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Conspecificity of *Phintella aequipeiformis* Zabka, 1985 and *P. lucai* Zabka, 1985 (Araneae: Salticidae) confirmed by DNA barcoding

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Abstract: *Phintella aequipeiformis* Zabka, 1985 was described from a male and *P. lucai* Zabka, 1985 from a female, but the opposite sex of each nominal species remained unknown. Both were collected from the same habitats of the same localities in our field work, and they share some similarities in the body color pattern. Moreover, our results from DNA barcoding show that both are one and the same species. We therefore place *P. lucai* in the synonymy of *P. aequipeiformis*, and provide a redescription of the species on the basis of both sexes.

Keywords: Taxonomy - jumping spider - synonyms - Vietnam.

INTRODUCTION

The genus *Phintella* Strand in Bösenberg and Strand, 1906 (Araneae: Salticidae) is comprised of small and colorful species (Zabka, 2012; Caleb, 2014). A total of 60 species has so far been described, 47 of them from Asia (World Spider Catalog, 2016). Most *Phintella* species, as well as other salticids, show distinct sexual dimorphism in morphology of the first pair of legs and chelicerae and of coloration in the adult instar, and subsequently both sexes are not yet known for nearly half of the nominal species in this genus (World Spider Catalog, 2016). This means that more than a few synonymies are likely hidden in the current classification of the genus *Phintella*.

DNA barcoding has been increasingly introduced into taxonomy and identification of various taxa in recent years (Hebert *et al*., 2003; Bickford *et al*., 2006; Fisher & Smith, 2008; Robinson *et al*., 2009; Pires & Marinoni, 2010). It is especially powerful for revealing male-female complementarity, and subsequently solving synonymies and/or unifying the male-based and female-based classifications (Barrett & Hebert, 2005; Robinson *et al*., 2009; Ekrem *et al*., 2010; Tanikawa, 2011; Glowska *et al*., 2014). Thus, we are in process of revising the classification of Asian species of the genus *Phintella* using DNA barcoding as well as a traditional morphological approach. Our first result is presented here.

Phintella aequipeiformis Zabka, 1985 and *P. lucai* Zabka, 1985 were described from northern Vietnam as different species. The holotype of the former is a male collected from Lao Cai Province, and that of the latter is a female from Yen Bai Province. The two type localities are separated by approximately 80 kilometers. Since their original description, the complementary sex remained unknown for both nominal species. However, conspecificity of *P. aequipeiformis* and *P. lucai* was indicated from the following results of our field work: i) males of *P. aequipeiformis* and females of *P. lucai* were collected from the same habitats of the same localities; ii) there are similarities in the body color pattern. Moreover, our result from DNA barcoding of specimens newly collected from northern and central Vietnam give further evidence for the conspecificity of *P. aequipeiformis* and *P. lucai*. We therefore propose *P. aequipeiformis* as the senior synonym of *P. lucai*, and redescribe *P. aequipeiformis* on the basis of both sexes.

MATERIALS AND METHODS

Specimen depositories: MHNG, Muséum d'histoire naturelle, Geneva, Switzerland; NSMT, National Museum of Nature and Science, Tokyo, Japan; IEBR, Institute of Ecology and Biological Resources, Hanoi, Vietnam; LPC, private collection of corresponding author; HNHM, Hungarian Natural History Museum, Budapest, Hungary.

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Sampling and specimen preparation: Specimens were collected from northern and central Vietnam (Fig. 1). Each spider was killed using acetyl acid, and, in fresh condition, a series of images of the body in dorsal view was captured at different focal planes by a Canon EOS 60D digital camera with a MPE Canon 65 mm lens. Then a multi-focused montage image was produced using Helicon 6.2.2 Pro from the source images. After the imaging, legs III and IV of the right side of each spider were removed from the body and preserved in 99% ethanol for DNA analysis. The remainder of the body was preserved in 75% ethanol for morphological examination.

Morphological examination: Specimens were examined under an Olympus SZX12 microscope. Description of coloration is based on the specimens preserved in ethanol. All measurements are in millimeters (mm).

Abbreviations used in the present paper are as follows: ALE, anterior lateral eye; AME, anterior median eye; PLE, posterior lateral eye; PME, posterior median eye; ERW, width of anterior/median/posterior eye rows between outer margins of eyes in dorsal view; ALE-PME, distance from anterior margin of ALE to posterior margin of PME, with carapace in dorsal view; ALE-PLE, distance from anterior margin of ALE to posterior margin of PLE, with carapace in dorsal view.

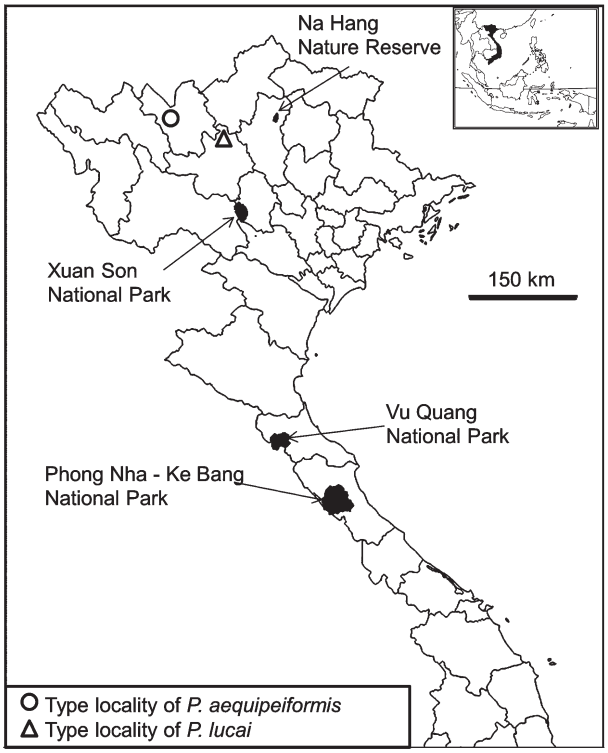

Fig. 1. Map of northern and central Vietnam showing four collection sites and the type localities of *Phintella aequipeiformis* and *P. lucai*.

Imaging of ethanol-preserved specimens: Multi-focused montage images of the body and male palp of *Phintella* specimens (Sal-LP-0325, Sal-LP-0329, Sal-LP-0490, Sal-LP-0491, Sal-LP-0586, Sal-LP-0587, Sal-LP-0622) were produced using Helicon 6.2.2 Pro from a series of source images taken by a Canon EOS X5 camera attached to a Nikon SMZ1270 microscope. Female genitalia were detached, and cleared by soaking them in 10% KOH solution at room temperature for about 24 hours. Multi-focused montage images of the genitalia were then produced using Helicon 6.2.2 Pro from a series of source images taken by a Canon EOS X5 camera attached to Nikon Eclipse E600 microscope.

Morphology-based identification: *Phintella* specimens were identified by referring to the original descriptions of the species known from Indochina and adjacent areas, and other taxonomic literature (Bösenberg & Strand, 1906; Zabka, 1985; Xie, 1993; Peng *et al*., 1993; Song *et al*., 1999; Yin *et al*., 2012; Prószyński, 2014). Nine males of *P. aequipeiformis* and twelve females of *P. lucai* were found in our collection. These specimens, together with three specimens of *P. versicolor* (Koch, 1846) and two specimens of *P. bifurcilinea* (Bösenberg & Strand, 1906), were used for DNA barcoding.

DNA barcoding: Genomic DNA was extracted from leg III or IV of the right side of each spider using the Chelex-TE extraction protocol. Each leg was transferred into a 1.5 mL microcentrifuge tube with 105 µL of extraction buffer (a mixture of 100 µL of 10% Chelex-TE solution and 5 µL Qiagen Proteinase K), incubated at 56˚C for about 24 hours, and then heated at 99˚C for 10 minutes for inactivating the Qiagen Proteinase K. A standard DNA barcoding region of the COI gene was amplified using the primer set C1-J-1718/C1-N-2776 (Hedin & Maddison, 2001). Each PCR contained 5 µL of 2xPCR buffer, 2 µL of dNTPs (final 0.4 mM), 0.3 µL of 10 pmol/µL forward and reverse primers (final 0.3 µM), 0.2 µL of 1.0 U/µL DNA polymerase KOD FX Neo (TOYOBO KFX-2015), and 0.5 µL of DNA template. The PCR thermal regime consisted of one cycle of 2 min at 94°C; 35 cycles of 10 sec at 98°C, 30 sec at 52°C and 45 sec at 68°C; and a final cycle of 7 min at 68°C. After confirming the PCR amplification on a 2.0% agarose gel, the amplified products were incubated at 37°C for 30 min and at 80°C for 20 min with an Illustra™ ExoStar (GE Healthcare, Buckinghamshire, UK) to remove any excess primers and nucleotides. The cycle sequencing reactions were run with an ABI PRISM BigDye Terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems). The sequencing reaction products were purified, concentrated by ethanol precipitation with sodium acetate, and their nucleotide sequences were determined using an automated sequencer (ABI PRISM 3100, Applied Biosystems). Forward and Reverse strands were assembled after trimming the primer regions and disarrayed parts, and questionable sites were manually

confirmed by referring to the chromatograms of the two strands. This process was done using ChromasPro 1.7.6 (Technelysium Pty Ltd., Australia). In total, 19 sequences of COI from four species of the genus *Phintella*, i.e., seven sequences of *P. aequipeiformis*, seven of *P. lucai*, three of *P. versicolor* and two of *P. bifurcilinea*, were confirmed and submitted to the DDBJ database (accession numbers: LC105655-LC105673) (Table 1).

These sequences, in addition to the homologue sequences of *Phintella piatensis* Barrion & Litsinger, 1995 (Accession No.: AY297396), *Cosmophasis micarioides* (Koch, 1880) (Accession No.: EU815580) and *Telamonia vlijmi* Prószyński, 1984 (Accession No.: KJ598073) provided by other authors (Maddison & Hedin, 2003; Maddison *et al*., 2008; Kim *et al*., 2014), were aligned using Muscle (Edgar, 2004) built in MEGA 6.06 (Tamura *et al*., 2013). Pairwise divergences were calculated using the p-distance (obtained by dividing the number of nucleotide differences by the total number of nucleotides compared) and the Kimura-two-parameter (K2P) distance model (Kimura, 1980). A neighbor-joining tree based on the K2P distance model was created using MEGA 6.06 (Fig. 2).

RESULTS AND DISCUSSION

DNA barcoding

The specimens of *Phintella aequipeiformis* and *P. lucai* were recognized as genetically closer to each other than to specimens of other species (Fig. 2). The pairwise divergence values among 14 individuals (seven males of *P. aequipeiformis* and seven females of *P. lucai*) were 0-0.83% in p-distance and 0-0.84% in K2P. Neither subgroups equivalent to the two nominal species nor any phylogeographic units were recognized within the *P. aequipeiformis* + *P. lucai* group. On the other hand, the pairwise divergence values between the *P. aequipeiformis* + *P. lucai* group and the three congeneric species, i.e., *P. vesicolor*, *P. bifurcilinea* and *P. piatensis*, were 9.35-10.18% in p-distance and 10.15-11.11% in K2P.

Through COI-based DNA barcoding, Blagoev *et al*. (2016) revealed that the mean nearest-neighbor distance based on species was 10 times higher than the mean intraspecific divergence in Canadian spiders (7.85% vs. 0.78%), and 7 times higher in Canadian salticid spiders (7.57% vs. 1.18%). Other studies on various arthropod taxa including spiders (Hebert *et al*., 2003 for Lepidoptera; Barrett & Hebert, 2005 for spiders; Smith *et al*., 2005 for Formicidae; Robinson *et al*., 2009 for spiders; Renaud *et al*., 2012 for Diptera; Glowska *et al*., 2014 for syringophilid mites; Doña *et al*., 2015 for feather mites) suggested that the interspecific divergence values of COI are usually greater than 2-3%, or the intraspecific divergence values of COI are usually less than 2-3%. When applying these thresholds to the present case, the conspecificity of *Phintella aequipeiformis* Zabka, 1985 and *P. lucai* Zabka, 1985 is strongly supported.

Taxonomic treatment

Phintella aequipeiformis and *P. lucai* were described in

Table 1. List of specimens used for DNA barcoding, with DDBJ/GenBank accession numbers.

Species	Sex	Specimen code	Locality	Accession number
Phintella aequipeiformis	Male	Sal-LP-0329	Vietnam, Phu Tho Province, Xuan Son National Park.	LC105658
Phintella aequipeiformis	Female	Sal-LP-0490	Vietnam, Tuyen Quang Province, Na Hang Nature Reserve.	LC105659
Phintella aequipeiformis	Male	Sal-LP-0491	Vietnam, Tuyen Quang Province, Na Hang Nature Reserve.	LC105660
Phintella aequipeiformis	Male	Sal-LP-0531	Vietnam, Tuyen Quang Province, Na Hang Nature Reserve.	LC105661
Phintella aequipeiformis	Male	Sal-LP-0586	Vietnam, Ha Tinh Province, Vu Quang National Park.	LC105662
Phintella aequipeiformis	Male	Sal-LP-0587	Vietnam, Ha Tinh Province, Vu Quang National Park.	LC105663
Phintella aequipeiformis	Female	Sal-LP-0588	Vietnam, Ha Tinh Province, Vu Quang National Park.	LC105664
Phintella aequipeiformis	Female	Sal-LP-0589	Vietnam, Ha Tinh Province, Vu Quang National Park.	LC105665
Phintella aequipeiformis	Male	Sal-LP-0622	Vietnam, Ha Tinh Province, Vu Quang National Park.	LC105666
Phintella aequipeiformis	Female	Sal-LP-0726	Vietnam, Ha Tinh Province, Vu Quang National Park.	LC105669
Phintella aequipeiformis	Female	Sal-LP-0727	Vietnam, Ha Tinh Province, Vu Quang National Park.	LC105670
Phintella aequipeiformis	Female	Sal-LP-0728	Vietnam, Ha Tinh Province, Vu Quang National Park.	LC105671
Phintella aequipeiformis	Female	Sal-LP-0729	Vietnam, Ha Tinh Province, Vu Quang National Park.	LC105672
Phintella aequipeiformis	Male	Sal-LP-0731	Vietnam, Ha Tinh Province, Vu Quang National Park.	LC105673
Phintella bifurcilinea	Male	Sal-LP-0148	Vietnam, Vinh Phuc Province, Me Linh Station.	LC105655
Phintella bifurcilinea	Female	Sal-LP-0681	Vietnam, Vinh Phuc Province, Me Linh Station.	LC105668
Phintella versicolor	Male	Sal-LP-0204	Vietnam, Hai Phong Province, Bach Long Vi island	LC105656
Phintella versicolor	Male	Sal-LP-0271	Vietnam, Lao Cai Province, Sapa Town.	LC105657
Phintella versicolor	Female	Sal-LP-0670	Vietnam, Hanoi city, Ba Vi District.	LC105667

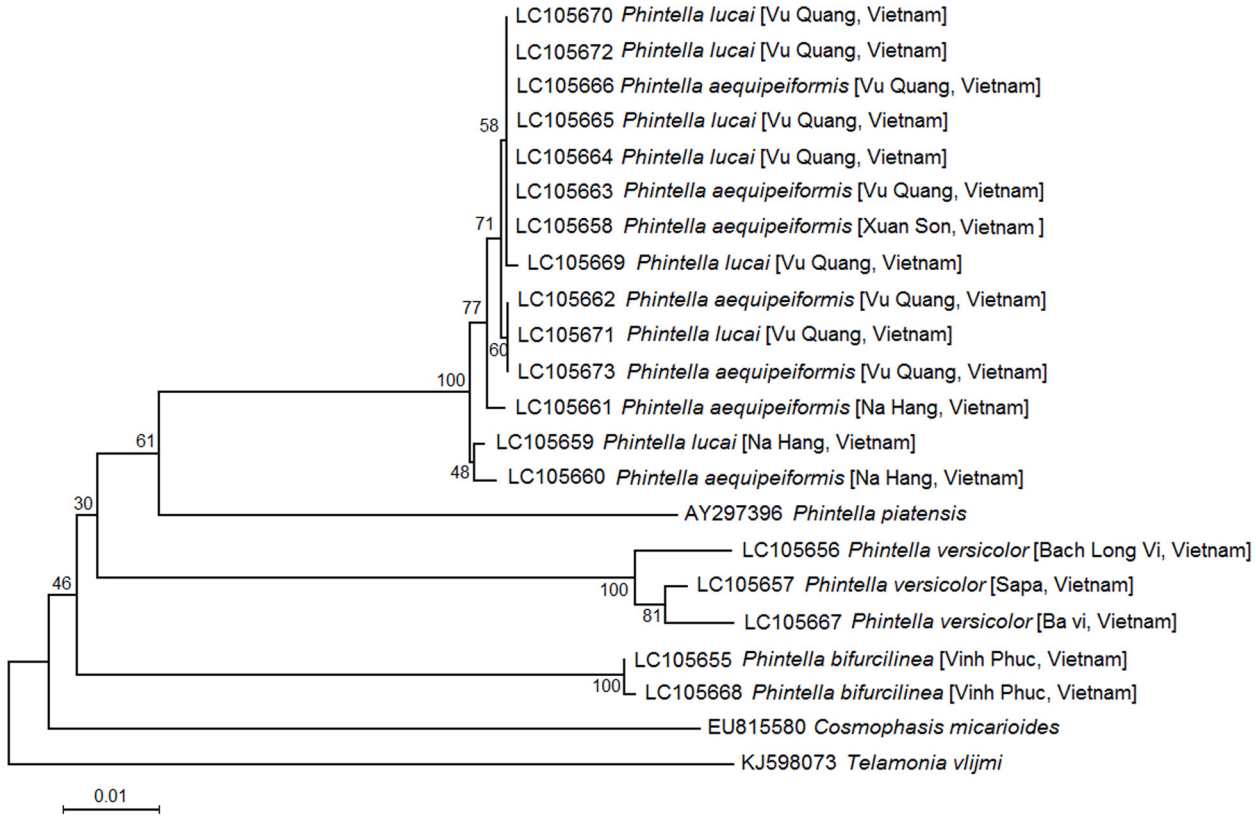

Fig. 2. Neighbor-joining tree generated under the K2P distance model, based on a dataset consisting of 851 bp sequences. Numbers beside nodes refer to bootstrap values (1000 replicates). DDBJ/Genbank accession numbers are placed before species names.

the same publication (Zabka, 1985). We treat *P. lucai* as a junior synonym of *P. aequipeiformis* because *P. aequipeiformis* was redescribed several times (Xie, 1993; Peng *et al*., 1993; Song *et al*., 1999, Yin *et al*., 2012), while *P. lucai* was not. Moreover, it is better to retain *P. aequipeiformis*, which is represented by a male holotype, because males possess more useful characters for identification than females.

***Phintella aequipeiformis* Zabka, 1985**

Figs 3-4

Phintella aequipeiformis Zabka, 1985: 427, figs 422-425, 450. – Xie, 1993: 358, figs 8-10. – Peng *et al*., 1993: 151, figs 518-523. – Song *et al*., 1999: 537, figs 307I, 328A. – Yin *et al*., 2012: 1423, fig. 775a-f. Holotype deposited in HNHM, not examined.

Phintella lucai Zabka, 1985: 430, figs 444-446. **Syn. nov**. Holotype deposited in HNHM, not examined.

Material examined: Vietnam: MHNG, LC105661, Sal-LP-0531; 1 male; Tuyen Quang Province, Na Hang Nature Reserve; 11.III.2015; leg. Luong & Yamasaki. – MHNG, LC105663, Sal-LP-0587; 1 male; LC105664, Sal-LP-0588; 1 female; LC105672, Sal-LP-0729; 1 female; Ha Tinh Province, Vu Quang National Park; 18.III.2015; leg. Luong & Yamasaki. – NSMT, LC105662, Sal-LP-0586; 1 male; LC105665, Sal-LP-0589; 1 female; Ha Tinh Province, Vu Quang National Park; 18.III.2015; leg. Luong & Yamasaki. – IEBR-AR-0272; 2 males; IEBR-AR-0331; 1 female; Ha Tinh Province, Vu Quang National Park; 18.III.2015; leg. Luong & Yamasaki. – IEBR-AR-0382; 1 female; Ha Tinh Province, Vu Quang National Park; 23.III.2015; leg. Luong & Yamasaki. – LPC, Sal-LP-0048; 1 female; Quang Binh Province, Phong Nha - Ke Bang National Park; 23.IV.2014; leg. Luong. – LPC, Sal-LP-0325; 1 female; LC105658, Sal-LP-0329; 1 male; Phu Tho Province, Xuan Son National Park; 10.VIII.2014; leg. Luong. – LPC, LC105659, Sal-LP-0490; 1 female; LC105660, Sal-LP-0491; 1 male; Tuyen Quang Province, Na Hang Nature Reserve; 10.III.2015; leg. Luong & Yamasaki. – LPC, Sal-LP-0730; 1 female; Ha Tinh Province, Vu Quang National Park; 18.III.2015; leg. Luong & Yamasaki. – LPC, LC105666, Sal-LP-0622; 1 male; Ha Tinh Province, Vu Quang National Park; 19.III.2015; leg. Luong & Yamasaki. – LPC, LC105669, Sal-LP-0726; 1 female; LC105670, Sal-LP-0727; 1 female; LC105671, Sal-LP-0728; 1 female; Ha Tinh Province, Vu Quang National Park; 23.III.2015; leg. Luong & Yamasaki. – LPC, LC105673, Sal-LP-0731; 1 male; Ha Tinh Province, Vu Quang National Park; 24.III.2015; leg. Luong & Yamasaki.

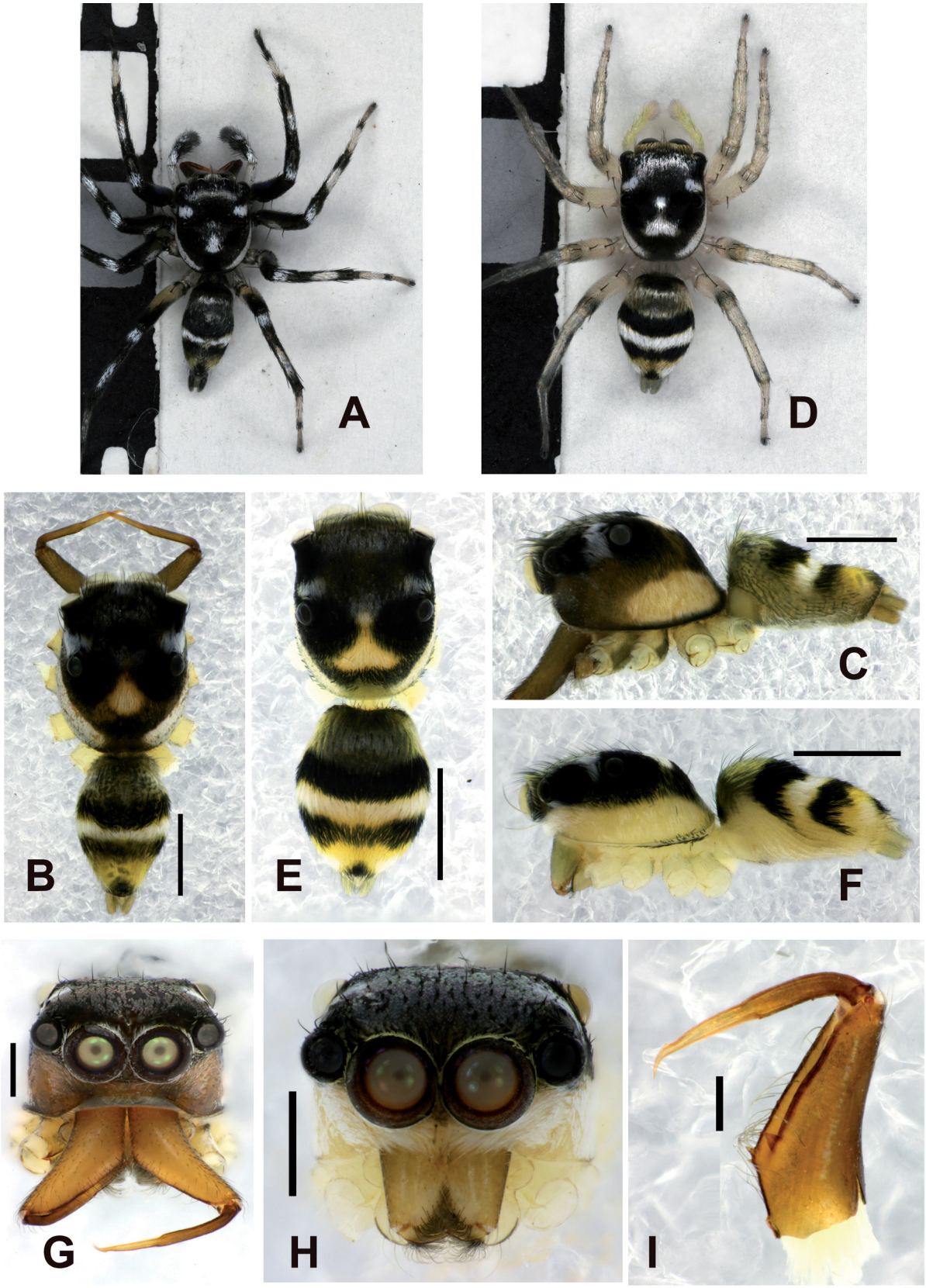

Fig. 3. *Phintella aequipeiformis* specimens from Vietnam; males (A-C, G, I), females (D-F, H). (A-B, D-E) Body, dorsal view. (C, F) Body, lateral view. (G-H) Prosoma, frontal view. (I) Chelicera and fang, ventral view. Scale lines 1 mm (B-C, E-F), 0.5 mm (G-H), 0.2 mm (I). Specimen codes: Sal-LP-0586 (A, I), Sal-LP-0329 (B-C), Sal-LP-0490 (D-F), Sal-LP-0622 (G), Sal-LP-0325 (H).

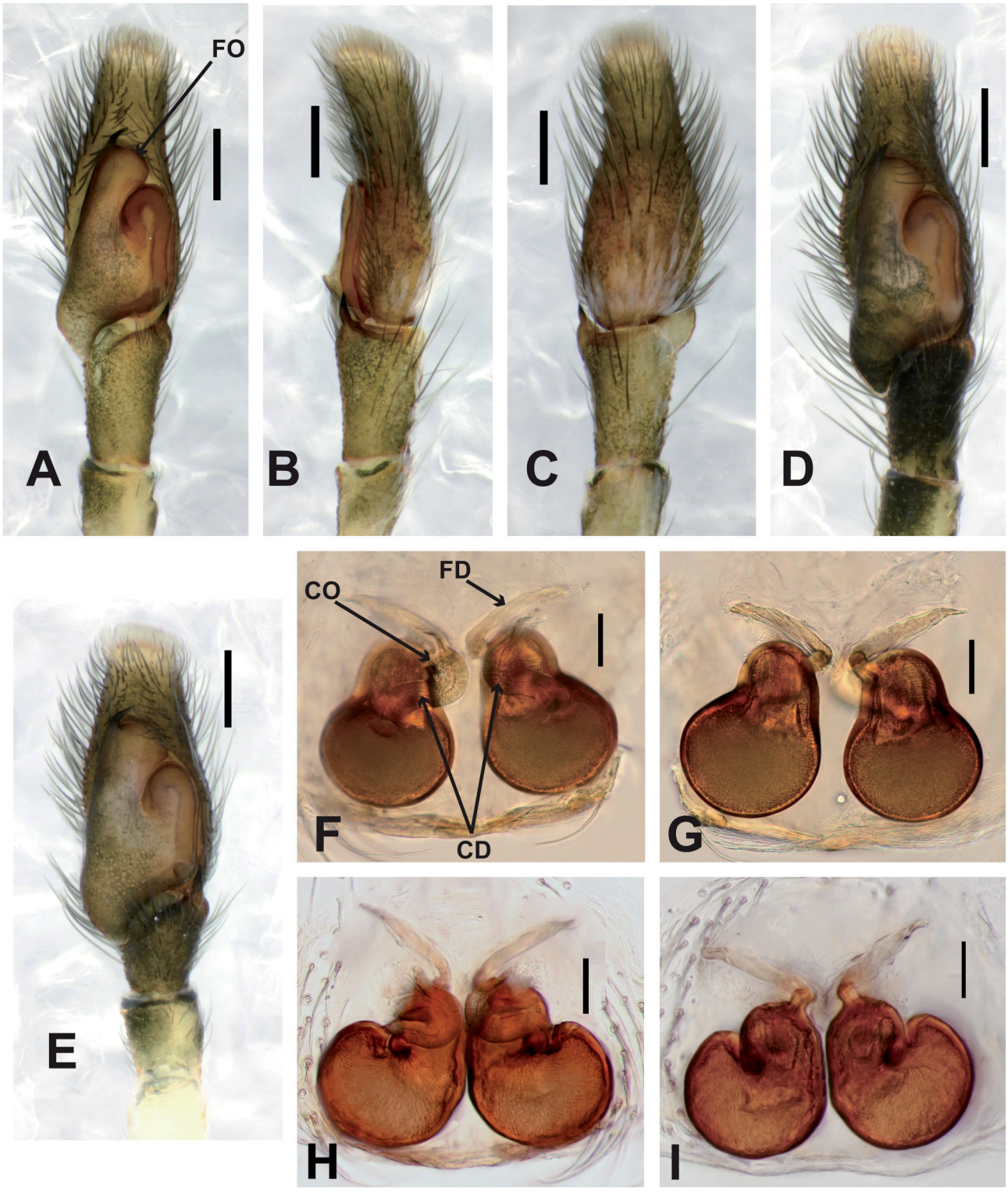

Fig. 4. *Phintella aequipeiformis* specimens from Vietnam; males (A-E), females (F-I). (A, D-E) Palp, ventral view. (B) Palp, retrolateral view. (C) Palp, dorsal view. (F, H) Epigyne, ventral view. (G, I) Epigyne, dorsal view. Abbreviations: CD, copulatory duct; CO, copulatory opening; FD, fertilization duct; FO, rounded flaky outgrowth. Scale lines 0.2 mm (A-E), 0.05 mm (F-I). Specimen codes: Sal-LP-0329 (A-C), Sal-LP-0491 (D), Sal-LP-0587 (E), Sal-LP-0048 (F-G), Sal-LP-0588 (H-I).

Diagnosis: Markings on abdomen of both sexes characteristic, i.e. one white and two black bands of hairs running transversally over dorsum. Embolus short and slightly bent, claw-like; retrolateral corner of anterior part of bulbus rounded, forming a so-called "rounded flaky outgrowth" (Zabka, 1985). Spermathecae large, weakly constricted, and divided into relatively small anterior part and large posterior part.

Measurements: *Male* (n=9). Body length 3.32-4.50; carapace length 1.72-2.25; abdomen length 1.53-2.21, width 0.94-1.36; ERW anterior 1.28-1.64; ERW median 1.06-1.41; ERW posterior 1.19-1.54; ALE-PME 0.38-0.55; ALE-PLE 0.79-1.09.
Female (n=12). Body length 3.24-4.83; carapace length 1.56-1.97; abdomen length 1.60-2.65, width 1.21-2.00; ERW anterior 1.28-1.51; ERW median 1.08-1.29; ERW posterior 1.24-1.44; ALE-PME 0.42-0.46; ALE-PLE 0.85-0.86.

Description: *Male* (Fig. 3A-C, G, I). Carapace mainly black on dorsal surface, yellowish cream tinged with black on lateral surface; AME fringed with whitish gray hairs; dorsum of cephalic part covered with shiny scale-like hairs; area between PME and PLE covered with white scale-like hairs forming white patch; fin-shaped area behind fovea yellowish cream, covered with white scale-like hairs; large yellowish area on lateral surface of thoracic part extending from above coxa II to coxa IV, covered with white scale-like hairs. Clypeus brown to yellowish brown, sparsely covered with whitish transparent scale-like hairs. Chelicera long, pale brown, weakly tinged with brown, with one retrolateral and two prolateral teeth at basal end of fang furrow. Fang long, pale brown. Maxilla and labium pale yellow, weakly tinged with gray or brown. Sternum pale yellow. Abdomen oval, covered with several kinds of scale-like hairs; anterior quarter of dorsum covered with transparent scale-like hairs; middle part of dorsum blackish, with white scale-like hairs forming one white and two black transversal bands, white band running between black bands; posterior part of dorsum yellowish cream, posterior end black, covered with transparent scale-like hairs. Legs with a pattern of black and pale yellow; small patches on dorsum of patellae and tibiae covered with white hairs.
Palp (Fig. 4A-E). Segments with pattern of black and pale yellow; dorsum of patella to cymbium covered with white scale-like hairs. Cymbium slender, tapering with long black hairs. Embolus short and slightly bent, claw-like. Retrolateral corner of anterior part of bulbus rounded, forming so-called "rounded flaky outgrowth" (see Zabka, 1985). Posterior lobe of bulbus well-developed. Sperm duct distally strongly curving behind rounded flaky outgrowth, proximally running along retrolateral margin of bulbus. Retrolateral tibial apophysis short, with relatively thin tip.
Female (Fig. 3D-F, H). Carapace almost as in male; its lower lateral surface pale yellow, lighter than that of male, covered with white hairs. Clypeus densely covered with white scale-like hairs. Chelicera, fang, maxilla and labium almost as in male, except for shorter chelicera. Abdomen almost as in male, but more rotund. Legs and palp mostly pale yellow.
Genitalia (Fig. 4F-I). Copulatory openings small, situated near anterior margin of spermathecae. Copulatory ducts narrow, sclerotized, strongly curving. Spermathecae large, weakly constricted, divided into relatively small anterior part and large posterior part; connection points with copulatory ducts located near weak constriction. Fertilization ducts connected to anterior part of spermathecae.

Distribution: Vietnam: Lao Cai, Yen Bai, Tuyen Quang, Phu Tho, Ha Tinh, Quang Binh (Zabka, 1985; present study); China: Hunan, Guangxi (Xie, 1993; Peng *et al*., 1993; Song *et al*., 1999; Yin *et al*., 2012).

Habitat: This species was found in secondary forests, often on shrubs along forest trails and along forest edges.

Remarks: This species shows some variations in the shape of the bulbus and in the length of the palpal tibia (Fig. 4A, D, E), as well as in the shape of the spermathecae (Fig. 4F-I). These varieties occur sympatrically. Our results from DNA barcoding (Fig. 2) strongly suggest that all these varieties belong to a single species.

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