THE DNA BARCODES FOR THE SPECIES DELIMITATION OF THE GENUS
*Tylopus* Jeekel, 1968 IN VIETNAM (Diplopoda: Polydesmida: Paradoxosomatidae)

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Received 16 December 2020, accepted 3 June 2021

ABSTRACT

The 680 bp fragment of the COI gene was employed for DNA barcodes of the millipede genus *Tylopus* in Vietnam. A total of 22 samples representing for 14 morphological *Tylopus* species were analyzed. The K2P genetic divergence between *Tylopus* species ranges from 12.2% to 18.9% with a mean of 15 ± 1%. The intraspecific divergences are slightly different between species, from 3% to 5%. The AGBD analysis and phylogenetic trees also support 14 morphological species. It is also suggested to have more COI sequences of more species for better barcode reference library and better molecular species identification.

Keywords: Molecular taxonomy, biodiversity, COI gene, millipedes.

*Citation: Nguyen Duc Anh, Nguyen Thi Thu Anh, Phung Thi Hong Luong, Dang Thi Hoa, Nguyen Giang Son, 2021. The DNA barcodes for the species delimitation of the genus *Tylopus* Jeekel, 1968 in Vietnam (Diplopoda: Polydesmida: Paradoxosomatidae). *Academia Journal of Biology*, 43(2): 37–45. https://doi.org/10.15625/15761

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INTRODUCTION

*Tylopus* is one of the largest genera in the family Paradoxosomatidae Daday, 1889, with 64 valid species distributed in mountainous areas in Indochina and the southern part of China (Golovatch & Enghoff, 1993; Likhitrakarn et al., 2010; Nguyen, 2012). Taxonomy of the genus has been revised by Golovatch & Enghoff (1993) and Likhitrakarn et al. (2010), but species occurring in areas other than Thailand have yet been poorly studied.

To date, 20 *Tylopus* species have been recorded in Vietnam (Attems, 1937, 1953; Golovatch, 1984; Enghoff et al., 2004; Nguyen, 2012; Golovatch, 2019). These species have been found only from the type localities in the original descriptions. Since Vietnam is located in the distribution center of the genus *Tylopus*, the number of *Tylopus* species is expected to be higher than in present. Vietnamese *Tylopus* species can be distinguished by morphological characters, mainly gonopod conformation. Some species are differentiated in minor gonopod structure, for example *Tylopus phanluongi* Nguyen, 2012 and *Tylopus hilaris* (Attems, 1937) (Nguyen, 2012). Thus, molecular data are suggested to be used for species description and recognition. Unfortunately, there are no published reports on molecular data of the genus *Tylopus* except 16S rRNA of several *Tylopus* species (Nguyen et al., 2017).

Molecular based taxonomy has been recently used with sequence diversities in the Folmer segment of the mitochondrial gene, *Cytochrome c Oxidase Subunit I* (*COI*). The COI barcodes have also been used to distinguish millipede species. For example, Wesener (2015) used the COI fragment to synonymize *Glomeris malmivaga* Verhoeff, 1912 with *Glomeris ornata* Koch, 1847. Zhao et al. (2020) used a COI fragment for additional data for new species description. The COI sequences are also used in many barcoding projects relating to myriapods, e.g. Barcoding Fauna Bavaria (Spelda et al., 2011). However, the database or reference library for DNA barcodes are still limited. In other words, for the identification of a wide set of species, reference barcode libraries are needed. Therefore, more COI data for millipede species should be sequenced and published.

This work is devoted to provide the COI barcodes for species delimitation of the genus *Tylopus* in Vietnam, and furthermore to build up the barcoding reference library for the Vietnamese millipede fauna.

METHOD AND MATERIALS

Taxon sampling, identification and DNA extraction

A total of 22 specimens was collected from various part of Vietnam and directly preserved in ethanol 85–90%. All were examined under microscope Olympus SZX10 to exactly identify members of the genus *Tylopus*. All materials and voucher specimens are kept in Department of Soil Ecology, Institute of Ecology and Biological Resources (IEBR, Vietnam), and Field Museum of Natural History (Chicago, USA).

Total genomic DNA was extracted from leg tissue using the DNAeasy Blood & Tissue Kit (Qiagen™).

DNA amplification and sequencing

A fragment of the mitochondrial gene, *Cytochrome c Oxidase Subunit I* (*COI*) was amplified using the polymerase chain reaction (PCR). The set of universal primers COI-1F20 (5'-ACTCTAATCATAAGG AT-3') and COI-1R19 (5'-TAAACCTCCGGTGAACCA-3') (Nguyen et al., 2017) was used to amplify a 680 bp fragment of the COI gene. The PCR conditions were as following: denaturation at 94 °C for 5 min., 38 cycles of 94 °C for 45 seconds, 42 °C for 45 seconds, 72 °C for 1 minute, and final 72 °C for 5 min. After thermal cycling, 2 µl PCR products was screened for potentially successful amplification of a fragment of 16 S or COI by using electrophoresis in 1.2% Agarose- TBE 1X. The electrophoresis was performed in conditions of 100 mA, 120 V and 20 minutes.
About 20 µl of successfully amplified PCR products were purified using the ExosapIT with the company protocol. The purified PCR products were sent to sequence using an Applied Biosystems automatic sequencer (ABI3130 XL) at the Institute of DNA Technology (Vietnam) using the same primer sets as for initial PCR.

Alignment and phylogenetic analysis

Each successful sequence was checked and edited manually using BioEdit ver.7.1 (Hall, 1999), and confirmed using BLASTN 2.6.0+ searches (Zhang et al., 2000). All confirmed sequences were aligned using Cluster X ver.2.0 (Larkin et al., 2007) the ambiguous nucleotide sites and gaps were removed using MEGA X (Kumar et al., 2018).

Table 1. Analyzed species/specimens, deposition voucher, collection data and GenBank accession number

| No. | Species                        | Locality              | Voucher      | COI            | Notes                      |
|-----|-------------------------------|-----------------------|--------------|----------------|----------------------------|
| 1   | *Tylopus crassipes*           | Sapa, Lao Cai         | IEBR-Myr 92 | KX096920       |                            |
| 2   | *Tylopus hilaroides*          | Cuc Phuong, Ninh Binh| IEBR-543    | MW384914       |                            |
| 3   | *Tylopus hilaroides*          | Cuc Phuong, Ninh Binh| IEBR-198    | MW384918       |                            |
| 4   | *Tylopus hilaroides*          | Cuc Phuong, Ninh Binh| SVE-149     | MW384905       |                            |
| 5   | *Tylopus hilaroides*          | Cuc Phuong, Ninh Binh| SVE-173     | MW384904       |                            |
| 6   | *Tylopus hilaroides*          | Tam Dao, Vinh Phuc    | SVE-55      | MW384903       |                            |
| 7   | *Tylopus nodulipes*           | Huong Son, Ha Tinh    | IEBR-105    | MW384919       |                            |
| 8   | *Tylopus nodulipes*           | Minh Hoa, Quang Binh  | IEBR-557    | MW384912       |                            |
| 9   | *Tylopus roseiparaterga*      | Ba Vi, Ha Noi         | SVE-70      | MW384902       |                            |
| 10  | *Tylopus roseiparaterga*      | Tam Dao, Vinh Phuc    | IEBR-185A   | KX096923       | Nguyen et al. (2017)       |
| 11  | *Tylopus sapaensis*          | Sa Pa, Lao Cai        | IEBR-93     | MW384908       |                            |
| 12  | *Tylopus spinisternus*        | Bi Doup - Nui Ba, Lam Dong | IEBR-234 | MW384916       |                            |
| 13  | *Tylopus sp.1*                | Ba Vi, Ha Noi         | SVE-73      | MW384901       |                            |
| 14  | *Tylopus sp.1*                | Ba Vi, Ha Noi         | SVE-74      | MW384900       |                            |
| 15  | *Tylopus sp.2*                | Phong Nha - Ke Bang, Quang Binh | IEBR-210 | MW384917       |                            |
| 16  | *Tylopus sp.2*                | Phong Nha - Ke Bang, Quang Binh | IEBR-IPE6 | MW384907       |                            |
| 17  | *Tylopus sp.3*                | Sa Pa, Lao Cai        | IEBR-556    | MW384913       |                            |
| 18  | *Tylopus sp.4*                | Son Dong, Bac Giang   | IEBR-509    | MW384915       |                            |
| 19  | *Tylopus sp.5*                | Hoang Lien, Lao Cai   | IEBR-558    | MW384911       |                            |
| 20  | *Tylopus sp.6*                | Tam Dao, Vinh Phuc    | IEBR-603    | MW384910       |                            |
| 21  | *Tylopus sp.7*                | Muong Nhe, Dien Bien  | IEBR-617    | MW384909       |                            |
| 22  | *Tylopus sp.8*                | Bach Ma, Thua Thien Hue | IEBR-740 | MW384906       |                            |
| 23  | *Oxidus gigas*                | Sapa, Lao Cai         | IEBR-Myr 113 | KX096921       |                            |
| 24  | *Oxidus gigas*                | Duc Xuan, Ha Giang    | IEBR-Myr 516 | KX096928       |                            |
| 25  | *Oxidus riukiaria*            | Ryukyu, Japan         | IEBR-H500   | KX096926       |                            |
| 26  | *Oxidus riukiaria*            | Ryukyu, Japan         | IEBR-H500J  | KX096927       |                            |
| 27  | *Oxidus gracilis*             | Taiwan                | IEBR-549    | KX096931       |                            |
| 28  | *Oxidus gracilis*             | Ryukyu, Japan         | IEBR-466    | KX096924       |                            |
| 29  | *Oxidus gracilis*             | Ryukyu, Japan         | IEBR-471    | KX096925       |                            |

Nguyen et al. (2017)
The reliability of the alignment was estimated using distance estimation and model of Kimura two parameters (K2P) performed in MEGA X (Kimura, 1980). The nucleotide frequencies were statistically calculated using MEGA X. The COI sequences were translated into amino acids for confirmation using transversion code in MEGA X.

The phylogenetic tree was reconstructed using the Maximum Likelihood (ML) analysis with the best model resulted from the ModelFinder (Kalyaanamoorthy et al., 2017) performed in IQTREE ver.1.6.2 for Windows (Minh et al., 2020). Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the best substitution pattern. Codon positions included were 1st+2nd+3rd+Noncoding. Bayesian Inference (BI) analysis was performed using the MrBayes ver 3.2 (Ronquist et al., 2012) with 10 million generations, heating parameter of 0.06 and sampling every 1,000 generations.

The AGBD analysis was performed using the online server (https://bioinfo.mnhn.fr/abi/public/abgd/abgd_web.html) to recognize the number of genetic groups. The parameters are default setting except the selected distance = Kimura80 and the relative gap with (X) = 1.0.

All specimens and DNA vouchers were deposited in the Department of Soil Ecology, Institute of Ecology and Biological Resources (IEBR), Vietnam Academy of Science and Technology (VAST), Hanoi, Vietnam.

All nucleotide sequences were deposited in GenBank with accession numbers. Collection localities, specimen vouchers, and GenBank accession numbers are presented in Table 1.

**RESEARCH**

**Dataset statement**

The final aligned dataset of the gene COI consists of 570 bp, and has nucleotide frequencies of 20.4, 42.1, 23.4 and 14.1 for A, T, G and C, respectively. The GC content accounts for 37% of total nucleotides. The COI dataset contains 157 (29.9%) parsimony informative and 177 (33.7%) variable sites.

**Genetic distances**

The K2P distance between the taxa ranges (Tylopus and Oxidus species) from 0% to 22.5% (Fig. 1); overall genetic distance is 16%. The mean interspecific distance between Tylopus species is about 15%±1%. The maximum divergence is 18.9% between Tylopus roseiparaterga and Tylopus sp.5, and the minimum distance is 12.2% between Tylopus sp.6 and Tylopus sp.7 (Table 2).

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**Figure 1.** Interspecific/intraspecific COI variability (K2P): distance to nearest neighbor
\begin{table}
\centering
\small
\begin{tabular}{lcccccccccc}
\hline
Species & (1) & (2) & (3) & (4) & (5) & (6) & (7) & (8) & (9) & (10) & (11) & (12) & (13) \\
\hline
Tylopus nodulipes (1) & & & & & & & & & & & & & \\
Tylopus hilaroides (2) & & & & & & & & & & & & 15.7 & \\
Tylopus sp.2 (3) & & & & & & & & & & 17.2 & 17.4 & & \\
Tylopus spinisternus (4) & & & & & & & & & 16.5 & 15.6 & 14.3 & & \\
Tylopus sp.4 (5) & & & & & & & & 13.8 & 15.7 & 15.7 & 13.9 & & \\
Tylopus sp.3 (6) & & & & 16.0 & 16.9 & 12.6 & 12.6 & 14.9 & & & & \\
Tylopus sp.5 (7) & & & 15.8 & 15.5 & 14.8 & 16.0 & 15.3 & 14.5 & & & & \\
Tylopus sp.6 (8) & & 15.4 & 15.5 & 14.0 & 13.9 & 12.6 & 13.1 & 14.1 & & & & \\
Tylopus sp.7 (9) & & 15.6 & 16.6 & 14.5 & 15.4 & 17.0 & 16.7 & 15.4 & 12.2 & & & \\
Tylopus sp.8 (10) & 18.4 & 18.6 & 15.8 & 17.7 & 15.6 & 15.6 & 17.3 & 15.6 & 15.8 & & & \\
Tylopus crassipes (11) & 18.3 & 17.6 & 17.1 & 16.7 & 16.3 & 15.6 & 18.1 & 15.5 & 17.5 & 18.4 & & \\
Tylopus sapaensis (12) & 17.5 & 14.9 & 15.1 & 17.4 & 16.7 & 17.9 & 15.6 & 14.7 & 15.6 & 16.9 & 17.9 & \\
Tylopus roseiparaterga (13) & 16.4 & 18.0 & 16.6 & 17.2 & 16.7 & 17.1 & 18.9 & 15.2 & 14.8 & 16.7 & 13.3 & 16.7 & \\
Tylopus sp.1 (14) & 17.9 & 15.2 & 14.7 & 15.8 & 16.3 & 15.6 & 16.2 & 14.1 & 14.6 & 18.5 & 17.0 & 13.1 & 16.2 \\
\hline
\end{tabular}
\caption{Estimates of Evolutionary Divergence over Sequence Pairs between \textit{Tylopus} species (\%)}
\end{table}
Within the genus *Tylopus*, intraspecific divergences are slightly different between species, from 3% (for *Tylopus* sp.3) to 5% (for *Tylopus hilaroides* and *Tylopus* sp.2). Within the genus *Oxidus*, three species have significantly genetic distances, 13.2–14.1% between *O. gigas* and *O. gracilis*; 14.0–15.1% between *O. gigas* and *O. riukiaria*; 12.1–13.2% between *O. gracilis* and *O. riukiaria*. The AGBD analysis also indicates 17 groups with prior maximal distance \( P = 0.022 \)–0.036. This result is more likely to satisfy with 17 morphological species including three *Oxidus* species.

**Phylogenetic analysis**

The phylogenetic trees were reconstructed for the gene COI using two methods of Maximum Likelihood (ML) and Bayesian Inference (BI). For ML analysis, we consider clades with bootstrap values below 65% to be weekly supported, between 65–85% to be moderately supported, and more than 85% to be strongly supported. For BI analysis, clades will be well supported if BI posterior probability is less than 0.65 bpp; be moderately supported if BI is between 0.65–0.85 bpp; and be well supported if BI is more than 0.85 bpp (Nguyen et al., 2017).

Both phylogenetic trees also show the distinct separation of all *Tylopus* species and *Oxidus* species. All *Tylopus* species are clearly distinct from each other (Fig. 2). Two large linages are obviously separated with strong supports of bootstrap and BI values (99% and 1.00 bpp, respectively). The first clade includes samples of three species: *Tylopus hilaroides*, *T. sapaensis* and *Tylopus* sp.1. The separation of three species is well to moderately supported by bootstrap and BI values (80–90% and 0.74–0.9 bpp, respectively). The second clade consists of samples from 11 species. However, the relationship between species in the second clade is not well resolved (Fig. 2b) or very poorly supported by bootstrap values (Fig. 2a). It seems not to have same common ancestor for 11 *Tylopus* species in the second clade. This problem is mainly due to lack of more species for deeper analysis. Therefore, it is suggested to have more samples from different species for further analysis.

**DISCUSSION**

The genus *Tylopus* is placed into the Sulciferini with typical characters of spiral solenophore completely sheathing solenomere, presence of postfemoral demarcation and processes (Jeekel 1968). Until now, no relationship analysis among sulciferine genera has been made, except only Golovatch & Enghoff (1993) reported the closer relationship among *Tylopus* species and *Oxidus gracilis*. They recommended that *Oxidus gracilis* or the genus *Oxidus* can be a sister of the genus *Tylopus* because both genera have similar gonopod characters, such as presences of lamina \( l \), process \( h \), spine \( z \). The close relationship was also strongly supported by molecular evidences (Nguyen et al., 2017).

This is the first report on using the COI gene for species identification in the genus *Tylopus* and the family Paradoxosomatidae. Prior to this study, the COI distances were calculated using uncorrected \( p \)-distance for *Sphaerobelum* species (from 20.2% to 24.4%) (Zhao et al., 2020), *Glomeris* species (from 11.5% to 17.1%) (Wesener, 2015). In comparison with these researches, the genetic distance between *Tylopus* species (from 12.2% to 18.9%, with means of 15%) is narrower than that of Zhao et al. (2020) but relatively similar to that of Wesener (2015). Regarding phylogenetic analysis, all researches also indicated the COI has limitation due to rapidly evolved mitochondrial gene, and recommend to have more genes, especially of nuclear origin, for evolutionary analysis in addition to COI for a better understanding of evolution (Spelda et al., 2011).
The DNA barcodes for the species delimitation

Figure 2. Phylogenetic diagram of the Tylopus species inferring from 570 bp COI fragment

a: Maximum Likelihood analysis; b: Bayesian Inference analysis.

Values at nodes show the bootstrap and BI
CONCLUSION

The COI barcodes reveals the genetic divergence of 14 *Tylopus* species. The mean interspecific distance is about 15 ± 1% (ranging from 12.2% to 18.9%). The intraspecific divergences are slightly different between species, from 3% to 5%.

A COI fragment is an efficient tool in helping to recognize the *Tylopus* species in particular and millipede species in general. However, a reference barcode library is needed and the genetic variation must be better known for the comprehensive molecular identification.

Acknowledgements: Authors sincerely thank Dr. Petra Sierwald for her permission to extract DNA from specimens deposited in the Field Museum of Natural History (Chicago, USA). This research is funded by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106.05-2019.320.

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