Integrative taxonomy of the primitively segmented spider genus *Ganthela* (Araneae: Mesothelae: Liphistiidae): DNA barcoding gap agrees with morphology

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Species delimitation is difficult for taxa in which the morphological characters are poorly known because of the rarity of adult morphs or sexes, and in cryptic species. In primitively segmented spiders, family Liphistiidae, males are often unknown, and female genital morphology – usually species-specific in spiders – exhibits considerable intraspecific variation. Here, we report on an integrative taxonomic study of the liphistiid genus *Ganthela* Xu & Kuntner, 2015, endemic to south-east China, where males are only available for two of the seven morphological species (two known and five undescribed). We obtained DNA barcodes (cytochrome c oxidase subunit I gene, COI) for 51 newly collected specimens of six morphological species and analysed them using five species-delimitation methods: DNA barcoding gap, species delimitation plugin [PID(Liberal)], automatic barcode gap discovery (ABGD), generalized mixed Yule-coalescent model (GMYC), and statistical parsimony (SP). Whereas the first three agreed with the morphology, GMYC and SP indicate several additional species. We used the consensus results to delimit and diagnose six *Ganthela* species, which in addition to the type species *Ganthela yundingensis* Xu, 2015, completes the revision of the genus. Although multi-locus phylogenetic approaches may be needed for complex taxonomic delimitations, our results indicate that even single-locus analyses based on the COI barcodes, if integrated with morphological and geographical data, may provide sufficiently reliable species delimitation.


INTRODUCTION

How a species is delimited has major implications, not only for taxonomy, but also for biology in general, because accurate species boundaries are crucial for understanding speciation patterns and processes (Sites & Marshall, 2004; Wiens, 2007; Camargo & Sites, 2013; Hedin, 2015), and will impact every subfield of the life sciences. Species delimitation is far from a trivial task for taxa that are morphologically similar (cryptic species or those lacking clear diagnostic features) or those rare in collections (missing sexes or adult forms), however.
As traditional taxonomy usually makes qualitative decisions on species delimitation, it is a fairly subjective field (de Queiroz, 2007; Hausdorf, 2011). On the other hand, modern species delimitation approaches treat species as hypotheses in a statistical framework, and use objective tests to delineate evolutionarily independent lineages as species (for a review, see Fujita et al., 2012; see also Agnarsson & Kuntner, 2007). An integrative approach to taxonomy increasingly uses several lines of evidence to delimit and describe taxa (Dayrat, 2005; Will, Mishler & Wheeler, 2005; Pante, Schoelincx & Puillandre, 2015). Of these, modern molecular-based species delimitation approaches are now routinely used in a variety of taxa to complement traditional taxonomy (Carstens et al., 2013; Fouquet et al., 2014; Shirley et al., 2014). Research on species delimitation is somewhat geopolitically biased (e.g. Harris & Froufe, 2005), however, as the majority of studies have been carried out using European and North American taxa (e.g. Hamilton, Formanowicz & Bond, 2011; Derkarabedian & Hedlin, 2014; Hamilton et al., 2014), with only very few studies performed in East and Southeast Asia (Huang et al., 2013). In spiders, a phylogenetic bias is also apparent, as most prior studies that used molecular-based species delimitation have focused on derived spider groups to the exclusion of the primitively segmented spiders, Liphistidiidae (e.g. Hamilton et al., 2011, 2014; Candek & Kuntner, 2015; Derkarabedian & Hedlin, 2014).

Whereas the use of single-locus data for delimiting species has been a source of some controversy (Hebert et al., 2003a; Moritz & Cicero, 2004; Blaxter et al., 2005; Rubinoff & Holland, 2005), many species-delimitation methods do use single-locus data in order to balance costs and benefits. For example, DNA barcoding gap analysis routinely uses single-locus DNA data (Hebert et al., 2003a; Hebert, Ratnasingham & deWaard, 2003b), as do other recently proposed techniques, such as ‘automatic barcode gap discovery’ (ABGD; Puillandre et al., 2012), the ‘generalized mixed Yule-coalescent model’ (GMYC; Pons et al., 2006), the species delimitation plugin [P ID(Liberal); Masters, Fan & Ross, 2011], the ‘statistics parsimony network analysis’ (SP; Templeton, Crandall & Sing, 1992), and the W&P method (Wiens & Penkrot, 2002). An increasing body of literature uses these approaches and confirms their usefulness in delimiting species to complement traditional (morphological) taxonomy (Hart & Sunday, 2007; Longhorn et al., 2007; Hamilton et al., 2011, 2014; Kuntner & Agnarsson, 2011; Weigand et al., 2011, 2013; Jörgler et al., 2012; Paz & Crawford, 2012; Fujisawa & Barraclough, 2013; Hendrixson et al., 2013; Kekkonen & Hebert, 2014; Opatova & Arnedo, 2014).

Approaches to species delimitation using single-locus data can be broadly categorised into two groups, species discovery versus species validation, depending on whether the samples are partitioned prior to analysis. Although it is unnecessary to partition the samples into putative species when discovering species through the use of ABGD (Puillandre et al., 2012), GMYC (Pons et al., 2006), and SP (Templeton et al., 1992), species validation requires the a priori assignment of putative species that can then be tested through DNA barcoding gap analysis (Hebert et al., 2003a, b) and P ID(Liberal) (Masters et al., 2011). Comparing the results from various species-delimitation methods combined with morphological-based species diagnoses should in theory yield more accurate taxonomic units compared with classical approaches, especially for species with high intraspecific variation.

The primitively segmented spiders, family Liphistidiidae, represent an ancient lineage in which members are restricted to Southeast and East Asia (World Spider Catalog, 2015). The family has recently been reviewed to contain eight genera, each endemic to a different geographical region (Xu et al., 2015a, b); however, species delimitation in liphistidiid females have simple genitals with extraordinarily intraspecific variation, blurring the distinction between geographic variation and species-level divergence, and thus making species delimitation based on female morphology extremely challenging (Haupt, 2003; Tanikawa, 2013; Tanikawa & Miyashita, 2015). This problem is exacerbated by the fact that it is relatively easier to find adult females than adult males (our own data suggest that the ratio of immature : adult female : adult male specimens collected in the field is 13 : 16 : 1), with 23 out of 89 liphistidiid species only being known from a single sex (20 from females and three from males only; World Spider Catalog, 2015). In the acute absence of male diagnostic features, liphistidiid taxonomy consequently suffers from common taxonomic errors such as over-splitting, over-lumping, and incorrect species assignment of individuals or populations (Rittmeyer & Austin, 2012), with potentially erroneous implications regarding species diversity, intraspecific variation, and gene flow (Funk & Omland, 2003; Bickford et al., 2007). Over-lumping, in particular, is a major concern to biodiversity research as the units of diversity, and therefore conservation decisions, are underestimated (Bickford et al., 2007). This is particularly relevant to liphistidiid, in which the species-level taxonomy has been nearly devoid of molecular analyses (but, see Tanikawa, 2013; Tanikawa & Miyashita, 2015), yet the known species are often island endemics or otherwise restricted in ranges (Xu et al., 2015a, b).

Here, we report on a species delimitation study that integrated morphological data with DNA nucleotide data in a group of primitively segmented spiders. We used...
the standard DNA barcoding region of the mitochondrial cytochrome c oxidase subunit I (COI; Hebert et al., 2003a, b), which has been shown to represent a rapid and accurate marker in testing species hypotheses in animals (Hebert et al., 2003a; b; Barrett & Hebert, 2005; Robinson et al., 2009; Paz & Crawford, 2012; Kekkonen & Hebert, 2014), and in particular in spiders (e.g. Hamilton et al., 2011, 2014; Čandek & Kuntner, 2015). We studied Ganthela Xu & Kuntner, 2015, a genus restricted to east China (Fujian and Jiangxi Provinces) that contains seven morphological species. In addition to the recently described type species, Ganthela yuningensis Xu, 2015, there is only one other known species, Ganthela cipingensis (Wang, 1989), and judging from female morphology five undescribed species (Xu et al., 2015a). As in other liphistiids, Ganthela female genital morphology is highly variable and the males of five new (morphological) species are not available from collections. We therefore generated DNA barcode data to devise a specimen phylogeny, and used the five species-delimitation methods listed above to test the morphology-based species identification. We then used the consensus results to delimit and diagnose the species diversity in Ganthela.

MATERIAL AND METHODS

TAXON SAMPLING

We sampled 51 individuals from five localities of one described and five new species of Ganthela in East China (Fujian and Jiangxi Provinces; see inset in Fig. 1), and used a specimen of Sinothela sinensis (Bishop & Crosby, 1932) as the out-group (Table 1). We collected adults and immature spiders by excavating them from their subterranean burrows. We found a single species per locality except for two species at Mount Wangjiang, Fujian Province. All specimens were collected alive and reared in the laboratory until maturation. Voucher specimens are deposesed at the Centre for Behavioural Ecology and Evolution (CBEE), College of Life Sciences, Hubei University, Wuhan, China, and all type specimens are deposesed in the National Zoological Museum, Chinese Academy of Sciences, Beijing, China.

MOLECULAR PROTOCOLS

Total genomic DNA was extracted from spider legs using the Animal Genomic DNA Isolation Kit (Dingguo, Beijing, China), following the manufacturer’s protocols. Following the standard polymerase chain reaction (PCR) settings (Bond & Hedin, 2006; Agnarsson, 2010; Kuntner et al., 2013; Zhang & Maddison, 2013), we amplified cytochrome c oxidase subunit I (COI) using the primer pairs LCO1490/HCO2198 (Folmer et al., 1994) with the following PCR reaction protocol: initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 1 min, annealing at 40 °C for 1 min, and elongation at 72 °C for 90 s; and final extension at 72 °C for 7 min. The 25-μL PCR reactions including 16.7 μL of double-distilled H2O, 2.5 μL of 10× Taq buffer (mixed with MgCl2; TianGen Biotech, Beijing, China), 2.5 μL of dNTP Mix (2.5 mM), 1 μL of each forward and reverse 10-μM primer, 1 μL of DNA template, and 0.3 μL Taq DNA polymerase (2.5 U μL−1; TianGen Biotech, Beijing, China). The double-stranded PCR products were visualized by agarose gel electrophoresis (1% agarose). PCR products were purified and sequenced by Sunny Biotechnology Co., Ltd (Shanghai, China) using the ABI 3730XL DNA analyser.

We manually edited the sequences using GENEIOUS 5.6.6 (Biomatters Ltd, 2012), translated nucleotide reads to amino acids to check for stop codons and to ensure the proper configuration of codon positions, and aligned the sequences in GENEIOUS with gap opening/extension penalties set to 24/3.

PHYLOGENETIC ANALYSES

The COI data were treated as a single partition, and the best COI substitution model, chosen based on the Akaike information criterion (AIC) in jMODELTEST 2.1.3 (Darriba et al., 2012), was GTR + I + G. We performed maximum-likelihood (ML) analyses in GARLI 2.01 (Zwickl, 2006), and assessed branch supports by 100 bootstrap replicates. We used SumTrees in the DendroPy phylogenetic Python library (Sukumaran & Holder, 2010) to generate a majority-rule bootstrap consensus tree. We conducted Bayesian-inference (BI) analyses in MrBayes 3.2.1 (Ronquist et al., 2012) by running Markov chain Monte Carlo (MCMC) with one independent chain for 50 million generations. We monitored stationarity in TRACER 1.6 (Rambaut et al., 2014) and discarded as ‘burn-in’ the first quarter of cold-chain samples with the remaining trees used to build a consensus. We used FigTree 1.4.0 (Rambaut, 2012) to visualize and manipulate trees, and manually summarized the results from different approaches using vector graphics in Adobe ILLUSTRATOR.

SPECIES DELIMITATION

We analysed the COI data set (see Appendix S1) using five different species-delimitation methods. As both DNA barcoding gap (Hebert et al., 2003a) and P ID(Liberal) require a priori species designation, we divided 51
**Ganthela** individuals into six putative species based on a combination of phylogenetic topology from gene trees, morphological characters, and geographic information. In the DNA barcoding gap analysis, we examined the overlap between the mean intraspecific and interspecific Kimura two-parameter (K2P) and uncorrected p-distance for each candidate species calculated in MEGA 5.2.2 (Tamura *et al*., 2011). The species-delimitation plug-in (Masters *et al*., 2011) in GENEIOUS 5.6.6 (Biomatters Ltd, 2012) was used to obtain P ID(Liberal) statistical values. P ID(Liberal) is based on the putative species groups to calculate the mean probability of intra-/intergenetic distance ratios for these candidate species groups. The BI tree was used as a guide tree to test species hypothesis.

The next three methods that we used do not require assigning terminals to putative species. The ABGD procedure (Puillandre *et al*., 2012) calculates all pairwise distances in the data set, evaluates intraspecific divergences, and then sorts the terminals into candidate species with calculated $P$ values. We performed the ABGD analyses online (http://wwwabi.snv.jussieu.fr/public/abgd/), using three different distance metrics: Jukes–Cantor (JC69; Jukes & Cantor, 1969), K2P (Kimura, 1980), and simple distance (p-distance; Nei & Kumar, 2000). We analysed the data using two different values for the parameters $P_{\text{min}}$ (0.0001 and 0.001), $P_{\text{max}}$ (0.1 and 0.2), and relative gap width ($X = 1$ or 1.5), with the other parameters set to default values.

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**Figure 1.** Bayesian COI gene tree for 51 terminals of *Ganthela*, with the results of five different species delimitation approaches, in addition to morphology (see legend). Numbers above branches show posterior probability and bootstrap supports, and values below branches show mean intraspecific (black) and interspecific genetic distances (red), calculated as Kimura two-parameter (K2P)/p-distance. Species names and locality group terminals (for specimen codes, see Table 1) according to consensus results of species delimitation approaches.
<table>
<thead>
<tr>
<th>Specimen code</th>
<th>Genus</th>
<th>Species</th>
<th>Haplotype</th>
<th>Locality</th>
<th>Country</th>
<th>Coordinates</th>
<th>Elevation (m a.s.l.)</th>
<th>COI GenBank accession</th>
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Table 1. Samples used in species delimitation: specimen label, taxon name, number of haplotypes, sample collection locality with coordinates, and GenBank accession numbers.
The GMYC methodology (Pons et al., 2006) uses likelihood to test for species boundaries by detecting the transition point of interspecific versus intraspecific rates of lineage coalescence. We performed GMYC analyses in the ‘splits’ package for R (Ezard, Fujisawa & Barraclough, 2009). We used the single-threshold model (Pons et al., 2006) because prior studies have shown that the output of the multiple-threshold model (Monaghan et al., 2009) is no better than the single-threshold model (Hamilton et al., 2011, 2014; Paz & Crawford, 2012; Fujisawa & Barraclough, 2013; Hendrixson et al., 2013; Talavera, Dinca & Vila, 2013; Kekkonen & Hebert, 2014). We used BEAST 1.8.0 (Drummond et al., 2012) to obtain an ultrametric gene tree that GMYC requires as a guide tree, in combination with a strict molecular clock (Zuckermandl & Pauling, 1962) and Yule speciation model (Yule, 1924; Gernhard, 2008). We used standard arthropod substitution rates (Brower, 1994) by setting the COI substitution rate parameter as a normal prior with a mean value of 0.0115, and ran 50 million generations, sampling every 5000 generations. We used TRACER 1.6 (Rambaut et al., 2014) to assess the chain convergence and correct mixing of each MCMC chain, then discarded as ‘burn-in’ 10% of the trees in each chain to settle on an ultrametric tree using TreeAnnotator (Drummond et al., 2012).

Finally, we employed SP haplotype network analysis (Templeton et al., 1992) to investigate intraspecific relationships among the terminals, and thus delimit species (Jörger et al., 2012; Weigand et al., 2013). We generated haplotype networks using TCS 1.21 (Clement, Posada & Crandall, 2000) with a 95% parsimony criterion.

**Taxonomy**

Within a liphistiid genus-level revision, we recently described (Xu et al., 2015a) the type species of Ganthela: *G. yundingensis* Xu, 2015. Additional Ganthela specimens were examined with an Olympus SZX16 stereomicroscope, and anatomical details were studied with a Leica M205C stereomicroscope. Male palps and female genitalia were examined and photographed with a Leica M205C stereomicroscope and Olympus BX51 compound microscope after being dissected from the spider bodies. Genitalia were cleared in boiling 10% KOH for a few minutes to dissolve soft tissues. Unless otherwise noted, left palps were depicted. All measurements are in millimetres. Leg and palp measurements are given in the following order: total length (femur + patella + tibia + metatarsus + tarsus).

Abbreviations used are: ALE, anterior lateral eyes; AME, anterior median eyes; BL, body length; CL, carapace length; Co, conductor; CT, contrategulum; CW, carapace width; E, embolus; OL, opisthosoma length; OW, opisthosoma width; PC, paracymbium; PLE, posterior or lateral eyes; PME, posterior median eyes; RC, receptacular cluster; T, tegulum.

**Results**

**Phylogenetic inference**

The COI matrix of 51 individuals of *Ganthela* of 656 bp had 210 variable and 209 parsimony-informative sites, and contained 13 haplotypes (Table 1; Appendix S1). The phylogenetic topologies from BI and ML analyses agreed on *Ganthela* monophyly, with the node being highly supported (Fig. 1; posterior probability, PP = 1; bootstrap value, BS = 1). The two highly supported (PP > 0.95, BS > 75%) sister clades within *Ganthela* correspond to two geographical regions: Jiangxi and Fujian provinces (Fig. 1). Within the Jiangxi and the Fujian clades, the individuals from each separate locality grouped into solidly supported clades (PP = 1, BS > 75%). These locality clades mostly comprise closely related individuals, except in the case of Mount Wangjiang, where a single locality harbours two distinct and highly supported species clades (Fig. 1).

**Species delimitation**

**DNA barcoding gap**

Based on our prior species hypotheses, the lowest mean interspecific distance was 12/11% (K2P/uncorrected p-distance) found between *G. cipingensis* and *Ganthela jianensis* sp. nov., which was about eight times the highest mean intraspecific distance (1.62/1.57% for K2P/uncorrected p-distance) estimated for *Ganthela xianyouensis* sp. nov. (Table 2). Histograms with pairwise distances visualize a barcoding gap in *Ganthela* of 4–12% (K2P)/4–11% (uncorrected p-distance; Fig. 2). Six species were identified by the barcoding gap range, of which one is known and five are new (Fig. 1).

**PID(Liberal)**

The results based on the Bayesian inference tree found high PID(Liberal) values of ≥0.97 (0.88–1.0) and low intra-/interspecific ratios of ≤0.1 for all species hypothesized (Table 2), thereby also supporting the taxonomy of six putative species (Fig. 1).

**ABGD analysis**

The ABGD results using different parameter combinations and the initial partition of six species (Table 3) agreed on those six species, whereas those under a recursive partition regime yielded more species (Table 3). The settings $P_{	ext{min}}/P_{	ext{max}} = 0.0001/0.2$ yielded the most significant $P$ values.

All analyses reported so far support six morphological species, with syntopic terminals being conspecific, except in the case of Mount Wangjiang, where two species coexist within an absolute distance of 1 km.
In contrast, the following two analyses suggest more species. The single-threshold model GMYC resulted in nine clusters and entities with different confidence intervals of 7–9 and 7–11, respectively (Table 4). These results indicate two species in each of the Ji’an, Xianyou, and Mount Qingyuan clades (Fig. 1). The SP haplotype networks also show similar results, but with a single species within the Mount Qingyuan clade (Figs 1, 3).

DISCUSSION

In this study, under an integrative framework, we analysed original DNA barcoding data with five different species-delimitation methods to test the validity of one described and five undescribed morphological species of the liphistiid genus *Ganthela* known to us, in addition to the type species *G. yundingensis* Xu, 2015. Our results show that three molecular species-delimitation methods – DNA barcoding gap, P ID(Liberal), and ABGD – all fully confirm the morphological understanding of *Ganthela* species diversity, and thus elegantly complement traditional taxonomy (Fig. 1); however, GMYC and SP found more species of *Ganthela*, and thus disagree with the morphology, a result that reinforces conclusions from previous studies (Esselstyn et al., 2012; Paz & Crawford, 2012; Miralles & Vences, 2013; Talavera et al., 2013; Hamilton et al., 2014). We use the consensus from our analyses for species delimitation and diagnoses to conclude the formal revision of *Ganthela* (see Taxonomy).

The traditional DNA barcoding gap and ABGD methods are both based on the identification of a significant difference between inter- and intraspecific genetic distances in samples of terminal taxa, in order to assign organisms into putative species. For the DNA barcoding gap analysis, we assigned *Ganthela* specimens into putative species considering the phylogenetic, morphological, and geographic information available. Our results are roughly comparable with those from the mygalomorph spider literature, which found a barcode gap of 5–6% (Hamilton et al., 2011, 2014); however, the gap is wider in *Ganthela*, ranging from 4 to 12% (K2P)/4 to 11% (uncorrected p-distance; Fig. 2). ABGD usually generates diverse outcomes (Jörgersen et al., 2012; Puillandre et al., 2012; Kekkonen & Hebert, 2014). In this study, however, all analyses under different assumptions confirmed the six species, thereby lending further support to the utility of the barcoding gap in delimiting species (Weigand et al., 2013; Čandek & Kuntner, 2015; Hamilton et al., 2014).

P ID(Liberal) is usually used to test species hypotheses on phylogenetic trees (Masters et al., 2011; Hamilton et al., 2014). According to our findings, a P ID(Liberal) value > 95% would be reasonable to distinguish *Ganthela* species, and thus the cut-off of 95% can be used to delimit *Ganthela* and probably other liphistiid species.
Figure 2. DNA barcoding gap for Ganthela. Histograms show division of intraspecific (grey) and interspecific (black) COI sequence variation based on Kimura two-parameter (K2P, A) and uncorrected p-distance (B).

Table 3. Results of the automatic barcode gap discovery (ABGD) analyses

| Substitution model | $P_{\text{min}}/P_{\text{max}}$ | $X$ | Prior intraspecific divergence ($P$) | 0.001 | 0.0017 | 0.0028 | 0.0046 | 0.0077 | 0.0129 | 0.0215 | 0.0359 | 0.0599 | 0.1 |
|-------------------|-------------------------------|-----|-------------------------------------|-------|--------|--------|--------|--------|--------|--------|--------|-----|
| JC                | 0.001/0.1                     | 1.5 | Initial                             | 6     | 6      | 6      | 6      | 6      | 6      | 6      | 6      | 6    | 6    |
|                   |                               |     | Recursive                           | 11    | 9      | 9      | 9      | 9      | 8      | 7      | 7      | 7    | 6    |
| K2P               | 0.001/0.1                     | 1.5 | Initial                             | 6     | 6      | 6      | 6      | 6      | 6      | 6      | 6      | 6    | 6    |
|                   |                               |     | Recursive                           | 11    | 9      | 9      | 9      | 9      | 8      | 7      | 7      | 7    | 6    |
| Simple            | 0.001/0.1                     | 1.5 | Initial                             | 6     | 6      | 6      | 6      | 6      | 6      | 6      | 6      | 6    | 6    |
|                   |                               |     | Recursive                           | 11    | 9      | 9      | 9      | 9      | 8      | 7      | 7      | 7    | 6    |
| JC                | 0.001/0.1                     | 1   | Initial                             | 6     | 6      | 6      | 6      | 6      | 6      | 6      | 6      | 6    | 6    |
|                   |                               |     | Recursive                           | 11    | 9      | 9      | 9      | 9      | 8      | 7      | 7      | 7    | 6    |
| K2P               | 0.001/0.1                     | 1   | Initial                             | 6     | 6      | 6      | 6      | 6      | 6      | 6      | 6      | 6    | 6    |
|                   |                               |     | Recursive                           | 11    | 9      | 9      | 9      | 9      | 8      | 7      | 7      | 7    | 6    |
| Simple            | 0.001/0.1                     | 1   | Initial                             | 6     | 6      | 6      | 6      | 6      | 6      | 6      | 6      | 6    | 6    |
|                   |                               |     | Recursive                           | 11    | 9      | 9      | 9      | 9      | 8      | 7      | 7      | 7    | 6    |
| JC                | 0.0001/0.2                    | 1.5 | Initial                             | 6     | 6      | 6      | 6      | 6      | 6      | 6      | 6      | 6    | 6    |
|                   |                               |     | Recursive                           | 11    | 11     | 11     | 11     | 9      | 9      | 8      | 7      | 7    | 6    |
| K2P               | 0.0001/0.2                    | 1.5 | Initial                             | 6     | 6      | 6      | 6      | 6      | 6      | 6      | 6      | 6    | 6    |
|                   |                               |     | Recursive                           | 11    | 11     | 11     | 11     | 9      | 9      | 8      | 7      | 7    | 6    |
| Simple            | 0.0001/0.2                    | 1.5 | Initial                             | 6     | 6      | 6      | 6      | 6      | 6      | 6      | 6      | 6    | 6    |
|                   |                               |     | Recursive                           | 11    | 11     | 11     | 11     | 9      | 9      | 8      | 7      | 7    | 6    |
| JC                | 0.0001/0.2                    | 1   | Initial                             | 6     | 6      | 6      | 6      | 6      | 6      | 6      | 6      | 6    | 6    |
|                   |                               |     | Recursive                           | 11    | 11     | 11     | 11     | 9      | 9      | 8      | 7      | 7    | 6    |

JC, Jukes–Cantor substitution model; K2P, Kimura two-parameter substitution model; Simple, p-distance; $X$, relative gap width.
Table 4. Results of the general mixed Yule-coalescent (GMYC) analyses

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Clusters (CI)</th>
<th>Entities (CI)</th>
<th>Likelihood (null)</th>
<th>Likelihood (GMYC)</th>
<th>Likelihood ratio</th>
<th>Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single</td>
<td>9 (7–9)</td>
<td>9 (7–11)</td>
<td>153.0361</td>
<td>166.2342</td>
<td>26.3962**</td>
<td>−0.3631672</td>
</tr>
</tbody>
</table>

Clusters, operational taxonomic units (OTUs) delineated by GMYC with more than one specimen; Entities, singleton OTUs delineated by GMYC; CI, confidence interval; Likelihood (null), likelihood of the null model; Likelihood (GMYC), likelihood of the GMYC model; Threshold, the threshold between speciation and coalescence processes; Single, single-threshold model; **P < 0.001.

As a species delimitation technique, the often used GMYC is known to overestimate the number of species (Esselstyn et al., 2012; Paz & Crawford, 2012; Miralles & Vences, 2013; Talavera et al., 2013; Hamilton et al., 2014; but see, Jörger et al., 2012). In our case GMYC indeed seemingly overestimates the Ganthela species, suggesting that there are nine. Because GMYC requires an input tree, a differently obtained ultrametric gene tree, particularly one resulting from several genes, would probably change these results (Talavera et al., 2013; Kekkonen & Hebert, 2014). Our starting tree was derived from a single mitochondrial gene, but the addition of nuclear genes would be useful (Jörger et al., 2012; Dellicour & Flot, 2015).

Whereas SP has been successfully used in delimiting species in some taxa (Pons et al., 2006; Sauer & Hausdorf, 2012), it is often used to investigate intraspecific relationships. In our study, it oversplits Ganthela species and thus may not be helpful in delimiting Ganthela species.

The peril of employing single-locus species delimitation techniques is the possibility of over-splitting taxa as a result of co-amplified nuclear mitochondrial pseudogenes (Song et al., 2008), incomplete lineage sorting and introgression (Hausdorf & Hennig, 2010; Edwards & Knowles, 2014), and particularly the deep genetic structuring of mtDNA sequences (Bond et al., 2001; Arnedo & Ferrández, 2007). To avoid spurious results, taxonomic studies ideally incorporate multilocus data and analyse it in a phylogenetic framework to test the validity of putative species (e.g. Satler, Carstens & Hedin, 2013; Edwards & Knowles, 2014; Opatova & Arnedo, 2014). Multi-locus approaches may be particularly useful to understand lineages with sedentary females and vagile males (Opatova & Arnedo, 2014; Hedin, 2015), where maternally inherited mtDNA may show deep genetic structuring, even in the absence of geographical barriers (Bond et al., 2001).

Showing poor dispersal abilities, primitively segmentated spiders are strongly endemic and range-restricted, and the high interspecific genetic distances in our study support the hypothesized low levels of gene flow among populations, which is typical of mygalomorphs (Hamilton et al., 2011, 2014; Hendrixson et al., 2013; Opatova & Arnedo, 2014). Nevertheless, our results indicate that even single-locus analyses based on the COI barcodes, if integrated with morphological and geographical data, may provide sufficiently reliable species delimitation, and that multi-locus phylogenetic approaches may not be necessary for reliable taxonomic decisions.

**TAXONOMY**

**GENUS GANTHELA** Xu & Kuntner, 2015

For genus diagnosis and description, see Xu et al., 2015a.

Type species by original designation, *G. yundingensis* Xu, 2015. For diagnosis and description, see Xu et al., 2015a.

**GANTHELA CIPINGENSIS** (Wang, 1989) (Fig. 4)

*Liphistius cipingensis* Wang, 1989: 30, figs 1–6 (description of female, type may be lost from Hunan Normal University, not available for examination).

*Heptathela cipingensis* Platnick, 1993: 77 (transferred from *Liphistius*).

*Songthela cipingensis* Ono, 2000: 150.

**Material examined**

Five females [XUX-2013-(508/510/511/512/514)] collected at Cemetery of Mount Jinggang Revolutionary Martyrs, Ciping Town, Jinggangshan City, Jiangxi Province, China, 26.58°N, 114.16°E, 900 m a.s.l., 21 October 2013; two females [XUX-2013-(515–518)] collected at Nanshan, Ciping Town, Jinggangshan City, Jiangxi Province, China, 26.57°N, 114.16°E, 90 m a.s.l., 22 October 2013, collected by F.X. Liu, C. Xu, and X. Xu. No male found.

**Diagnosis**

Females of *G. cipingensis* resemble *G. xianyouensis* sp. nov., but differ by the details in genitalia, with relatively longer genital stalks (Fig. 4B, C). *Ganthela cipingensis* differs from all other *Ganthela* species by the following unique nucleotide substitutions in the standard DNA barcode alignment: G (20), G (26), C (77), C (326), and C (413).

**Description**

Female (Fig. 4A). Carapace and opisthosoma dark brown; with clear fovea; chelicerae robust, with
Figure 3. Haplotype networks of *Ganthela* under a 95% parsimony criterion. The size of each open circle indicates haplotype frequency, numbers preceded by ‘H’ indicate haplotype number, and numbers in brackets indicate population sizes. Open dots on lines connecting haplotypes indicate a substitution. Dashed lines enclosing haplotype networks correspond to morphological and consensus species.
promargin of cheliceral groove with between ten and 13 strong denticles of variable size; legs with strong hairs and spines; opisthosoma with 12 tergites, with tergite 5 being largest; seven spinnerets. Measurements: BL 8.70–14.70, CL 4.13–6.40, CW 3.70–5.00, OL 4.65–8.00, and OW 3.25–5.40; ALE > PLE > PME > AME; palp 9.72 (3.47 + 1.70 + 2.15 + 2.40), leg I 12.00 (3.75 + 2.15 + 2.40 + 2.30 + 1.40), leg II 12.06 (3.70 + 2.13 + 2.23 + 2.50 + 1.50), leg III 13.30 (3.60 + 2.20 + 2.25 + 3.35 + 1.90), and leg IV 19.07 (5.30 + 2.67 + 3.45 + 5.20 + 2.44).

Female genitalia
The posterior part of the genital area wide, rectangular (Fig. 4B, C), a pair of receptacular clusters close to each other and more or less parallel, with short genital stalks (Fig. 4B, C).

Distribution
Jiangxi (Jinggangshan) Province, China.

GANTHELA JIANENSIS Xu, Kuntner & Chen sp. nov. (Fig. 5)

Holotype
Female (XUX-2013-534), Mount Qingyuan, Ji’an City, Jiangxi Province, China, 27.06°N, 115.05°E, 100 m a.s.l., 23 October 2013, collected by F.X. Liu, X. Xu, and Z.T. Zhang.

Paratypes
Ten females (XUX-2013-530-531/533–536/538-541A) collected at the same locality, 23 October 2013, collected by F.X. Liu, X. Xu, and Z.T. Zhang. No male found.

Etymology
‘Jian’ refers to the type locality of this species.

Diagnosis
Females of G. jianensis sp. nov. differ from other species of Ganthela by details in genitalia, with short and thick genital stalks (Fig. 5B, C), and by the slightly curved posterior part of the genital area (Fig. 5B, C). Ganthela jianensis sp. nov. differs from all other Ganthela species by the following unique nucleotide substitutions in the standard DNA barcode alignment: G (59), A (131), A (155), C (245), T (249), G (317), C (398), C (528), C (581), and C (638).

Description
Female (holotype) (Fig. 5A). Carapace and opisthosoma, dark brown; chelicerae robust, with promargin of cheliceral groove with between ten and 13 strong denticles of variable size; legs with strong hairs and spines; opisthosoma with 12 tergites, with tergite 5 being largest; seven spinnerets. Measurements: BL 11.80–15.50, CL 5.60–7.40, CW 5.30–6.50, OL 5.95–9.10, and OW 4.46–6.70; ALE > PLE > PME > AME; palp 11.55 (4.00 + 2.10 + 2.45 + 3.00), leg I 13.25 (4.15 + 2.50 + 2.35 + 2.65 + 1.60), leg II 12.88 (3.73 + 2.37 + 2.25 + 2.75 + 1.78), leg III 13.82 (3.75 + 2.15 + 2.50 + 2.65 + 1.90), leg IV 19.07 (5.30 + 2.67 + 3.45 + 5.20 + 2.44).
Female genitalia
Posterior part of genital area slightly curved (Fig. 5B, C), with pair of receptacular clusters close to each other, and with very short and thick genital stalks (Fig. 5B, C).

Distribution
Jiangxi (Ji’an) Province, China

GANTHELA QINGYUANENSIS Xu, Kuntner & Liu sp. nov. (Fig. 6)

Holotype
Male (XUX-2012-288, matured 18 July 2013 at CBEE, College of Life Sciences, Hubei University), around TV Tower, Mount Qingyuan, Quanzhou City, Fujian Province, China, 24.95°N, 118.60°E, 475 m a.s.l., 11 December 2012, collected by D. Li, F.X. Liu, M. Kuntner, and X. Xu.

Paratypes

Etymology
‘Qingyuan’ refers to the type locality of this species, Mount Qingyuan.

Diagnosis
Males of G. qingyuanensis sp. nov. differ from G. yundingensis by anatomical details in the palps, which have a small posterior apophysis on the conductor, in addition to the spiniform apex (Fig. 6G). Females of G. qingyuanensis sp. nov. can be distinguished from all other Ganthela species by details in the genitalia, with longer genital stalks (Fig. 6B, C). Ganthela qingyuanensis sp. nov. differs from all other Ganthela species by the following unique nucleotide substitutions in the standard DNA barcode alignment: C (42), C (50), C (104), G (108), C (158), G (197), C (218), A (269), C (380), G (339), C (353), C (383), C (449), C (504), C (575), and C (625).

Description
Male (holotype). Carapace brown; opisthosoma light brown, with dark brown tergites; sternum narrow, much longer than wide; a few long pointed hairs running over ocular mound in a longitudinal row; chelicerae robust, with promargin of cheliceral groove with 11 denticles of variable size; legs with strong hairs and spines; opisthosoma with 12 tergites, with tergites 2–6 larger than others, and with tergite 4 being largest; seven spinnerets. Measurements: BL 10.55, CL 5.00, CW 4.85, OL 5.60, and OW 3.63; ALE > PLE > PME > AME; leg I (4.00 + 2.42 + 2.17 + 3.23 + 2.00), leg II (3.61 + 3.00 + 3.45 + 5.53 + 2.78).

Female genitalia
Posterior part of genital area slightly curved (Fig. 5B, C), with pair of receptacular clusters close to each other, and with very short and thick genital stalks (Fig. 5B, C).

Palp
Cymbium with a projection; prolateral side of paracymbium unpigmented and unsclerotized, numerous setae and spines at the tip of paracymbium (Fig. 6F, G). Contrategulum has two marginal apophyses with smooth margin and blunt distal end (Fig. 6H). Tegulum with a dentate, wide edge (Fig. 6F, G). Conductor with a spiniform apex and a small apophysis at posterior part, parallel with embolus (Fig. 6G). Embolus largely sclerotized, with a wide and flat opening, and distal embolus slightly sclerotized (Fig. 6G).

Figure 5. Ganthela jianensis Xu, Kuntner & Chen sp. nov. A, female (XUX-2013-536). B, C, female genitalia (XUX-2013-534): B, dorsal view; C, ventral view; RC, receptacular cluster. Scale bar 0.5 mm.
Female (Fig. 6A). Carapace and opisthosoma of female similar to male, except coloration lighter than male; chelicerae robust, with promargin of cheliceral groove with between ten and 12 strong denticles of variable size; legs with strong hairs and spines; opisthosoma with 12 tergites, similar to male; seven spinnerets. Measurements: BL 10.68–14.48, CL 5.48–7.05, CW 4.52–6.00, OL 5.77–7.62, and OW 4.13–5.68; ALE > PLE > PME > AME; palp 9.51 (3.40 + 1.70 + 2.00 + 2.41), leg I 11.43 (3.85 + 1.95 + 2.08 + 2.25 + 1.30), leg II 11.08 (3.40 + 1.95 + 1.96 + 2.45 + 1.32), leg III 11.81 (3.46 + 2.05 + 1.92 + 2.70 + 1.68), and leg IV 17.46 (4.95 + 2.48 + 3.05 + 4.68 + 2.30).

**Female genitalia**

Posterior part of genital area rectangular (Fig. 6B–E), with pair of receptacular clusters with genital stalks (Fig. 6B, C), or with paired receptacular clusters fused into a big cluster (Fig. 6D, E).
Variation
Female receptacular clusters show considerable intraspecific variation, with the pair of receptacular clusters fused.

Distribution
Fujian (Quanzhou) Province, China.

**GANTHELA WANGJIANGENSIS** Xu, Kuntner & Liu sp. nov. (Fig. 7)

**Holotype**
Female (XUX-2013-159), Mount Wangjiang, Huangyang Village, Zhuangbian Town, Hanjiang District, Putian City, Fujian Province, China, 25.66°N, 118.87°E, 680 m a.s.l., 10 July 2013, collected by F.X. Liu, X. Xu, and Z.T. Zhang. No male found.

**Etymology**
’Wangjiang’ refers to the type locality of this species, Mount Wangjiang.

**Diagnosis**
Females of *G. wangjiangensis* sp. nov. differ from all other *Ganthela* species by the W-shaped posterior part of the genital area, and can be further distinguished from *G. venus* sp. nov. by the connected basal genital stalks (Fig. 7A, B). *Ganthela wangjiangensis* sp. nov. differs from all other *Ganthela* species by the following unique nucleotide substitutions in the standard DNA barcode alignment: G (54), C (101), C (179), C (320), A (336), T (372), C (386), C (422), G (476), T (512), and C (632).

**Description**
Female (holotype). Carapace and opisthosoma light brown; tergites slightly dark brown; sternum narrow, length more or less twice of width; a few long pointed hairs running over ocular mound in a longitudinal row; chelicerae robust, with promargin of cheliceral groove with 12 strong denticles of variable size; legs with strong hairs and spines; opisthosoma with 12 tergites, with tergites 2–6 larger than others, and the with tergite 4 being largest; seven spinnerets. Measurements: BL 11.50, CL 4.90, CW 4.30, OL 6.40, OW 5.55; ALE > PLE > PME > AME; palp 7.94 (2.78 + 1.50 + 1.48 + 2.18), leg I 9.10 (3.05 + 1.35 + 1.70 + 1.80 + 1.20), leg II 9.50 (2.80 + 1.67 + 1.71 + 2.02 + 1.30), leg III 10.00 (2.75 + 1.76 + 1.57 + 2.45 + 1.47), and leg IV 13.62 (3.83 + 1.92 + 2.28 + 3.52 + 2.07).

**Female genitalia**
The posterior part of genital area W-shaped (Fig. 7A, B), with a pair of receptacular clusters separated from each other, and with the basal parts of short genital stalks fused (Fig. 7A, B).

**Distribution**
Fujian (Putian) Province, China.

**Remarks**
*Ganthela wangjiangensis* sp. nov. was collected within 1 km of *G. venus* sp. nov., but at a higher altitude.

**GANTHELA XIANYOUENSIS** Xu, Kuntner & Chen sp. nov. (Fig. 8)

**Holotype**
Female (XUX-2013-153), Qingfengge, Dongshi Village, Yuanzhuang Town, Xianyou County, Putian City, Fujian Province, China; 25.24°N, 118.70°E, 336 m a.s.l., 10 July 2013; collected by F.X. Liu, X. Xu, and Z.T. Zhang.

**Paratypes**
Two females (XUX-2013-153/154) collected at the same locality, 10 July 2013; collected by F.X. Liu, X. Xu, and Z.T. Zhang. No male found.

**Etymology**
’Xianyou’ refers to the type locality of this species.

**Diagnosis**
Females of *G. xianyouensis* sp. nov. can be distinguished from all other *Ganthela* species by details of...

Figure 7. *Ganthela wangjiangensis* Xu, Kuntner & Liu sp. nov. A, B, female genitalia (XUX-2013-159). A, dorsal view; B, ventral view. RC, receptacular cluster. Scale bar: 0.5 mm.
the genitalia, with very short but slender genital stalks (Fig. 8B, C). *Ganthela xianyouensis* sp. nov. further differs from all other *Ganthela* species by the following unique nucleotide substitutions in the standard DNA barcode alignment: G (96), G (146), C (277), C (314), T (338), C (393), C (428), and T (572).

**Description**

Female (holotype) (Fig. 8A). Carapace and opisthosoma light brown; tergites dark brown; sternum narrow, length more or less twice of width; a few long pointed hairs running over ocular mound in a longitudinal row; chelicerae robust, with promargin of cheliceral groove with 11 or 12 strong denticles of variable size; legs with strong hairs and spines; opisthosoma with 12 tergites, with tergites 2–6 larger than others, and with tergite 4 being largest; seven spinnerets. Measurements: BL 13.60, CL 6.10, CW 4.96, OL 6.56, and OW 5.45; ALE > PLE > PME > AME; palp 9.48 (3.26 + 1.66 + 2.06 + 2.50), leg I 11.58 (3.72 + 1.92 + 2.15 + 2.32 + 1.47), leg II 11.45 (3.64 + 1.93 + 2.05 + 2.25 + 1.58), leg III 12.58 (3.55 + 2.10 + 2.10 + 3.00 + 1.83), and leg IV 17.53 (4.88 + 2.25 + 3.15 + 4.55 + 2.70).

**Female genitalia**

The posterior part of genital area rectangular (Fig. 8B, C), with a pair of receptacular clusters close to each other, and with very short but slender genital stalks (Fig. 8B, C).

**Distribution**

Fujian (Putian) Province, China.

**GANTHELA VENUS** Xu sp. nov. (Fig. 9)

**Holotype**

Female (XUX-2013-160), Mount Wangjiang, Huangyang Village, Zhuangbian Town, Hanjiang District, Putian City, Fujian Province, China, 25.66°N, 118.88°E, 459 m a.s.l., 11 July 2013, collected by F.X. Liu, X. Xu, and Z.T. Zhang. No male found.

**Etymology**

Named after the Greek goddess of love, Venus.

**Diagnosis**

Females of *G. venus* sp. nov. can be distinguished from *G. wangjiangensis* sp. nov. by details in the genitalia, which have separated stalks on the anterior part (Fig. 9A, B). *Ganthela venus* sp. nov. furthermore differs from all other *Ganthela* species by the following unique nucleotide substitutions in the standard DNA barcode alignment: C (36), A (80), T (227), C (341), G (347), T (354), C (431), and T (539).

**Description**

Female (holotype). Carapace and opisthosoma light brown; tergites slightly dark brown; sternum narrow, length more or less twice of width; a few long pointed hairs running over ocular mound in a longitudinal row;
chelicerae robust, with promargin of cheliceral groove with ten strong denticles of variable size; legs with strong hairs and spines; opisthosoma with 12 tergites, with tergites 2–6 larger than others, and with tergite 4 being largest; seven spinnerets. Measurements: BL 9.80, CL 4.38, CW 4.20, OL 5.6, and OW 5.00; ALE > PLE > PME > AME; palp 7.63 (2.50 + 1.31 + 1.65 + 2.17), leg I missing, leg II 8.49 (2.71 + 1.50 + 1.68 + 1.50 + 1.10), leg III 9.34 (2.52 + 1.58 + 1.56 + 2.30 + 1.38), and leg IV 13.70 (3.75 + 1.60 + 2.55 + 3.68 + 2.12).

Female genitalia
Posterior part of genital area W-shaped (Fig. 9A, B), with pair of receptacular clusters with short stalks on the anterior part of genital separated from one another (Fig. 9A, B).

Distribution
Fujian (Putian) Province, China.

Remarks
See comments under G. wangjiangensis sp. nov.

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of this article at the publisher’s web-site:

**Appendix S1.** Aligned COI sequences used for species delimitation (separate nexus file).