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Genetic diversity calibration in species of the genus *Frankliniella*: new cases of cryptic species

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Abstract

Frankliniella Karny is the third most speciose genus within Thysanoptera, with 236 valid species. Despite its high economic relevance and remarkable diversity, the Linnean and Wallacean deficits, as well as the paucity of biological and ecological data are evident, especially due to the confusing taxonomy of the group. In this sense, we carried out a molecular analysis based on the Cytochrome C Oxidase subunit I (COI) gene and an extensive data set towards understanding the molecular delimitation, as well as establishing genetic diversity thresholds among *Frankliniella* species. Our study included 348 specimens (20 from the present study) referring to 43 BINs (Barcode Index Numbers) available in BOLD. For that, the traditional analysis of DNA barcode (Neighbor-Joining and genetic distances) and the determination of the diversity threshold were carried out, together with two types of phylogenetic analyses (Maximum Likelihood and Bayesian Inference) and five species delimitation algorithms (ABGD, PTP, GMYC, PhyloMap, BIN). Our analyses identified with high confidence 42 putative species in *Frankliniella*, suggesting cases of taxonomic misidentification, and a threshold of 2.35%, which allowed us to recognize a cryptic diversity and/or species complexes in five species of the genus: *F. insularis* (Franklin), *F. intonsa* (Trybom), *F. occidentalis* (Pergande), *F. schultzei* (Trybom) and *F. tritici* (Fitch). The present data demonstrate the efficiency of molecular markers for the identification of independent evolutionary units in *Frankliniella*, reinforcing the need to carry out an extensive taxonomic review in this important group of thrips since different studies already pointed to incongruity in the diagnosis of species through traditional taxonomy.

Key words: BOLD, DNA barcode, delimitation algorithms, taxonomic misidentification, thrips.

Introduction

The Thysanoptera order comprises about 6,337 valid species (ThripsWiki, 2022), being very diverse in the Neotropics (Mound and Marullo, 1996), mainly in Brazil, where approximately 617 species occur (Monteiro and Lima, 2020). Among their members, *Frankliniella* Karny is the third most speciose genus, including 236 valid species (ThripsWiki, 2022) 90% of them which occur in the neotropical region (Mound and Kibby, 1998). The genus has high economic importance, since, among the 16 species of Thysanoptera that are vectors of *Orthotospovirus*, nine belong to *Frankliniella* (Rotenberg *et al.*, 2015; Keough *et al.*, 2016).

Some studies were of great importance for knowledge of the genus, such as taxonomic identification keys for *Frankliniella* species (Cavalleri and Mound, 2012; Lima and Miyasato, 2017), as well as some studies on the biology of the genus (Berzosa and Maroto, 2003; Zhang *et al.*, 2007; Alves-Silva *et al.*, 2013; Gilbertson *et al.*, 2015; Lima *et al.*, 2016). Nevertheless, taxonomic studies on Thysanoptera are still scarce, as well as data regarding the biology, behaviour, and geographic distribution (Mound, 2002). The lack of such information may be attributed to issues in the taxonomic classification of the order. In general, specimen identification is based on morphological characters, which in many cases, are not informative due to a low phenotypic variation among species (Brunner *et al.*, 2004). The tiny size of the specimens jointly with the fact that the identification keys are elaborated considering solely adult individuals' morphology (Brunner *et al.*, 2002; Glover *et al.*, 2010), the occurrence of sexual dimorphism, cryptic species, and species complex (Tyagi *et al.*, 2008; Iftikhar *et al.*, 2016) are the main factors that hamper the diagnosis of this group. Additionally, the slide preparation process for taxonomic identification is meticulous and time-consuming and often depend on specialists in the taxonomy of this group, considering that the morphological differences among species may be very subtle and susceptible to errors. Some authors even suggest the use of scanning electron microscopy, which together with molecular techniques can be used for the precise identification of species, but the former is expensive (Kumar *et al.*, 2014).

Therefore, molecular identification markers such as DNA barcode can be considered an interesting alternative to traditional methods of identification, representing a valuable tool to assist in taxonomic issues (Karimi *et al.*, 2010). The identification system, based on the mitochondrial Cytochrome C Oxidase subunit I (COI) gene has been used effectively (Hebert *et al.*, 2003), becoming a universal marker in molecular taxonomy (Bueno-Silva, 2012). Within the order, the COI gene has been shown to be efficient and necessary for taxonomy, helping to elucidate taxonomic problems, uncover new species, detect cryptic species and even species complexes (Rebijith *et al.*, 2014; Iftikhar *et al.*, 2016; Tyagi *et al.*, 2017), besides enabling the creation of a barcode reference library that is essential for accurate identification (Marullo *et al.*, 2020). Thus, our study was conducted to determine thresholds of genetic distance among *Frankliniella* species using a DNA barcode, which can be used as an indicator of cryptic diversity and detect morphological misidentification within this emblematic thrips genus.

Materials and methods

Collection and identification of biological material

A total of 20 specimens of *Frankliniella* (11 *F. tritici* (Fitch), one *F. gardeniae* Moulton, three *F. schultzei* (Trybom) and five *F. insularis* (Franklin), were collected in Maracás (13.4411°S 40.4308°W), Dario Meira (14.4364°S 39.9078°W) and Boa Nova (14.3625°S 40.2075°W), all municipalities situated in the southwest of Bahia state, Brazil. The collection technique used was the "beating tray" method and then the specimens were stored in 2 ml microtubes with ethanol pro analysis. The specimens had the DNA extracted, and then were mounted on slides and stored at the Insect Biology Lab - Universidade Estadual do Sudoeste da Bahia UESB. They were identified by Adriano Cavalleri, an expert taxonomist of Thysanoptera.

Obtaining of the gene fragments mtDNA COI-5p

For total DNA extraction, we used the "salting-out" protocol detailed in Sunnucks and Hales, (1996), and adapted by Rugman-Jones *et al.*, (2006) it is a non-destructive extraction method, which makes it possible to mount the specimens on slides for morphological identification after DNA extraction. The amplification reaction was performed using universal primers LCO-1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO-2198 (5′- TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) (Folmer *et al.*, 1994) and it consisted of a total volume of 25 µL containing: 0.08 pmol of each primer, 3 mM MgCl2, 0.2 mM dNTPs, 2.5 µL of 10× buffer, 2.5 U of Taq polymerase and 1 µL of extracted DNA (not quantified).

The amplification consisted in an initial denaturation step of 94 °C for 3 minutes, followed by 35 cycles: Denaturation at 92 °C for 1 minute, primer annealing at 54 °C for 2 minutes and extension at 72 °C for 2 minutes, with final extension at 72 °C for 7 minutes. All reactions were accompanied by a negative control. The amplification products were verified by electrophoresis on agarose gel at 1.2%, after staining with bromophenol blue and GelRed (Biotium, USA) in the proportion of 3:1, visualized in ultraviolet light and photo documented with the LPix device (Loccus®).

The sequencing was performed by the companies: ACTGene and Fiocruz. The PCR products were purified in 20% polyethylene glycol (PEG) followed by two washes with 80% alcohol and rehydration using the solution from Wizard® Genomic DNA Purification (Promega). The sequencing reactions were performed according to the terminal dideoxynucleotide method (Sanger *et al.*, 1977) using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems/Life Technologies, Foster City, CA, USA). The samples were analysed in an automatic sequencer ABI 3500XL (Applied Biosystems, Foster City, CA, USA). The sequencing was performed in both directions, and the same primers from the PCR step were used.

Data analyses

The twenty sequences were obtained using the "DNA Baser Sequence Assembler" v. 4.16 program (Heracle Biosoft, 2014) and deposited in the Barcode Of Life Data Systems (BOLDSYSTEMS, 2019) platform in the project named "Thysanoptera of Bahia - THYS", and automatically assigned to a Barcode Index Number - BIN (group of sequences corresponding to a single taxon), following the analytical procedures established by Ratnasingham and Hebert (2013).

In order to carry out an accurate genetic calibration, sequences of all *Frankliniella* species available in BOLD were added to the database, totalling 347 sequences referring to 17 morphological taxa (*Frankliniella* sp., *F. australis* Morgan, *F. aztecus* (Crawford), *F. bispinosa* (Morgan), *F. borinquen* Hood, *F. citripes* Hood, *F. fortissima* Priesner, *F. gardeniae*, *F. insularis*, *F. intonsa* (Trybom), *F. minuta* (Moulton), *F. occidentalis* (Pergande), *F. panamensis* Hood, *F. schultzei*, *F. tritici*, *F. tenuicornis* (Uzel) and *F. unicolor* Morgan, together with one sequences of *Pseudanaphothrips achaetus* (Bagnall) which were used as outgroup (table 1). The minimum size (> 500 base pairs) was the criterion adopted to select the sequences of *Frankliniella* available on the platform since this is a necessary condition to validate the sequences deposited as barcode tags and all BINs. Additionally, aiming to cover all genetic diversity, the public BOLD sequences were subsequently selected based on the haplotypes of each BIN, using the DnaSP v5 software (Librado and Rozas, 2009), totalling 40 of the 41 BINS of *Frankliniella* present in BOLD system. The sequence alignment was performed in Mega X (Kumar *et al.*, 2018), using the ClustalW tool (Thompson *et al.*, 1994), as well as their translation, to verify possible stop codons. In the same software, a dendrogram was generated using the Neighbor-Joining distance method (NJ), based on the Kimura-2-parameters model (K2P) (Kimura, 1980) and 1000 "bootstrap" pseudo-replicates (Felsenstein, 1985).

Phylogenetic analyses were performed using Bayesian Inference (BI) and Maximum Likelihood (ML) in the software Mr. Bayes 3.2.6 (Ronquist and Huelsenbeck, 2003) and RAxML-HPC BlackBox 8.2.10 (Stamatakis, 2014), respectively, both implemented at the CIPRES Science Gateway 3.3 (http://www.phylo.org/index.php). For the BI reconstruction, two independent series with four Markov chains and 10,000,000 generations were used, with 10% burn-in and GTR + G nucleotide evolution model obtained in Kakusan v.xx (Tanabe, 2007). The performance of this analysis was verified using Tracer v. 1.6 (Rambaut *et al.*, 2013) and the trees generated were edited in FigTree v.1.4.2 (Rambaut, 2014) and ©Adobe Photoshop CC v.14.0 to build a summary tree.

In order to delimit evolutionary units in *Frankliniella*, several species delimitation algorithms were tested, using only the barcode sequences of the species obtained in the present study, together with the haplotypes selected from the free platforms (BOLD and GenBank). For the "Automatic Barcode Gap Discovery" (ABGD) algorithm (Puillandre *et al.*, 2012) the pairwise distance matrix, obtained from MEGA X (Kumar *et al.*, 2018), was used as input on the online server (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) with settings: Pmin = 0.001, Pmax = 0.1, steps = 10, X = 1.0 and Kimura 80 model.

The other algorithms were tested using a subset of the data containing a sequence for each haplotype. For "Poisson Tree Processes" (PTP) (Zhang *et al.*, 2013) and

Table 1. List of species included in BINs of the genus *Frankliniella*, and outgroup used in the database, as well as their respective BINs and countries of origin.

Species	BIN	Origin countries
F. australis	ACD4752	-
F. bispinosa	AAL2827	USA
F. gardeniae	ADG9125	Brazil
F. insularis	ADG9126	Brazil
F. insularis *F. fortissima* *F. aztecus* *F. citripes*	AAM8084	Singapore, Mexico, Costa Rica, Puerto Rico, Colombia, Malaysia
F. intonsa	AAF6737	Japan, China, Norway, USA, Serbia, UK, Germany, India, Russia, Bangladesh, Belgium
F. intonsa	AAN4977	USA
F. occidentalis	ACB4865	USA, Canada
F. occidentalis	ACZ4231	USA, Canada, China, Mexico
F. occidentalis *F. borinquen*	AAB8717	Canada, Germany, USA, China, Netherlands, Australia, New Zealand, Japan, Croatia, South Africa, Norway, Serbia, UK, Kenya, Colombia, India, Zambia, Chile, Mexico
F. panamensis	ADH2227	Colombia
F. schultzei	ACD4589	Canada, USA, Australia, India, Pakistan
F. schultzei	AAN6620	Pakistan, Australia, India, Kenya, Brazil, South Africa
F. schultzei	ACK5130	South Africa, Kenya
F. schultzei	ACL1106	South Africa
F. schultzei	ACY9272	India
F. schultzei	ADH9591	India
F. schultzei *Thrips palmi* *T. parvispinus*	ACP1643	Indonesia, Malaysia
F. schultzei *Scirtothrips dorsalis* *F. minuta*	AAM8089	Australia, India
F. tenuicornis	AAT9732	China, Norway
F. tritici	ADG9127	Brazil
F. tritici	AAM7855	USA, Canada, Indonesia
F. tritici	AAG0734	Canada, USA
F. tritici	ACS6765	Kenya
F. unicolor	ACR6111	India
Frankliniella	AAL2817	Canada, USA
Frankliniella	AAM5264	Canada
Frankliniella	AAN4978	Canada
Frankliniella	AAN4979	USA
Frankliniella	AAP8870	Mexico, Honduras, Costa Rica
Frankliniella	ACC0793	Canada
Frankliniella	ACC5047	USA
Frankliniella	ACF5981	Honduras
Frankliniella	ACI5056	Canada
Frankliniella	ACK2804	Canada
Frankliniella	ACL2814	Canada
Frankliniella	ACT0860	Honduras
Frankliniella	ACT3264	Honduras, Costa Rica
Frankliniella	ACT3733	Honduras
Frankliniella	ACU2813	Canada
Frankliniella	ACY3569	Canada
Frankliniella	ADA9841	Costa Rica
Pseudonaphothrips achaetus	ACD6122	-

Phylogenetic Map PTP (PhyloMap) analysis (Zhang *et al*., 2011) we used an ML tree, obtained in RAxML-HPC BlackBox 8.2. 10, implemented at CIPRES, as an entry on online servers (http://mptp.h-its.org. and http://species.h-its.org/). For the Single-rate PTP analysis, a single value (p = 0.001) is assumed as a threshold for genetic diversity, while in PhyloMap combines the PTP data with Principal Coordinate Analysis (PCoA), discriminating species from a rooted phylogenetic tree.

For the multiple and simple analyses of the General Mixed Yule-Coalescent (GMYC) algorithm (Fujisawa and Barraclough, 2013; Zhang *et al*., 2013), available on the platform https://species.h-its.org/gmyc/, it was incorporated as an input file, an ultrametric phylogenetic tree, obtained in BEAST 1.8.2 (Drummond *et al*., 2012) according to the criteria: GTR + G substitution model, strict molecular clock, arbitrary mutation rate (1.0 substitution / site / Ma), Yule's coalescence method a priori, 10 million generations with sampling every 1,000 generations. In this method, species are identified by changes in the rate of branching in the input tree.

Based on the groupings obtained by most analyses, the intra and interspecific distances were calculated according to the K2P model (Kimura, 1980) and 1000 "bootstrap" psdeudoreplics (Felsenstein, 1985), in the Mega X software (Kumar *et al*., 2018). The interspecific distance matrix, was converted in a heat map using the online tool Heatmapper (Babicki *et al*., 2016). Then, a potential threshold was established among *Frankliniella* species / lineages, considering the distance matrix based on the K2P

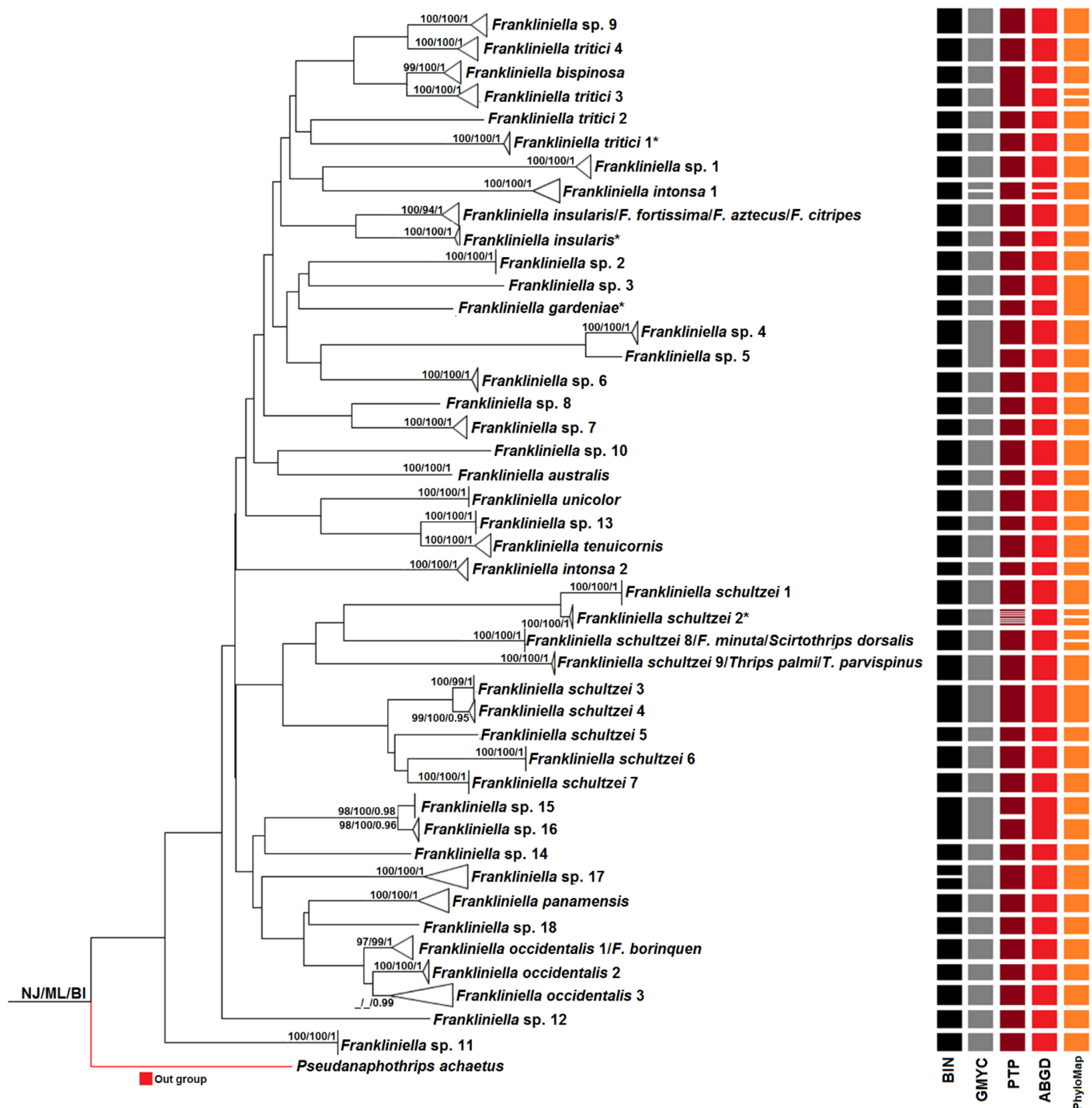

Figure 1. Summary tree based on NJ, ML and BI with their respective support values indicated in each branch. Values below 96% of bootstrap and 0.9 probability posteriori were not shown. The columns present the results obtained by PTP, ABGD, GMYC, BIN and PhyloMap analyses. (*) Refers to groups that have sequences generated in this study.

Table 2. Intraspecific genetic distance of the Molecular Operational Taxonomic Units (MOTUs) defined within the genus *Frankliniella* by the majority of the analyses performed in the present study and obtained based on the COI gene, K2P and 1000 bootstrap pseudo-replicates (n/c refers to species with only one sequence).

Species	Intraspecific Distance (%)
Frankliniella intonsa 1	0.88
Frankliniella sp. 1	0.34
F. schultzei 1	0
F. schultzei 2	0.13
F. insularis / F. fortissima / F. aztecus / F. citripes	0.49
F. insularis	0.11
Frankliniella sp. 2	0
Frankliniella sp. 3	n/c
F. gardeniae	n/c
Frankliniella sp. 4	0.17
Frankliniella sp. 5	n/c
Frankliniella sp. 6	0.10
Frankliniella sp. 7	0.45
Frankliniella sp. 8	n/c
F. tritici 1	0.20
F. tritici 2	n/c
F. tritici 3	0.34
F. bispinosa	0.37
F. tritici 4	0.68
Frankliniella sp. 9	0.43
Frankliniella sp. 10	n/c
F. australis	n/c
Frankliniella sp. 11	0.08
F. intonsa 2	0.28
Frankliniella sp. 12	n/c
F. tenuicornis	0.40
Frankliniella sp. 13	0
F. unicolor	0
Frankliniella sp. 14	n/c
Frankliniella sp. 15 / *Frankliniella* sp. 16	1.13
Frankliniella sp. 17	0.86
F. panamensis	0.67
Frankliniella sp. 18	n/c
F. occidentalis 1 / *F. borinquen*	0.54
F. occidentalis 2	0.08
F. occidentalis 3	1.23
F. schultzei 3 / *F. schultzei* 4	0.89
F. schultzei 5	n/c
F. schultzei 6	0
F. schultzei 7	0
F. schultzei 8 / *F. minuta / Scirtothrips dorsalis*	0
F. schultzei 9 / *Thrips palmi / T. parvispinus*	0.14
Mean	0.33

model using the localMinima function available in the SPIDER v1.3-0 package (Brown *et al*., 2012) in R program (R Development Core Team, 2013).

To understand the relationship and geographic distribution of genetic variation among species haplotypes that showed cryptic diversity/morphological misidentification, haplotype networks were generated using the Median-Joining Network method (Bandelt *et al*., 1999) implemented in Population Analysis with Reticulate Trees (POPART) (Leigh and Bryant, 2015), excluding missing and ambiguous data.

Results

The analysed database was composed of 348 sequences, with a size of 590 base pairs, totalling 43 BINs, being 3 exclusive BINs (ADG9125, ADG9127, and ADG9126) referring to *F. insularis*, *F. gardeniae*, and *F. tritici* 1, which were generated from the sequences of this study.

The ABGD algorithm delimited 43 species, PTP (Poisson Tree Processes) 48, PhyloMap (Phylogenetic Map PTP) 44, BIN 43, and GMYC (General Mixed Yule-Coalescent) 42 species. Molecular analyses revealed the presence of 42 Molecular Operational Taxonomic Units

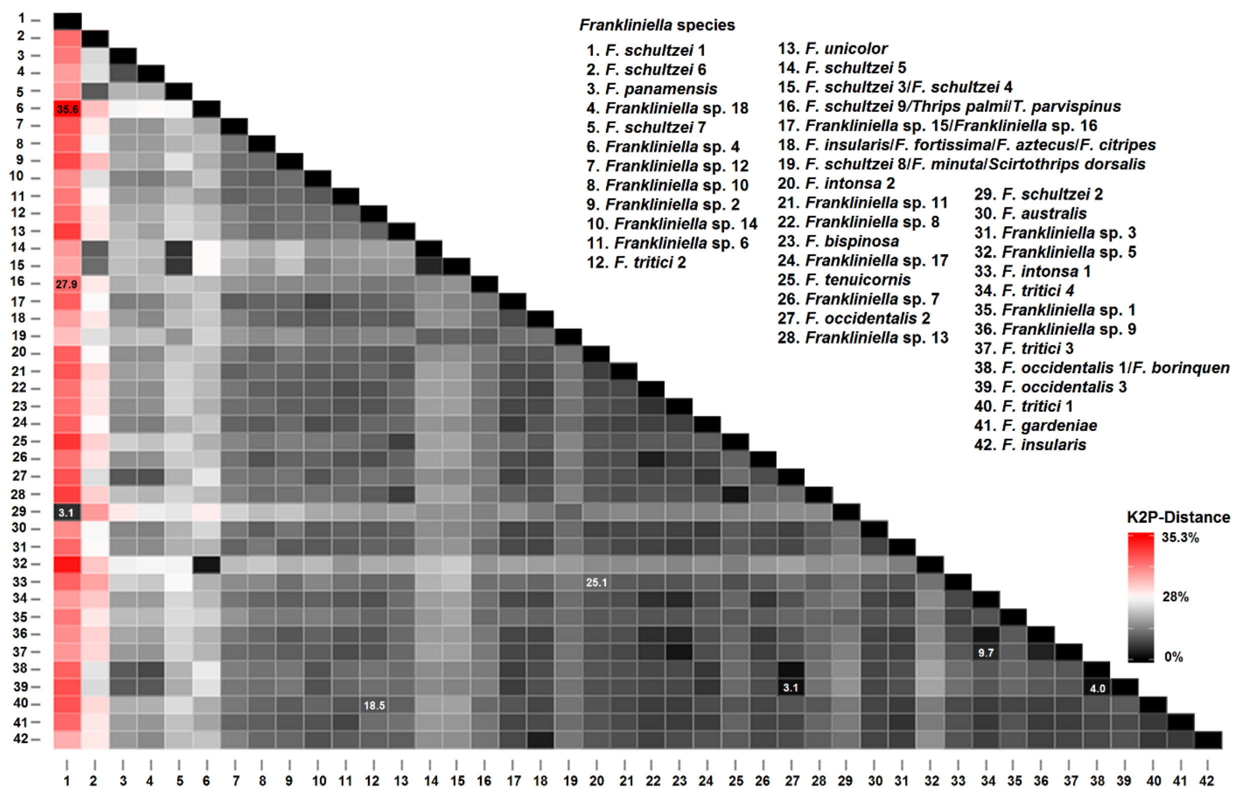

Figure 2. Heatmap showing the interspecific distances among the *Frankliniella* MOTUs, based on the K2P in COI sequences. The minimum and maximum values (0 to 35.3%), as well as values among cryptic species are highlighted in the image.

(MOTUs) defined based on groupings with high support values (≥ 99 bootstrap and ≥ 0.9 of posterior probability) in at least two of the methodologies used for tree reconstruction (NJ, ML and BI) and / or at least 3 species delimitation algorithms (figure 1, table 2).

However, incongruences were observed in the delimitation of some MOTUs: *F. intonsa* 1 identified as two species in ABGD and GMYC; *F. schultzei* 2 divided into seven taxonomic units in PTP and two in PhyloMap; *Frankliniella* sp. 3 and *F. gardeniae* considered as a single species on PhyloMap; *Frankliniella* sp. 4 and *Frankliniella* sp. 5 treated like a single taxon in the GMYC; *F. tritici* 3 divided in two taxonomic units in PhyloMap but grouped with *F. bispinosa* after PTP analyse; *Frankliniella* sp. 15 / *Frankliniella* sp. 16 separated into two units by PTP and PhyloMap; *Frankliniella* sp. 12 and *F. tenuicornis* as a single taxon on PhyloMap; *Frankliniella* sp. 17 separated into two units by BIN; *F. schultzei* 8 / *F. minuta* / *Scirtothrips dorsalis* (Hood) considered as two evolutionary units by PhyloMap (figure 1).

The intraspecific distances of COI-5p ranged from 0% (*F. schultzei* 1, *Frankliniella* sp. 2, *Frankliniella* sp. 13, *F. unicolor*, *F. schultzei* 6, *F. schultzei* 7 e *F. schultzei* 8 / *F. minuta* / *S. dorsalis*) to 1.23% (*F. occidentalis* 3) with an average value of 0.33% (table 2). On the other hand, the interspecific distances ranged from 3.1% (between *F. occidentalis* 3 e *F. occidentalis* 2, and *F. schultzei* 1 and *F. schultzei* 2) to 35.3% (between *F. schultzei* 1 and *Frankliniella* sp. 4), with average value of 21.6% (figure 2, and supplemental material table S1). The average intraspecific distance was 65.4 times less than the average interspecific distance, indicating the existence of barcode gap.

The threshold graph determined a value of 2.35% for *Frankliniella* (figure 3), which indicates that species / lineages with intraspecific values above this can be considered distinct taxonomic units. All intraspecific values are below to the established threshold.

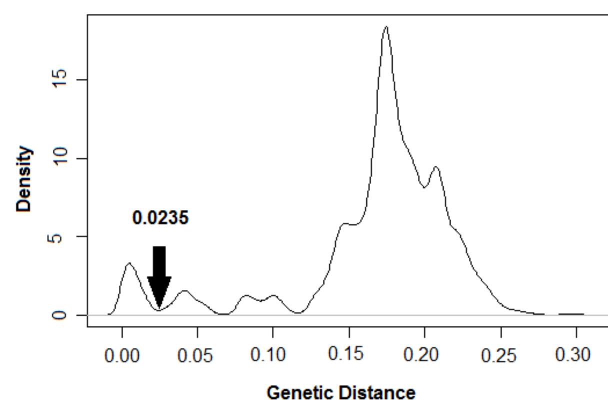

Figure 3. Graph obtained from a distance matrix based on the K2P model using the localMinima function available in the SPIDER v1.3-0 package demonstrating the genetic distance threshold of species of the genus *Frankliniella*.

In the haplotype network, twenty six haplotypes were obtained for *F. intonsa*, with *F. intonsa* 1 and *F. intonsa* 2 separated by 91 mutations (figure 4A); eight haplotypes for *F. insularis*, being *F. insularis* / *F. fortissima* / *F. aztecus* / *F. citripes* and *F. insularis* separated by 37 mutations (figure 4B); 27 haplotypes in *F. tritici*, separating *F. tritici* 3 from *F. tritici* 1 by 73 mutations, *F. tritici* 2 by 70 mutations and *F. tritici* 4 by 43 nucleotide changes (figure 4C); 50 haplotypes in *F. occidentalis*, separating *F. occidentalis* 3 from *F. occidentalis* 2 and *F. occidentalis* 1 by 9 and 10 mutations, respectively (figure 4D); 12 haplotypes for *F. schultzei*, and their MOTUs were separated by 3 to 77 mutations (figure 4E).

Discussion

Through the DNA barcode analysis carried out in the present study, it was possible to delimit with high efficiency 42 MOTUs among the 16 evaluated taxa, which were corroborated by most analyses (figure 1). However, inconsistencies were detected when the results of the algorithms were compared, as observed in *F. intonsa* 1, *F. schultzei* 2, *Frankliniella* sp. 3, *F. gardeniae*, *Frankliniella* sp. 4, *Frankliniella* sp. 5, *F. bispinosa*, *Frankliniella* sp. 15 / *Frankliniella* sp. 16, *Frankliniella* sp. 12, *F. tenuicornis*, *Frankliniella* sp. 17 and *F. schultzei* 8 / *F. minuta* / *S. dorsalis*. Studies that include divergences between algorithms are common and have already been described in the literature Thysanoptera species (Tyagi *et al*., 2017) and in insects of other orders such as Hemiptera (Martoni *et al*., 2018; Zheng, *et al*., 2020).

In fact, some algorithms tend to underestimate or overestimate the number of species in large data sets due to sampling and intraspecific diversity. Several studies compare the performance of GMYC, and PTP, and suggested that, in the absence of gene flow, such algorithms are influenced by the ratio between the population size and the divergence time, while the number of loci and the sample size of each species have less significant effects (Lou *et al*., 2018). GMYC and PTP generally act efficiently in scenarios involving more than one probable species in the absence of gene flow, but PTP outperforms GMYC when fewer species are involved (Fujisawa and Barraclough, 2013; Lou *et al*., 2018). On the other hand, ABGD detects the barcode gap as a significant gap and uses it to partition data, being sensitive to recent speciation events (Puillandre *et al*., 2012), so it is recommended the combined use of algorithms to generate greater reliability in the separation of potential species (MOTUs), as performed in the present study.

Considering the different operational criteria for the biological unit delimitation for biodiversity characterization, the internal algorithm of the BOLD database has been widely used. The BIN analyses all new sequences that are deposited, and groups them by clusters, that is, sequences belonging to the same genetic profile, corresponding to the species (Ratnasingham and Hebert, 2013). This algorithm provides a unique identification index, generating a page containing all the aggregated data of specimens belonging to each BIN, containing all the information with its associated metadata (Ratnasingham and Hebert, 2013). In this way, BIN is an important tool, since as all the metadata of the sequences are compiled in a single page, which makes it possible to detect taxonomic errors.

The efficiency of this analysis in the recovery of species limits has been evaluated by examining the correspondence between the recovered MOTUs and the Linnean species present in each data set, allowing the identification of four categories: a) match, when all specimens of the same species are grouped in a single BIN; b) split, when specimens of the same species are grouped in different BINs; c) merge, when different species are grouped in the same BIN and d) mixture, when the species present merge and split at the same time (Ratnasingham and Hebert, 2013). Based on this metric classification, it was possible to observe all the categories above mentioned (table 1), with emphasis on the cases of merge and mixture observed in the BINs: AAB8717 which includes *F. occidentalis* and *F. borinquen*; AAM8084 referring to *F. insularis*, *F. fortissima*, *F. citripes* and *F. aztecus*, the first two being considered synonymous (Mound, 2015); ACP1643 includes *F. schultzei*, *Thrips palmi* Karny, and *Thrips parvispinus* (Karny); while AAM8089 includes *F. schultzei*, *F. minuta* and *S. dorsalis*. Such clusters were corroborated by most of the analyses and presented intraspecific distances of 0.54%, 0.49%, 0.14% and 0%, respectively, values significantly lower than the genetic diversity threshold of 2.35% determined by the present study (figure 3). These results indicate serious morphological misidentification since they include not only distinct species but also different genera, probably as a reflection of the complex taxonomy of the group. As a consequence, such errors compromise the accuracy of the group's biodiversity inventories.

On the other hand, situations in which the same species has more than one BIN (split), corroborated by most of the algorithms and trees reconstructed in the present study, are observed in *F. occidentalis* (BINs AAB8717, ACB4865 and ACZ4231), *F. intonsa* (BINs AAF6737 and AAN4977), *F. tritici* (BINs AAG0734, AAM7855, ADG9127 and ACS6765) and *F. schultzei* (BINs ACL1106, ADH9591, AAM8089, AAN6620, ACD4589, ACK5130, ACP1643, ACV9287) (figure 1). These data are also supported by the interspecific distances calculated between the MOTUs identified in these taxa, ranging from 3.1% to 4% in *F. occidentalis*, 25.1% in *F. intonsa*, 9.7% to 18.5% in *F. tritici* and 3.1% to 27.9% in *F. schultzei* (figure 2), values above the genetic diversity threshold of 2.35% (figure 3) established for the *Frankliniella* genus. It is worth mentioning that the data of intraspecific distance of taxonomic units based only on morphology, disregarding the MOTUs determined here, varied from 2.59% in *F. occidentalis* to 18.07% in *F. schutzei* (table 3). In this sense, our results may indicate, in addition to errors of taxonomic identification, the existence of a complex of species/cryptic species and/or morphological misidentification in the group.

In recent years, several cases of species complex and cryptic species have been reported in Thysanoptera, mainly in the genera *Scirtothips*, *Thrips*, *Frankliniella*, and *Pseudophilothrips* (Mound *et al*., 2010; Rebijith *et al*., 2014; Iftikhar *et al*., 2016). Considering that the *Frankliniella*

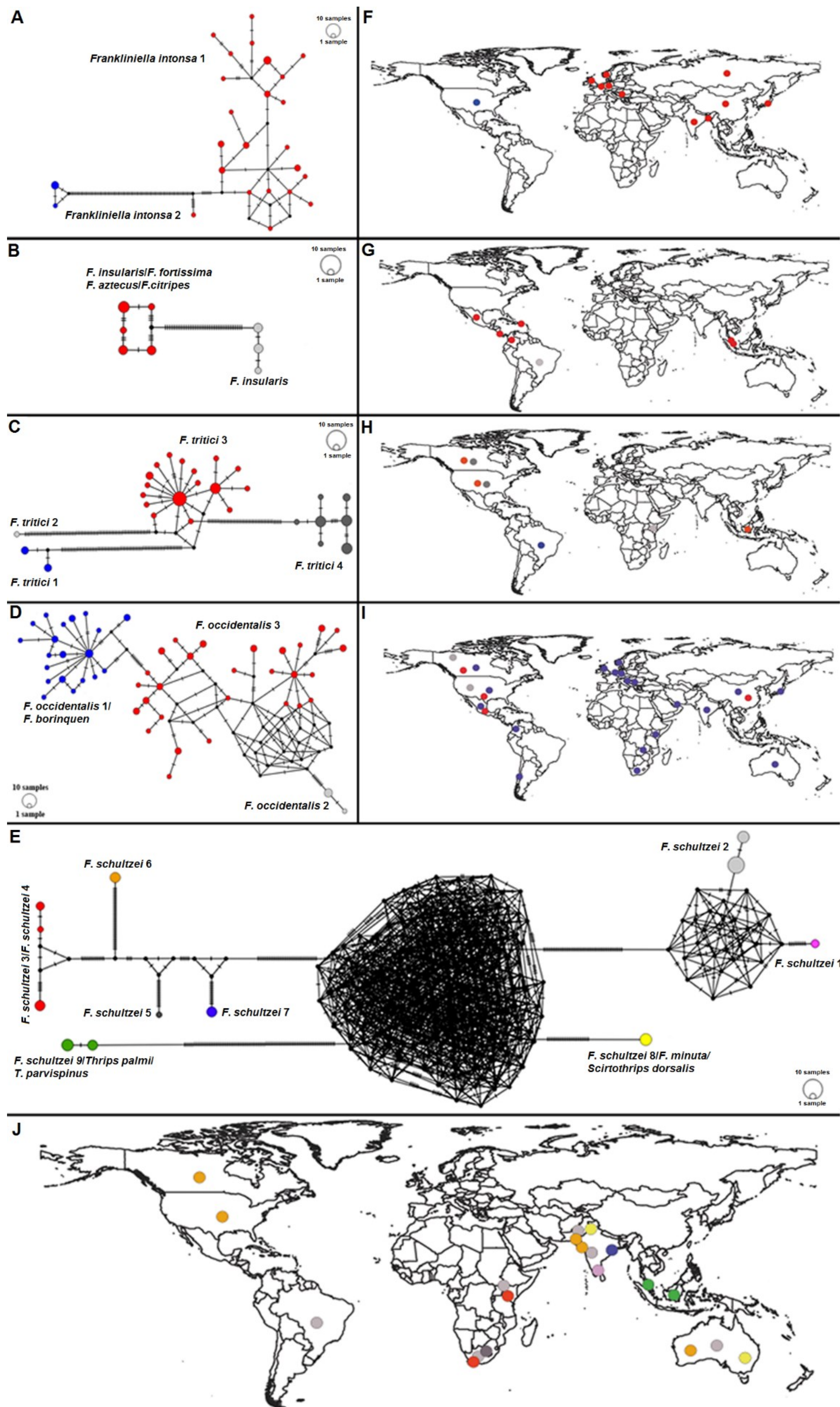

Figure 4. Haplotypes network demonstrating the relationships among *Frankliniella* cryptic species lineages as well morphological misidentification and the geographic distribution of lineages. (**A**) (**F**) *F. intonsa*; (**B**) (**G**) *F. insularis*; (**C**) (**H**) *F. tritici*; (**D**) (**I**) *F. occidentalis*; (**E**) (**J**) *F. shultzei*. The distinct colours in the circles indicate the different MOTUs, the size of the circles is proportional to the frequency of the data set haplotypes, small black circles represent hypothetical haplotypes (median vectors) and the perpendicular marks on the lines connecting the circles represent the number of steps mutational between haplotypes.

Table 3. Intraspecific genetic distance for *Frankliniella* species, defined by morphological identification, obtained based on the COI gene and K2P.

Species	Intraspecific distance (%)
F. occidentalis	2.59
F. tritici	7.9
F. intonsa	9.72
F. schultzei	18.07

species diagnosis is very problematic, the determination of a threshold indicator of cryptic diversity (figure 3) proves to be essential for the detection of new species, helping to reduce the taxonomic limitations. For example, in the case of *F. occidentalis*, in which a study using the COI and 28S genes indicated that this taxon actually includes two species that have undergone sympatric speciation and is therefore considered a case of cryptic species (Rugman-Jones *et al*., 2010). Previous studies, using COI and rRNA, ITS2 in specimens collected in China, already indicated such a possibility (Wu *et al*., 2009), which corroborates this hypothesis and still points to the possibility of up to five different genetic lineages (Rebijith *et al*., 2014), thus justifying the existence of more than one BIN in this taxon. The presence of different lineages was also observed in the present study, which determined three lineages from Canada, Germany, USA, China, The Netherlands, Australia, New Zealand, Japan, Croatia, South Africa, Norway, Serbia, UK, Kenya, Colombia, India, Zambia, Chile, and Mexico, separated by up to ten mutations (figure 4D).

Cases of cryptic species in the genus also include *F. schultzei*, which, in India, has no morphological differentiation other than variations in colour (dark, light, and brown), and comprises three genetic lineages (Tyagi *et al*., 2017), determined based on DNA barcode analysis and four delimitation algorithms (ABGD, GMYC, PTP and BIN) similarly to what has been described for this species in Australia (Hereward *et al*., 2017). Another recent study, using an integrative taxonomy approach in *F. schultzei* (morphometry, molecular and ecological data) in Kenya, also corroborates these data (Gykonio *et al*., 2017). In the present study, this species has eight divergent lineages supported by most analyses (figure 1) and are separated by a large number of mutations (figure 4E). It is worth mentioning that although the lineages defined here as 3 to 7 by *F. schultzei* are related to each other, the haplotype network did not recover a clear relationship among these lineages, as observed by a large number of median vectors, and the others represented in the network (*F. schultzei* 1, *F. schultzei* 2, *F. schultzei* 8 / *F. minuta* / *S. dorsalis*, *F. schultzei* 9 / *Thrips palmi* / *T. parvispinus*) due to the great genetic divergences among them, suggesting the existence of errors in morphological identification that can involve phylogenetically very distant species.

Unprecedented data of cryptic species or morphological misidentification in the genus *Frankliniella* were observed in the present study, referring to *F. intonsa*, *F. insularis*, and *F. tritici*. In the first case, two genetic lineages (*F. intonsa* 1 and *F. intonsa* 2) were supported by most analyses, where *F. intonsa* 1 (BIN AAF6737) includes mostly individuals from European and Asian countries, while *F. intonsa* 2 (BIN AAN4977) is restricted to USA (table 2). Both MOTUs were separated by 91 mutations, with *F. intonsa* 1 showing a greater haplotypic diversity (figure 4A). The second case includes two lineages of *F. insularis*, in which *F. insularis* / *F. fortissima* / *F. aztecus* / *F. citripes* (BIN AAM8084) include specimens from Singapore, Mexico, Costa Rica, Puerto Rico, Colombia and Malaysia and *F. insularis* (BIN ADG9126) with specimens from Brazil, they were separated by 37 mutations, however, there are caveats, since BIN AAM8084 groups 3 species (figure 4B), which can be not only a case of cryptic species but also misidentification.

Another species that has cryptic diversity or morphological misidentification is *F. tritici*, in which the presence of four genetic lineages was found, being *F. tritici* 4 (BIN AAG0734) from Canada and the USA, *F. tritici* 3 (BIN AAM7855) from the USA, Canada and Indonesia, *F. tritici* 2 (BIN ACS6765) from Kenya and *F. tritici* 1 (BIN ADG9127) from the present study, exclusive to Brazil, which accumulates 73 mutations in relation to *F. tritici* 3. This latter species, with high genetic variability, is separated from the other lineages (2 and 4) by 70 and 43 mutations, respectively (figure 4C). These lineages divergences can be explained by the great geographical extension that resulted in the isolation by distance and consequently reduction of the gene flow among populations. However, the absence of intermediate haplotypes (presence of median vector) observed among all MOTUs demonstrates that possible addictive events may be responsible for the "genetic break" observed in the network (figure 4), not ruling out the possibility of sampling deficit. In fact, in many cases, geographic isolation constitutes an effective barrier that contributes to speciation (McLeish *et al*., 2011), which can be a plausible explanation for the species above mentioned. In the case of *Frankliniella* species, their spread over wide geographical distances, as observed by mapping the lineages (figure 4F-J), and occurred mainly due to anthropic action (via international vegetable trade) (Rugman-Jones *et al*., 2006). This characteristic, added to the polyphagous nature of most species, allows the use of available resources in a more effective way, making them crop pests all over the world (Kirk and Terry, 2003).

On the other hand, for the *F. tritici* 4 and *F. tritici* 3 lineages, which occur in Canada and the USA (figure 4H), it is possible that it is a case of sympatric speciation, as already described in Hemiptera, the sister group of Thysanoptera (Li *et al*., 2015), where a genetic relationship between choice and performance in the habitat led to the reproductive isolation of populations and consequently speciation (Via, 1999).

Conclusion

In general, the identification system based on the COI gene as used in the present study, proved to be highly effective for *Frankliniella* MOTUs delimitation, detection of cryptic species, as well as in the identification of taxonomic errors.

The threshold (2.35%) determined for *Frankliniella* represents an important addition to the already established methods of species delimitation, acting as an indicator of cryptic diversity for this genus of great economic importance. The combination of different algorithms, together with the genetic threshold, will provide more reliable and robust results for species separation and possible description. The approaches used in the present study proved to be very efficient for the thrips identification and detection of cryptic species and misidentification. However, it is still necessary for a thorough taxonomic review on the vouchers of the sequences deposited in the BOLD, as well as a taxonomic review in Thysanoptera as a whole, since different studies of integrative taxonomy have pointed out inconsistencies in the identification of some groups considering only the traditional taxonomic approach.

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