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DNA Barcodes of Pholcid Spiders (Araneae : Pholcidae) in Bukidnon and Camiguin, Philippines

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ABSTRACT

DNA barcoding is a global initiative to standardize species identification of every living organism. This study aims to examine the effectiveness of DNA barcoding in species identification using 61 CO1 sequences from 12 species of pholcid spiders. CO1 barcodes of pholcid spiders demonstrate 95.08% diagnostic effectiveness as shown in clusters of neighbor-joining (NJ) tree. Barcode Gap Analysis (GBA) of 12 pholcid species exhibits the presence of barcode gap, with mean distance to nearest neighbor species of 11.27% (range 0.16-19.69%). Distance Analysis (DA) also revealed that mean intraspecific divergence of 12 species identified was 3.05%. Moreover, six species exhibit deep divergence with greater than 2% threshold value. The result implies that DNA barcoding can be utilized to facilitate species level identification leading towards deeper understanding of species diversity among pholcid spiders.

Key words: Barcode gap, Concordant, Cytochrome c oxidase 1, Genetic divergence, Neighbor-joining tree.

INTRODUCTION

DNA Barcoding provides immediate diagnosis for species identification using a short, standardized gene region of 658 bp long in the mitochondrial gene - cytochrome c oxidase sub-unit 1 (CO1) by creating a comprehensive library that links barcodes and organisms (Stoeckler & Hebert, 2008; Savolainen *et al*., 2005).

The applications of DNA Barcoding have been successfully demonstrated in various works, such as distinguishing species of Indian mosquitoes (Diptera: Culicidae), vector of human malaria; *Aphis glycines*, pest of soybean in Asia and North America, in distinguishing species of tropical Lepidoptera and discovery of cryptic species that are closely related and are similar morphologically. DNA barcoding can also be used to link different life stages, e.g. larvae, pupae, and adults which is particularly useful where multiple species co-occur, or larvae are difficult to rear (Zhou *et al*., 2014; Kumar *et al*., 2007; Hajibabaei *et al*., 2006).

In spiders, identification is mostly based on morphology, particularly the genitalia (Huber, 2004). However, identification of juvenile is often difficult without detailed examination of the genitalia.Some groups like the female of spider *Trochosa* is not easily distinguishable with morphology-based taxonomy (Nentwig *et al*., 2015).

In this study, DNA barcodes of pholcid spiders were utilized to examine species assemblages according to the CO1 sequences generated. Data analysis includes determination of CO1 sequence recovery, Distance Analysis of Intra- and interspecific divergence, Barcode Gap analysis, and Neighbor-Joining (NJ) Tree.

MATERIALS AND METHODS

The specimens were collected at three sampling sites (Figure 1) that includes CEDAR (*Community Ecological Development and* Recreation), located at 8.251°N latitude and 125.034°E longitude in Brgy,Impalutao, Impasug-ong, Bukidnon; MKAETDC (Mt. KitangladAgri-Ecological and Techno-Demo Center), located at 8.1033°N latitude and 125.034°E longitude in Brgy. Imbayao, Malaybalay, Bukidnon at the vicinity of Katibawasan falls, located at 9.213°N latitude and 124.718°E longitude in Brgy. Pandan, Mambajao, Camiguin.

Collection of Samples

Using a combination of cruising and visual search methods, pholcid spiders were collected individually using a wide- mouth plastic container and then transferred to 2ml Eppendorf tubes containing 95% ethanol for storage at 20°C. Collection information such as locality, coordinates, and elevation were obtained for each site. All specimens were sent to Bernhard A. Huber at Alexander Koenig Zoological Research Museum for identification. Detailed information about the specimens used in this study is accessible through Pholcid Spiders of Bukidnon and Camiguin, Philippines (Project code: PPHBC) project on the Barcode of Life Data Systems (BOLD) website (http://www.bold systems.org/).

DNA extraction, PCR amplification, and Sequencing

DNA extraction, PCR amplification, and sequencing were performed at the Centre for Biodiversity Genomics, Biodiversity Institute of Ontario, University of

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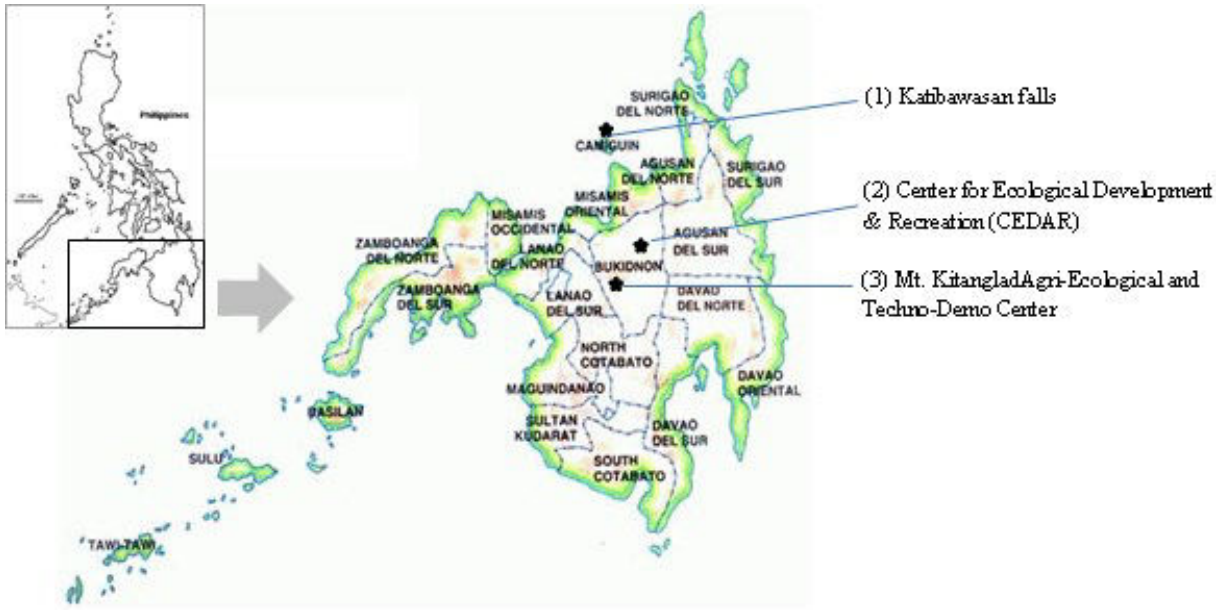

Figure 1. Map showing the three sampling sites.

Guelph, Canada following the standard protocols (Ivanova *et al.,* 2007, Ivanova and Grainger, 2007a, 2007b, 2007c). DNA extractions for larger specimens were performed using their single legs while for smaller specimens voucher specimen recovery procedure was adopted. PCR amplification was performed using the standard primer pair LepF1: ATTCAAC-CAATCATAAAGATATTGG and LepR1: TAAACTTCTGGATGTCCAAAAAATCA (Hebert *et al.,* 2004a), and the CO1fast regime was initiated as follows: initial de-naturation at 94°C for 1 min, five cycles of 94°C for 40 sec, annealing at 45°C for 40 sec, and extension at 72°C for 1 min, followed by 35 cycles of 94°C for 40 sec, 51°C for 40 sec, and 72°C for 1 min, with a final extension at 72°C for 5 min, followed by indefinite hold at 4°C (Ivanova and Grainger, 2006). The PCR products were visualized on a pre-cast agarose E-Gel (Invitrogen™) inside the trans- illuminator and digital image of E-Gel bands was captured for records. The image was analyzed and aligned using the E-editor™ 2.0 software.

Furthermore, sequencing reactions were carried out using sequencing mix comprised of 10% trehalose (Sigma, 90210) for sequencing, BigDye® Terminator mix v3.1, 5X sequence buffer (ThermoFisher Scientific, USA), and molecular grade ddH_2O.Cycle sequencing was performed at 96°C for 1 min, 35 cycles of 96°C for 10 sec, 55°C for 5 sec, 60°C for 2:30 min, and last cycle of 60°C for 5 min. Amplicons were sequenced bidirectionally on an ABI3730XL. The generated sequences were edited using CodonCode Aligner v. 3.0.2 software to generate a single consensus sequence.

Statistical Analysis

All CO1 sequences of pholcid spiders were analyzed using analytical tools at the Barcode of Life Data Systems (www.boldsystems.org) version 4.0 including Distance Analysis and Barcode Gap analysis. Neighbor-joining (NJ) tree was also constructed from the COI sequences using the Kimura-2-Parameter (K2P) (Kimura, 1980) in Mega ver.6.0 (Tamura *et al.,* 2011), with bootstrap values of 500 replications.

RESULTS AND DISCUSSION

Ninety-nine spider specimensfrom six genera were analyzed and 81.8% COI sequences were successfully recovered (Table1). However, members of the family Ochyroceratidae and Theridiidae were excluded for further analysis due to insufficient number of individuals, hence, the study focused mainly on the family Pholcidae.

The 61 CO1 sequences of pholcid spiders were compared with BOLD and NCBI databases. The result showed no matching records for all the query sequences. Morphology-based identification showed 12 morphospecies representing six pholcid genera. These include: *Aetana sp., Aetana kiukoki, Belisana sp., Belisana PSt1158, Belisana Phi129, Belisana Phi94, Calapnita nunezae, Holocneminus PSt1291, Panjange Phi111, Panjange camiguin, Panjange marilog* and *Spermophora Phi109.*

Moreover, 58 COI sequences of pholcid spiders with average of six sequences range from 2-13 were subjected to genetic distance analysis. The length of the nucleotide sequences ranged from 440 to 658 base pairs (bp) and 82.8% of them were greater than 600bp. In addition, 13.1% lacked Barcode Index Numbers (BINs) because they did not meet the BIN criteria (>500bp, no stop codons, <1% ambiguous bases) specified on the Barcode of Life Data Systems (BOLD).

The extent of sequence divergence between species of pholcid spiders with greater than two specimens increases with taxonomic rank (Table 2). Intraspecific divergence ranged from 0 to 22.8% with mean of 3.1, while congeneric divergence ranged from 2.2 to 27.6% with mean of 20.6%.

Species were evaluated for the presence of Barcode gap using BOLD (Ratnasingham and Hebert, 2007) and Automated Barcode Gap Discovery (Puillander *et al.,* 2011). The result showed that the mean intra-specific

Table 1. Percent success in recovery of CO1 sequence for specimens (n=74) in six genera of Pholcidae in Bukidnon and Camiguin provinces.

Genera	No. of Specimen	Sequence Success (%)	BINs	Mean GC (%)	Std. Err.
Aetana	14	57.14	6	32.7	0.14
Belisana	16	87.5	14	34.5	0.38
Calapnita	13	100	9	36.8	0.14
Holocneminus	1	100	1	32.1	-
Panjange	21	80.95	16	35.3	0.44
Spermophora	9	88.89	8	33.9	0.26

Table 2. Sequence divergence at the CO1 barcode region among Pholcid spiders conducted in BOLD.

	n	Taxa	Comparisons	Min.Dist. (%)	Mean Dist. (%)	Max. Dist. (%)	SE Dist. (%)
Within Species	58	9	203	0	3.05	22.8	0.03
Within Genus	39	3	158	2.22	20.59	27.6	0.03
Within Family	61	1	1469	0.16	22.40	34.1	0

Table 3. Species comparison of intraspecific values and the nearest neighbor distance among Pholcid spiders conducted on BOLD.

Species	Mean Intra-Sp	Max. Intra-Sp	Nearest Species	Distance to NN
Aetana sp.	N/A	0	*Spermophora Phi109*	0.16
Aetana kiukoki	3.44	7.37	*Spermophora Phi109*	16.68
Belisana sp.	N/A	0	*Belisana PSt1158*	19.69
Belisana PSt1158	2.37	2.37	*Belisana Phi94*	2.22
Belisana Phi129	8.32	14.42	*Belisana PSt1158*	18.63
Belisana Phi94	0.1	0.15	*Belisana Pst1158*	2.22
Calapnitanunezae	1.07	3.61	*Belisana PSt1158*	18.19
Holocneminus PSt1291	N/A	0	*Aetana sp.*	0.31
Panjange Phi111	0.11	0.46	*Aetana kiukoki*	18.34
Panjange camiguin	0.33	0.77	*Panjange marilog*	19.36
Panjange marilog	2.86	7.48	*Panjange camiguin*	19.36
Spermophora Phi109	6.94	22.82	*Aetana sp.*	0.16

distance for each species is 2.8% and the nearest Neighbor distance for each species is 11.3% (Table 3). It has been shown that six pholcid species exhibit high intraspecific divergence. These are: *Spermophora Phi109* (22.82%), *Belisana Phi129* (14.42%), *Panjange marilog* (7.48%), *Aetana kiukoki* (7.37%), *Calapnita nunezae* (3.61%) *and Belisana PSt1158* (2.37%).

As shown in figure 2, CO1 sequence divergence is directly proportional with the taxonomic rank, and within species divergence being the lowest (Ashfaq *et al.,* 2013; Kartavtsev, 2013; Ward *et al.,* 2005; Hebert *et al.,* 2003; Nei and Kumar, 2000; Johns *et al.,*1998).

Cluster analysis using neighbor-joining (Saitou and Nei, 1987) showed that the five pholcid genera clearly separated from each cluster; however, three specimens have shown taxonomic discordance (Figure 2). These are: *Aetana sp.* (GenBank Accession KX038715), *Holocneminus PSt1291* (KX038750) *and Spermophora Phi109* (KW038794). The taxonomic discordance between barcode sequence clusters and species designations at the genus level is not conclusive since these are represented by only one specimen and the sequence length is greater than 600bp that is sufficient enough for species-level discrimination because the level of interspecific divergence tends to be large relative to the magnitude of the intraspecific variation (Hebert *et al.,* 2003). In addition, more samples are needed to verify that these species can be separated consistently and reliably by DNA barcodes (Hebert *et al. 2010*). Moreover, six species demonstrated deep intraspecific divergences with greater than 2% threshold (Figure 3). In addition, clusters in neighbor-joining tree indicated that most species were distinct from other taxa despite a very low distance to their nearest neighbors/species.

Despite the insignificant geographical variation in barcode sequences, the high intraspecific divergence of *Spermophora Phi109*, *Belisana Phi129*, *Panjange marilog, Aetana kiukoki, Calapnita nunezae and Belisana PSt1158* is possibly associated with genetic and geographical distance. It might be that the microhabitats played a role in the extent of divergence between pholcid spider species because they are known to occupy

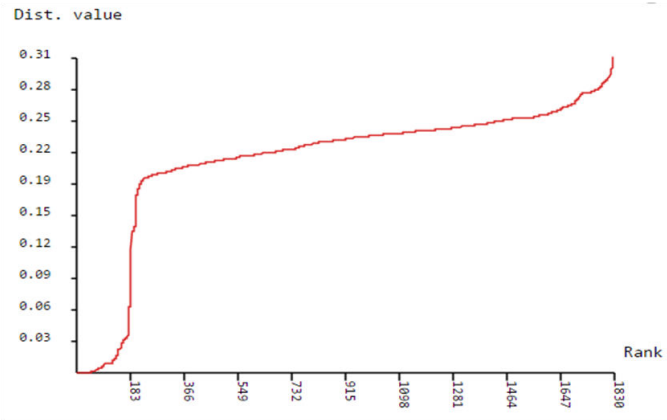

Figure 2. Plot showing the Barcode gap generated using the Automated Barcode Gap Discovery method.

different microhabitats and exhibit cryptic coloration to avoid predators (Huber, 2014).

CONCLUSION

DNA Barcoding can be an effective tool in species identification of pholcid spiders. However, larger sample size of specimens will potentially increase the taxonomic magnification and defining boundaries of each species.

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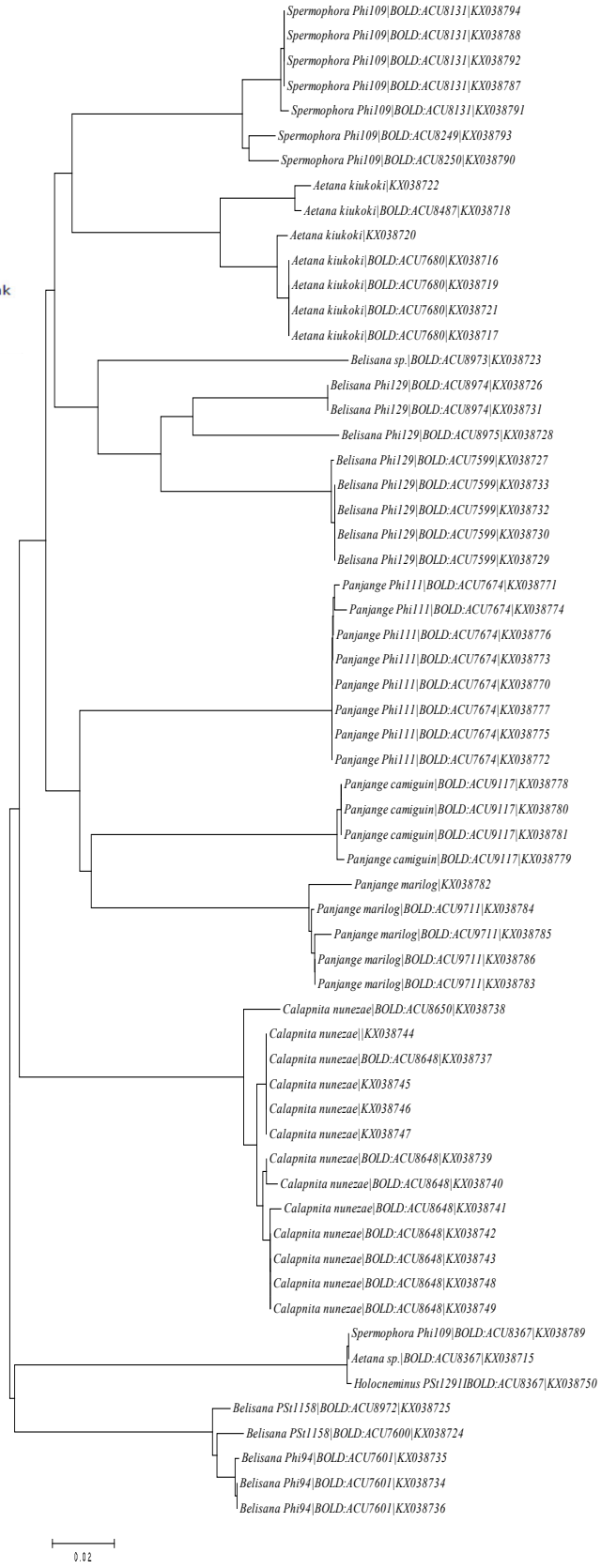

Figure 3. Neighbor-joining tree (K2P) of Pholcid spiders showing the evolutionary distance in substitutions per site at 0.02 scale.

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