL: Rio de Janeiro. Nova Friburgo	SEQ	SEQ
INGROUP		0	,		
Heteropodagrion croizati	ODO-LABSIA 660	2	COLOMBIA: Churumbelos Putumavo	SEQ	SEO
Heteropodagrion croizati	ODO-LABSIA 659	Z	COLOMBIA: Churumbelos Putumayo		SEO
Heteropodagrion cuvebri sp. nov	ODO-LABIA 326	0	COLOMBIA: Quindio Brehmen	SEO	-
Heteronodegrion cuyabri sp. nov.		Ŧ	COLOMBIA: Quindio, Brohmon	SEO	_
Heteropodagrion cuyabri sp. nov.		0	COLOMBIA: Quindio, Brohmon	SEQ	
Heteropodagrion cuyabri sp. nov.		0	COLOMBIA: Quindio, Dieninen		
Heteropouagrion cuyabri sp. nov.		ď		SEQ	SEQ
Heteropouagriori cuyabri sp. nov.		¥		-	SEQ
neteropodagrion cuyabri sp. nov.	ODO-LABSIA 663	ď	COLOIVIBIA: Quindio, Brenmen	SEQ	-
neteropodagrion cuyabri sp. nov.		¥,	COLOMBIA: Quindio, Brehmen	SEQ	SEQ
Heteropodagrion diabolum sp. nov.	ODO-LABSIA 639	ð	COLOMBIA: Tutunendo, Choco	SEQ	-
Heteropodagrion diabolum <b>sp. nov</b> .	ODO-LABSIA 640	ð	COLOMBIA: Tutunendo, Choco	SEQ	SEQ
Heteropodagrion diabolum <b>sp. nov.</b>	ODO-LABSIA 641	ę	COLOMBIA: Tutunendo, Choco	SEQ	-
Heteropodagrion donnellyi <b>sp. nov.</b>	ODO-LABSIA 645	8	PANAMA: Cerro Azul	SEQ	SEQ
Heteropodagrion donnellyi <b>sp. nov.</b>	ODO-LABSIA 646	3	PANAMA: Cerro Azul	SEQ	SEQ
Heteropodagrion donnellyi sp. nov.	ODO-LABSIA 647	3	PANAMA: Cerro Azul	SEQ	SEQ
Heteropodagrion donnellyi sp. nov.	ODO-LABSIA 648	3	PANAMÁ: Cerro Azul	SEQ	SEQ
Heteropodagrion donnellyi sp. nov.	ODO-LABSIA 649	3	PANAMÁ: Cerro Azul	SEQ	-
Heteropodagrion donnellyi sp. nov.	ODO-LABSIA 650	Ŷ	PANAMÁ: Cerro Azul	SEQ	SEQ
Heteropodagrion donnellvi sp. nov.	ODO-LABSIA 651	ð	PANAMÁ: Cerro Azul	SEQ	SEQ
Heteropodagrion donnellvi sp. nov.	ODO-LABSIA 658	ð	PANAMÁ: Cerro Azul	SEQ	-
Heteropodagrion paramillo sp. nov.	ODO-LABSIA 655	ð	COLOMBIA: Peque, Antioquia	-	SEQ
Heteropodagrion paramillo sp. nov.	ODO-LABSIA 652	ð	COLOMBIA: Peque, Antioquia	-	SEQ
Heteropodagrion paramillo sp. nov	ODO-LABSIA 653	ž	COLOMBIA: Peque, Antioquia	SEQ	SEQ
Heteropodagrion paramillo sp. nov	ODO-LABSIA 654	õ	COLOMBIA: Peque Antioquia	SEQ	SEO
Heteronodagrion paramillo sp. nov.		+ 1	COLOMBIA: Peque Antioquia	-	SEO
Heteropodagrion sanguinines	AGY95128	U	ECUADOR: Quito	AGY95128	-
Heteronodagrion santuario sp. nov	ODO-LABSIA 322	2	COLOMBIA: Risaralda, Santuario	SEO	SEO
Heteronodagrion santuario sp. nov.	ODO-LABSIA 636	2	COLOMBIA: Risaralda, Santuario	SEO	SEO
Heteronodegrion sentuario sp. nov		2	COLOMBIA: Risaralda, Santuario	SEO	SEO
Heteropodagrion santuario sp. nov.		0	COLOMBIA: Risaralda, Santuaria	SEQ	SEQ
Heteropodagrion confusion on new		¥	COLOMPIA: INStratua, Satiluatio		
neteropodagrion santuario sp. nov.		¥		SEQ	-
neteropodagrion superbum		Q,		SEQ	SEQ
neteropodagrion superbum	ODO-LABSIA 6//	ď	COLOMBIA: Cauca, Calima	SEQ	SEQ
neteropodagrion superbum	ODO-LABSIA 685	ď	COLOMBIA: Cauca, San Antonio	SEQ	-
Heteropodagrion superbum	ODO-LABSIA 678	ð	COLOMBIA: Cauca, Calima	SEQ	-
Heteropodagrion superbum	ODO-LABSIA 679	õ	COLOMBIA: Cauca, Calima	SEQ	-
Heteropodagrion superbum	UDU-LABSIA 680	3	COLOMBIA: Cauca, Calima	SEQ	-
Heteropodagrion superbum	ODO-LABSIA 681	3	COLOMBIA: Cauca, San Antonio	SEQ	-
Heteropodagrion superbum	UDO-LABSIA 682	3	COLOMBIA: Cauca, San Antonio	SEQ	SEQ
Heteropodagrion superbum	ODO-LABSIA 683	3	COLOMBIA: Cauca, San Antonio	SEQ	-
Heteropodagrion superbum	ODO-LABSIA 684	3	COLOMBIA: Cauca, San Antonio	SEQ	-
Heteropodagrion varipes	ODO-LABSIA 319	3	ECUADOR: Morona, Santiago	-	SEQ
Heteropodagrion varipes	ODO-LABSIA 656	3	ECUADOR: Morona, Santiago	SEQ	SEQ
Mesagrion leucorrhinum	ODO-LABSIA 657	Ŷ	COLOMBIA: Churumbelos, Putumavo	SEQ	SEQ
Mesagrion leucorrhinum	ODO-LABSIA 669	3	COLOMBIA: Rio Claro, Antioquia	SEQ	SEQ
Mesagrion leucorrhinum	ODO-LABSIA 668	ð	COLOMBIA: Rio Claro. Antioquia	SEQ	SEQ
Mesagrion leucorrhinum	ODO-LABSIA 667	ð	COLOMBIA: Chirajara, Cundinamarca	-	SEQ
Mesagrion leucorrhinum	ODO-LABSIA 670	ð	COLOMBIA: Alban, Cundinamarca	SEQ	SEQ
Mesagrion leucorrhinum	ODO-LABSIA 317	3	COLOMBIA: Antioquia, Rio Claro	SEQ	SEQ

The command used for ML in COI datas set was iqtree2 -nt 6 -bnni -s infile.txt st DNA -m TESTNEWMERGE -alrt 1000 --sprrad 6 -B 1000 --prefix output with initial seed 188243 using SPRNG. For ITS2 the command used was iqtree2 -nt 6 -bnni -s infile.txt -st DNA -m TESTNEWMERGE -alrt 1000 --sprrad 6 -B 1000 --prefix output. Finally with the concatenated matrix was used the command iqtree2 -nt 6 -bnni -s infile.txt -bsam GENE -keep\_empty\_seq -p partition\_file.txt -st DNA -rclusterf 50 -m TESTMERGE -alrt 1000 --sprrad 6 -B 1000 --prefix output –symtest with initial seed 389373 using SPRNG.

#### Molecular species delimitation

Three distinct approaches of species delimitation/validation based on molecular data were applied (1) Automatic Barcode Gap Discovery (ABGD, Puillandre et al., 2012), for (2) Assemble Species by Automatic Partitioning (ASAP, Puillandre et al., 2020), and Bayesian Poisson Tree Processes (bPTP, Zhang et al., 2013). For the first two that are based only on pairwise divergences, matrices of both markers were pruned of outgroup taxa. For the third method, the resulting ML gene trees were given.

#### Morphological analysis and terminology

Images of morphological structures were obtained either with the aid of a LEICA MZ16 stereomicroscope, equipped with a Canon camera, and source images combined with LAS MONTAGE (Version 4.7) or using a DSLR camera equipped with a 100 mm dedicated macro lens with help of Helicon Remote (4.4.3 W) and source images merged to obtain focus stacking images in Helicon Focus (8.8.2).

Identification and revision of the morphology were undertaken by comparison with museum specimens, original descriptions, and literature data, for example Garrison et al., (2010) and photos from type series when necessary (e.g., *H. sanguinipes* and *M. leucorrhinum*). External morphological terminology adopted in descriptions are based on standard proposals (Tillyard, 1917; Cowley, 1941; Garrison et al., 2010). Nomenclature for wing venation follows Riek & Kukalová-Peck (1984).

Anatomical structure submitted to scanning electron microscopy (SEM) were dissected and cleaned, but not metallized. Images were obtained under low vacuum

TESCAN VEGA3 LMU Microscope at the Centro de Microscopia Eletrônica (CME) of the Federal University of Paraná (UFPR).

The following abbreviations are used: RP3-4 = Radial posterior; IR2= Intercalar posterior radial; RP2= Posterior radial 2; Ax = antenodal crossveins; CuP = cubitus posterior; Fw = fore wing; Hw = hind wing; Pt = pterostigma; S1–10 = abdominal segments.

# 4.3 Results

#### Sequence data matrix

Sequence data was newly generated herein for 54 specimens of those 49 belonging to the *Mesagrion-Heteropodagrion* complex (Table 1). The final COI alignment included 58 sequences with 444 bp, 295 distinct patterns, 179 parsimony-informative, 29 singleton sites, and 236 constant sites. The final ITS2 alignment included 42 sequences with 485 sites, 397 variable sites, 237 parsimony-informative, and 1876 constant sites. The concatenated matrix included 67 taxa with 929 sites, 692 variable sites, 416 parsimony-informative and 423 constant sites.

# Phylogenetic and species delimitation analyses

The concatenated ML analysis recovered a well-supported (UFBoot = 88) *Heteropodagrion–Mesagrion* complex clade (Fig. 3), however the monophyly of *Heteropodagrion* is not recovered. *Mesagrion* is intermingled in *Heteropodagrion* clade, recovered as more related to all other species except of *H. croizati* and *H. varipes*, with high support (UFBoot = 90). The topologies of COI and ITS2 partitions independently support this pattern too (Supplementary Material 1-2).

All *Mesagrion* sequences were clustered together, with splitting into two geographic groups where the Antioquia sequences are separated from those of Meta, Cundinamarca and Putumayo.

*Heteropodagrion* is divided into five subclades: I (*H. croizati* + *H. varipes*); II (*H. diabolum* **sp. nov.**); III (*H. paramillo* **sp. nov**. + *H. donnellyi* **sp. nov.**); and IV (*H. cuyabri* **sp. nov**. (*H. superbum* + *H. santuario* **sp. nov.**).

It is notable that in the ML individual partitions are not congruent in all, ITS2 partition shown a clade than recovered *Mesagrion leucorrhinum* as the sister group of *Heteropodagrion* (Suppl. Mat. 3).

The internal relationships of the clades corresponding to the putative species are congruent between separate and concatenated analyzes of genes, even with the sequence in Genbank of *H. sanguinipes* which, by not integrating into any of the clades of other species, offers insights into its divergence within the new sequences obtained herein.

The ABGD, ASAP, and bPTP were mostly congruent in the results, resulting in the delimitation of 10- 12 species of the *Heteropodagrion* and *Mesagrion* complex (Fig. 3). These results support the discovery of five new species that appear distinct from *H. croizati*, *H. sanguinipes*, *H. varipes*, and *H. superbum* (red and black morphs), also included in the analyses. Thus, they are formally described below. The only incongruence found was between analyses based on COI and ITS2, where ITS2 does not support the separation of *H. santuario* **sp. nov.** from *H. superbum*. However, based on the COI evidence and morphological characters, this entity is described herein.

# Genetic distances

Pairwise genetic distances between sequences of the two markers studied are shown in Tables 2 and 3. In COI intraspecific distances showed little variation, on average <1%, with *H. donnellyi* **sp. nov**., *H. santuario* **sp. nov**., and *H. cuyabri* **sp. nov.** with less than 1%. The maximum value for *M. leucorrhinum* involved specimens from Putumayo (8%). With ITS2, the variation was greater, *H. santuario* **sp. nov**. with %, *H. donnellyi* **sp. nov.** with 2%, *H. cuyabri* **sp. nov.** with 1%, *H. paramillo* **sp. nov.** less than 1%, and the highest value was 10% in *H. superbum*. In *M. leucorrhinum* the maximum pairwise value was 5%.

The five species described herein showed high interspecific genetic distance values in both data sets. Interspecific distances in COI sequences ranked between 10% (*H. superbum* and *H. cuyabri* **sp. nov.**) and 25% (*H. santuario* **sp. nov.** and *H. varipes*). In ITS2 data set the values were slightly higher, ranked between 5% (*H. santuario* **sp. nov.** and *H. superbum*) and 54% (*H. donnellyi* **sp. nov.** and *H. varipes*).



0.07

**Fig 3.** Maximum likelihood tree of the concatenated dataset (COI and ITS2) of *Heteropodagrion- Mesagrion* complex and outgroup taxa. Node associated values are SH-like aLRT with 1000 replicates and bootstrap values. Arrow indicates the studied ingroup, *Heteropodagrion- Mesagrion* complex. Colored bars to the right refer to results of molecular species delimitation methods based on each marker.
Table 2. COI matrix with intra- and interspecific genetic distances estimated by the Kimura 2parameter method, between different species in the *Heteropodagrion* and *Mesagrion* generic complex.

	1	2	3	4	5	6	7	8
1. M. leucorrhinum	0.00-0.01							
2. H. sanguinipes	0.19-0.23	-						
3. H. donnellyi <b>sp. nov.</b>	0.22-0.24	0.16-0.17	0.00-0.01					
4. <i>H. santuario</i> <b>sp. nov.</b>	0.21-024	0.17-0.18	0.15-0.20	0.00-0.01				
5. H. cuyabri <b>sp. nov.</b>	0.21-0.26	0.16-0.19	0.15-0.21	0.12-0.16	0.00-0.01			
6. H. superbum	0.19-0.22	0.14-0.15	0.16-0.21	0.10-0.13	0.11-0.13	0.00-0.01		
7. H. paramillo <b>sp. nov.</b>	0.21	0.17	0.14-0.21	0.19-0.20	0.16-0.19	0.14-0.15	0.00-0.01	
8. H.diabolum <b>sp. nov.</b>	0.19-0.20	0.17-0.18	0.17-0.20	0.19-0.21	0.17-0.19	0.17-0.19	0.19-0.20	0.00-0.01
9. H. varipes	0.25	0.20	0.19-0.22	0.24-0.25	0.23-0.24	0.21-0.23	0.20-0.21	0.22-0.23

Table 3. ITS2 matrix with intra- and interspecific genetic distances estimated by the p- distance method, between the different species in the *Heteropodagrion* and *Mesagrion* generic complex.

	1	2	3	4	5	6	7
1. M.leucorrhinum	0.00-XXX						
2. H. donnellyi <b>sp. nov.</b>	0.37-0.47	0.00-0.03					
3. <i>H. santuario</i> <b>sp. nov.</b>	0.36-0.42	0.17-0.21	0.00-0.03				
4. H. cuyabri <b>sp. nov.</b>	0.34-0.40	0.24-0.28	0.16-0.19	0.00-0.01			
5. H. superbum	0.39-0.44	0.17-0.27	0.05-0.10	0.13-0.18	0.00-0.10		
6. <i>H. paramillo</i> sp. nov.	0.36-0.43	0.20-0.24	0.14-0.18	0.15-0.19	0.13-0.15	0.00-0.01	
7. <i>H.diabolum</i> <b>sp. nov.</b>	0.38-0.43	0.24-0.29	0.23-0.26	0.19-0.23	0.23-0.27	0.25-0.27	-
8. H. varipes	0.30-0.37	0.30-0.38	0.26-0.29	0.23-0.30	0.29-0.30	0.25-0.27	0.28

# 4.4 Taxonomy

## 4.4.1 Type material examined

*Heteropodagrion croizati* (5 ♂, 1♀). male Holotype Colombia. Putumayo Department: Churumbelos waterfalls, National Natural Park Serrania de los Churumbelos AIKA- WASI. 19-I-2010 L. Pérez & Montes Fontalvo leg. UARC

*Mesagrion leucorrhinum* holotype  $3^{\circ}$  examined by photos available at <u>https://data.nhm.ac.uk/dataset/collection-specimens/resource/05ff2255-c38a-40c9-b657-</u> <u>4ccb55ab2feb/record/8980501</u>). *Heteropodagrion sanguinipes* syntypes examined by photos Type series (2 $^{\circ}$ , 2 $^{\circ}$ ) in the drawer collection in IRSN.

Besides, material collected in type localities of species listed below.

*Heteropodagrion varipes* (2♂) Type locality, Morona Santiago, 15.7 km W of Macas on road to Guamote. 2°06'47" S 78° 18'24" W. 2567m Leg. L. Perez, 2018. UARC.

*Heteropodagrion superbum* COLOMBIA, (16♂ 4♀) Type Locality. Cauca Department, Cali km 8 San Antonio way to Tocotá (Lat N 3,5243 Lon W -76,6255 1757 m.a.s.l.), 5-vi-2022. Leg. L. Perez. UARC.

*Heteropodagrion* cf. *superbum* Donnelly PANAMÁ, Cerro Azul, Agua Fria waterfalls, alt. 438m, (lat 9,2723668 lon -79,3741325), 20-II-2022. L. Perez Leg. UARC

### 4.4.1 Family Heteragrionidae Rácenis, 1959

### 4.4.1.1 Genus Heteropodagrion Selys, 1885

Heteropodagrion Selys, (1885: 6) (Reprint, mention); -Selys, 1886: 48,49, 50, 51 (description of Holotype from, Quito, ECUADOR) (Selys, 1885- Holotype male: lost, Sintypes "Quito" 2 males, 2 females, leg.? In IRSNB); —Kirby, 1890: 123 (mention in catalogue); —Calvert, 1913 (Key); — Ris, 1918 (Description H. superbum) ); —Rácenis (1959:339 Classification); —Paulson, 1983 (List of South American Odonata) ); -Bridges, 1994 Catalogue); -Davies & Tobin, 1984 (Classification); —Tsuda (1986, 1991); —Steinmann (1997: 153 as "N. mysticum"); Cotapaxi Province); —Garrison & von Ellenrieder, 2005 (Synonymy of *N. mysticum* with *H. sanguinipes*); -Heckman (2006: 267, key, reproduction of illustrations from Ris, 1918); -Tennessen, 2010 (description of the larva H. sanguinipes); -Kalkman et al., 2010: 123 (mention); -Garrison et al., 2010: 92 (keys, illustrations of cerci in dorsal and lateral views); --Pérez-Gutierrez, 2011 (description of *H. croizati*; Key for the complex); —Dijkstra et al., 2013 (Molecular phylogeny related to Dimeragrion); -Daigle, 2014 (description of H. varipes and H. nigripes); - Mauffray & Tenessen, 2019 (catalog of Odonata from Ecuador); -Bybee et al., 2021 (classification of Heteragrionidae and Mesagrionidae based on phylogenomic analyses); —Paulson et al., 2022 (list of world Odonata https://www2.pugetsound.edu/academics/academic-resources/slatermuseum/biodiversity-resources/dragonflies/world-odonata-list2/. 20/06/2022).

Generic diagnosis (Adapted of Garrison et al., 2010)

Medium to large-sized (37–51 mm) body coloration with combinations of red, black, red-brown, white, and yellow pale markings, legs, and other parts of body (Figs. 1a, 32-40). Frons angulated, smoothly curved ridge between median ocellus and inner eye margin, most posterior point of head at level posterior margin of ocular lobes (Fig. 4-5). Posterolateral margins of prothorax rounded; posterior lobe rounded (Fig. 6-10). Legs with hind femur reaching mid-length of S2 or shorter (Fig. 41). No accessory crossveins basal or distal to CuP-crossing; CuP-crossing closer to antenodal 1 than to 2, petiolation ending at level of second antenodal as long as CuP or longer; discoidal field of Hw with one or two cells between quadrangle and subnodus; RP3-4 recessed for a length equal or shorter to subnodus or just proximal to subnodus; IRP2 arising at subnodus or just to it; two or more supplementary between IRP1 and RP2 (absent in H. varipes), and two supplementary sectors between RP2 and IR2 and IR2 and RP3-4; pterostigma as long as two underlying cells or slightly longer, with anterior margin short, about as long as <sup>1</sup>/<sub>2</sub> length of posterior margin (Fig. 2a, 11-15, 27-29). Genital ligula with inner fold well developed and paired flagella on distal segment (Fig. 23-25a). Dorsum of male S10 with a low tubercle, tergum with median cleft; male cerci forcipate with teeth, denticles and spines (Fig. 16a, 17-22); paraproct rudimentary (except in *H. croizati* is long and smooth) (Fig. 21a). Ovipositor not reaching posterior margin of S10, its ventral margin finely serrulated (Fig. 31a-f); tergum of female S8 almost completely divided into two triangular sclerites.

Type species: *Heteropodagrion sanguinipes* Selys, 1885 by original designation.

#### Species account

#### Heteropodagrion croizati Pérez-Gutiérrez & Montes-Fontalvo, 2011

(Fig. 4a, 6a, 11a, 16a, 21a, 23a, 26a, 27a, 31a, 32a, 37a)

Heteropodagrion croizati Pérez-Gutiérrez & Montes-Fontalvo, 2011: 63-68 (description of Holotype male, COLOMBIA. Serrania de Los Churumbelos, Mocoa, Putumayo Deparment); —Mauffray and Tennessen 2019: (mention, record from Ecuador).

**Material examined.** *Type material*: Holotype ♂, COLOMBIA, Department of Putumayo, Mocoa, Churumbelos Aika-Wasi National Natural Park (1°09′03.43" N 76°

38'46.72" 613 m.a.s.l.), 19-I-2010. L. Perez & Montes- Fontalvo leg. Paratypes (43, 22) same data as holotype but 19-I-2013. UARC

**Diagnosis:** This species is easily distinguished, because it is the only one of the genus, so far, with smooth paraproct longer than cercus (Fig. 16a, 21a) and ligula with flattened with short flagella directed backwards (Fig. 23a), in addition to being distinctive in males for having a yellow ring at the base of each abdominal segment and dark brown distally (Fig. 32a), poststernum pale purple (Fig. 6a) unlike *H. cuyabri* in which this is pale yellow (Fig. 6b), and distributed towards the south of west Andean mountains in Colombia and north of Ecuador.

**IUCN category:** This species is considered Least Concern (Bota- Sierra et al., 2016).

#### Heteropodagrion cuyabri sp. nov.

(Figs. 4b, 6b, 11b, 17a, 23b, 26b, 27b, 31b, 32b, 37b, 41a)

Material examined. *Type material*: Holotype ♂, Colombia. Quindío Department, Brehmen Private Reserve, Second Order Stream in andean cloud forest, Brehmen Private Natural Reserve, 2nd Order Stream, (Lat N 4,6998 Lon W -75,6564, 1.765 m.a.s.l.), 2015, L. Pérez leg UARC 31 voucher LABSIA 665). Paratypes: 8♂ 3♀ same data. Colombia, Quindío Department, Barbas stream (Lat N 04° 40′ 30" Lon W 75° 36′12" 2000 m.a.s.l.), 03.01. 2009. N. Uribe & C. Bota leg., CEUA.

**Etymology**. Specific name in genitive singular as a reference to "*cuyabro*", popular gentilism with than Colombian people of Quindio Department is traditionally called, and to whom this species is dedicated.

**Diagnosis.** This is a large species with vivid red abdomen, with pale yellow venter (Fig. 32b, 6b), distinguishable by cercus with crest of fused denticles on inner dorsal ridge (Fig. 17a, 21b). Very similar in appearance to *H. superbum* in San Antonio locality but with a poststernum pale yellow, unlike the dark pattern present in *H. superbum* complex (Fig. 9a).

**Description of male holotype.** *Head* (Fig. 4b): Labrum ivory yellow, with anterior rear dark brown (40% of its width), mandibles caramel, gena brown, anteclypeus ivory yellow, postclypeus brown, frons angulate brown with pale spots caramel near epistomal suture, a yellow bar below the middle eye, antennae base brown, scape yellow, yellow pedicel with brown posterior edge, brown flagellum, caramel spots between the base of the antennae and the edge of compound eyes, a pair of very distinctive caramel-colored bars that descend obliquely from the lateral ocelli there is the base of the antennae. Region that comprises the vertex from the middle eyes to the posterior part of the occiput with an opaque and shiny appearance like the rest of the epicranial suture. Rear of head yellow, caramel in the ventral part. Occipital bar slightly concave.

**Thorax** (Fig. 6b): Prothorax dark brown laterally with yellow tergal region that extends wide of the prothorax and syntorax disc of circular anterior lobe, pronotum globose, middle lobe with dark brown proepimeron like two black projections, posterior lobe with convex semicircular disc.

Synthorax dorsally dark brown, mesepisternum with longitudinal yellow stripe 0.23 curved up to the alar sclerite, mesepimeron largely dark brown paler near pleural suture, dark interpleural suture, metepimeron largely brown with yellow stripe encompassing the middle of the spiracle not reaching up to the alar sclerite, mesinfraepisternum dark brown, pale pleural suture but obscure in the proximal, a pale medial region leading to the alar sclerite, metepimeron largely pale brown with yellow anterior region, poststernum pale yellow.

**Legs** (Fig. 41a) Pro, meso and metacoxa pale red, coxa red, femur with dorsal carina red and white, in the one charge 10 setae, femur and tibiae articulation dark brown, tibiae, and tarsi red.

*Wings* (Fig. 11b): hyaline, costal and subcostal sectors pale amber towards tip of wings, pterostigma dark brown to black, covering 1<sup>1/2</sup> cell, MA arising near to second antenodal, RP3-4 arising before subnodus, IR2 arising after subnodus, RP2 arising in 6<sup>th</sup> Px, IR1 arising in 10<sup>th</sup> Px. Two supplementary sectors between IR1 and RP2, the upper covering 10 cells, the lower 9. Two supplementary sectors between RP2 and

IR2, the upper covering 2 cells, the lower 3. Two supplementary sectors between IR2 and RP3-4 the upper covering 9 cells, the lower 6.

**Abdomen** (Fig. 32b, 41a) abdominal segments largely vivid red, dorsally brown, cerci with prominent major black teeth, followed by a crest of fused denticles in the inner ridge of the tip (Fig. 17a, 21b), ligula with inner fold well developed, tip of ligula rounded with median cleft and flagella short laterally projected with extremes very concave (Fig. 23b).

*Measurements (mm)*: Total length (incl. caudal appendages) 45.84; abdomen length (excl. caudal appendages) 38.12; cerci 1.03; head maximum width 5.40; Fw length 29.32; Hw length 29.04; Fw maximum width 6.05; Hw maximum width 5.85; Pt length on Fw 1.97 and on Hw 2.05; length of metathoracic femur 4.71; metathoracic tibia 5.30.

**Variation of males paratypes.** All type series is very similar in appearance, minor differences are the number of cells in the supplementary sectors between IR1-RP2 (12- 10), In some individuals, pruinescence can be seen on the thorax, which gives an opaque appearance.

*Measurements (mm)*: Total length (incl. caudal appendages) 36.83- 45.84; abdomen length (excl. caudal appendages) 37.15- 38.12; cerci 0.95- 1.03; head maximum width 5.02- 5.40; Fw length 28.5-30; Hw length 28.3-29.5; Fw maximum width 5.91- 6.05; Hw maximum width 5.84; Pt length on Fw 1.67-1.81 and on Hw 1.70- 1.72; length of metathoracic femur 4.17- 4.30; metathoracic tibia 4.40- 4.67.

**Female:** (Fig. 37b) Coloration pattern in head and thorax are very similar to male, but largely brown in general appearance, with dorsum of abdominal segments dark brown, wing venation same as in the male but RP2 arising after 7th Px, IR1 arising between 10<sup>th</sup> and 11<sup>th</sup> PX (Fig. 27b); ovipositor valves surpassing the level of the cercus tip (Fig. 31b), ventral surface of ovipositor finely serrulate, size of stigma approximately as the cerci.

**Measurements:** Total length (incl. caudal appendages) 41.31; abdomen length (excl. caudal appendages) 31.75; head maximum width 4.85; Fw length 29.89; Hw length 29.95; Fw maximum width 5.82; Hw maximum width 6.07; Pt length on Fw 2.02 and on Hw 2.00; length of metathoracic femur 4.69; metathoracic tibia 5.02.

Larva: Unknown

**Ecology and behavior:** Cloud Forest, riparian forest of first and second-order stream, the type locality for this species is in the Brehmen private natural protected reserve at 1900 m.a.s.l.

**IUCN category:** Not evaluated.

#### Heteropodagrion diabolum sp. nov.

(Fig. 4c, 7a, 12a, 17b, 21c, 23c, 33a, 38a, 41b)

Material examined. *Type material*: Holotype ♂, COLOMBIA. Department of Chocó, Tutunendo, Quebrada El Diablo (Lat 5.7386083 lon -76.5309723 77 m. a. s. l.), I-20-2013. L. Pérez leg., UARC Paratypes: 8♂ 1♀ same data.

**Etymology.** Specific name is an adjective masculine in reference to type locality Quebrada El Diablo, in Tutunendo, los "*Heteropodagrion* del diablo" the unique locality where this species has been collected.

**Diagnosis.** This small species is distinguishable by the white cercus curved and somewhat angled in 0.20 with a row of four small black denticles equidistantly separated at outer edge plus two smaller on inner edge, no major teeth recognizable (Fig. 17b), posternum largely yellow with small brown dark convex mark 0.75 distal (Fig. 7a), is too distinguishable by the vivid red pterostigma (Fig. 12a) similar in the *H. donnellyi* but with red borders, the terminal segment of ligula with prominent keel ending in a prominent tip and long flagella laterally projected (Fig. 23c), in *H. nigripes* Daigle the cerci are similar but denticles on outer edge decrease in size, the most proximal is the largest, inner edge holds two pair of small denticles, nonetheless *H. nigripes* is a black and yellow species without red on anywhere on his body (Fig. 43).

**Description of male holotype.** *Head* (Fig. 4c): labrum white with anterior dark brown border 0.20 max. width, anteclypeus yellow, posclypeus dark brown, light epistomal suture, genae ocher, frons divided by a deep depression, escape and pedicel yellow, compound eyes dorsally reddish (alive specimens), ventrally yellow, ocelli yellow with white yellow stripes extending to middle length toward antennae, occipital bar ochraceous.

**Thorax** (Fig. 7a): Alive mesepisternum with a distinctive pale brown stripe on each side of median dorsal carina plus in the middle along its entire length a yellow band approximately 0.15 the thickness of the sclerite, metepisternum crossed by white band that passes above the spiracle, the lower portion dark brown to the pleural suture, towards the alar sclerite is ocher yellow, metepimeron largely yellow with middle region dark. Poststernum largely yellow with convex dark brown stripe.

*Legs* (Fig. 33a, 41b): Coxae red and yellow, metafemur with carina red and white, hold 13 fine setae almost with the same size, tibiae, and tarsi red with yellow.

**Wing** (Fig. 12a): hyaline, MA arising near to second antenodal, RP3-4 arising before subnodus, IR2 arising almost in the subnodus, RP2 arising between 6th and 7thPx nearest 7th, IR1 arising in 9th Px. Two supplementary sectors between IR1 and RP2, the upper covering 6 cells, the lower 3. One spurious supplementary sector between RP2 and IR2. Two supplementary sectors between IR2 and RP3-4 the upper covering 7 cells, the lower 3. Pterostigma light red, subcostal side covering 1<sup>1/2</sup> cell.

**Abdomen** (Fig. 33a, 41b): abdominal segments dorsally largely dark brown to black, bases and extremes of segments black, laterally, and ventrally each segment largely yellow, ligula with prominent tip and long flagella laterally projected (Fig. 23c), cercus white with a row of 4 black denticles equidistally separated, plus two more in the tip (Fig. 17b, 21c).

*Measurements (mm)*: Total length (incl. caudal appendages) 30.15; abdomen length (excl. caudal appendages) 29.01; head maximum width 4.22; Fw length 22.00;

Hw length 22.23; Fw maximum width 4.67; Hw maximum width 4.73; Pt length on Fw 1.75 and on Hw 1.53; length of metathoracic femur 3.43; metathoracic tibia 3.82.

Variation in males paratypes: Minor differences was detected in measurements below.

*Measurements (mm)*: Total length (incl. caudal appendages) 30.15-31.7; abdomen length (excl. caudal appendages) 29.01-30.46; head maximum width 4.22-4.73; Fw length 22.00-23.3; Hw length 22.23-23.1; Fw maximum width 4.67-4.8; Hw maximum width 4.73-4.97; Pt length on Fw 1.75-1.8 and on Hw 1.53-1.6; length of metathoracic femur 3.43-3.5; metathoracic tibia 3.82-4.0.

**Female** (Fig. 38a): Unfortunately, the specimen is a teneral that was preserved in very poor condition, another specimen was photographed but it was not possible to collect it.

Larva. Unknown.

**Ecology and behavior.** Pluvial Forest in Choco Central, the few individuals that were collected were on the margins of small tributary streams of the Tutunendo River. The area is highly threatened by artisanal mining and damage to the smaller streams due to excess sedimentation has been evidenced in  $f_{\circ}$  ield.

**IUCN category:** Not evaluated.

#### Heteropodagrion donnellyi sp. nov.

(Figs. 4d, 7b, 12b, 18a, 21d, 24a, 26c, 28a, 31c, 33b, 38b, 41c)

Heteropodagrion cf. superbum Donnelly (1992, biological data).

Material examined. *Type material*: Holotype ♂ PANAMÁ, Cerro Azul, Agua Fria waterfalls, (lat 9,2723668 lon -79,3741325, 438 m.a.s.l.), 20-II-2022. L. Pérez leg., UARC; PANAMÁ (15♂ 5♀), Cerro Azul, Rio Piedras, 300 m.a.s.l.), 25-II-2022. Paratypes: 19♂ 5♀ Rio Piedras, Cerro Azul, Panamá.

**Etymology**. Specific name in reference to Thomas Donnelly, eminent North American Odonatologist who in 1992 mentioned the species in the canal zone of Panama and defined it as *cf. superbum*.

**Diagnosis.** A small species with a distinctive colour pattern in head (Fig. 4d), does not have supplementary sectors between RP2 and IR2, pterostigma small, red with dark borders (Fig. 12b), posternum red with dark brown pattern (Fig. 7b), cercus dark like those of *H. superbum*, but largest tooth more prominent and pointed (Fig. 18a, 21d) with truncated tip. Ligula with longitudinal keel in the terminal segment, flagella with ventrally extended rounded tip (Fig. 24a)

**Description of male holotype.** *Head* (Fig. 4d) Labrum ivory yellow, with anterior rear dark brown, mandibles dark brown, gena brown, anteclypeus ivory yellow, postclypeus light brown, frons angulate brown with pales spots caramel near epistomal suture, a yellow bar below middle ocellus, base of antennae brown, scape yellow, pedicel half yellow, posterior half brown, flagellum brown, caramel spots between the base of the antennae and the edge of the compound eyes, a pair of very distinctive caramel-colored bars descending obliquely from the lateral ocelli towards the base of the antennae. Region that includes the vertex from the middle ocelli to the back of the occiput with an opaque, non-shiny appearance like the rest of the epicranium. Rear of head yellow, caramel towards the ventral part. Occipital bar slightly concave.

**Thorax** (Fig. 7b): Prothorax laterally brown with yellow tergal region extending along prothorax and synthorax circular anterior lobe disk, globose pronotum, middle lobe with dark brown proepimeronon as two blunt projections, posterior lobe with convex circular disk. Synthorax dorsally dark brown, mesepisternum with longitudinal yellow stripe curved to the alar sclerite, mesepimeron completely dark brown, interpleural suture dark, metepimeron largely brown with yellow stripe covering half of the spiracle not reaching the alar sclerite, mesinfraepisternum dark brown, pleural suture pale darker in the proximal middle part and paler reaching the wing sclerite, metepimeron largely pale brown, poststernum pale yellow. Procoxa dark red to brown, meso and metacoxa pale red. *Legs* (Fig. 33b, 41c): Coxa red, femur with carina red and white, in the one charge 9 setae, articulation femur-tibiae black, tibiae and tarsi black dorsally.

*Wing* (Fig. 12b): hyaline, costal and subcostal sectors ochraceous towards tip of wings, pterostigma red, subcostal side covering 2 cell, MA arising near to second antenodal, RP3-4 arising before subnodus, 17 Px (FW), 15 Px (HW), IR2 arising after subnodus, RP2 arising in 6th complete Px, IR1 arising in 8th Px. Two supplementary sectors between IR1 and RP2, the upper covering 9 cells, the lower 6. Two supplementary sectors between RP2 and IR2, the upper covering 6 cells, the lower 4. Two supplementary sectors between IR2 and RP3-4 the upper covering 7 cells, the lower 4 (5 HW).

**Abdomen** (Fig. 33b): abdominal segments largely vivid red, with anterior and posterior dark ring, dorsally darker, a yellow fine line extends in each segment, ventrally the 8S-10S yellowish. Ligula with protuberance in the distal segment (Fig. 24a)

*Measurements (mm):* Total length (incl. caudal appendages) 39.43; abdomen length (excl. caudal appendages) 31.90; cerci 1.24; head maximum width 4.21; Fw length 22.84; Hw length 22.5; Fw maximum width 4.20; Hw maximum width 4.32; Pt length on Fw 1.48 and on Hw 164; length of metathoracic femur 4.05; metathoracic tibia 4.62.

**Variation in male paratypes.** In teneral the red portion on abdominal segments is followed by yellow and just at the joint of the segments it darkens.

**Female** (Fig. 38b): The appearance is similar as in male, the coloration pattern in head, thorax and abdomen is similar too but largely brown and yellow, in the FW MA raised beyond second antenodal, RP3-4 raised before subnodus, IR2 raised after subnodus, RP2 raised in 5th posnodal, IR1 raised 9th posnodal, number of posnodals 20 (complete 5, 15 misaligned) (Fig. 28a), ovipositor (Fig. 31c).

*Measurements (mm):* Total length (incl. caudal appendages) 36.31; abdomen length (excl. caudal appendages) 29.33; head maximum width 4.33; Fw length 23.57;

Hw length 23.34; Fw maximum width 4.87; Hw maximum width 5.09; Pt length on 1.57 and on Hw 1.60; length of metathoracic femur 4.23; metathoracic tibia 4.87.

Larva. Unknown.

**Ecology and behavior.** Adults are in canyons of streams and first-order lotic systems, due abrupt topography in channel zone basins, are commons in splash zones in waterfalls, with abundant palms, ferns, and other typical vegetation of humid zones in this habitat.

**Remarks.** Donnelly in 1992 refers to the question of whether "*superbum*" is a variable taxon or whether it represents "several taxa" based on 10 specimens collected in the Panama Canal area (p. 82), Rio Agua Salud; Pipeline Road (no year) (see Donnelly, 1992: p 60).

According to that Chagres Rivers, Agua Fria waterfalls, and Pacora hills correspond to the same basin of what is now known as Canal Zone and comparing with the material collected in this study, we found sufficient reasons to ensure that *H*. cf. *superbum* of Donnelly is an undescribed species, which we herein name it as *H*. *donnellyi* **sp. nov.** 

A total of 45 specimens were examined, between tenerals and matures. It is possible to rule out that postmortem effects are causing deformations that could induce erroneous identification because specimens collected remained almost unchanged with treatment in 98% alcohol and acetonated.

**IUCN category:** Not evaluated.

### Heteropodagrion nigripes Daigle, 2014

(Figs. 17c, 43)

Heteropodagrion nigripes Daigle, 2014. Daigle 2014: 35 description of Holotype male, ECUADOR. Morona Santiago Province, N of Indanza 07-XI-1957, illustrations of cerci in dorsal view) in FSCA); —Mauffray & Tenessen, 2019 (mention, record from Ecuador). **Diagnosis:** its notable the black and light yellow coloration in this species, with absence of red, makes it quite easy to differentiate (Daigle, 2014), its white rings resemble those present in *H. diabolum* **sp. nov.**, outer dorsal edge of cercus with three neighbor denticles followed by 2-3 very small ones. Inner edge with 4 black denticles grouped in pairs (Fig. 17c).

**Remarks**: This species is only mentioned from its type locality Ecuador, Morona: Santiago. Material for comparative studies could not be obtained of this species.

**IUCN category:** This small distribution and secretive habitat led *H. nigripes* to be considered threatened species according to IUCN, in VU categories (Mauffray & Tennessen 2020). All these denotes that reported populations are small and probably highly localized in these specialized and fragile habitats like other species of the genus.

#### Heteropodagrion paramillo sp. nov.

(Figs. 5a, 8a, 13a, 18b, 22a, 24b, 26d, 28b, 31d, 34a, 39a, 41d)

Material examined. *Type material*: Holotype ♂ COLOMBIA, Antioquia, Km 15 Via Juntas de Uramita-Peque (N lat 6,980 lon W -75,9779, 2676 m.a.s.l) L. Perez leg., 31-12-2018. Paratypes: 4♂ 3♀ same data. UARC.

**Etymology**. Specific name in reference to Cerro Paramillo near Peque town, type locality of this new species. It is a noun in apposition.

**Diagnosis.** A species with dark thorax and vivid red abdomen, cercus with major teeth followed by two minor teeth (Fig. 18b), pterostigma dark brown (Fig. 28b). Ligula with distal segment constrained in middle, flagella bent directed upwards (Fig. 24b).

**Description of male holotype.** *Head* (Fig. 5a): Labrum ivory yellow, with anterior rear dark brown, mandibles brown, gena brown, anteclypeus yellow, postclypeus pale brown, frons angulate brown with pales spots caramel near epistomal suture, a yellow bar below middle ocellus, base of antennae brown, scape yellow,

pedicel half yellow, posterior middle brown, flagellum brown, caramel spots between base of antennae and edge of compound eyes, a pair of very distinctive caramelcolored bars that descend obliquely from the lateral ocelli towards the base of the antennae. Region that includes the vertex from the middle ocelli to the back of the occiput with an opaque, non-shiny appearance like the rest of the epicranium. Rear of head yellowish, caramel towards the ventral part. Occipital bar slightly concave.

**Thorax** (Fig. 8a): Prothorax laterally brown with yellow in region tergal extending along the prothorax and synthorax, anterior lobe disk circular, globose pronotum, middle lobe with dark brown proepimeron as two blunt projections, posterior lobe with convex circular disk. Synthorax dorsally dark brown, mesepisternum with yellow longitudinal stripe darker to dorsal carina, mesepimeron completely dark brown, interpleural suture dark, metepimeron largely brown with yellow stripe covering half of the spiracle not reaching to the alar sclerite, mesinfraepisternum dark brown, pleural suture pale darker in the proximal middle part and paler reaching the alar sclerite, metepimeron largely pale brown, poststernum largely dark with apical part red. Procoxa dark red, meso and metacoxa pale red.

*Legs* (Fig. 34a, 41d): Coxa red, femur with carina red and white, in the first charge 10 setae, tibiae and tarsi red.

*Wing* (Fig. 13a): hyaline, with costal and subcostal sectors amber towards tip of wings, pterostigma dark brown to black, covering 1.5 cell, MA arising near to second antenodal, RP3-4 arising before subnodus, IR2 arising after subnodus, RP2 arising between 5th and 6thPx, IR1 arising in 9th Px. Two supplementary sectors between IR1 and RP2, the upper covering 10 cells, the lower 9. Two supplementary sectors between RP2 and IR2, the upper covering 3 cells, the lower 3. Two supplementary sectors between between IR2 and RP3-4, the upper covering 9 cells, the lower 7.

**Abdomen** (Fig. 34a): abdominal segments largely vivid red, dorsally brown, ligula with distal segment constrained in middle and flagella directed upwards with extremes concave and expanded (Fig. 24b), cerci with major teeth followed by two minor teeth (Fig. 18b, 22a).

*Measurements (mm)*: Total length (incl. caudal appendages) 46.89; abdomen length (excl. caudal appendages) 38.89; cerci 0.96; head maximum width 4.82; Fw length 28.35; Hw length 29.20; Fw maximum width 5.49; Hw maximum width 5.21; Pt length on Fw 1.85 and on Hw 1.96; length of metathoracic femur 4.31; metathoracic tibia 4.49.

Variation in male paratypes. Minor variation in the series was detected.

**Female** (Fig. 39a): Largely brown, head largely brown (Fig.26d), ovipositor (Fig. 31d); wings (Fig. 28b)

*Measurements (mm)*: Total length (incl. caudal appendages) 43.32; abdomen length (excl. caudal appendages) 31.98; head maximum width 4.90; Fw length 32.07; Hw length 32.14; Fw maximum width 5.49; Hw maximum width 5.21; Pt length on Fw 2.06 and on Hw 2.07; length of metathoracic femur 5.03; metathoracic tibia 5.38.

Larva. Unknown

**Ecology and behavior.** All specimens were collected in the same small stream, in a cloud forest, with dense tree and shrub vegetation, all active along the bank of the channel.

**IUCN category:** Not evaluated.

### Heteropodagrion sanguinipes Selys, 1885

(Figs. 19a, 34b, 42)

Heteropodagrion Selys, 1885: 6 (mention, preprint); —Selys, 1886: 48 (description of Holotype from, Quito, ECUADOR) (Selys, 1885- Holotype male: lost, Sintypes "Quito" 2 males, 2 females, leg. M. Emile Deville In IRSNB); —Kirby, 1890 (Catalogue); — Calvert, 1913 (Key); —Ris, 1918 (Description *H. superbum*); —Rácenis (1959:339 Classification); — Paulson, 1983 (List of South American Odonata); —Bridges, 1994 (Catalogue); — Davies & Tobin, 1984 (Classification); —Tsuda (1986, 1991); —Steinmann, 1997: 153 (as "*N. mysticum*"); Cotapaxi Province); —Garrison & von Ellenrieder, 2005 (Synonymy of *N. mysticum* with *H. sanguinipes*) ); —Heckman, 2006: 267 (key, reproduction of illustrations from Ris, 1918); —Tennessen, 2010 (description of the larva *H.*

*sanguinipes*); —Kalkman et al., 2010: 123 (mention); —Garrison et al., 2010 (Genus keys); —Pérez-Gutierrez & Montes-Fontalvo, 2011 (description of *H. croizati*; Key for the complex); —Dijkstra et al., 2013 (Molecular phylogeny related to *Dimeragrion*); —Daigle, 2014 (description of *H. varipes* and *H. nigripes*); — Mauffray & Tenessen, 2019 (catalog of Odonata from Ecuador); —Bybee *et al.*, 2021 (classification of Heteragrionidae and Mesagrionidae based on phylogenomic analyses); —Paulson et al., 2022 (list of world Odonata https://www2.pugetsound.edu/academics/academic-resources/slater-museum/biodiversity-resources/dragonflies/world-odonata-list2/. 20/06/2022)

**Diagnosis:** Tip of the cercus rounded compared to *H. superbum* (truncated), *H. sanguinipes* has a small denticle closer to the larger tooth than in *H. superbum* (see Fig. 2 in Daigle, 2014: p41.)

**Remarks:** In Selys (1885), based on the single male from Ecuador, Selys states that the measurements are: male (38 mm) and female (32 mm) abdomen and male (25 mm) and female (26 mm) hind wings. Head black, lower lip and behind the eyes livid. "Abdomen largely yellowish", joints of segments 2 to 7 largely blackish, denotes that the color pattern is clearly not dark as in *H. superbum*, when it describes "Red or reddish feet", it does not refer to the very noticeable white lines present in San Antonio specimens.

This species was considered a senior synonym of *Neuragrion mysiticum* by Garrison & Ellenrieder (2005), as indicated by the data *H. sanguinipes* is a species distributed in the south of the distribution range of the genus, in Ecuador (Mauffray & Tennessen, 2020), presence in Colombia not yet has been confirmed.

Photographs of the cerci (Daigle, 2014) correspond to the illustrations?? of Garrison et al. (2010), based on specimens from Quito, Ecuador.

**IUCN category:** This species is considered Least Concern Bota-Sierra et al., 2021.

*Heteropodagrion santuario* sp. nov. (Figs. 1a, 5b, 8b, 13b, 19b, 22b, 24c, 26e, 29a, 31e, 39b, 41e) *Heteropodagrion superbum* Pérez- Gutiérrez & Montes- Fontalvo, 2011: 68 (misidentification of larvae as *H. superbum*).

**Material examined** COLOMBIA,  $(12 \stackrel{\circ}{\circ} 6 \stackrel{\circ}{\circ})$  Type Locality. Risaralda Deparment, Santuario, San Rafael river (5.074722, -75.964 2000 m.a.s.l.), 5-vi-2012. L. Pérez leg., UARC.

**Diagnosis:** A black species with dark brown cerci, dorsal edge of cercus flattened (prominent and outstanding in *H. superbum*) with major tooth pointed without contiguous teeth, posterior medial dorsal edge with 5 minute teeth (Fig. 19b), venter of thorax largely reddish-ochre with middle region pale, apical region of metasternum with two big dark spots than extends towards apical region of sclerite (Fig. 8b), basal region lighter, reddish near the base of the abdomen. Distal segment of ligula with tip bulky, flagella latero-posteriorly directed backwards in 45°, with extreme concave and expanded (Fig. 24c).

**Description of male holotype.** *Head* (Fig. 5b): labrum yellow with anterior black border, anteclypeus yellow, postclypeus dark brown, light epistomal suture, genae ocher, frons divided by a shallow depression, escape and pedicel brown with yellow articulation, compound eyes dorsally red, ventrally yellow, ocelli yellow with small pale spots, occipital bar brown, posterior region of occiput caramel.

**Thorax** (Fig. 8b): Prothorax largely dark brown and dorsally with yellow regions in the anterior lobe, median lobe and a little darker in the posterior lobe, margin of posterior lobe smooth and gently convex. Mesepisternum with yellow stripes extending to the alar sinus, medium suture dark brown. Mesepimeron completely dark brown, dorsal portion of metepisternum with yellow stripe from the metaspiracle to shortly before the alar sinus, ventral portion dark brown to the pleural suture, towards the alar sclerite is red, metepimeron largely brown with dorsal region yellow near to alar sinus. Metasternum yellow, basal portion of posternum with two large spots paler in the ventral suture, apical portion of posternum pale with brown longitudinal spot.

**Legs** (Figs. 35a, 41e): pro, meso-, and metacoxae red, red femur with dorsal white carina.

**Wing** (Fig. 13b): hyaline, costal and subcostal sectors amber towards tip of wings, pterostigma dark red with black frame, covering almost 2 cells, costal side 0.75 of the subcostal side, proximal side almost parallel to costa. Two antenodals complete, 1 supplementary incomplete in HW). MA arising just after to second antenodal for a lenght equal to its size, RP3-4 arising before subnodus for a length equal to first postnodal, IR2 arising little after subnodus, in the HW IR2 arising below the subnodus, RP2 arising between 5th and 6thPx nearest 5th, IR1 arising in 8th Px. Two supplementary sectors entre IR1 and RP2, the upper covering 10–11 cells, the lower 9. Two supplementary sectors between RP2 and IR2, the upper covering 4 cells, the lower 4–5. Two supplementary sectors between IR2 and RP3-4, the upper covering 13 cells, the lower one 8–9. CuP to length from the first Ax equal to three times its size.

**Abdomen** (Fig. 35a): Largely dark brown to black, lateral, and ventrally pale brown. Cerci dark brown with dorsal edge flattened with major tooth pointed without contiguous teeth, posterior medial dorsal edge with 3-5 small teeth additionally in the inner edge.

*Measurements (mm):* Total length (incl. caudal appendages) 48.35; abdomen length (excl. caudal appendages) 39.02; cerci 1.25; head maximum width 4.79; Fw length 30.99; Hw length 30.33; Fw maximum width 5.29; Hw maximum width 5.09; Pt length on Fw 1.86 and on Hw 1.75; length of metathoracic femur 4.38; metathoracic tibia 4.80.

**Variation in males paratypes.** The type series is very homogeneous in size, colour, and morphology like as in holotype, minor differences were noticeable in wings venation.

**Female** (Fig. 40a): Largely brown in general appearance, paler areas in the head (Fig. 26e), coxae and metepimeron, abdominal segments with dorsal surface darker, distal portion of S7 tergum membranized yellow, tergum of S8 divided in triangular esclerites (Fig. 31e). Px more misaligned compared with the male ones (Fig. 29a).

*Measurements (mm)*: Total length (incl. caudal appendages) 44.92; abdomen length (excl. caudal appendages) 37.63; head maximum width 4.88; Fw length 29.65; Hw length 29.28; Fw maximum width 5.71; Hw maximum width 5.69; Pt length on Fw 1.76 and on Hw 1.82; length of metathoracic femur 4.50; metathoracic tibia 5.18.

Larva: unknown.

**Ecology and behavior.** This species is abundant along riparian forest in Andean cloud forest, females and males were collected in rocky segments, with closed forest canopy, same as larvae that were found on rocky walls covered with ferns, mosses, and plant material with detritus.

**IUCN category:** Not evaluated.

#### Heteropodagrion superbum Ris, 1918

(Figs. 2a, 5c, 9a, 14, 15a, 20a-b, 22c, 25a, 26f, 29b, 31f, 35b, 40a, 41f)

Heteropodagrion superbum Ris, 1918: 89-90 (description of Holotype male, COLOMBIA, San Antonio, Cauca Department, Arch. Naturg. Illustrations of caudal appendages in dorsal and lateral view); —Perez- Gutierrez & Palacino- Rodriguez, 2010 (mention in Checklist);
—Perez-Gutierrez & Montes-Fontalvo, 2011 (misidentification larvae); —Mauffray & Tenessen, 2019 (catalog of Odonata from Ecuador).

**Material examined** COLOMBIA,  $(16 \stackrel{\circ}{\circ} 4 \stackrel{\circ}{\circ})$  Type Locality. Cauca Deparment, Cali km 8 San Antonio way to Tocotá (Lat N 3,5243 Lon W -76,6255 1757 m.a.s.l.), 5-vi-2022. L. Perez leg., UARC;  $(10 \stackrel{\circ}{\circ})$  Cauca Deparment, Calima, Rio Bravo Canyon Lat N 3,8823 Lon W -76, 5658 1433 m. a. s. l., L. Perez leg., UARC;  $(1 \stackrel{\circ}{\circ})$  Valle del Cauca Departament, Anchicayá-Dagua Hidroelectric, Antenas de la Riqueza. Oct.-17-2016. 1180 m. a. s. l. C. Bota & C. Flores leg., CEUA.

**Diagnosis:** A vivid red abdomen species with dark brown cerci, little curved, outer dorsal edge of cercus protruding with major tooth pointed without contiguous teeth with prominent edge than extends towards to cercus tip, inner edge with 5 minute teeth (Fig. 22c), venter of thorax largely red with middle region pale, apical region of

metasternum with two big dark spots than extends towards apical region of sclerite (Fig. 9a), basal region lighter, reddish near the base of the abdomen. Distal segment of ligula with carina bifurcated in middle, flagella latero-posteriorly directed backwards in 40°, with extreme concave and expanded (Fig. 25a).

**Description of male.** *Head* (Fig. 5c): labrum yellow with anterior black border, anteclypeus yellow, postclypeus dark brown, light epistomal suture, genae ocher, frons divided by a shallow depression, escape and pedicel brown with yellow articulation, compound eyes dorsally red, ventrally yellow, ocelli yellow with small pale spots, occipital bar brown, posterior region of occiput caramel.

**Thorax** (Fig. 9a): Prothorax largely brown dorsally with yellow regions in the anterior lobe, median lobe and a little darker in the posterior lobe, margin of posterior lobe smooth and gently convex. Mesepisternum with yellow stripes extending to the alar sinus, medium suture dark brown. Mesepimeron completely dark brown, dorsal portion of metepisternum with yellow stripe from the metaspiracle to shortly before the alar sinus, ventral portion dark brown to the pleural suture, towards the alar sclerite is red, metepimeron largely brown with dorsal region yellow near to alar sinus. Metasternum yellow, basal portion of posternum with two large spots paler in the ventral suture, apical portion of posternum pale with brown longitudinal spot. Legs with pro, meso-, and metacoxae red, red femur with dorsal white carina. (Fig. 35b, 41f).

**Wing** (Fig. 15a): hyaline, costal and subcostal sectors ambar towards tip of wings, pterostigma dark brown with black veins, covering almost 2 cells, costal side 0.75 of the subcostal side, proximal side almost parallel to costa. Two antenodals complete, 1 supplementary incomplete in HW. MA arising just after to second antenodal for a lenght equal to its size, RP3-4 arising before subnodus for a length equal to first postnodal, IR2 arising little after subnodus, in the HW IR2 arising below the subnodus, RP2 arising between 5th and 6thPx nearest 5th, IR1 arising in 8th Px. Two supplementary sectors entre IR1 and RP2, the upper covering 10–11 cells, the lower 9. Two supplementary sectors between RP2 and IR2, the upper covering 4 cells, the lower 4–5. Two supplementary sectors between IR2 and RP3-4 the upper covering 13 cells, the lower 8–9. CuP to length from the first Ax equal to three times its size.

**Abdomen** (Fig. 35b): Vivid red, abdominal segments without markings and spots that form some pattern, only a slim black ring over articulations, largely vivid red laterally, dorsally dark, ventrally S8–10 yellow, cerci with prominent teeth in the 0.60 of length continuous with smooth edge, inferior edge with 5 fine denticles (Fig. 22c), terminal segment of ligula with bifurcated keel and flagella laterally expanded (Fig. 25a).

*Measurements (mm):* Total length (incl. caudal appendages) 46.93; abdomen length (excl. caudal appendages) 40.88; cerci 1.37; head maximum width 4.89; Fw length 29.63; Hw length 29.67; Fw maximum width 5.17; Hw maximum width 5.47; Pt length on Fw 1.81 and on Hw 1.99; length of metathoracic femur 4.52; metathoracic tibia 4.77.

**Variation in the males.** This species has black and red dimorphic males, in the Calima specimens (black morph) was observed a third AX, but it is incomplete, between the two normally AX present.

The vivid red color in San Antonio corresponds with Selys notation, in the Calima and Anchicayá populations the color is dark red to black. The variation can be summarized below.

**San Antonio and Rio Bravo Specimens**: Total length (incl. caudal appendages) 45.15; abdomen length (excl. caudal appendages) 35.31; cerci 1.15; head maximum width 4.64; Fw length 25.33; Hw length 25.63; Fw maximum width 4.80; Hw maximum width 4.66; Pt length on Fw 1.50 and on Hw 1.37; length of metathoracic femur 4.23; metathoracic tibia 4.28. have differences in the wing venation, in FW the vein RP3-4 arises 0.45 closer to the subnode than those from San Antonio, this difference is notable, in HW the trend is also maintained.

**Anchicayá specimens**: Total length (incl. caudal appendages) 32.5; abdomen length (excl. caudal appendages) 24.05; cerci 1.42; head maximum width 4.15; Fw length 22.85; Hw length 23.29; Fw maximum width 4.18; Hw maximum width 4.37; Pt length on Fw 1.60 and on Hw 1.62; length of metathoracic femur 3.93; metathoracic tibia 4.14.

**Female** (Fig. 40a): Largely pale brown in general appearance, head dark brown, labrum and clypeus with anterior border largely black (Fig. 26f), paler areas in the coxae and metepimeron, abdominal segments with anterior and posterior portions darker, distal portion of S7 tergum membranized yellow, tergum of S8 divided in triangular esclerites, valves of ovipositor extend posteriorly to cercus tip (Fig. 31f). Px more misaligned compared with the male ones (Fig. 29b).

*Measurements (mm):* Total length (incl. caudal appendages) 44.92; abdomen length (excl. caudal appendages) 37.63; head maximum width 4.88; Fw length 31.60; Hw length 31.90; Fw maximum width 6.32; Hw maximum width 6.54; Pt length on Fw 1.93 and on Hw 2.17; length of metathoracic femur 5.30; metathoracic tibia 5.40.

Larva. Unknown.

**Ecology and behavior.** In San Antonio, they are commonly seen perched on walls of small waterfalls, pending in lianas and roots due to their red and black color on the back of the abdomen, they are very difficult to spot.

**IUCN category:** This species is considered Least Concern (Bota- Sierra *et al.* 2021)

### Heteropodagrion varipes Daigle, 2014

(Figs. 5d, 9b, 15b, 20c, 36a)

*Heteropodagrion varipes* Daigle, 2014: 35 description of Holotype male, ECUADOR. Morona Santiago Province, N of Indanza 07-XI-1957, illustrations of cerci in dorsal view) in FSCA); Mauffray & Tenessen, 2019 (catálogo de Odonata do Equador).

Material examined: ECUADOR (2♂) Type locality, Morona Santiago, 15.7 km W of Macas on road to Guamote. (2°06'47" S 78° 18'24" W. 2567 m.a.s.l.) 2018 L. Perez leg., UARC.

**Diagnosis** Color pattern black-white, variation consistent with original description, material collected in the type locality. The diagnostic character for the

species is the prominent middle tooth on the inner edge of cercus (Fig. 20c). This sp. in FW it does not have additional sectors between RP2 and IR2, in HW it is just one cell (Fig. 15b).

*Measurements (mm):* Total length (incl. caudal appendages) 47.23; abdomen length (excl. caudal appendages) 39.05; cerci 1.13; head maximum width 5.13; Fw length 27.84; Hw length 28.21; Fw maximum width 5.06; Hw maximum width 5.24; Pt length on Fw 1.66 and on Hw 1.75; length of metathoracic femur 4.08; metathoracic tibia 4.40.

**IUCN category:** This species is considered Endangered (Mauffray and Tennessen 2020).

### 4.4.2 Family Mesagrionidae

### 4.4.2.1 Genus Mesagrion Selys, 1885

(Fig. 2b, 6, 11, 17b, 23d, 26b, 27g, 31, 32g, 39a, 43b)

Mesagrion Selys, 1885: 6 (Mesagrion leucorrhinum by original designation, description of Holotype male, Colombia, Bogotá, Cundinamarca Department); —Selys, 1886: 50 (key, additions to description, comparison with Heteropodagrion) ; — Kirby, 1890: 123 (mention in catalogue);—Ris, 1918: 76, 90 (additions to description, illustrations of caudal appendages in dorsal and lateral view); —Ris, 1918b: 188 (Mention); —Bridges, 1994 (Family, genus and species group catalogue) ; —Steinman, 1997: 153 (mention in catalog); —Paulson, 1983 (Odonata list for or country) ; —Garrison & Ellenrieder, 2005 (comparison with Neuragrion mysticum) ; —Heckman (2006: 220 Reproduction of illustrations from Garrison & von Ellenrieder, 2005); —Garrison et al., 2010: 92 (mention, key for Megapodagrionidae genera); Perez- Gutierrez & Palacino- Rodriguez, 2010: 209 (mention in Checklist); —Pérez-Gutierrez, 2011a (Key) —Pérez-Gutiérrez & Montes-Fontalvo (2011b: Description of female and larva); —Mauffray & Tenessen (2019: mention, reported in Ecuador).

Species account

### Mesagrion leucorrhinum Selys, 1885

(Figs. 1b, 2b, 5e, 10, 16b, 22d, 25b, 26g, 30, 31g, 36b, 40b, 43b)

Material Examined. Type material. Holotype, Colombia, Bogotá, Cundinamarca Department (15 ♂, 5 ♀) COLOMBIA, Cundinamarca Department, Guayabetal, Chirajara stream (04° 15' 45 "N 073° 49' 34" W alt. 1276 m.a.s.l), 13-X-2012. L.A. Pérez leg., UARC. COLOMBIA, (123, 39) Cundinamarca Departament, Alban, padre Luna farm, Sector bocatoma- cascadas (04° 53' 53" N 074° 25' 31" W 2000 ma.s.l) 15-X-2012. L.A. Pérez leg., UARC. COLOMBIA, (5 3) Antioquia, Departament, River Claro, 800 m.a.s.l, 15-X-2016. L.A. Pérez leg., UARC. COLOMBIA, (3 ♂, 1♀) Departamento de Putumayo, Mocoa, Parque Nacional Natural Churumbelos Aika- Wasi (1°09'03.43" N 76° 38'46.72" O alt. 613m.a.s.l) 19-I-2013. L.A. Pérez leg., UARC. COLOMBIA, (4∂, 1♀) Santander, Zapatoca Vereda Palo Blanco, Cuenca Quebrada El Ramo C. Bota leg., CEUA. COLOMBIA, (13) Departamento de Antioquia, Remedios Finca La Brillantina, Vereda La Cruz 1 C. Bota leg., CEUA. COLOMBIA, (1<sup>3</sup>) Departamento de Antioquia, Santo Domingo, Santiago locality, Vereda La Quiebra C. Bota leg., CEUA. COLOMBIA, (1<sup>(1</sup>) Antioquia, Maceo, Vereda San Pedro, Finca San Pedro, C. Bota leg., CEUA. COLOMBIA, (1<sup>(1</sup>) Antioquia, Anorí, Vereda El Zafiro, Finca EL Pital Leg. C. Bota leg., CEUA. 04-17-19; same data but (1<sup>2</sup>) La Forzosa reserve. COLOMBIA, (1<sup>2</sup>) Caldas, Norcasia El Tigre reserve. COLOMBIA, (1<sup>2</sup>) Meta, Bosque Bavaria; Holotype (only https://data.nhm.ac.uk/dataset/collection-specimens/resource/05ff2255-c38a-40c9images b657-4ccb55ab2feb/record/8980501), Mesagrion leucorrhinum COLOMBIA, (15♂, 5♀) Cundinamarca department, Guayabetal, Chirajara stream (04° 15' 45 "N 073° 49' 34" 1276 m.a.s.l.) 13-X-2012. L.A. Pérez leg., UARC.

#### Generic diagnosis (Adapted from Garrison et al., 2010)

**Description of male.** *Head* (Fig. 5e) Head black with white labrum, frons angulated, postclypeus white with brown medially, pale stripe transverse forward to medial ocellus, between ocelli dark brown and shiny, location of most posterior point of head at level of compound eyes, posterior region including occiput pale, with smoothly curve ridge dorsally between ocelli. Alive with compound eyes black dorsally and green ventrally.

**Thorax** (Fig. 10) Prothorax with posterolateral projections rounded with red spots in the middle lobe, synthorax largely black, mesepisternum with yellow stripes 0.35 width, mesepimeron black, dorsal portion of metepisternum crossed complete by

yellow stripe extends since metinfraepisternum to alar sinus. Distal portion of metapleural suture with yellow spot. Metepimeron largely black. Metasternum and posternum pale yellow.

*Legs* (Fig. 36b)

*Wing* (Fig. 2b) No accessory crossveins basal or distal to CuP, CuP closer to 1 Ax than 2; petiolation ending well beyond CuP for a distance as long as Cup or longer. two cells between quadrangle and subnodus in discoidal field of HW. RP3-4 recessed at subnodus in FW, just before in the HW. IR2 arising below 1th Px, two or more supplementary sectors between IR1 and RP2 and two supplementary sectors, no supplementary sectors between RP2 and IR2, two supplementary sectors between IR2 and RP3-4. Subcostal side of Pt just 1 underlying cells longer, costal side 0,5 as long or shorter as subcostal side.

**Abdomen** (Fig. 36b) Vivid red laterally, dark dorsally, thin black ring in each articulation of abdominal segments, ligula with inner fold well developed and paired latero-apical flagella cornua or lobes with bent tip (Fig. 25b). Dorsum of S10 of male a little depressed and flat. Male cercus forcipate, dorso inner edge with 10- 11 small denticles stiil cercus tip (Fig. 16b, 22d). Paraprocts as long as cercus with 8-9 serrulated and rounded pointed teeth.

*Measurements (mm):* Total length (incl. caudal appendages) 45.19; abdomen length (excl. caudal appendages) 37.58; cerci 1.20; head maximum width 4.85; Fw length 28.02; Hw length 27.96; Fw maximum width 5.0; Hw maximum width 5.07; Pt length on Fw 1.50 and 1.85 on Hw; length of metathoracic femur 3.72; metathoracic tibia 4.02.

**Rio Claro specimens**: Total length (incl. caudal appendages) 37.65; abdomen length (excl. caudal appendages) 31.80; cerci 1.02; head maximum width 4.42; Fw length 22.04; Hw length 21.83; Fw maximum width 4.42; Hw maximum width 4.40; Pt length on Fw 1.48 and 1.5 on Hw; length of metathoracic femur 3.09; metathoracic tibia 3.89.

*Female* (Perez-Gutierrez & Montes-Fontalvo, 2011) (Fig. 40b). Ovipositor not reaching posterior margin of S10, valves with minute denticles ventrad (Fig. 31g). Tergum of S8 almost completely divided into two triangular sclerites, females are dimorphic, apparently when teneral exhibit a similar male red coloration, although mature females with coloration like the male were also recorded.

Meta Department, Bavaria Forest specimens female: Total length (incl. caudal appendages) 29.46; abdomen length (excl. caudal appendages) 26.82; head maximum width 4.43; Fw length 24.69; Hw length 24.47; Fw maximum width 4.83; Hw maximum width 4.79; Pt length on Fw and 1.85 on Hw 1.48; length of metathoracic femur 3.44; metathoracic tibia 4.17.

**Larva.** (Fig. 1d): Description and other information's in Perez-Gutiérrez and Montes-Fontalvo (2011).

**Ecology and behavior**: The Rio Claro specimens were collected in creeks and rock walls in tributaries of Rio Claro Canyon River in low abundances, in Alban and Guayabetal the specimens were abundant along waterfalls, rock walls covered with mosses, lianas, liverworts and ferns, tandems were too common in different dates throughout the year.

**IUCN category:** This species is considered Least Concern (Bota-Sierra et al., 2016)

**Remarks:** There is no reference to the use of the name "*Heteropodagrion*" *leucorrhinum*, however it is worth noting that one of the Holotype labels has crossed out the generic name "*Heteropodagrion*" (Fig. 43b), perhaps before using the name *Mesagrion* Selys called the entire group of species under the generic name *Heteropodagrion*.

# KEY FOR IDENTIFICATION OF MALES OF HETEROPODAGRION- MESAGRION

- RP3-4 in FW arising at or little beyond subnodus, IR2 arising below 1<sup>st</sup> AX (Fig. 2b), long paraprocts dorsally serrulated (Fig. 16b) ..... *Mesagrion leucorrhinum*
- 3. (2) Supplementary sectors in FW between RP2- IR2 absent (Fig. 12) ...... 4
- 3'. Supplementary sectors in FW between RP2- IR2 present (Fig. 11) ...... 6
- 4. (3) Prominent teeth in the inner side of cerci (Fig. 20c) ...... H. varipes
- 4'. Inner side of cerci smooth (Fig. 20a-b) ..... 5
- 5. (4) Cercus curved and somewhat angled in 0.20 with a row of four small black denticles equal in size and equidistantly separated at outer edge of tip, plus two smaller on inner edge (Fig. 17b), Pterostigma totally light red (Fig. 12a), posternum largely yellow with small brown dark convex mark 0.75 distad ....... *H. diabolum* sp. nov.
- 6. (3) Inner edge of cercus tip with black crest of fused denticles (Fig. 17a), tip of ligula rounded with median cleft and short flagella laterally projected with extremes very concave (Fig. 23b), poststernum pale yellow without markings or spots (Fig. 6b). *H. cuyabri* sp. nov.

- (7) Outer dorsal edge of cercus with three neighbor denticles followed by 2-3 very small ones, inner edge with 4 black denticles grouped in pairs, black and yellow species (Fig. 17c) ...... *H. nigripes*

- 9'. Outer dorsal edge of cercus with major teeth alone without neighbor teeth ..... 10
- (9) Outer edge of cercus protruding free without teeth, inner edge with 5 small denticles (Fig. 20a-b), distal segment of ligula with carina bifurcated in middle, flagella latero-posteriorly directed backwards in 40°, with extreme concave and expanded (Fig. 25a)
- 10' Outer edge of cercus flattened, posterior medial dorsal edge with 4-minute teeth **(Fig. 19b)**, distal segment of ligula with tip bulky, flagella latero-posteriorly directed backwards in 45°, with extreme concave and expanded **(Fig. 24c)** ......*H. santuario* sp. nov.



Heteropodagrion croizati



Heteropodagrion cuyabri



Heteropodagrion donnellyi

**Fig. 4. Frontal and dorsal view of male head.** (*a*) *Heteropodagrion croizati.* (*b*) *Heteropodagrion cuyabri* **sp. nov**. (*c*) *Heteropodagrion diabolum* **sp. nov.** (*d*) *Heteropodagrion donnellyi* **sp. nov.** Photos by L.P.



Heteropodagrion paramillo



Heteropodagrion santuario



Heteropodagrion superbum



Heteropodagrion varipes



Mesagrion leucorrhinum

**Fig. 5.** Male, head: frontal and dorsal view. (*a*) *Heteropodagrion paramillo* **sp. nov.** (*b*) *Heteropodagrion santuario* **sp. nov.** (*c*) *Heteropodagrion superbum.* (*d*) *Heteropodagrion varipes.* (*e*) *Mesagrion leucorrhinum.* Photos by L.P



Heteropodagrion croizati

Heteropodagrion cuyabri

**Fig. 6.** Male, thorax: dorsal, ventral and lateral view. (*a*) *Heteropodagrion croizati*. (*b*) *Heteropodagrion cuyabri* **sp. nov**. Photos by L.P



**Fig. 7.** Male, thorax: dorsal, ventral and lateral view. (*a*) *Heteropodagrion diabolum* **sp. nov.** (*b*) *Heteropodagrion donnellyi* **sp. nov**. Photos by L.P



**Fig. 8.** Male, thorax: dorsal, ventral and lateral view. (*a*) *Heteropodagrion paramillo* **sp. nov.** (*b*) *Heteropodagrion santuario* **sp. nov.** Photos by L.P



**Fig. 9.** Male, thorax: dorsal, ventral and lateral view. (*a*) *Heteropodagrion superbum*. (*b*) *Heteropodagrion varipes.* Photos by L.P



**Fig. 10.** Male, thorax: dorsal, ventral and lateral view in *Mesagrion leucorrhinum*. Photos By L.P



Fig. 11. Male, wings: dorsal view. (a) *Heteropodagrion croizati*. (b) *H. cuyabri* sp. nov. Photos by L.P




Heteropodagrion diabolum



**Fig. 12.** Male, wings: dorsal view. (*a*) *Heteropodagrion diabolum* **sp. nov**. (*b*) *Heteropodagrion donnellyi* **sp. nov.** Photos by L.P



**Fig. 13.** Male, wings: dorsal view. (*a*) *Heteropodagrion paramillo* **sp. nov**. (*b*) *Heteropodagrion santuario* **sp. nov**. Photos by L.P



(a)

(b)



**Fig. 14.** Male, wings: dorsal view of *Heteropodagrion superbum (a)* (Colombia, Cauca: San Antonio, SAIA681) (*b*) (Colombia, Cauca: Anchicayá, CEUA94670). Photos by L.P



**Fig. 15.** Male, wings: dorsal view (a) *Heteropodagrion superbum*. (b) *Heteropodagrion varipes*. Photos by L.P



Heteropodagrion croizati



Mesagrion leucorrhinum

**Fig. 16.** Male, cerci: dorsal view and tip detail. (*a*) *Heteropodagrion croizati*. (*b*) *Mesagrion leucorrhinum.* Photos by L.P.



Heteropodagrion cuyabri



Heteropodagrion diabolum



**Fig. 17.** Male, cerci: dorsal view and tip detail. (*a*) *Heteropodagrion cuyabri* **sp. nov.** (*b*) *Heteropodagrion diabolum* **sp. nov.** (*c*) *Heteropodagrion nigripes* (Photo by J. Daigle). Photos by L.P



Heteropodagrion donnellyi



Heteropodagrion paramillo

**Fig. 18.** Male, cerci: dorsal view and tip detail. (*a*) *Heteropodagrion donnellyi* **sp. nov**. (*b*) *Heteropodagrion paramillo* **sp. nov.** Photos by L.P.



Heteropodagrion sanguinipes



**Fig. 19.** Male, cerci: dorsal view and tip detail. (*a*) *Heteropodagrion sanguinipes (*Extracted from Daigle, 2014). (*b*) *Heteropodagrion santuario* **sp. nov.** Photos by L.P.





Heteropodagrion varipes

**Fig. 20**. Male, cerci: dorsal view and tip detail. (a) *Heteropodagrion superbum*. (a) Type Locality in San Antonio, Cauca. (b) *Heteropodagrion superbum*. Rio Bravo (c) *Heteropodagrion varipes*. Photos by L.P.





Heteropodagrion diabolum

Heteropodagrion donnellyi

**Fig. 21**. Male, cerci: Scanning electron micrographs showing sculpture of cerci. (*a*) *Heteropodagrion croizati*. (*b*) *Heteropodagrion cuyabri* **sp. nov.** (*c*) *Heteropodagrion diabolum* **sp. nov.** (*d*) *Heteropodagrion donnellyi* **sp. nov.** 



Heteropodagrion superbum

Mesagrion leucorrhinum

**Fig. 22**. Male, cerci: Scanning electron micrographs showing sculpture of cerci. (*a*) *Heteropodagrion paramillo* **sp. nov**. (*b*) *Heteropodagrion santuario* **sp. nov**. (*c*) *Heteropodagrion superbum*. (*d*) *Mesagrion leucorrhinum*.



Heteropodagrion croizati



Heteropodagrion cuyabri



Heteropodagrion diabolum

**Fig. 23.** Scanning electron micrographs showing sculpture of male ligula: ectal, tip detail and lateral views. (*a*) *Heteropodagrion croizati*. (*b*) *Heteropodagrion cuyabri* **sp. nov**. (*c*) *Heteropodagrion diabolum* **sp. nov**.



Heteropodagrion donnellyi



Heteropodagrion paramillo



Heteropodagrion santuario

**Fig. 24.** Scanning electron micrographs showing sculpture of male ligula: ectal, tip detail and lateral views. (a) *Heteropodagrion donnellyi* **sp. nov.** (b) *Heteropodagrion paramillo* **sp. nov**. (c) *Heteropodagrion santuario* **sp. nov**.



Heteropodagrion superbum



Mesagrion leucorrhinum

**Fig. 25.** Scanning electron micrographs showing sculpture of male ligula: ectal, tip detail and lateral views. (*a*) *Heteropodagrion superbum.* (*b*) *Mesagrion leucorrhinum.* 





(d)

(a)

Mesagrion leucorrhinum =

**Fig. 26.** Female, head: frontal view. (a) *Heteropodagrion croizati.* (b) *Heteropodagrion cuyabri* **sp. nov**. (c) *Heteropodagrion donnellyi* **sp. nov**. (d) *Heteropodagrion paramillo* **sp. nov**. (e) *Heteropodagrion santuario* **sp. nov**. (f) *Heteropodagrion superbum.* (g) *Mesagrion leucor rhinum.* Photos by L.P.





**Fig. 27.** Female, left pair wings: dorsal view. (*a*) *Heteropodagrion croizati*. (*b*). *Heteropodagrion cuyabri* **sp. nov**. Photos by L.P.







Heteropodagrion paramillo

**Fig. 28.** Female, left pair wings: dorsal view. (*a*) *Heteropodagrion donnellyi* **sp. nov.** (*b*) *Heteropodagrion paramillo* **sp. nov.** Photos by L.P.



**Fig. 29.** Female, left pair wings: dorsal view. (*a*) *Heteropodagrion santuario* **sp. nov.** (*b*) *Heteropodagrion superbum.* Photos by L.P.

Heteropodagrion superbum



Fig. 30. Female, left pair wings: dorsal view in Mesagrion leucorrhinum. Photos by L.P.



**Fig. 31.** Female, ovipositor: lateral view. (*a*) *Heteropodagrion croizati*. (*b*) *Heteropodagrion cuyabri* **sp. nov**. (*c*) *Heteropodagrion donnellyi* **sp. nov**. (*d*) *Heteropodagrion paramillo* **sp. nov**. (*e*) *Heteropodagrion santuario* **sp. nov**. (*f*) *Heteropodagrion superbum.* (*g*) *Mesagrion leucorrhinum*. Photos by L.P.

Mesagrion leucorrhinum=



Heteropodagrion croizati



Heteropodagrion cuyabri =

**Fig. 32.** Habitus of live specimens. Male. (*a*) *Heteropodagrion croizati* (Colombia, Putumayo: Serrania de los Churumbelos Aika Wasi). (*b*) *Heteropodagrion cuyabri* **sp. nov** (Colombia, Quindio: Private reserve Brehmen). Photos by L.P.



Heteropodagrion diabolum



Heteropodagrion donnellyi

**Fig. 33.** Habitus of live specimens. Male. (*a*) *Heteropodagrion diabolum* **sp. nov.** (Colombia, Chocó: Tutunendo, El Diablo Stream). (*b*) *Heteropodagrion donnellyi* **sp.nov.** (Panamá, Ciudad de Panamá: Cerro Azul, Agua fría waterfalls LABSIA650). Photos by L.P.



Heteropodagrion paramillo



Heteropodagrion sanguinipes =

**Fig. 34.** Habitus of live specimens. Male. (*a*) *Heteropodagrion paramillo* **sp. nov.** (Colombia, Antioquia: Via Juntas de Uramita- Peque). Photo by L.P. (*b*) *Heteropodagrion sanguinipes* (Ecuador, Quito: Santo Domingo de los Tsachilas Province). Photo by K. Tennessen, 2010 extracted of Mauffray and Tennessen, 2019.)



Heteropodagrion santuario



Heteropodagrion superbum -

**Fig. 35.** Habitus of live specimens. Male. (*a*) *Heteropodagrion santuario* **sp. nov.** (*Colombia: Risaralda, Santuario*). (*b*) *Heteropodagrion superbum* (Colombia, Cauca: Cali, San Antonio, Km 18 via Dagua). Photos by L.P.



Heteropodagrion varipes



**Fig. 36.** Habitus of live specimens. Male (*a*) *Heteropodagrion varipes* male in hand (Ecuador, Morona Santiago: Abanico stream LABSIA319). (*b*) *Mesagrion leucorrhinum.* (Colombia: Rio Claro, Antioquia) Photos by L.P.



Heteropodagrion croizati



Heteropodagrion cuyabri

**Fig. 37.** Habitus of live specimens. Female (*a*) *Heteropodagrion croizati* (Colombia, Putumayo: Serrania de los Churumbelos Aika Wasi). (*b*)*Heteropodagrion cuyabri* **sp. nov.** (Colombia, Quindio: Filandia, Hacienda Brehmen, Barvas River). Photos by L.P.



Heteropodagrion donnellyi

**Fig. 38.** Habitus of live specimens. Female (*a*) *Heteropodagrion diabolum* **sp. nov.** (Colombia, Chocó: Tutunendo, El Diablo Stream). (*b*) *Heteropodagrion donnellyi* **sp. nov.** (Panamá, Ciudad de Panamá: Cerro Azul, Agua fría waterfalls). Photos by L.P.



Heteropodagrion paramillo



Heteropodagrion santuario

**Fig. 39.** Habitus of live specimens. Female. (*a*) *Heteropodagrion paramillo* **sp. nov.** (Colombia, Antioquia: Via Juntas de Uramita- Peque). (*b*) *Heteropodagrion santuario* **sp. nov** A: Female laying eggs under leaf (Colombia, Risaralda, Santuario, Rio San Rafael). Photos by L.P.



Heteropodagrion superbum



Mesagrion leucorrhinum=

**Fig. 40.** Habitus of live specimens. Female (*a*) *Heteropodagrion superbum*. Colombia, Cauca: Cali, San Antonio, Km 18 via Dagua). (*b*) *Mesagrion leucorrhinum*. (Colombia, Cundinamarca, Alban). Photos by L.P.



**Fig. 41.** Habitus in lateral view of males of *Heteropodagrion*. (*a*) holotype of *Heteropodagrion cuyabri* **sp. nov.** (*b*) holotype of *Heteropodagrion diabolum* **sp. nov.** (*c*) holotype of *Heteropodagrion donnellyi* **sp. nov.** (*d*) holotype of *Heteropodagrion paramillo* **sp. nov.** (*e*) holotype of *Heteropodagrion santuario* **sp.nov.** (*f*) *Heteropodagrion superbum.* Photos by L.P.



**Fig. 42.** Labels of name-bearing specimens of *Heteropodagrion sanguinipes.* (*a*) Type series (2a, 29) in the box collection in ISRN. (*b*) Lectotype. (*c*) syntype female. (*d*) syntype male. (Photos by A. Pinto)



**Fig. 42.** Labels of name-bearing specimens of *Heteropodagrion sanguinipes.* (*a*) Type series  $(2\sigma, 2\varphi)$  in the box collection in ISRN. (*b*) Lectotype. (*c*) syntype female. (*d*) syntype male.

## 4.5 Discussion

### 4.5.1 Morphological data

After examination of specimens of the putative species of *Heteropodagrion*, it is concluded that they are remarkably similar in appearance and structure of the male's caudal appendages, but noticeable characters permit diagnoses the species groups. Cerci are more direct source of differentiation, the row of denticles on the median-posterior edge can be single or paired. Garrison & Ellenrieder (2005) examined the syntypes of *H. sanguinipes* and *H. superbum* and stated that they could be synonyms without making this designation formally.

In this work, analyzes are consistent in showing that *H. superbum* is a consistent valid species from Cauca with variable populations in the color phenotype and size. In the original description, Ris (1918) mentions the red color of the abdomen, however there is a notorious difference between the red of San Antonio (type locality) and the black morph of Anchicayá and Calima (Fig. 20a-b).

Analysis also shows that *H. santuario* **sp. nov.** is distinguishable from the populations of Cauca (*H. superbum*) with the color pattern of the ventral thorax (Fig. 8b) and the species *H. cuyabri* **sp. nov.** which is part of the *superbum* complex, in this way the superbum complex is configured by *H. superbum*, *cuyabri* **sp. nov.**, and *santuario* **sp. nov**).

New *Heteropodagrion* species have been previously misidentified as *H. superbum* due to the similarity mainly of the cercus, (i.e *H.* cf. *superbum* Donnelly, 1992). Herein *H. paramillo* **sp. nov.** is not considered as part of the *superbum* complex because of its distinct cercus morphology, according to complex of species sensu Bickford et al., (2006), therefore difficult to diagnose (Días Moreira et al., 2022). Morphological stasis has been claimed to explain this pattern (Sanchez et al., 2014; Pfeninger & Schwenk, 2007).

# 4.5.2 Species delimitation

Analyses based on COI solved better and more effectively than ITS2 the internal affiliations between *Heteropodagrion* spp. and *Mesagrion* and give full solved

delimitation between species. *Heteropodagrion* and *Mesagrion* showed intrageneric genetic distances ~20-28% in accordance with reference values for this level in other studies in Odonata (Pimenta et al., 2021).

Intraspecific distances in *Heteropodagrion* ranked between 0- 1% according to general values in another with studies with zygopterans; *Calopteryx* Leach, 1815 max. (8%); *Forcepcioneura* (<2%) (Pimenta et al., 2021); in Anisopterans as *Cordulegaster* (5-6%) (Sneider et al., 2021); *Palpopleura* (0.6-5.3%) Mitchell & Samways 2005). ITS2 failed mainly in separating *H. superbum* and *H. santuario* **sp. nov;** in all other clusters both topologies solved efficiently the same groups, thus congruence was achieved.

The clade *M. leucorrhinum* showed genetic intraspecific distances of 2-8 % (COI) and 1-5% (ITS2) so here we continue to consider *Mesagrion* as a single species in the entire distributional range studied, these genetic distances are slightly greater than averages recorded by other groups with incipient speciation, such as the andean *Polythore procera* (Selys, 1869) with ~3% K2P intraspecific distances (Sanchez *et al.* 2010); as too in *Xantocnemis tuanuii* Rowe, 1987 (Marynov *et al.* 2016); 5% in *Megalestes* Selys, 1862 (Yu & Xue, 2020); between 0– 1.65% in *Paracercion* Weekers & Dumont, 2004 (Zhang et al., 2021); and Anisopterans as *Palpopleura* Rambur, 1842 (Mitchel & Samways, 2005).

Species concepts are a "framework" rather than a theoretical imposition to be assumed when speciation processes have not taken place in a way that consequently produces divergence between metapopulations (Burbrink & Ruane, 2021; Winston, 1999). Species delimitation methods can detect fringes of the so-called "grey area", even species can have an independent evolutionary trajectory and maintain even a slight gene flow, a product of their ancestry/descent, in some parts of their distribution range according to de Queiroz (1998, 2007).

Hence the barcode region in COI marker by itself is not a guarantee of efficient delimitation (e.g., Marynov et al., 2016; Vilela et al., 2019; Vega-Sanchéz et al., 2019) it must be used in conjunction with other data sources in groups with complex taxonomy when species delimitation is the main purpose (Bourguignon et al., 2013; Zhang et al., 2021; Jones et al., 2022). It is a mistake to think of it as the last instance of the taxonomic cycle (Meier et al., 2021; Yu et al., 2015).

Garrison et al., (2010 p. 92) suggests the possibility of considering *H. superbum* as a synonym of *H. sanguinipes* based in high similarity in cercus morphology. To rule out the effects of morphological variation due to geographic gradients, an attempt was

made to cover the entire distribution range of the *superbum* complex. In the separate and combined analyses these sequences were grouped together suggesting color polymorphism in *H. superbum*. For its part, *H. sanguinipes* (accession number genbank AGY95128) appears divergent with respect to *H. superbum* (0.18 in COI) confirming the primary morphological hypothesis of valid species.

The use of DNA for species delimitation has shown that the rate of introgression between mtDNA>nDNA, then, combined ITS and morphological data have resolved species delimitation because it avoids bias from possible introgression (Da Silva et al., 2017; Zhang et al., 2021). The use of a single locus does not allow to carry out a critical delimitation, it is highly probable to assume limits that only explain the history of the gene (in part only) (De Queiroz, 2007).

# 4.6 Conclusions

Finally, using an integrative approach based on morphological and molecular data we were able to propose new taxa within the *Heteropodagrion* and confirm with multiple evidence that *H. cuyabri* **sp. nov.**, *H. diabolum* **sp. nov.**, *H. donnellyi* **sp. nov**., *H. paramillo* **sp. nov**., *H. santuario* **sp. nov**. and *H. superbum* are distinct evolutionary species, therefore, they are objectively valid.

On the other hand, the closely relationship among species is revealed, species groups can be addressed as follows: *H. croizati* + *H. varipes*; *H. paramillo* + *H. donnellyi*, and the group conformed for *H. santuario* **sp. nov.** + *H. superbum* with *H. cuyabri* **sp. nov** as species more closely related.

Nominate new species in *Heteropodagrion* was possible because we combined at least two sources of molecular data, which made it possible to reconcile species hypotheses in addition to the morphological study of type material from 4 of the 5 valid species. Previous dubious taxa were established, diagnosed, and described.

Diagnosing species and naming taxa were a problematic task in this group but were confidently done with the addition of molecular evidence from different cellular compartments (mtDNA and nDNA), which drastically overcame the impediment for discriminate the species.

Our study covering the entire range of distribution of *Mesagrion* and *Heteropodagrion* reveals that individual molecular partitions are not conclusive to

resolve phylogenetic relationships among species, thus a concatenated analysis with morphological examination was necessary for the accurate delimitation of species.

In an integrative protocol, sequence concatenation was determinant for closing our taxonomic cycle of the taxonomic cycle, inclusion of both nuclear marker ITS2 and mitochondrial COI for species delimitation allowed revealing possible scenarios of ancestry/descendance between these genera.

The evolutionary history of the genes (COI and ITS2) herein seems to correspond to that of species scrutinized with two types of delimitation methods, both concluded same limits between the *Heteropodagrion* and *Mesagrion* clade. However, *Mesagrion leucorrhinum* showing intraspecific divergences >4% in COI deserve future analyses to clarify the diversity in the clade.

Decisions based on the concatenated analysis confirmed the status *H. superbum* and *H. sanguinipes* as valid species. However, the concept of "complex of species" for *Heteropodagrion superbum* clade is clarified, as the species is morphologically variable and exhibits different morphs along its distribution range.

#### Acknowledgements

The authors are indebted to all curators of collections and odonatologist colleagues that made this study possible, special thanks are due to Jerome Constant (RBINS), Rosser Garrison (CDFA), and Jürg De Marmels (MIZA). Special thanks to the professional guide Fabian Cuesta and Mr Tito Cuesta, owner of the farm where the Agua Fria waterfalls are located, for their hospitality during the days of stay at Cerro Azul in Altos de Pacora, Panama. This study was partially supported by grants to APP by the project "Insetos e a metrópole" (Edital #02/2020, #06/2021, #04/2023 - PESQUISA/PRPPG/UFPR, Apoio a Atividades de Pesquisa); TaxOnline - Network of Biological Collections of Paraná through grant by NAPI - Fundação Araucária de Apoio ao Desenvolvimento Científico e Tecnológico do Estado do Paraná (FA, process #103/2020). DMT is a research productivity fellow from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Proc. 314557/2021-0) and a Cientista do Nosso Estado fellow from Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ, Proc. E-26/200.503/2023).
#### 4.7 References

- Azevedo JAR, Guedes TB, Nogueira CdC, Passos P, Sawaya RJ, Prudente ALC, Barbo FE, Strüssmann C, Franco FL, Arzamendia V, Giraudo AR, Argôlo AJS, Jansen M, Zaher H, Tonini JFR, Faurby S, Antonelli A. 2020 Museums and cradles of diversity are geographically coincident for narrowly distributed Neotropical snakes. *Ecography*, 43: 328–339. doi: <u>10.1111/ecog.04815</u>
- Brower AVZ, Schuh RT. 2021 Biological systematics: Principles and applications, 3rd. Edition. Ithaca: Cornell University Press.
- Bybee SM, Ogden TH, Branham, MA, Whiting, MF. 2008 Molecules, morphology and fossils: a comprehensive approach to odonate phylogeny and the evolution of the odonate wing. Cladistics 23, 1–38.doi:
- Bybee S, Kalkman V, Erickson J, Frandsen PB, Breinholt, JW, Suvorov A, Dijkstra KDB, Cordero-Rivera A, Skevington JH, Abbott JC, Sanchez-Herrera M, Lemmon AR, Lemmon E, Ware JL. 2021 Phylogeny and classification of Odonata using targeted genomics, *Molecular Phylogenetics and Evolution* **160**, 107115. <u>https://doi.org/10.1016/j.ympev.2021.107115</u>.
- Bourguignon T, Šobotník J, Hanus R, Krasulová J, Vrkoslav V, Cvacka J, Roisin Y. 2013 Delineating species boundaries using an iterative taxonomic approach: The case of soldierless termites (Isoptera, Termitidae, Apicotermitinae). *Molecular Phylogenetics and Evolution* **69**, 694–703.
- Bota-Sierra C, Palacino F, Rache L, Tennessen K Mauffray W. 2016 Heteropodagrion croizati. The IUCN Red List of Threatened Species 2016:
  e.T49254609A49256265. https://dx.doi.org/10.2305/IUCN.UK.2016-1.RLTS.T49254609A49256265.en. Accessed on 04 November 2023.
- Bota-Sierra, C., Palacino, F., Rache, L., Ellenrieder, N. Paulson, D. 2016 *Mesagrion leucorrhinum*. The IUCN Red List of Threatened Species 2016: e.T60294A66887433. https://dx.doi.org/10.2305/IUCN.UK.2016-1.RLTS.T60294A66887433.en. Accessed on 04 November 2023.
- Bota-Sierra, CA, Montes-Fontalvo JM, Perez L, Sandoval-H J Florez C. 2021 *Heteropodagrion superbum*. The IUCN Red List of Threatened Species 2021: e.T49254451A49256269. https://dx.doi.org/10.2305/IUCN.UK.2021-2.RLTS.T49254451A49256269.en. Accessed on 04 November 2023.

- Clement M, Snell Q, Walker P, Posada D, Crandall, K. 2002 TCS: Estimating gene genealogies. Parallel and Distributed Processing Symposium, International Proceedings, 2, 184.
- Bridges CA. 1994 Catalogue of the family-group, genus-group, and species-group names of the Odonata of the world (Third Edition). *Urbana*, xlvi+905 pp.
- Calvert P. 1913 The fossil Odonate *Phenacolestes,* with a discussion of the venation of the legion *Podagrion* Selys. *Proceedings of the Academy of Natural Sciences of Philadelphia* **65**, 225–272.
- Clausnitzer V, Kalkman V, Ram M, Collen B, Baillie J, Bedjanic M, Darwall W, Dijkstra K, Dow R, Hawking J, Karube H, Malikova E, Paulson D, Schütte K, Suhling F, Villanueva RJ, Ellenrieder N, Wilson K. 2009 Odonata enter the biodiversity crisis debate: The first global assessment of an insect group. *Biological Conservation*. 142. 1864–1869. 10.1016/j.biocon.2009.03.028.
- Cordero A, Andrés JA. 1996 Colour polymorphism in Odonates: females that mimic males? *Journal of the British Dragonfly Society* **12**: 50–60.
- Cordero-Rivera A. Lorenzo- Carballa MO. 2010 Three sisters in the same dress: cryptic speciation in African odonates. *Molecular Ecology*, 19: 3840 3841. <u>https://doi.org/10.1111/j.1365-294X.2010.04721.x</u>
- Cowley J. 1941 The descriptive terms applied to the pterothorax and penis of odonata. *Proceedings Royal Entomological Society of London*. (*B*) 10(1): 5-7
- Daigle JJ. 2014 Two new *Heteropodagrion* species from Ecuador (Odonata: Megapodagrionidae) *Odonatologica* **43**, 35–42.
- Davies DAL, Tobin P. 1984 The dragonflies of the world: a systematic list of the extant species of Odonata. Vol 1 Zygoptera, Anisozygoptera. *Societas Internationalis Odonatologica Rapid Communications* **3**, 1–127.
- De Marmels JC. 2001 Revision of *Megapodagrion* Selys, 1886 (*Insecta, Odonata: Megapodagrionidae*). Dissertation, Universität Zürich, Zürich.
- Deng J, Guo Y, Cheng Z, Lu C, Huang X. 2019 The Prevalence of Single-Specimen/Locality Species in Insect Taxonomy: An Empirical Analysis. *Diversity*, **11**, 106. https://doi.org/10.3390/d11070106
- Dijkstra KDB, Bechly G, Bybee SM, Dow RA, Dumont HJ, Fleck G, Garrison RW, Hämäläinen M, Kalkman VJ, Karube H, May ML, Orr AG, Paulson DR, Rehn AC, Theischinger G, Trueman JWH, Tol JV, Von Ellenrieder N, Ware J. 2013 The classification and diversity of dragonflies and damselflies (Odonata). In: Zhang,

Z.-Q. (Ed.) Animal Biodiversity: An Outline of Higher-level Classification and Survey of Taxonomic Richness (Addenda 2013). *Zootaxa* **3703**, 36–45. <u>https://doi.org/10.11646/zootaxa.3703.1.9</u>

- Dijkstra KDB, Kalkman VJ, Dow RA, Stokvis FR, Van Tol, J. 2014 Redefining the damselfly families: a comprehensive molecular phylogeny of Zygoptera (Odonata). *Systematic Entomology* **39**, 68–96.
- Djan M, Ståhls G, Velickovic N, Acanski J, Obreht VD, Rojo S, Pérez-Bañón C, Radenkovic S, Vujic A. 2020 The *Merodon planifacies* subgroup (Diptera, Syrphidae): Congruence of molecular and morphometric evidence reveal new taxa in Drakensberg mountains valleys (Republic of South Africa). *Zoologischer Anzeiger* 287, 105–120.
- Donnelly, TW. 1992 The Odonata of central Panamá and their position in the Neotropical odonate fauna, with a checklist, and descriptions of new species. In Insects of Panama and Mesoamerica: selected studies. Edited by D. Quintero and A. Aiello. Oxford University Press, Oxford. pp. 52– 90.
- Dupuis JR, Roe AD, Sperling FA. 2012 Multi-locus species delimitation in closely related animals and fungi: one marker is not enough. *Molecular Ecology*. (18): 4422-4436. doi: 10.1111/j.1365-294X.2012.05642.x.
- Folmer O, Black Mb, Hoeh Wr, Lutz R, Vrijenhoek R. 1994 DNA primers for amplification of mitochondrial Cytochrome C oxidase subunit I from diverse metazoan invertebrates. *Molecular marine biology and biotechnology.* 3: 294-9.
- Garrison RW, Von Ellenrieder N. 2005 *Neuragrion mysticum* (Odonata: Megapodagrionidae) demystified. *Canadian Entomologist* **137**, 169–163.
- Garrison RW, Von Ellenrieder N, Louton JA. 2010 Damselfly genera of the New World. An Illustrated and Annotated Key to the Zygoptera. The Johns Hopkins University Press xiv + 490 pp, + 24 pls.
- Guo B, Kong L. 2022 Comparing the Efficiency of Single-Locus Species Delimitation Methods within Trochoidea (Gastropoda: Vetigastropoda). *Genes 13*, 2273. https://doi.org/10.3390/genes13122273
- Hillis D, Chambers E, Devitt T. 2021 Contemporary Methods and Evidence for Species Delimitation. *Ichthyology & Herpetology*. 109. 10.1643/h2021082.
- Jones R, Brock PD, Mantovani B, Beasley-Hall P, Yeates DK., Lo N. 2022 Integrative taxonomy of the stick insect genus *Austrocarausius* Brock, 2000 (Phasmatodea:

Lonchodidae) reveals cryptic species in remnant Queensland rainforests. *Invertebrate Systematics* **36**, 849-873.

- Kalkman VJ, Choong ChY, Orr AG, Schütte K. 2010 Remarks on the taxonomy of Megapodagrionidae with emphasis on the larval gills (Odonata) *International Journal of Odonatology* **13**, 119–135.
- Kalyaanamoorthy S, Quang BM, Wong TKF, von Haeseler A, Jermiin LS. 2017 ModelFinder: Fast model selection for accurate phylogenetic estimates. Nature Methods, 14:587–589. https://doi.org/10.1038/nmeth.4285.
- Katoh K, Rozewicki J, Yamada KD. 2019 MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization, *Briefings in Bioinformatics*, Volume 20, Issue 4, July, Pages 1160–1166, <u>https://doi.org/10.1093/bib/bbx108</u>
- Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M. Sturrock S. Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B. Meintjes P, Drummond A. 2012 Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, 28(12), 1647– 1649. https://doi.org/10.1093/bioinformatics/bts199
- Karsch F. 1891 Neue Odonaten von Ecuador. Societas Entomologica 6, 105.
- Kirby WF. 1890 A Synonymic Catalogue of Neuroptera Odonata, or Dragonflies, with an Appendix of Fossil Species. Guerney and Jackson, London, 202 pp. <u>https://doi.org/10.5962/bhl.title.5534</u>.
- Lam-Tung, Heiko AS, von Haeseler A, Quang Minh B. 2015 IQ-TREE: A fast and effective stochastic algorithm for estimating maximum likelihood phylogenies. *Molecular Biology Evolution*, 32:268-274. <u>https://doi.org/10.1093/molbev/msu300</u>
- Lorenzo-Carballa MO, Garrison, RW, Encalada AC, Cordero-Rivera A. 2020 Darwin Returns to the Galapagos: Genetic and Morphological Analyses Confirm the Presence of *Tramea darwini* at the Archipelago (Odonata, Libellulidae). *Insects, 12*.
- Marinov M, Amaya-Perilla C, Holwell GI, Varsani A, Van Bysterveldt K, Kraberger S, Stainton D, Dayaram A, Curtis N, Cruickshank RH, Paterson A. 2016 Geometric morphometrics and molecular systematics of *Xanthocnemis sobrina* (McLachlan, 1873) (Odonata: Coenagrionidae) and comparison to its congeners. *Zootaxa* 4078, 84–120.

- Mauffray WF, Tenessen KJ. 2019 A catalogue and historical study of the Odonata of Ecuador. *Zootaxa*, **4628**, 001–265.
- Mauffray W. Tennessen K. 2020 Heteropodagrion nigripes. The IUCN Red List of Threatened Species 2020: e.T119974946A119974959.
   https://dx.doi.org/10.2305/IUCN.UK.2020-2.RLTS.T119974946A119974959.en.
   Accessed on 04 November 2023.
- Mauffray, W, Tennessen, K. 2020 Heteropodagrion varipes. The IUCN Red List of Threatened Species 2020: e.T119975038A119975059.
  https://dx.doi.org/10.2305/IUCN.UK.2020-2.RLTS.T119975038A119975059.en.
  Accessed on 04 November 2023.
- McLachlan R. 1897 *Thaumatoneura inopinata*, a new genus and species of Calopteryginae. *The Entomologist Monthly Magazine* (2)8: 130-131.
- Meier R, Blaimer BB, Buenaventura HE, Vonrintelenb T, Srivathsana A, Yeo D. 2021 A re-analysis of the data in Sharkey *et al.*'s (2021) minimalist revision reveals that BINs do not deserve names, but BOLD Systems needs a stronger commitment to open science. *Cladistics*,1–12.
- Miller MA., Pfeiffer W. Schwartz T. 2010 "Creating the CIPRES Science Gateway for inference of large phylogenetic trees". In: *Proceedings of the Gateway Computing Environments Workshop* (GCE), 14 Nov. 2010, New Orleans, LA pp 1 - 8. DOI: 10.1109/GCE.2010.5676129
- Mitchell A, Samways MJ. 2005 The morphological 'forms' of *Palpopleura lucia* (Drury) are separate species as evidenced by DNA sequencing Anisoptera: Libellulidae). *Odonatologica* **34**, 173–178.
- Orr MC, Ferrari RR, Hughes AC. 2021 Taxonomy must engage with new technologies and evolve to face future challenges. *Nature Ecology and Evolution* **5**, 3–4 https://doi.org/10.1038/s41559-020-01360-5
- Ortega- Salas H, Gonzalez- Soriano E, Jocque, M. 2022 Untangling the waterfall damsels: a review of the Mesoamerican genus *Paraphlebia* Selys in Hagen, 1861 (Odonata: Thaumatoneuridae) with descriptions of 11 new species. *Zootaxa* 5089, 001–066.
- Osozawa S, Sato F, Wakabayashi J. 2017 Quaternary Vicariance of Lotic *Coeliccia* in the Ryukyu-Taiwan Islands Contrasted with Lentic *Copera*. *Journal of Heredity*, 280–287 doi:10.1093/jhered/esx007

- Pinto ÂP. 2024 Cap. 15, Odonata Fabricius, 1793, pp. 187-233. In: Rafael, J.A.; Melo, G.A.R.; Carvalho, C.J.B. de; Casari, S. & Constantino, R. (eds). Insetos do Brasil: Diversidade e Taxonomia. 2<sup>a</sup> ed. Instituto Nacional de Pesquisas da Amazônia, Manaus. 880 pp. https://doi.org/10.61818/56330464c15
- Pinto ÂP, Bota-Sierra CA, Marinov M. 2022 Species identification and description, in: Cordoba-Aguilar A, Beatty Ch, Bried J (eds), Dragonflies and Damselflies: Model Organisms for Ecological and Evolutionary Research, 2nd edn (Oxford, 2022; online edn, Oxford Academic, 15 Dec. 2022) https://doi.org/10.1093/oso/9780192898623.003.0019, accessed 13 Nov. 2023.
- Pinto, ÂP. 2016 A fauna de libélulas da América do Sul: a última fronteira a ser desvendada. *Informativo Sociedade Brasileira de Zoologia*. 117. 7-9.
- Pfenninger M, Schwenk K. 2007 Cryptic animal species are homogeneously distributed among taxa and biogeographical regions. BMC Evol Biol 7: 121. BMC evolutionary biology. 7. 121. 10.1186/1471-2148-7-121.
- Pérez-Gutiérrez LA, Montes-Fontalvo JM. 2011a *Heteropodagrion croizati* **sp. nov.** (Odonata: Megapodagrionidae) with a key to the known species of the genus. *Zootaxa* **2810**, 63–68.
- Pérez-Gutiérrez LA, Montes-Fontalvo J. 2011b Rediscovery of Mesagrion leucorrhinum (Zygoptera: Megapodagrionidae): a "formal" description of female and ultimate stadium of larva with notes on habits. International Journal of Odonatology, 14, 91–100.
- Pimenta AL, Pinto A, Takiya D. 2019 Integrative taxonomy and phylogeny of the damselfly genus *Forcepsioneura* Lencioni, 1999 (Odonata: Coenagrionidae: Protoneurinae) with description of two new species from the Brazilian Atlantic Forest. Arthropod Systematics and Phylogeny 77, 397–415.
- Pons J, Barraclough TG, Gomez-Zurita J, Cardoso A, Duran DP, Hazell S, Kamoun S, Sumlin WD. 2006 Sequence-Based Species Delimitation for the DNA Taxonomy of Undescribed Insects. *Systematic Biology* **55**, 595–609.
- Puillandre N, Lambert A, Brouillet S, Achaz G. 2012 ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology* 21, 1864–1877. doi: 10.1111/j.1365–294X.2011.05239.x.
- Rácenis J. 1959 Notas taxonomicas sobre la familia Megapodagrionidae (Odonata: Zygoptera) con las espécies venezolanas. *Acta Biologica Venezuelica* 2, 335– 367.

- Riek ER, Kukalová-Peck J. 1984 A new interpretation of dragonfly wing venation based upon Early Upper Carboniferous fossils from Argentina (Insecta: Odonatoidea) and basic character states in pterygote wings. *Canadian Journal of Zoology* 62(6): 1150-1 166.
- Ris F. 1918 Libellulin (Odonata) aus der Region der amerikanischen Kordilleren von Costarica bis Catamarca.: *Archiv für Naturgeschichte (Abteilung A)* **82**, 1–197.
- Sánchez- Herrera M, Realpe E, Salazar C. 2010 A Neotropical polymorphic damselfly shows poor congruence between genetic and traditional morphological characters in Odonata, *Molecular Phylogenetics and Evolution*, 57( 2): 912-917, https://doi.org/10.1016/j.ympev.2010.08.016.
- Sánchez- Guillén R, Ceccarelli S, Villalobos F, Neupane S, Rivas- Torres A, Sanmartín-Villar I, Wellenreuther M, Bybee S, Velasquez-Vélez M, Realpe E, Chávez-Ríos J, Dumont H, Cordero-Rivera A. 2020 The evolutionary history of colour polymorphism in *Ischnura* damselflies (Odonata: Coenagrionidae). *Odonatologica*. 49: 333-370. 10.5281/zenodo.4268559.
- Selys-Longchamps E. 1862 Synopsis des *Agrionines*. 3e legion: *Podagrion. Bulletin Academie Royale Belgique* Serie **2**, 5–44.
- Selys-Longchamps E De. 1885 Programme d'une revision des Agrionines. *Comptes Rendus de la Societe Entomologique de Belgique* **29**, cxli– cxlvi (1–8 separate).
- Selys-Longchamps E De. 1886 Revision du Synopsis des Agrionines. Première partie comprenant les légions *Pseudostigma–Podagrion–Platycnemis* et *Protonevra*. *Memoires couronnes et autres memoires, l'Academie royale de medecine de Belgique* 38, 1–233.
- Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P. 1994 Evolution, Weighting, and Phylogenetic Utility of Mitochondrial Gene Sequences and a Compilation of Conserved Polymerase Chain Reaction Primers, *Annals of the Entomological Society of America*, 87, 651-701, <u>https://doi.org/10.1093/aesa/87.6.651</u>
- Schneider T, Vierstraete A, Müller O, Van Pelt GJ, Caspers M, Ikemeyer D, Snegovaya N, Dumont HJ. 2021 Taxonomic Revision of Eastern Part of Western Palaearctic *Cordulegaster* Using Molecular Phylogeny and Morphology, with the Description of Two New Species (Odonata: Anisoptera: Cordulegastridae). *Diversity* 13, 667.https://doi.org/10.3390/d13120667
- Steinmann H. 1997 World catalogue of Odonata. Volume I. Zygoptera. In: H. Wermuth and M. Fischer (eds.), Das Tierreich. The Animal Kingdom. Eine

Zusammenstellung und Kennzeichnung der rezenten Tierformen. Walter de Gruyter, Berlin, **110**, xxi + 500 pp.

- Tamura K, Stecher G, Kumar S. 2021 MEGA11: Molecular Evolutionary Genetics Analysis version 11. *Molecular Biology and Evolution* 38:3022-3027.
- Tennessen KJ. 2010 The madicolous nymph of *Heteropodagrion sanguinipes* Selys (Odonata: Megapodagrionidae). *Zootaxa* **2531**, 29–28.
- Tillyard RJ. 1917 The Biology of Dragonflies (Odonata or Paraneuroptera). Cambridge University Press, Cambridge Zoological Series, London, xii + 396 pp.
- Tsuda S. 1986 A distributional list of world Odonata, Preliminary edition. Published by author, Osaka, 246 pp.
- Vaidya G, Lohman D, Meier R. 2010 SequenceMatrix: Concatenation software for the fast assembly of multi-gene datasets with character set and codon information. *Cladistics*. 27. 171 - 180. 10.1111/j.1096-0031.2010.00329.x.
- Vega-Sánchez YM, Mendoza-Cuenca LF, González-Rodríguez A. 2019 Complex evolutionary history of the American Rubyspot damselfly, *Hetaerina americana* (Odonata): Evidence of cryptic speciation, *Molecular Phylogenetics and Evolution*139, https://doi.org/10.1016/j.ympev.2019.106536.
- Villela DS, Koroiva R, Cordero-Rivera A, Guillermo-Ferreira R. 2019 A further study on *Franciscobasis* Machado and Bedê, 2016 (Odonata: Coenagrionidae), a newly described genus from Minas Gerais, Brazil. *PLoS One* 14, e0223241.<u>https://doi.org/10.1371/journal.pone.0223241</u>
- Wheeler QD. 2007 Invertebrate systematics or spineless taxonomy? In: Zhang ZQ and Shear WA (eds) Linnaeus Tercentenary: Progress in Invertebrate Taxonomy. *Zootaxa*, 1668, 1-766.
- Yu X, Xue JL, Hämäläinen M, Liu Y, Bu WJ. 2015 A revised classification of the genus *Matrona* Selys, 1853 using molecular and morphological methods (Odonata: Calopterygidae). *Zoological Journal of the Linnean Society* **174**, 473–486. <u>https://doi.org/10.1111/zoj.12253</u>
- Yu X, Xue J. 2020 A review of the damselfly genus *Megalestes* Selys, 1862 (Insecta: Odonata: Zygoptera: Synlestidae) using integrative taxonomic methods. *Zootaxa* 4851, 245–270.
- Zhang J, Kapli P, Pavlidis P, Stamatakis A. 2013 A General Species Delimitation Method with Applications to Phylogenetic Placements. *Bioinformatics* (Oxford, England) 29, 2869–2876.

Zhang H, Ning X, Yu X, Wen-Jun Bu. 2021 Integrative species delimitation based on COI, ITS, and morphological evidence illustrates unique evolutionary history of the genus *Paracercion* (Odonata: Coenagrionidae). *PeerJ* 9, e11459 DOI 10.7717/peerj.11459.

# 5 Chapter 3 - A MOLECULAR PHYLOGENY OF *HETEROPODAGRION* SELYS, 1885 AND *MESAGRION* SELYS, 1885: A COMPLEX OF NEOTROPICAL DAMSELFLIES (ODONATA: ZYGOPTERA: HETERAGRIONIDAE AND MESAGRIONIDAE)<sup>2</sup>

León Andrés Pérez-Gutiérrez<sup>, B, C</sup>, Daniela Maeda Takiya<sup>D</sup>, Maria Paula Rozo<sup>D</sup>, and Ângelo Parise Pinto<sup>B</sup>

<sup>A</sup>Laboratorio de Entomologia, Research Group "Biodiversidad del Caribe Colombiano. Programa de Ciências Biológicas, Universidad del Atlántico, Barranquilla, Colombia.

<sup>B</sup>Laboratory of Systematics on Aquatic Insects (LABSIA), Departamento de Zoologia, Universidade Federal do Paraná, P. O. Box 19020, 81531-980, Curitiba, PR, Brazil.

<sup>c</sup> Programa de Pós-graduação em Entomologia, Departamento de Zoologia, Universidade Federal do Paraná.

<sup>D</sup>Laboratório de Entomologia, Instituto de Biologia, Universidade Federal do Rio de Janeiro, Caixa Postal 68044, Cidade Universitária, 21941971, Rio de Janeiro, RJ, Brazil. Corresponding author: E-mail: <u>leonperez@mail.uniatlantico.edu.co</u>

L.PG: https://orcid.org/0000-0002-2775-3040

DMT: https://orcid.org/0000-0002-6233-3615

MPR: https://orcid.org/0000-0003-2792-2091

APP: https://orcid.org/0000-0002-1650-5666

# Abstract

The first molecular phylogenetic analysis of genera *Heteropodagrion* Selys, 1885 and *Mesagrion* Selys, 1885 including nine of the ten valid species is here presented with the aim of resolving their systematic positions under a phylogenetic context, based on analysis of nucleotide sequences of regions of three molecular markers, a nuclear (28S) and two mitochondrial (COI and 16S rRNA). Concatenated analysis with maximum likelihood criteria found *Heteropodagrion* and *Mesagrion* sister groups with high support, however, not support the monophyly of genera *Heteropodagrion*. The evidence suggests too that *Dimeragrion* is the sister genera of *Mesagrion*-

<sup>&</sup>lt;sup>2</sup> To be submitted to Zoological Journal of the Linnean Society (Percentile WOS Clarivate 94%, Qualis: A1) author guidelines available at: <u>https://academic.oup.com/zoolinnean/pages/General\_Instructions</u>

*Heteropodagrion* complex and joined can represent an entire monophyletic group under same circumscription. The monophyly of the Heteragrionidae family is not recovered and confirm previously statement than only the genera *Heteragrion* + *Oxystigma* supported this clade.

Key words: Megapodagrionidae, Zygoptera, phylogeny, monophyletic.

## 5.1 Introduction

The systematics and evolutionary history among zygopterans remain inconclusive for the "recalcitrant" taxa, also named "*incertae sedis*", among them are many genera once collectively known as Megapodagrionidae *s.l.*, the megapod damselflies (see chapters 2 and 1). Recent results from phylogenomic studies (Bybee et al., 2021) suggest that the largest genera among the megapods, *Heteragrion* Selys, 1862 with 62 species along with *Oxystigma* Selys, 1862 and *Dimeragrion* Calvert, 1913 form a clade named Heteragrionidae, originally stablished by Rácenis (1959).

All these genera previously pertained to a large concept of megapods or Megapodagrionidae *s.l.*, and other genera, such as, *Heteropodagrion* Selys, 1885 were reallocated in Heteragrionidae while *Mesagrion* Selys, 1885 was placed on its own family Mesagrionidae, and claimed to be distantly related to Heteragrionidae (see Bybee *et al.* 2021). This idea was contrary to the traditional idea of a close relationship between *Heteropodagrion* and *Mesagrion* since its generic erection, and reinforced by Dijkstra et al. (2013), although *Mesagrion* has not yet been included in a combined analysis with *Heteropodagrion*.

The systematic position of *Dimeragrion* is also unstable in the group. Previously, it was excluded from the "fan megapods" (Argiolestidae) for not being closely related to that clade (Kalkman & Theischinger, 2013). Morphologically the setae in the ligula are shared by *Dimeragrion*, *Heteropodagrion*, and *Mesagrion*, as well as the membranization of the tergum of the eighth abdominal segment of females, however, these characters have not been rigorously tested phylogenetically.

Although the data obtained with Sanger sequencing are by far more abundant and available, many so-called recalcitrant nodes in the phylogeny of Zygoptera have not been resolved because they suffer from the same problem of poor taxon sampling, e.g., the comprehensive analysis by Dijkstra et al. (2014) only includes sequences from *Heteropodagrion sanguinipes* Selys, 1885 (one of the 10 currently known species in the genus). Hitherto, the position of *Mesagrion* with respect to *Heteropodagrion* was not evaluated.

For this reason, in this phylogenetic study, the focus was to test the systematic position of *Heteropodagrion* and *Mesagrion* and clarifying the phylogenetic relationships between species once included in the Heteragrionidae clade. For this, 9 of the 10 species of *Heteropodagrion* and the single species of *Mesagrion* have been included to test the monophyly of genera.

## 5.2 Methods

#### 5.2.1 Specimen and sample acquisition

Specimens were collected with entomological nets, occasionally directly by hand. The collected specimens were identified using genus key by Garrison et al. (2010) and at species level based on revision such as Pérez-Gutiérrez et al. (Chapter 2). Entire specimen or tissue sample were fixed and preserved in absolute ethanol at -20°C for molecular analysis.

#### 5.2.2 DNA extraction and amplification and alignment

Genomic DNA was extracted from thoracic muscle or leg with muscular bundle detached with forceps with the remainder of the specimen kept as voucher and fully available for other studies. The DNeasy® Blood and Tissue kit (Qiagen Inc., Hilden, Germany) was used by optimizing the original protocol for incubating the tissue for lysis for 48h and generating two separate elutions of 50ul of DNA extract, instead of one of 100ul.

For amplification of COI, 16S rDNA, and 28S rDNA fragments, the following pairs of primers were used: C1-J-1718 (Simon et al., 1994) and HCO-2198 (Folmer et al., 1994); ODO-16S+ and -; and 28S\_FR2 and FR3 (Dijkstra et al., 2014). All PCR reactions had a total volume of 25µl, containing 5µl of 5x Taq buffer (Promega), 3.5µl MgCl2 (50nM, Promega), 2µl BSA (Promega), 1µl dNTP mix (10uM, Promega), 0.5µl of each 10µM primer (Invitrogen), 0.2µl of the enzyme Go®Taq DNA polymerase (Promega), and 1.0 to 2.0µl of genomic DNA. The thermocycler profile consisted of 3

min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C, with a final step of 7 min at 72°C.

PCR products were stained with GelRed<sup>™</sup> (Biotium), subjected to agarose gel electrophoresis in 1.0% TBE and visualized under UV light. Amplicons were purified using ExoSAP-IT® (USB Affymetrix) and sequenced in forward and reverse directions using the same PCR primers by Macrogen (Seoul, South Korea).

Electropherograms were assembled in Geneious® v9.1.2 (Kearse et al., 2012) to generate consensus sequences. The identity of all sequences was checked using BLAST on Genbank.

In addition to other sequences available at GenBank, consensus sequences of COI were aligned in MEGA X (Kumar et al., 2018) using the MUSCLE algorithm and amino acid translations were conducted to check for the absence of stop codons in the alignment., while the 16S and 28S sequences were aligned in MAFFT online version with the L-INS-i algorithm and Q-Ins respectively. Alignments were further checked visually in MEGAX (Supplementary material).

## 5.2.3 Taxon sampling

In addition to sequences generated herein, sequences publicly available from GenBank were included in the alignment to a total of 98 sequences of 33 taxa (Table 1). Six species were included as outgroup viz. Lestes helix Selys, 1918 (Lestidae), Polythore aurora Selys, 1879 (Polythoridae), Hetaerina brightwelli Kirby, 1823 (Calopterygidae), Metaleptobasis selysi 1956 (Coenagrionidae), Santos, Forcepsioneura sancta Hagen in Selys, 1860 (Protoneurinae), and Acanthagrion gracile Rambur, 1842 (Coenagrionidae). Representatives of Heteragrionidae (Heteragrion) and Allopodagrion contortum were included for more accuracy in the analysis. The trees were rooted with Lestes helix (Lestidae) first used for rooting according to Dijkstra et al. (2014). Specimens of *Heteropodagrion* included (9 species of 10 described) and of *Mesagrion leucorrhinum* were included for test monophyly of family level.

Table 1. Species included for the analysis of the *Mesagrion-Heteropodagrion* complex and outgroups with respective voucher specimen code, adult gender (G), collecting data, and GenBank accession numbers for 16S, 28S and COI sequences.

Taxon	Voucher	16S	28S	COI
Amphypterygidae				
Amphipteryx agrioides	-	KF369616	KF370014	KY773652
Philogeniidae				
Archaeopodagrion armatum	-	KF369622	KF370020	KF369302
Philogenia cassandra				
Philogenia iguita				
Philogenia ferox	GENBANK	KF369842	KF370241	KF369492
Coenagrionidae				
Acanthagrion gracile				
Forcepsioneura sancta	-	472 (95 indels)	584 (15 indels)	390
Metaleptobasis selysi		· · · ·	, ,	
Dicteriadidae				
Heliocharis amazona	-	KF369725	KF370124	KF369392/468
Calopterygidae				
Hetaerina brightwelli	ODO MZNRJ 0117	MZNRJ 117	MZNRJ 117	MZNRJ 117
Heteragrionidae		_	_	—
Dimeragrion percubitale	-	KF369689	KF370087	KF369360
Heteragrion aurantiacum	DNA M3060416-10	DNA M3060416-10	DNA M3060416-10	DNA M3060416-10
Heteragrion bariai	DNA ODO LABSIA 675	THIS STUDY	THIS STUDY	THIS STUDY464
Heteragrion bickorum		KF369731	KF370130	KF369396
Heteragrion inca	-	KF369733	KF370132	KF369398
Heteragrion sp.	LABIA 153		556	THIS STUDY463
Heteragrion chrysops	-	KF369732	KF370131	KF369397
Heteragrion triangulare	-	NO DATA	NO DATA	KY947445
Heteropodagrion croizati	DNA ODO LABSIA 660	THIS STUDY	THIS STUDY	THIS STUDY
Heteropodagrion cuyabri	DNA ODO LABSIA 642	THIS STUDY	THIS STUDY	THIS STUDY
Heteropodagrion diabolum	DNA ODO LABSIA 641	THIS STUDY	THIS STUDY	THIS STUDY
Heteropodagrion donnellyi	DNA ODO LABSIA 650	THIS STUDY	THIS STUDY	THIS STUDY
Heteropodagrion paramillo	DNA ODO LABSIA 654	THIS STUDY	THIS STUDY	THIS STUDY
Heteropodagrion sanguinipes		KF369734	KF370133	KF369399
Heteropodagrion santuario	DNA_ODO_LABSIA_322	THIS STUDY	THIS STUDY	THIS STUDY
Heteropodagrion superbum	DNA_ODO_LABIA_320	THIS STUDY	THIS STUDY	THIS STUDY
Heteropodagrion varipes	ODO_LABIA_319	THIS STUDY	THIS STUDY	THIS STUDY
Oxystigma sp.		KF369817	KF370216	KF369470
Hypolestidae				
<i>Hypolestes</i> sp.	-	KF369737	KF370135	KF369402
Lestidae				
Lestes helix	-	KF369756	KF370155	KF369420
Mesagrionidae				
Mesagrion leucorrhinum	DNA_ODO_LABSIA_318	THIS STUDY	NO DATA	THIS STUDY
Thaumatoneuridae				
Paraphlebia quinta				
Paraphlebia zoe	GENBANK	KF369830	KF370229	KF369481
Thaumatoneura inopinata	GENBANK	KF369933	KF370332	KF369572
Polythoridae				

Polythore aurora	GENBANK	KF369859	KF370257	KF369508
Incertae sedis				
Sciotropis cyclanthorum	GENBANK	KF369898	KF370296	NO DATA
Megapodagrionidae s.e				
Allopodagrion contortum				
Teinopodagrion meridionale				
Teinopodagrion venale	GENBANK	KF369925	KF370324	KF369565

## 5.2.4 Data analysis

Matrices were built including all lineages hypothesized by Dijkstra et al. (2014) that are related to Heteragrionidae, phylogenetic analysis was carried out on separated and concatenated matrices, initially partitioned by gene and codon position for COI. Phylogenetic analyses were performed using Maximum Likelihood (ML; Felsenstein, 1981) inferences as implemented by IQTREE (Miller et al., 2015; Nguyen et al., 2014). Most appropriate partition scheme and substitution model per partition was found by BIC using ModelFinder (Kalyaanamoorty et al., 2017). Ultrafast bootstrap supports (UFBoot) for the resulting ML topology were calculated using 1000 replicates, the same for separated matrices. For the combined analyses, all sequence alignments were concatenated into a single dataset using Sequence Matrix (Vaidya et al., 2010). Trees were edited in Figtree version 1.3.1 (Rambaut, 2007).

ML analyses were performed on the portal server CIPRES GATEWAY using IQ-TREE multicore version 1.6.12 (Miller et al., 2015; Nguyen et al., 2014). For the COI data set the command used was iqtree2 -nt 6 -bnni -s infile.txt -st DNA -m TESTNEWMERGE -alrt 1000 --sprrad 6 -B 1000 --prefix output using SPRNG - Scalable Parallel Random Number Generator), 1000 samples for ultrafast bootstrap (UFBoot), 1000 replicates for Shimoidara-Hasegawa-like approximate likelihood ratio test (SH-aLRT), and with value of initial seed 6822. For the 28S matrix the parameters used were iqtree2 -nt 6 -bnni -s infile.txt -st DNA -m TESTNEWMERGE -alrt 1000 -- sprrad 6 -B 1000 --prefix output with seed value 354735, branch support was tested using 1000 replicates of ultrafast bootstrap and 1000 replicates of SH-aLRT. Finally, the command used for concatenated data set was iqtree2 -nt 6 -bnni -s infile.txt -bsam GENE -keep\_empty\_seq -p partition\_file.txt -st DNA -rclusterf 50 -m TESTMERGE - alrt 1000 -- sprrad 6 -B 1000 -- prefix output \_-symtest with initial seed 12743 using SPRNG.

#### 5.3 Results

The matrices information could be summarized as follows: for COI = 36 taxa, Conserved sites = 252/468, Variable sites = 2731/468, Parsimony Informative Sites = 193/468; for 16S = 31 taxa, 563 sites, Conserved sites = 284 Parsimony informative Sites= 217, conserved sites =284 and for 28S = 36 taxa, 626 sites, Conserved sites= 516, variable sites = 173, Parsimony Informative Sites =56. Finally, the concatenated matrix has 38 taxa, 1657 sites, variable sites= 771, parsimony informative= 466, and conserved sites= 1042. Exploratory arrangements were first scrutinized in ML separated trees of each marker (Figs. 1–3). The phylogenetic reconstruction performed with combined data set of the three markers are summarized in Fig. 4.

Concatenated and separated analysis shown the genera representing Megapodagrionidae s.e. *Teinopodagrion* and *Allopodagrion* do not form a monophyletic group, in fact *Allopodagrion* appear more related to *Heteragrion* and *Oxystigma* (Heteragrionidae) with high support (97%), the COI tree shown this relationship (Fig. 1) while 28S places it phylogenetically closer to *Hypolestes* and *Sciotropis* (Fig. 2).

*Heteragrion* is a stable group with at least two distinguishable internal subgroups within the genus (BS 97%), that is linked with *Oxystigma* as sister taxa with high support (94%) forming a monophyletic group, this pattern was obtained in all analysis scrutinized.

The close relationship between *Sciotropis* and *Hypolestes* (UFBoot = 98%) had not been obtained until now, however, in all the analyzes this linkage was recovered, joined with Dicteriadidae (*Heliocharis amazona*) and Polythoridae (*Polythore aurora*) (BS=83%).

Philogeniidae (*Archaeopodagrion* + *Philogenia*) is recovered as monophyletic with *Thaumatoneura* (Thaumatoneuridae) as sister taxa in concatenated analysis.

*Mesagrion-Heteropodagrion* complex appear monophyletic taxa in all analysis, in concatenated high UFBoot support (94%) analysis of COI, 16S, and 28S, individually gene trees are congruent showing the same grouping (Fig. 1-4).

The internal relationships in *Heteropodagrion* shown a paraphyletic position of *Heteropodagrion croizati*, in all topologies obtained this taxa emerged as the sister group to *Mesagrion* + the remaining *Heteropodagrion s.s.* (UFBoot = 80%), rendering the genus as non-monophyletic.



**Fig. 1** Maximum likelihood gene tree of taxa studied included *Heteropodagrion* and *Mesagrion* based on COI data set. The phylogenetic tree is a consensus from 1000 replicates in IQTREE. Node-associated values refer to bootstrap percentages.



**Fig. 2** Maximum likelihood gene tree of taxa studied included *Heteropodagrion* and *Mesagrion* based on 28S data set. The phylogenetic tree is a consensus from 1000 replicates in IQTREE. Node-associated values refer to bootstrap percentages.



**Fig. 3** Maximum likelihood gene tree of taxa studied included *Heteropodagrion* and *Mesagrion* based on 16S data set. The phylogenetic tree is a consensus from 1000 replicates in IQTREE. Node-associated values refer to bootstrap percentages.



**Fig. 4** Maximum likelihood consensus tree of the concatenated data set (COI, 28S and 16S) gene tree of taxa studied included *Heteropodagrion* and *Mesagrion* based on 16S data set. The phylogenetic tree is a consensus from 1000 replicates in IQTREE. Node-associated values refer to bootstrap percentages.

## 5.4 Discussion

## Heteropodagrion- Mesagrion complex monophyly

Currently, the most complete phylogenetic hypothesis available with molecular data within Zygoptera are those of Dijkstra et al. (2014) and the phylogenomic one of Bybee et al. (2021), although the latter has an important bias of taxonomic sub-sampling for our corroboration purposes.

The fact that none of the analyses performed recovered the relationship between *Heteropodagrion* and *Dimeragrion* with *Heteragrion* and *Oxystigma* indicates that the circumscription of these genera into Heteragrionidae is artificial. In the same way, data sets of previous works such as Bybee et al. (2008) and Dumont et al. (2010) have also bias either due to taxonomic subsampling or due to the lack of homology between the sequenced genic regions, which did not allow them to be concatenated in a single matrix. Dijkstra et al. (2014), for their part, only included in their matrices a sequence of *Heteropodagrion (H. sanguinipes*) and did not include *Mesagrion,* in our analyses for the first time the two genera are widely represented taxonomically, and their sister taxa status is revealed in all analyses performed.

The gene trees give an approximation of clustering among *Heteropodagrion* and *Mesagrion* species showing congruence between the topologies of the three markers. The concatenated tree with the three markers represents the phylogeny of species of the *Heteropodagrion* and *Mesagrion* complex, with internal relationships well solved. a group with the superbum complex is corroborated in fact, (*H. cuyabri*, *H. santuario* and *H. superbum*).

Contrarily, *H. croizati* appears as a sister lineage and is not included in the clade that groups the rest of *Heteropodagrion*, decisions about the status of *H. croizati* will probably require resolving its paraphyletic status given our results.

Here we assume that the merging of *Mesagrion* into *Heteropodagrion* is not convenient because *Mesagrion* is now at family level (Mesagrionidae), with *Mesagrion* as type genus, in which case the least disturbing option in the taxonomy of the group would be to nominate under *Mesagrion* the whole group. For the time being we refrain from making nomenclatural changes that might cause taxonomic inflation until the analyses presented here have been refined.

#### Heteragrionidae monophyly

Comparisons with Bybee et al. (2021) cannot be made because they did not include *Oxystigma*, even so, they make additions to Heteragrionidae, which does not fit to the evidence presented.

All analyzes failed to recover the monophyly of the included lineages that comprise Heteragrionidae sensu Bybee et al., 2021 (*Dimeragrion*, *Heteropodagrion*, *Heteragrion*, and *Oxystigma*), due all analyses show that these groups of genera belong to different groups within the neotropical diversity of megapods.

Heteragrionidae according to our results should be restricted to the clade *Heteragrion* + *Oxystigma*. Support values for the Heteragrionidae clade (PP80%), in Dijkstra et al. (2014) is low, in our study with the addition of sequences of 9 of 10

species belonging to *Heteropodagrion* together with *Mesagrion* we achieved greater resolution to conclude the close relationship between these Andean genera with high support.

Considering that the *Heteragrion* sequences obtained in this study grouped with those available in Genbank, we can be confident in the accuracy of our results. For example, *Oxystigma* forms a monophyletic clade with the "*Heteragrion*" clade, which confirms the homology concordance in the sequences used.

Allopodagrion contortum was closely related to the clade (*Heteragrion* + *Oxystigma*), although more data will be necessary to confirm the relationship between Allopodagrion and *Heteragrion*, in none of the topologies a relationship with *Teinopodagrion* is recovered as suggested by De Marmels (2001).

Amphipterygidae appeared close to the Thaumatoneuridae + Philogeniidae clade are a consolidated group and recently evidence of larval morphology adds data that suggests such relationship (Novelo-Gutiérrez et al., 2020).

Combined analysis showed a node than grouped the rest of genera including *Thaumatoneura* as sister taxon of a monophyletic Philogeniidae (*Archaeopodagrion* + *Philogenia*), this relationship is support by low BS values and their systematic position placed *Paraphlebia* close to *Heteropodagrion* and *Mesagrion* complex.

## 5.5 Conclusions

These phylogenetic analyses based on molecular data of the three most used markers (COI, 28S rDNA, and 16S rDNA) offer strong support the sister relationship between *Mesagrion* and *Heteropodagrion*, which was our main goal.

The internal relationships among *Heteropodagrion* shown species groups welldefined, but the paraphyly of *H. croizati* and *H. varipes*, therefore *Heteropodagrion* is not a monophyletic group since *Mesagrion* fall into intermingled in *Heteropodagrion*.

The current frame of knowledge about relationships among Neotropical clades suggests with greater support that relationships are reticulated, the low support that some relationships present scenarios still open space for research in the cladogenesis of stem groups of this Zygoptera, as suggests the splits groups than divided Heteragrionidae p.e.

*Mesagrion* and *Heteropodagrion* represent lineages with discrete species but with a shared history from ancestry/descent, which is important in directing decisions

that can be made regarding Heteragrionidae and Mesagrionidae we assume as a more conservative decision to add *Heteropodagrion* to Mesagrionidae, since all analyzes would be suggesting grouping by close relationship.

Besides our results reinforced previous phylogenetic relationships in the internal clades, like Philogeniidae and Thaumatoneuridae, and it is pertinent to note that with these markers the close relationship between *Heteragrion* and the *Heteropodagrion* + *Mesagrion* clade was not corroborated, certainly with this evidence and with the analyzes shown here the monophyly is formally rejected.

## 5.6 REFERENCES

- Bybee S, Kalkman V, Erickson J, Frandsen PB, Breinholt, JW, Suvorov A, Dijkstra KDB, Cordero-Rivera A, Skevington JH, Abbott JC, Sanchez-Herrera M, Lemmon AR, Lemmon E, Ware JL. 2021 Phylogeny and classification of Odonata using targeted genomics, *Molecular Phylogenetics and Evolution* **160**, 107115. <u>https://doi.org/10.1016/j.ympev.2021.107115</u>.
- Chernomor O, von Haeseler A, Quang MB. 2016 Terrace aware data structure for phylogenomic inference from supermatrices. *Systematic Biology*, 65:997-1008. <u>https://doi</u>.org/10.1093/sysbio/syw037
- Dijkstra KDB, Bechly G, Bybee SM, Dow RA, Dumont HJ, Fleck G, Garrison RW, Hämäläinen M, Kalkman VJ, Karube H, May ML, Orr AG, Paulson DR, Rehn AC, Theischinger G, Trueman JWH, Tol JV, Von Ellenrieder N, Ware J. 2013 The classification and diversity of dragonflies and damselflies (Odonata). In: Zhang, Z.-Q. (Ed.) Animal Biodiversity: An Outline of Higher-level Classification and Survey of Taxonomic Richness (Addenda 2013). *Zootaxa* 3703, 36–45. https://doi.org/10.11646/zootaxa.3703.1.9
- Dumont HJ, Vierstraete A, Vanfleteren JR. 2010 A molecular phylogeny of the Odonata (Insecta). *Systematic Entomology*, 35: 6–18.
- Garrison RW, von Ellenrieder N, Louton JA. 2010 Damselfly Genera of the New World: An Illustrated and Annotated Key to the Zygoptera. Johns Hopkins University Press.
- Kalyaanamoorthy S, Quang MB, Wong KFT, von Haeseler A, Jermiin LS. 2017 ModelFinder: Fast model selection for accurate phylogenetic estimates. Nature Methods, 14:587–589. <u>https://doi</u>.org/10.1038/nmeth.4285

- Kalkman VJ, Theischinger G. 2013 Generic revision of Argiolestidae (Odonata), with four new genera. *International Journal of Odonatology*, 16: 1–52.
- Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A, 2012 Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data, *Bioinformatics*, Volume 28, Issue 12, 1647–1649, <u>https://doi</u>.org/10.1093/bioinformatics/bts199
- Katoh K, Rozewicki J, Yamada KD. 2019 MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization, Briefings in *Bioinformatics*, Volume 20, Issue 4, July, Pages 1160–1166, <u>https://doi.org/10.1093/bib/bbx108</u>
- Kimura M. 1980. Simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. – *Journal of Molecular Evolution* 16: 111 – 120.
- Novelo-Gutiérrez R, Bota-Sierra CA, Amaya-Vallejo V. 2020 Description of the larva of the genus Archaeopodagrion Kennedy, 1939 (Zygoptera: Philogeniidae). Zootaxa 4816(3): 325–332.

Rambout, A. GitHub. Available online: <u>http://tree.bio.ed.ac.uk/software/figtree/</u>

Quang MB, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, von Haeseler A, Lanfear R. 2020 IQ-TREE 2: New models and efficient methods for phylogenetic inference in the genomic era. *Molecular Biology and Evolution*, 37(5): 1530-1534. <u>https://doi</u>.org/10.1093/molbev/msaa015

#### **6 GENERAL CONCLUSIONS**

The relationships among lineages of Heteragrionidae *sensu* Dijkstra et al. (2014) and Bybee et al. (2021) are not supported, because they are not monophyletic. The Heteragrionidae as proposed herein should be represented only by core heteragrionids genera *Heteragrion* and *Oxystigma*.

The complex *Mesagrion-Heteropodagrion* was recovered as monophyletic with high support, however *Heteropodagrion* is non-monophyletic, with *H. croizati* as sister to *Mesagrion* plus remaining *Heteropodagrion*, thus it represents a divergent lineage.

Morphologically, the characters in the wing venation were diagnostic at the genus level with RP3-4 arising proximal to the subnodus in *Heteropodagrion* and distal in *Mesagrion*. The costal margin of pterostigma is also useful in generic diagnosis, being as long as  $\frac{1}{2}$  length posterior margin *Mesagrion* and as long as  $\frac{3}{4}$  length posterior margin in *Heteropodagrion*. At the species level, wing venation could be less effective in delimiting species, although there are characteristics that allowed sorting the species into 4 groups.

The trends in the modification of the venation show a reduction of the supplementary sectors probably with the most derived form of the trait, which shows its greater heterogeneity within *Heteropodagrion*, the wing venation in *Mesagrion* is noticeably reduced in reticulation compared to its sister group *Heteropodagrion*.

The markers used had the resolution for the delimitation of the species, consolidating the secondary hypotheses of species obtained during the analysis cycles that were executed, the decisions made here on the species status of 5 new entities that correspond to the *Heteropodagrion* lineage could be established with integration by congruence in most of the analyses.

The distances that were found correspond to those observed in ITS2, which on this occasion allowed us to recognize geographical phenotypic variation between *Heteropodagrion superbum*, but it is the same species according to the evidence of concatenated analyses.

With respect to related groups the analyses fail to be conclusive in defining which is the sister group of *Heteropodagrion* since the relationships place intermingled Thaumatoneuridae and Philogeniidae.

The analyzes show with considerable support that *Heteropodagrion* and *Mesagrion* are part of a closely related evolutionary group, then these genera are not heteragrionids. Based on the integrative analyses and based on criteria of first reviser we considering *Mesagrion* a junior synonym of *Heteropodagrion*, thus we formally propose the merger of the species names under the generic name *Heteropodagrion*. Mesagrionidae is composed by *Mesagrion* and *Heteropodagrion* plus *H. croizati* that circumscription is pending further studies.

## 7 REFERENCES

- Archibald SB, Cannings RA, Erickson RJ, Bybee SM, Mathewes RW. 2021 The Cephalozygoptera, a new, extinct suborder of Odonata with new taxa from the early Eocene Okanagan Highlands, western North America. *Zootaxa*, 4934 (1), 1–133. <u>https://doi.org/10.11646/zootaxa.4934.1.1</u>
- Azevedo JAR, Guedes TB, Nogueira CdC, Passos P, Sawaya RJ, Prudente ALC, Barbo FE, Strüssmann C, Franco FL, Arzamendia V, Giraudo AR, Argôlo AJS, Jansen M, Zaher H, Tonini JFR, Faurby S, Antonelli A. 2020 Museums and cradles of diversity are geographically coincident for narrowly distributed Neotropical snakes. *Ecography*, 43: 328–339. doi: <u>10.1111/ecog.04815</u>
- Bourguignon T, Šobotník J, Hanus R, Krasulová J, Vrkoslav V, Cvacka J, Roisin Y.
   2013 Delineating species boundaries using an iterative taxonomic approach: The case of soldierless termites (Isoptera, Termitidae, Apicotermitinae). *Molecular Phylogenetics and Evolution* 69, 694–703.
- Bota-Sierra C, Palacino F, Rache L, Tennessen K Mauffray W. 2016 Heteropodagrion croizati. The IUCN Red List of Threatened Species 2016:
  e.T49254609A49256265. https://dx.doi.org/10.2305/IUCN.UK.20161.RLTS.T49254609A49256265.en. Accessed on 04 November 2023.
- Bota-Sierra, C., Palacino, F., Rache, L., Ellenrieder, N. Paulson, D. 2016 *Mesagrion leucorrhinum.* The IUCN Red List of Threatened Species 2016: e.T60294A66887433. https://dx.doi.org/10.2305/IUCN.UK.2016-1.RLTS.T60294A66887433.en. Accessed on 04 November 2023.
- Bota-Sierra CA, Montes-Fontalvo JM, Perez L, Sandoval-H J Florez C. 2021 *Heteropodagrion superbum*. The IUCN Red List of Threatened Species 2021:

e.T49254451A49256269. https://dx.doi.org/10.2305/IUCN.UK.2021-2.RLTS.T49254451A49256269.en. Accessed on 04 November 2023.

- Brower AVZ, Schuh RT. 2021 Biological systematics: Principles and applications, 3rd. Edition. Ithaca: Cornell University Press.
- Bridges CA. 1994 Catalogue of the family-group, genus-group, and species-group names of the Odonata of the world (Third Edition). *Urbana*, xlvi+905 pp.
- Büsse S. 2016 Morphological re-examination of *Epiophlebia laidlawi* (Insecta: Odonata) including remarks on taxonomy. International Journal of Odonatology 19(4): 221–238. https://doi.org/10.1080/13887890.2016.1257442
- Büsse S, Ware, J. 2022 Taxonomic note on the species status of *Epiophlebia diana* (Insecta, Odonata, Epiophlebiidae), including remarks on biogeography and possible species distribution. **ZooKeys**. 1127. 79-90. 10.3897/zookeys.1127.83240.
- Bybee SM, Ogden TH, Branham, MA, Whiting, MF. 2008 Molecules, morphology and fossils: a comprehensive approach to odonate phylogeny and the evolution of the odonate wing. Cladistics 23, 1–38.doi:
- Bybee S, Kalkman V, Erickson J, Frandsen PB, Breinholt, JW, Suvorov A, Dijkstra KDB, Cordero-Rivera A, Skevington JH, Abbott JC, Sanchez-Herrera M, Lemmon AR, Lemmon E, Ware JL. 2021 Phylogeny and classification of Odonata using targeted genomics, *Molecular Phylogenetics and Evolution* **160**, 107115. <u>https://doi.org/10.1016/j.ympev.2021.107115</u>.
- Calvert PP. 1903 Odonata, pp. 129-144. In: *Biologia Centrali-Americana*: *Insecta Neuroptera*. R.H. Porter and Dulau Co., London
- Calvert P. 1913 The fossil Odonate *Phenacolestes,* with a discussion of the venation of the legion *Podagrion* Selys. *Proceedings of the Academy of Natural Sciences of Philadelphia* **65**, 225–272.
- Chernomor O, von Haeseler A, Quang MB. 2016 Terrace aware data structure for phylogenomic inference from supermatrices. *Systematic Biology*, 65:997-1008. <a href="https://doi.org/10.1093/sysbio/syw037Clausnitzer">https://doi.org/10.1093/sysbio/syw037Clausnitzer</a> V, Kalkman V, Ram M, Collen B, Baillie J, Bedjanic M, Darwall W, Dijkstra K, Dow R, Hawking J, Karube H, Malikova E, Paulson D, Schütte K, Suhling F, Villanueva RJ, Ellenrieder N, Wilson K (2009) Odonata enter the biodiversity crisis debate: The first global assessment of an insect group. *Biological Conservation*. 142. 1864–1869. 10.1016/j.biocon.2009.03.028.

- Clement M, Snell Q, Walker P, Posada D, Crandall, K. 2002 TCS: Estimating gene genealogies. Parallel and Distributed Processing Symposium, International Proceedings, 2, 184.
- Cordero A, Andrés JA. 1996 Colour polymorphism in Odonates: females that mimic males? *Journal of the British Dragonfly Society* **12**: 50–60.
- Cordero-Rivera A. Lorenzo- Carballa MO. 2010 Three sisters in the same dress: cryptic speciation in African odonates. *Molecular Ecology*, 19: 3840 3841. https://doi.org/10.1111/j.1365-294X.2010.04721.x
- Cowley J. 1941 The descriptive terms applied to the pterothorax and penis of odonata. *Proceedings Royal Entomological Society of London*. (B) 10(1): 5-7
- Daigle JJ. 2014 Two new *Heteropodagrion* species from Ecuador (Odonata: Megapodagrionidae) *Odonatologica* **43**, 35–42.
- Davies DAL, Tobin P. 1984 The dragonflies of the world: a systematic list of the extant species of Odonata. Vol 1 Zygoptera, Anisozygoptera. Societas Internationalis Odonatologica Rapid Communications 3, 1–127.
- Deacon C, Samways MJ. 2021 A Review of the Impacts and Opportunities for African Urban Dragonflies. Insects. 12(3):190. doi: 10.3390/insects12030190.
- De Marmels JC. 2001 Revision of *Megapodagrion* Selys, 1886 (*Insecta, Odonata: Megapodagrionidae*). Dissertation, Universität Zürich, Zürich.
- De Queiroz K. 2007 Species concepts and Species Delimitation, *Syst. Biol*. 56 (6): 879–886.
- Deng J, Guo Y, Cheng Z, Lu C, Huang X. 2019 The Prevalence of Single-Specimen/Locality Species in Insect Taxonomy: An Empirical Analysis. *Diversity*, **11**, 106. https://doi.org/10.3390/d11070106
- Dijkstra KDB, Bechly G, Bybee SM, Dow RA, Dumont HJ, Fleck G, Garrison RW, Hämäläinen M, Kalkman VJ, Karube H, May ML, Orr AG, Paulson DR, Rehn AC, Theischinger G, Trueman JWH, Tol JV, Von Ellenrieder N, Ware J. 2013 The classification and diversity of dragonflies and damselflies (Odonata). In: Zhang, Z.-Q. (Ed.) Animal Biodiversity: An Outline of Higher-level Classification and Survey of Taxonomic Richness (Addenda 2013). *Zootaxa* 3703, 36–45. <u>https://doi.org/10.11646/zootaxa.3703.1.9</u>
- Dijkstra KDB, Kalkman VJ, Dow RA, Stokvis FR, Van Tol, J. 2014 Redefining the damselfly families: a comprehensive molecular phylogeny of Zygoptera (Odonata). *Systematic Entomology* **39**, 68–96.

- Djan M, Ståhls G, Velickovic N, Acanski J, Obreht VD, Rojo S, Pérez-Bañón C, Radenkovic S, Vujic A. 2020 The *Merodon planifacies* subgroup (Diptera, Syrphidae): Congruence of molecular and morphometric evidence reveal new taxa in Drakensberg mountains valleys (Republic of South Africa). *Zoologischer Anzeiger* 287, 105–120.
- Donnelly TW. 1992 The Odonata of central Panamá and their position in the Neotropical odonate fauna, with a checklist, and descriptions of new species. In Insects of Panama and Mesoamerica: selected studies. Edited by D. Quintero and A. Aiello. Oxford University Press, Oxford. pp. 52– 90.
- Dumont HJ, Vierstraete A, Vanfleteren JR. 2010 A molecular phylogeny of the Odonata (Insecta). *Systematic Entomology*, 35: 6–18.
- Dupuis JR, Roe AD, Sperling FA. 2012 Multi-locus species delimitation in closely related animals and fungi: one marker is not enough. *Molecular Ecology*. (18): 4422-4436. doi: 10.1111/j.1365-294X.2012.05642.x.
- Folmer O, Black Mb, Hoeh Wr, Lutz R, Vrijenhoek R. 1994 DNA primers for amplification of mitochondrial Cytochrome C oxidase subunit I from diverse metazoan invertebrates. *Molecular marine biology and biotechnology.* 3: 294-9.
- Garrison RW, Von Ellenrieder N. 2005 *Neuragrion mysticum* (Odonata: Megapodagrionidae) demystified. *Canadian Entomologist* **137**, 169–163.
- Garrison RW, Von Ellenrieder N, Louton JA. 2010 Damselfly genera of the New World. An Illustrated and Annotated Key to the Zygoptera. The Johns Hopkins University Press xiv + 490 pp, + 24 pls.
- Grigoropoulou A, Ab Hamid S, Acosta R, Akindele, EO, Al-Shami S, Altermatt F, Amatulli G, Angeler D, Arimoro F, Aroviita J, Astorga-Roine A, Bastos R, Bonada N, Boukas N, Brand C, Bremerich V, Bush A, Cai Q, Domisch S. 2023 The global EPTO database: Worldwide occurrences of aquatic insects. *Global Ecology and Biogeography.* 32. 10.1111/geb.13648.
- Grimaldi D. Engel, MS. 2005 Evolution of the Insects. Cambridge University Press, New York, New York.
- Guo B, Kong L. 2022 Comparing the Efficiency of Single-Locus Species Delimitation Methods within Trochoidea (Gastropoda: Vetigastropoda). *Genes 13*, 2273. https://doi.org/10.3390/genes13122273
- Hillis D, Chambers E, Devitt T. 2021 Contemporary Methods and Evidence for Species Delimitation. *Ichthyology & Herpetology*. 109. 10.1643/h2021082.

- Jones R, Brock PD, Mantovani B, Beasley-Hall P, Yeates DK., Lo N. 2022 Integrative taxonomy of the stick insect genus *Austrocarausius* Brock, 2000 (Phasmatodea: Lonchodidae) reveals cryptic species in remnant Queensland rainforests. *Invertebrate Systematics* **36**, 849-873.
- Kalkman V, Clausnitzer V, Dijkstra KD, Orr A, Paulson D, Tol J. 2008 Global diversity of dragonflies (Odonata) in freshwater. 10.1007/978-1-4020-8259-7\_38.
- Kalkman VJ, Choong ChY, Orr AG, Schütte K 2010 Remarks on the taxonomy of Megapodagrionidae with emphasis on the larval gills (Odonata) *International Journal of Odonatology* **13**, 119–135.
- Kalkman VJ, Theischinger G. 2013 Generic revision of Argiolestidae (Odonata), with four new genera. *International Journal of Odonatology*, 16: 1–52.
- Kalyaanamoorthy S, Quang BM, Wong TKF, von Haeseler A, Jermiin LS. 2017 ModelFinder: Fast model selection for accurate phylogenetic estimates. Nature Methods, 14:587–589. https://doi.org/10.1038/nmeth.4285.
- Katoh K, Rozewicki J, Yamada KD. 2019 MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization, *Briefings in Bioinformatics*, Volume 20, Issue 4, July, Pages 1160–1166, <a href="https://doi.org/10.1093/bib/bbx108">https://doi.org/10.1093/bib/bbx108</a>
- Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M. Sturrock S. Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B. Meintjes P, Drummond A. 2012 Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, 28(12), 1647– 1649. https://doi.org/10.1093/bioinformatics/bts199
- Karsch F. 1891 Neue Odonaten von Ecuador. Societas Entomologica 6, 105.
- Kimura M. 1980 Simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. – *Journal of Molecular Evolution* 16: 111 – 120.
- Kirby WF. 1890 A Synonymic Catalogue of Neuroptera Odonata, or Dragonflies, with an Appendix of Fossil Species. Guerney and Jackson, London, 202 pp. <u>https://doi.org/10.5962/bhl.title.5534</u>.
- Kohli M, Letsch H, Greve C, Béthoux O, Deregnaucourt I, Liu S, Zhou X, Donath A,Mayer C, Podsiadlowski L, Gunkel S, Machida R, Niehuis O, Rust J, Wappler T,Yu X, Misof B, Ware J. 2021 Evolutionary history and divergence times of

Odonata (dragonflies and damselflies) revealed through transcriptomics. *iScience*. 24(11):103324. doi: 10.1016/j.isci.2021.103324.

- Lam-Tung, Heiko AS, von Haeseler A, Quang Minh B. 2015 IQ-TREE: A fast and effective stochastic algorithm for estimating maximum likelihood phylogenies. *Molecular Biology Evolution*, 32:268-274. https://doi.org/10.1093/molbev/msu300
- Lorenzo-Carballa MO, Garrison, RW, Encalada AC, Cordero-Rivera A. 2020 Darwin Returns to the Galapagos: Genetic and Morphological Analyses Confirm the Presence of *Tramea darwini* at the Archipelago (Odonata, Libellulidae). *Insects, 12*.
- Marinov M, Amaya-Perilla C, Holwell GI, Varsani A, Van Bysterveldt K, Kraberger S, Stainton D, Dayaram A, Curtis N, Cruickshank RH, Paterson A. 2016 Geometric morphometrics and molecular systematics of *Xanthocnemis sobrina* (McLachlan, 1873) (Odonata: Coenagrionidae) and comparison to its congeners. *Zootaxa* 4078, 84–120.
- Mauffray WF, Tenessen KJ. 2019 A catalogue and historical study of the Odonata of Ecuador. *Zootaxa*, **4628**, 001–265.
- Mauffray W. Tennessen K. 2020 Heteropodagrion nigripes. The IUCN Red List of Threatened Species 2020: e.T119974946A119974959.
   https://dx.doi.org/10.2305/IUCN.UK.2020-2.RLTS.T119974946A119974959.en.
   Accessed on 04 November 2023.
- Mauffray, W, Tennessen, K. 2020 Heteropodagrion varipes. The IUCN Red List of Threatened Species 2020: e.T119975038A119975059.
  https://dx.doi.org/10.2305/IUCN.UK.2020-2.RLTS.T119975038A119975059.en.
  Accessed on 04 November 2023.
- McLachlan R. 1897 *Thaumatoneura inopinata*, a new genus and species of Calopteryginae. *The Entomologist Monthly Magazine* (2)8: 130-131.
- May, ML. 2019 Odonata: Who They Are and What They Have Done for Us Lately: Classification and Ecosystem Services of Dragonflies. *Insects* 10, no. 3: 62. <u>https://doi.org/10.3390/insects10030062</u>
- Meier R, Blaimer BB, Buenaventura HE, Vonrintelenb T, Srivathsana A, Yeo D. 2021 A re-analysis of the data in Sharkey *et al.*'s (2021) minimalist revision reveals that BINs do not deserve names, but BOLD Systems needs a stronger commitment to open science. *Cladistics*,1–12.

- Miller MA., Pfeiffer W. Schwartz T. 2010 "Creating the CIPRES Science Gateway for inference of large phylogenetic trees". In: *Proceedings of the Gateway Computing Environments Workshop* (GCE), 14 Nov. 2010, New Orleans, LA pp 1 - 8. DOI: 10.1109/GCE.2010.5676129
- Mitchell A, Samways MJ. 2005 The morphological 'forms' of *Palpopleura lucia* (Drury) are separate species as evidenced by DNA sequencing Anisoptera: Libellulidae). *Odonatologica* **34**, 173–178.
- Misof B, Liu S, Meusemann K, Peters RS, Donath A, Mayer C, Frandsen PB, Ware J, Flouri T, Beutel RG, Niehuis O, Petersen M, Izquierdo-Carrasco F, Wappler T, Rust J, Aberer AJ, H, Bartel D, Blanke A, Berger S, Bohm A, Buckley TR, Calcott B, Chen J, Friedrich F, Fukui M, Fujita M, Greve C, Grobe P, Gu S, Huang Y, Jermiin LS, Kawahara AY, Krogmann L, Kubiak M, Lanfear R, Letsch H, Li Y, Li Z, Li J, Lu H, Machida R, Mashimo Y, Kapli P, McKenna DD, Meng G, Nakagaki Y, Navarrete-Heredia JL, Ott M, Ou Y, Pass G, Podsiadlowski L, Pohl H, von Reumont BM, Schutte K, Sekiya K, Shimizu S, Slipinski A, Stamatakis A, Song W, Su X, Szucsich NU, Tan M, Tan X, Tang M, Tang J, Timelthaler G, Tomizuka S, Trautwein M, Tong X, Uchifune T, Walzl MG, Wiegmann BM, Wilbrandt J, Wipfler B, Wong TK, Wu Q, Wu G, Xie Y, Yang S, Yang Q, Yeates DK, Yoshizawa K, Zhang Q, Zhang R, Zhang W, Zhang Y, Zhao J, Zhou C, Zhou L, Ziesmann T, Zou S, Li Y, Xu X, Zhang Y, Yang H, Wang J, Wang J, Kjer KM, Zhou X. 2014 Phylogenomics resolves the timing and pattern of insect evolution. *Science*, 346:763-767.
- Newton L, Tolman E, Kohli M, Ware JL. 2023 Evolution of Odonata: genomic insights.CurrentOpinioninInsectScience,58<a href="https://doi.org/10.1016/j.cois.2023.101073">https://doi.org/10.1016/j.cois.2023.101073</a>
- Novelo-Gutiérrez R, Bota-Sierra CA, Amaya-Vallejo V. 2020 Description of the larva of the genus Archaeopodagrion Kennedy, 1939 (Zygoptera: Philogeniidae). Zootaxa 4816(3): 325–332.
- Orr MC, Ferrari RR, Hughes AC. 2021 Taxonomy must engage with new technologies and evolve to face future challenges. *Nature Ecology and Evolution* **5**, 3–4 https://doi.org/10.1038/s41559-020-01360-5
- Ortega- Salas H, Gonzalez- Soriano E, Jocque, M. 2022 Untangling the waterfall damsels: a review of the Mesoamerican genus *Paraphlebia* Selys in Hagen, 1861

(Odonata: Thaumatoneuridae) with descriptions of 11 new species. *Zootaxa* **5089**, 001–066.

- Osozawa S, Sato F, Wakabayashi J. 2017 Quaternary Vicariance of Lotic *Coeliccia* in the Ryukyu-Taiwan Islands Contrasted with Lentic *Copera*. *Journal of Heredity*, 280–287 doi:10.1093/jhered/esx007
- Padial JM, Miralles A, De la Riva I, Vences M. 2010 The integrative future of taxonomy. *Frontiers in Zoology* 7:16.
- Pante E, Puillandre N, Viricel A, Arnaud-Haond S, Aurelle D, Castelin M, Chenuil A, Destombe C, Forcioli D, Valero M, Viard F & Samadi S. 2015 Species are hypotheses: Avoid connectivity assessments based on pillars of sand. *Molecular Ecology*. 24. 525–544. 10.1111/mec.13048.
- Paulson DR, Marinov M. 2021 Zootaxa 20th Anniversary Celebration: Odonata section. *Zootaxa*, 4979(1), 218–221. https://doi.org/10.11646/zootaxa.4979.1.21
- Pfenninger M, Schwenk K. 2007 Cryptic animal species are homogeneously distributed among taxa and biogeographical regions. BMC Evol Biol 7: 121. BMC evolutionary biology. 7. 121. 10.1186/1471-2148-7-121.
- Pérez-Gutiérrez LA, Montes-Fontalvo JM (2011a) *Heteropodagrion croizati* **sp. nov.** (Odonata: Megapodagrionidae) with a key to the known species of the genus. *Zootaxa* **2810**, 63–68.
- Pérez-Gutiérrez LA, Montes-Fontalvo JM. 2011b Rediscovery of *Mesagrion leucorrhinum* (Zygoptera: Megapodagrionidae): a "formal" description of female and ultimate stadium of larva with notes on habits. *International Journal of Odonatology*, **14**, 91–100.
- Pimenta AL, Pinto A, Takiya D. 2019 Integrative taxonomy and phylogeny of the damselfly genus *Forcepsioneura* Lencioni, 1999 (Odonata: Coenagrionidae: Protoneurinae) with description of two new species from the Brazilian Atlantic Forest. Arthropod Systematics and Phylogeny 77, 397–415.
- Pinto, AP. 2016 A fauna de libélulas da América do Sul: a última fronteira a ser desvendada. *Informativo Sociedade Brasileira de Zoologia*. 117. 7-9.
- Pinto AP. 2024 Cap. 15, Odonata Fabricius, 1793, pp. 187-233. In: Rafael, J.A.; Melo, G.A.R.; Carvalho, C.J.B. de; Casari, S. & Constantino, R. (eds). Insetos do Brasil: Diversidade e Taxonomia. 2<sup>a</sup> ed. Instituto Nacional de Pesquisas da Amazônia, Manaus. 880 pp. https://doi.org/10.61818/56330464c15

- Pinto AP, Bota-Sierra CA, Marinov M. 2023 Species identification and description, in: Cordoba-Aguilar A, Beatty Ch, Bried J (eds), Dragonflies and Damselflies: Model Organisms for Ecological and Evolutionary Research, 2nd edn. Oxford, Oxford Academic press, <u>https://doi.org/10.1093/oso/9780192898623.003.0019</u>.
- Pons J, Barraclough TG, Gomez-Zurita J, Cardoso A, Duran DP, Hazell S, Kamoun S, Sumlin WD. 2006 Sequence-Based Species Delimitation for the DNA Taxonomy of Undescribed Insects. *Systematic Biology* **55**, 595–609.
- Puillandre N, Lambert A, Brouillet S, Achaz G. 2012 ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology* 21, 1864–1877. doi: 10.1111/j.1365–294X.2011.05239.x.
- Quang MB, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, von Haeseler A, Lanfear R. 2020 IQ-TREE 2: New models and efficient methods for phylogenetic inference in the genomic era. *Molecular Biology and Evolution*, 37(5): 1530-1534. <u>https://doi</u>.org/10.1093/molbev/msaa015
- Rácenis J. 1959 Notas taxonomicas sobre la familia Megapodagrionidae (Odonata: Zygoptera) con las espécies venezolanas. *Acta Biologica Venezuelica* 2, 335– 367.
- Rambout, A. GitHub. Available online: http://tree.bio.ed.ac.uk/software/figtree/
- Riek ER, Kukalová-Peck J. 1984 A new interpretation of dragonfly wing venation based upon Early Upper Carboniferous fossils from Argentina (Insecta: Odonatoidea) and basic character states in pterygote wings. *Canadian Journal of Zoology* 62(6): 1150-1 166.
- Ris F. 1918 Libellulin (Odonata) aus der Region der amerikanischen Kordilleren von Costarica bis Catamarca.: *Archiv für Naturgeschichte (Abteilung A)* **82**, 1–197.
- Sánchez- Herrera M, Realpe E, Salazar C. 2010 A Neotropical polymorphic damselfly shows poor congruence between genetic and traditional morphological characters in Odonata, *Molecular Phylogenetics and Evolution*, 57(2): 912-917, <u>https://doi.org/10.1016/j.ympev.2010.08.016</u>.
- Sánchez- Guillén R, Ceccarelli S, Villalobos F, Neupane S, Rivas- Torres A, Sanmartín-Villar I, Wellenreuther M, Bybee S, Velasquez-Vélez M, Realpe E, Chávez-Ríos J, Dumont H, Cordero-Rivera A. 2020 The evolutionary history of colour polymorphism in *Ischnura* damselflies (Odonata: Coenagrionidae). *Odonatologica*. 49: 333-370. 10.5281/zenodo.4268559.

- Schlick-Steiner B, Steiner F, Seifert B, Stauffer C, Christian E, Crozier R. 2010 Integrative taxonomy: a multisource approach to exploring biodiversity. Annual Review of Entomology 55: 421–38.
- Selys-Longchamps E De. 1862 Synopsis des *Agrionines*. 3e legion: *Podagrion*. *Bulletin Academie Royale Belgique* Serie **2**, 5–44.
- Selys-Longchamps E De. 1885 Programme d'une revision des Agrionines. *Comptes Rendus de la Societe Entomologique de Belgique* **29**, cxli– cxlvi (1–8 separate).
- Selys-Longchamps E De. 1886 Revision du Synopsis des Agrionines. Première partie comprenant les légions *Pseudostigma–Podagrion–Platycnemis* et *Protonevra*. *Memoires couronnes et autres memoires, l'Academie royale de medecine de Belgique* 38, 1–233.

Silsby J. 2001 Dragonflies of the World. CSIRO Publishing, Collingwood.

- Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P. 1994 Evolution, Weighting, and Phylogenetic Utility of Mitochondrial Gene Sequences and a Compilation of Conserved Polymerase Chain Reaction Primers, *Annals of the Entomological Society of America*, 87, 651-701, <u>https://doi.org/10.1093/aesa/87.6.651</u>
- Schneider T, Vierstraete A, Müller O, Van Pelt GJ, Caspers M, Ikemeyer D, Snegovaya N, Dumont HJ. 2021 Taxonomic Revision of Eastern Part of Western Palaearctic *Cordulegaster* Using Molecular Phylogeny and Morphology, with the Description of Two New Species (Odonata: Anisoptera: Cordulegastridae). *Diversity* **13**, 667.https://doi.org/10.3390/d13120667
- Steinmann H. 1997 World catalogue of Odonata. Volume I. Zygoptera. In: H. Wermuth and M. Fischer (eds.), Das Tierreich. The Animal Kingdom. Eine Zusammenstellung und Kennzeichnung der rezenten Tierformen. Walter de Gruyter, Berlin, **110**, xxi + 500 pp.
- Tamura K, Stecher G, Kumar S. 2021 MEGA11: Molecular Evolutionary Genetics Analysis version 11. *Molecular Biology and Evolution* 38:3022-3027.
- Tennessen KJ. 2010 The madicolous nymph of *Heteropodagrion sanguinipes* Selys (Odonata: Megapodagrionidae). *Zootaxa* **2531**, 29–28.
- Tillyard RJ. 1917 The Biology of Dragonflies (Odonata or Paraneuroptera). Cambridge University Press, Cambridge Zoological Series, London, xii + 396 pp.
- Tsuda S. 1986 A distributional list of world Odonata, Preliminary edition. Published by author, Osaka, 246 pp.

- Vaidya G, Lohman D, Meier R. 2010 SequenceMatrix: Concatenation software for the fast assembly of multi-gene datasets with character set and codon information. *Cladistics*. 27. 171 - 180. 10.1111/j.1096-0031.2010.00329.x.
- Vega-Sánchez YM, Mendoza-Cuenca LF, González-Rodríguez A. 2019 Complex evolutionary history of the American Rubyspot damselfly, *Hetaerina americana* (Odonata): Evidence of cryptic speciation, *Molecular Phylogenetics and Evolution*139, https://doi.org/10.1016/j.ympev.2019.106536.
- Villela DS, Koroiva R, Cordero-Rivera A, Guillermo-Ferreira R. 2019 A further study on *Franciscobasis* Machado and Bedê, 2016 (Odonata: Coenagrionidae), a newly described genus from Minas Gerais, Brazil. *PLoS One* 14, e0223241.<u>https://doi.org/10.1371/journal.pone.0223241</u>
- Wheeler QD. 2007 Invertebrate systematics or spineless taxonomy? In: Zhang ZQ and Shear WA (eds) Linnaeus Tercentenary: Progress in Invertebrate Taxonomy. *Zootaxa*, 1668, 1-766.
- Yu X, Xue JL, Hämäläinen M, Liu Y, Bu WJ. 2015 A revised classification of the genus Matrona Selys, 1853 using molecular and morphological methods (Odonata: Calopterygidae). Zoological Journal of the Linnean Society **174**, 473–486. <u>https://doi.org/10.1111/zoj.12253</u>
- Yu X, Xue J. 2020 A review of the damselfly genus *Megalestes* Selys, 1862 (Insecta: Odonata: Zygoptera: Synlestidae) using integrative taxonomic methods. *Zootaxa* 4851, 245–270.
- Zhang J, Kapli P, Pavlidis P, Stamatakis A. 2013 A General Species Delimitation Method with Applications to Phylogenetic Placements. *Bioinformatics* (Oxford, England) **29**, 2869–2876.
- Zhang H, Ning X, Yu X, Wen-Jun Bu. 2021 Integrative species delimitation based on COI, ITS, and morphological evidence illustrates unique evolutionary history of the genus *Paracercion* (Odonata: Coenagrionidae). *PeerJ* 9, e11459 DOI 10.7717/peerj.11459.