Identifying spiders through DNA barcodes

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Abstract: With almost 40 000 species, the spiders provide important model systems for studies of sociality, mating systems, and sexual dimorphism. However, work on this group is regularly constrained by difficulties in species identification. DNA-based identification systems represent a promising approach to resolve this taxonomic impediment, but their efficacy has only been tested in a few groups. In this study, we demonstrate that sequence diversity in a standard segment of the mitochondrial gene coding for cytochrome *c* oxidase I (*COI*) is highly effective in discriminating spider species. A *COI* profile containing 168 spider species and 35 other arachnid species correctly assigned 100% of subsequently analyzed specimens to the appropriate species. In addition, we found no overlap between mean nucleotide divergences at the intra- and inter-specific levels. Our results establish the potential of *COI* as a rapid and accurate identification tool for biodiversity surveys of spiders.

Résumé : Avec presque 40 000 espèces, les araignées constituent un modèle important pour l'étude de la vie sociale, des systèmes d'accouplement et du dimorphisme sexuel. Cependant, la recherche sur ce groupe est souvent restreinte par les problèmes d'identification des espèces. Les systèmes d'identification basés dur l'ADN présentent une solution prometteuse à cette difficulté d'ordre taxonomique, mais leur efficacité n'a été vérifiée que chez quelques groupes. Nous démontrons ici que la diversité des séquences dans un segment type du gène mitochondrial de la cytochrome *c* oxydase I (*COI*) peut servir de façon très efficace à la reconnaissance des espèces d'araignées. Un profil de *COI* contenant 168 espèces d'araignées et 35 autres espèces d'arachnides a permis de placer correctement à l'espèce appropriée 100 % des spécimens qui ont alors été analysés après coup. De plus, il n'y a pas de chevauchement entre les divergences moyennes des nucléotides au sein d'une même espèce et d'une espèce à l'autre. Ces résultats confirment le potentiel de *COI* comme un outil rapide et précis d'identification pour les inventaires de biodiversité des araignées.

[Traduit par la rédaction]

Introduction

Although there are approximately 37 000 described spider species, this is thought to represent only a fraction of their total diversity (Adis and Harvey 2000). Certainly spiders are ubiquitous in terrestrial ecosystems, and some taxa also occur in freshwater and marine environments (Foelix 1996). Adults range in size from the 0.4-mm *Patu marplesi* Forster, 1959 to bird-eating tarantulas, but efforts to identify spiders through morphology are often problematic. There are a variety of reasons for this complexity. One of the most important impediments to identification is that most keys rely on the examination of adults, therefore most life stages are unidentifiable. A second complication arises because many species show striking sexual dimorphism, therefore separate criteria are required to identify females and males. In a number of taxa, detailed morphological analyses have failed to reveal diagnostic traits in one sex, limiting identifications to a single gender. The frequent occurrence of marked sexual dimorphism has created serious problems of synonomy, particularly because the original descriptions for roughly half of all spiders were based on a single specimen (Coddington and Levi 1991). For example, females and males in some species of the orb-weaving genera *Witica* O. Pickard-Cambridge, 1895 and *Micrathena* Sundevall, 1833 were originally placed in different genera because they are so morphologically different (Levi 1986*a*, 1986*b*).

Complications in arachnid identification also arise because many species continue to molt and grow as adults, leading to gross size differences. For example, some *Nephilia* males are twice the length of others (Cohn 1990). In general, species-level identifications are only possible with adults and they often require thorough analyses of genitalic morphology. The recent discovery of genitalic polymorphisms in some spider species has added a new layer of complexity to taxonomic decisions (Huber and Gonzalez 2001; Jocque 2002). In fact, Huber and Gonzalez (2001) have argued that the overwhelming reliance on genitalia for the determination of species boundaries has imposed a bias against the discovery of intra-specific polymorphisms in these traits. For these reasons, spiders are a group where it is critical to develop an accurate identification system that is broadly accessible. It has recently been proposed that DNA-based systems will soon provide a general solution to the problem of species identification in many groups (Tautz et al. 2002, 2003; Hebert et al. 2003*a*; Blaxter and Floyd 2003). DNA-based identification systems were first adopted for microbes and such organisms are now routinely identified using sequence data (Theron and Cloete 2000). The desirability of extending this approach to all of life is increasingly recognized (Hebert

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et al. 2003*a*; Tautz et al. 2003). However, the move from concept to implementation requires the critical step of identifying efficient protocols that enable species identification. In the case of the animal kingdom, a solution appears to have been identified.

Recent studies have indicated that sequence divergence in a standard region of the mitochondrial gene coding for cytochrome *c* oxidase I (*COI*) can provide species-level resolution in several animal groups (Hebert et al. 2003*a*, 2003*b*, 2004*a*, 2004*b*; Hogg and Hebert 2004). The *COI* gene is a good target because it is present in all animals, and sequence comparisons are straightforward because insertions and deletions are rare. Moreover, *COI* appears to possess enough sequence divergence to regularly allow differentiation between closely related species (Hebert et al. 2003*b*). However, comprehensive tests are still needed to confirm that *COI* can resolve species boundaries in groups with varied life histories, mating systems, and patterns of dispersal. As such, it is important to test the ability of *COI* to diagnose species boundaries in a diverse range of animal taxa. Here we investigate whether it is possible to reliably identify a wide variety of spider taxa using the *COI* gene.

Our study is not the first to use molecular methods to discriminate arachnid species. The Acari have been the target of considerable molecular work, because of the difficulties in their identification through morphological approaches and their economic and medical importance (reviewed in Navajas and Fenton 2000). Our study is also not the first attempt to create a means of arachnid identification for nonspecialists. Do et al. (1999) achieved 81% success in identifying six spider species employing a pattern-recognition system similar to that used in other taxonomic groups (Bungay and Bungay 1991; Yu et al. 1992; Weeks et al. 1997). Although these studies have highlighted the potential of automated methods for identification, they are strongly limited by the specificity of their target organisms. By contrast, DNA-based approaches potentially have widespread taxonomic applicability. In this study, we evaluate the effectiveness of *COI* to provide species-level identifications for an assemblage of spider species, mainly from the temperate-zone regions of North America. In addition, we also assess the magnitude of sequence divergence within and between species.

Methods and materials

Sequences

We examined 327 *COI* sequences from 203 arachnid species. The emphasis of this study was the order Araneae (168 species), but we also included 35 species from the arachnid orders Acari and Scorpiones. These were included to gain a preliminary sense of whether it will be possible to extend our findings on spiders to other arachnids. Approximately one third of these sequences (111 of 327) were obtained from GenBank in April 2003. This constituted all of the 600+ base pair (bp) arachnid sequences from the 5′ region of *COI* that were available in GenBank at the time. The remaining sequences were obtained through the sequence analysis of specimens from collections at the University of Alberta (Edmonton), the Canadian National Collection (Ottawa), and the Royal Ontario Museum (Toronto). Voucher specimens for all of the taxa are preserved at the Department of Zoology, University of Guelph. All specimens were either dried or preserved in 70%–95% EtOH. GenBank accession codes, identifications, and geographical provenance of samples can be found at www.barcodinglife.org.

We isolated total DNA from 1 to 9 individuals per species, using the GenElute™ genomic DNA miniprep kit (Sigma Co.) following the manufacturer's instructions. The polymerase chain reaction (PCR) (Saiki et al. 1988) was subsequently utilized to amplify a 660-bp segment of the mitochondrial *COI* gene using two primer pairs. We employed one standard forward primer (Folmer et al. 1994) — LCO1490 (5′-GGTCAACAAATCATCATAAAGATATTGG-3′) — and one new forward primer and two new reverse primers. Their sequences were as follows: chelicerate forward1 (5′-TACTCTACTAATCATAAAGACATTGG-3′), chelicerate reverse1 (5′-CCTCCTCCTGAAGGGTCAAAAAATGA-3′), and chelicerate reverse2 (5′-GGATGGCCAAAAAATCAA-AATAAATG-3′). Each PCR contained 5 μL of 10× PCR buffer at pH 8.3 (10 mmol/L of Tris HCl at pH 8.3, 1.5 mmol/L of $MgCl_2$, 50 mmol/L of KCl, 0.01% NP-40), 2.2 μL of $MgCl_2$, 0.26 μmol/L of each dNTP (C, G, A, T), 1.0 μL of each primer, 1.0 U (1 U ≈ 16.67 nkat) of *Taq* DNA polymerase (Qiagen Inc.), and 1 μL of DNA template. The PCR thermal regime consisted of 1 cycle of 1 min at 94 °C; 5 cycles of 1 min at 94 °C, 1.5 min at 45 °C, and 1.5 min at 72 °C; 35 cycles of 1 min at 94 °C, 1.5 min at 50 °C, and 1 min at 72 °C; and followed by a final cycle of 5 min at 72 °C. Each PCR product was gel purified using the Qiaex II kit (Qiagen Inc.) and was then subjected to dye terminator cycle-sequencing reactions (25 cycles, 55 °C annealing) and Ampli*Taq* R DNA polymerase FS (Perkin Elmer). It was then sequenced in one direction on an ABI 377 automated sequencer (Applied Biosystems) using the Big Dye version 3.1 sequencing kit. We checked the accuracy of sequencing by duplicate sequencing some individuals. We verified that all sequences were from Arthropoda using the GenBank BLAST algorithm.

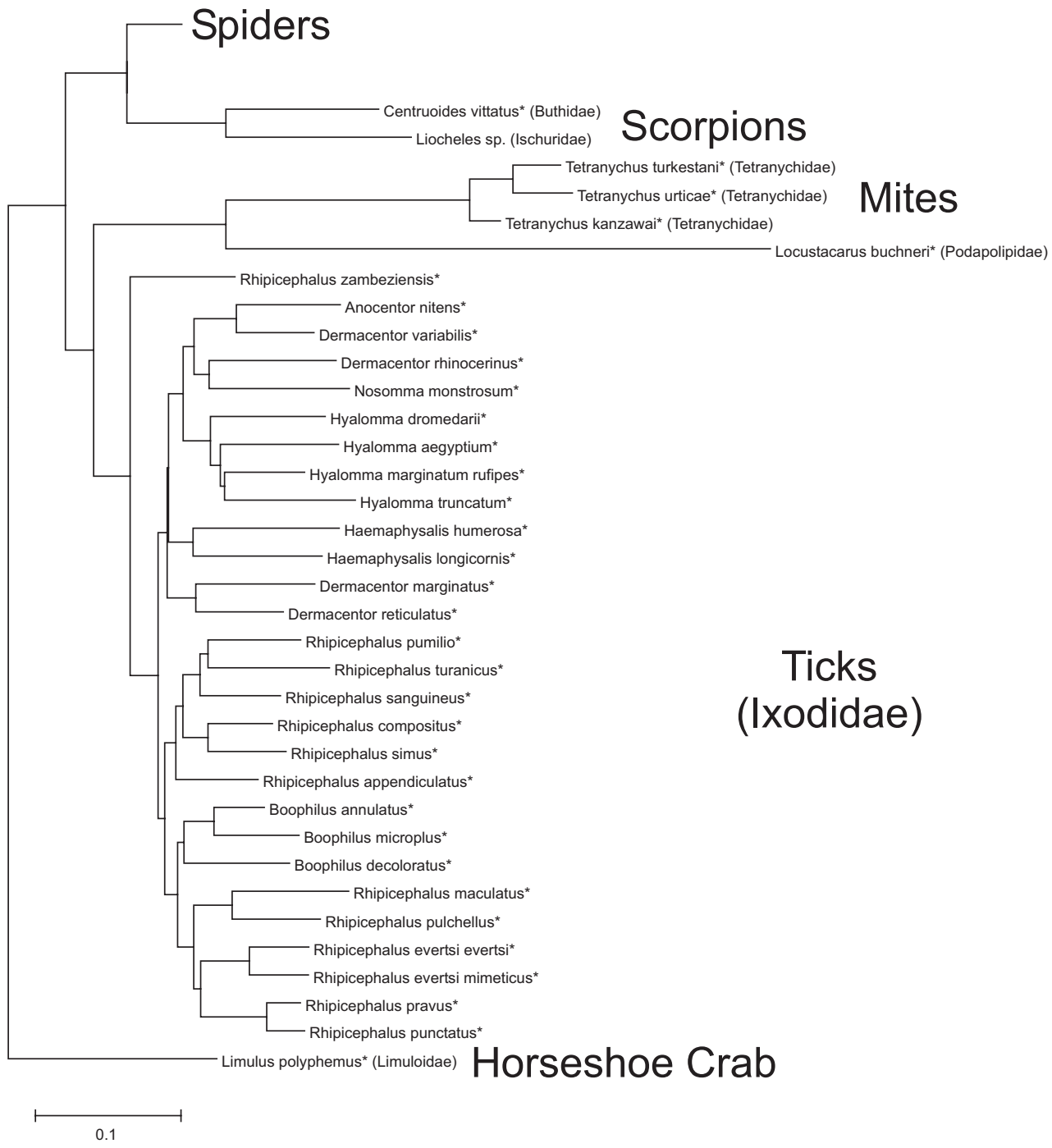
COI arachnid species profile and test taxa

Of the 327 sequences examined in this study, we randomly selected 1 sequence from each of the 203 arachnid species for inclusion in a *COI* species profile. The fewer taxa are included in a tree, the more difficult it is to place newly added taxa into the correct group (see Zwickl and Hillis 2002). Thus, by including just one representative of each species in the profile, we provided the most rigorous possible test of the ability of *COI* to distinguish between the taxa represented in our study. We tested the ability of this species profile to recognize additional sequences from those species that were represented in our study by several individuals. We used a horseshoe crab (*Limulus polyphemus* (L., 1748)) sequence as the outgroup because it is placed in Xiphosura, which together with Arachnida make up Eucheliceratа, and therefore represents a close relation to all arachnids (Weygoldt 1998).

Data analysis

We manually aligned sequences with the Bioedit sequence editor (Hall 1999) and subsequently pruned the alignment to 600 bp. We calculated nucleotide-sequence divergences using Kimura's two parameter model (Kimura 1980) for two

Fig. 1. Neighbour-joining analysis of a profile of DNA sequences from 203 arachnid species. Sequence divergence in 600 base pair (bp) of the *COI* gene calculated using Kimura's two parameter model model. * indicates the profile representative for a species.



reasons. First, it makes fewer assumptions about the nature of sequence change than more heavily parameterized models (Hebert et al. 2003*a*). This is an important attribute when transition rules governing nucleotide substitution vary among taxa. Second, it provides a conservative estimate of long branches because it underestimates the number of multiple hits in comparison to more complex models (Nei and Kumar 2000). We subsequently employed neighbour-joining analysis, implemented in MEGA version 2.1 (Kumar et al. 2001), to examine relationships among taxa. The neighbour-joining method has been shown to be computationally efficient and has a record of recovering trees that are at least as good as those generated by alternate methods (Nei and Kumar 2000).

The neighbour-joining profile contained 203 terminal nodes, each representing a different species. This profile was subsequently used as a classification tool by re-running the analysis following the repeated addition of a single "test" taxon to the data set. We used 75 additional representatives of species already present in the tree as test taxa. We measured the success of their classification by determining whether each test sequence grouped most closely with the single representative

Fig. 2. Neighbour-joining analysis of combined profile (n = 203), test (n = 75), and supplementary (n = 49) sequence divergence in 600 bp of the *COI* gene, using Kimura's two parameter model. * indicates the profile representative for a species.

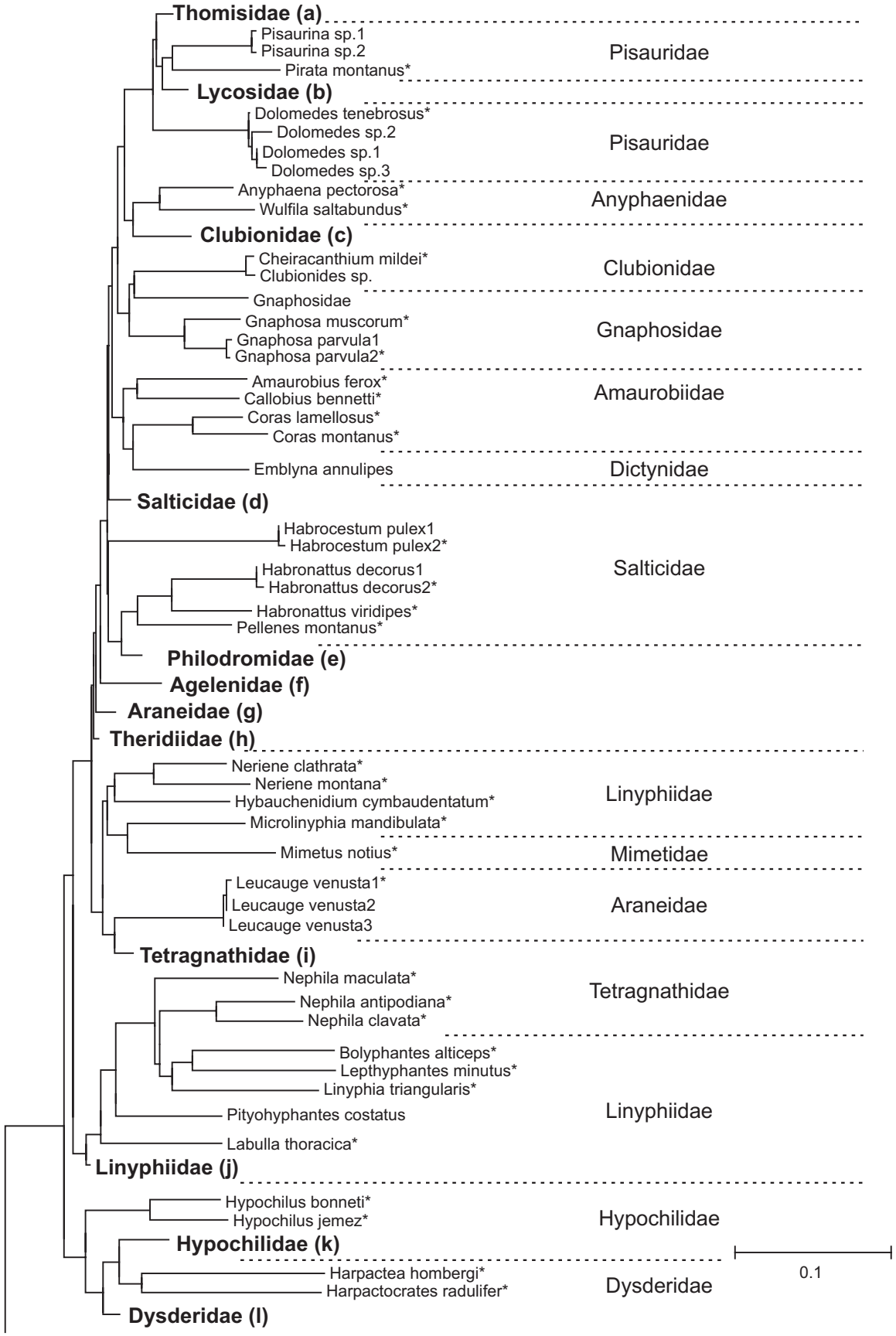


Fig. 2 (*continued*).

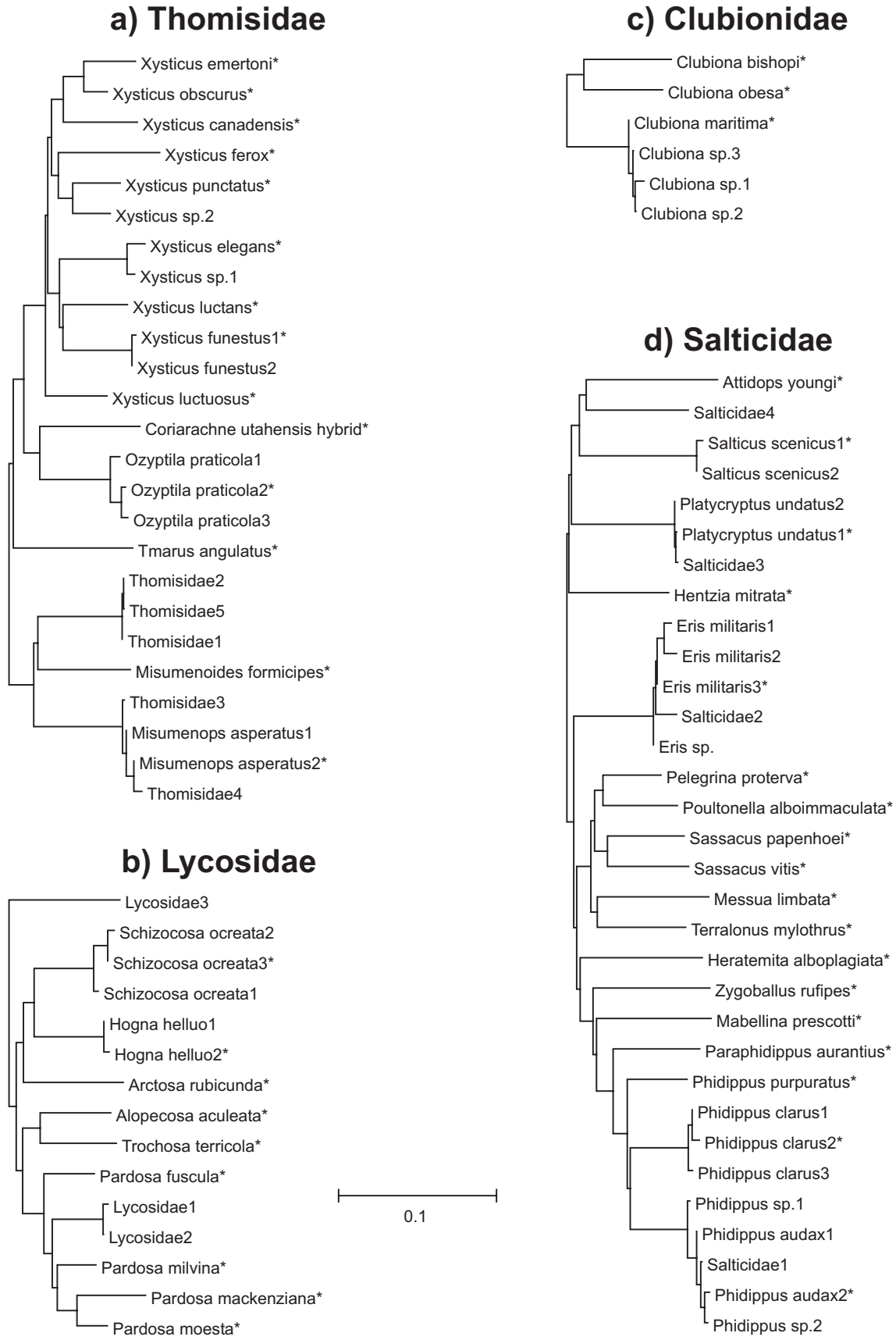


Fig. 2 (*continued*).

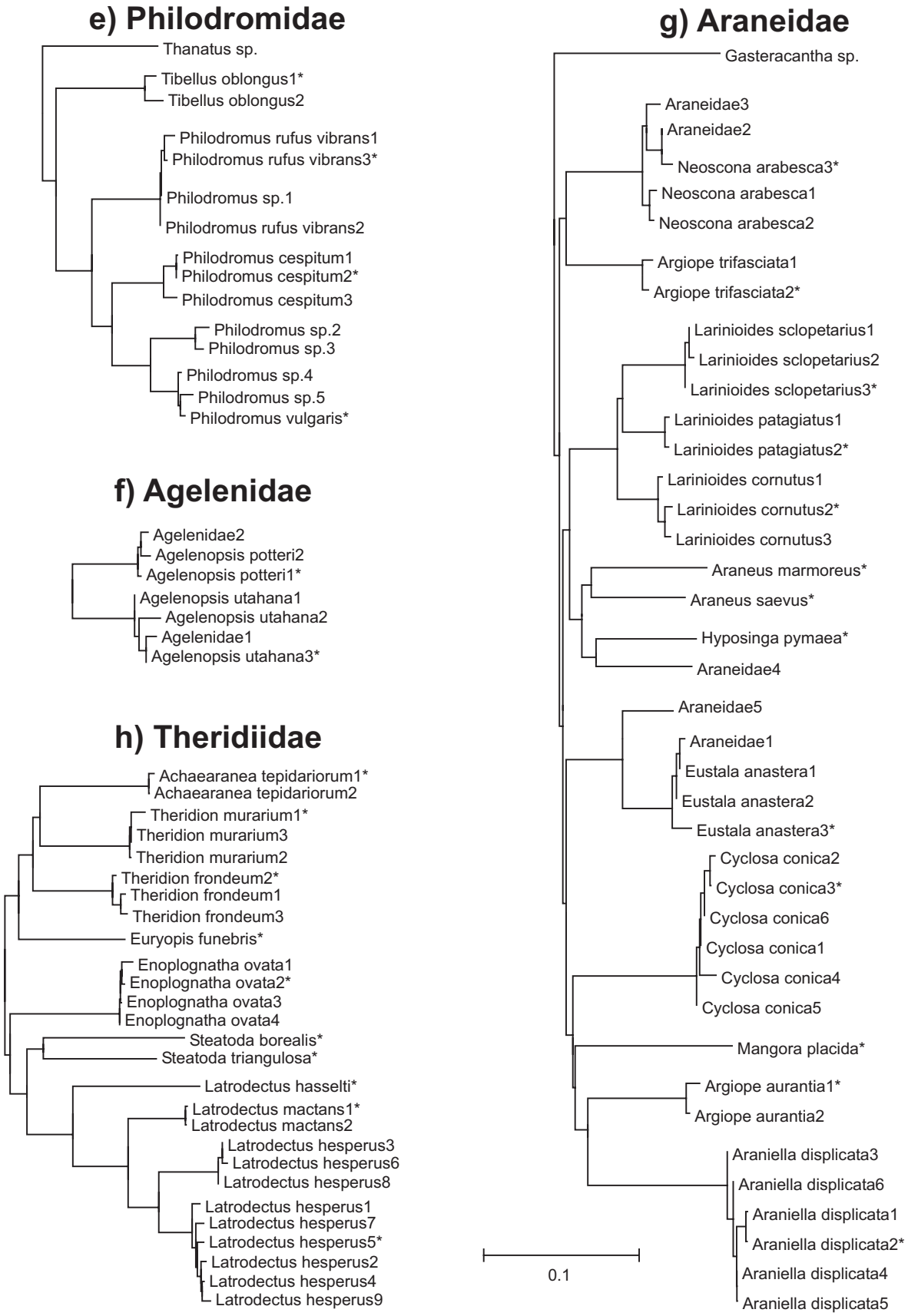


Fig. 2 (*concluded*).

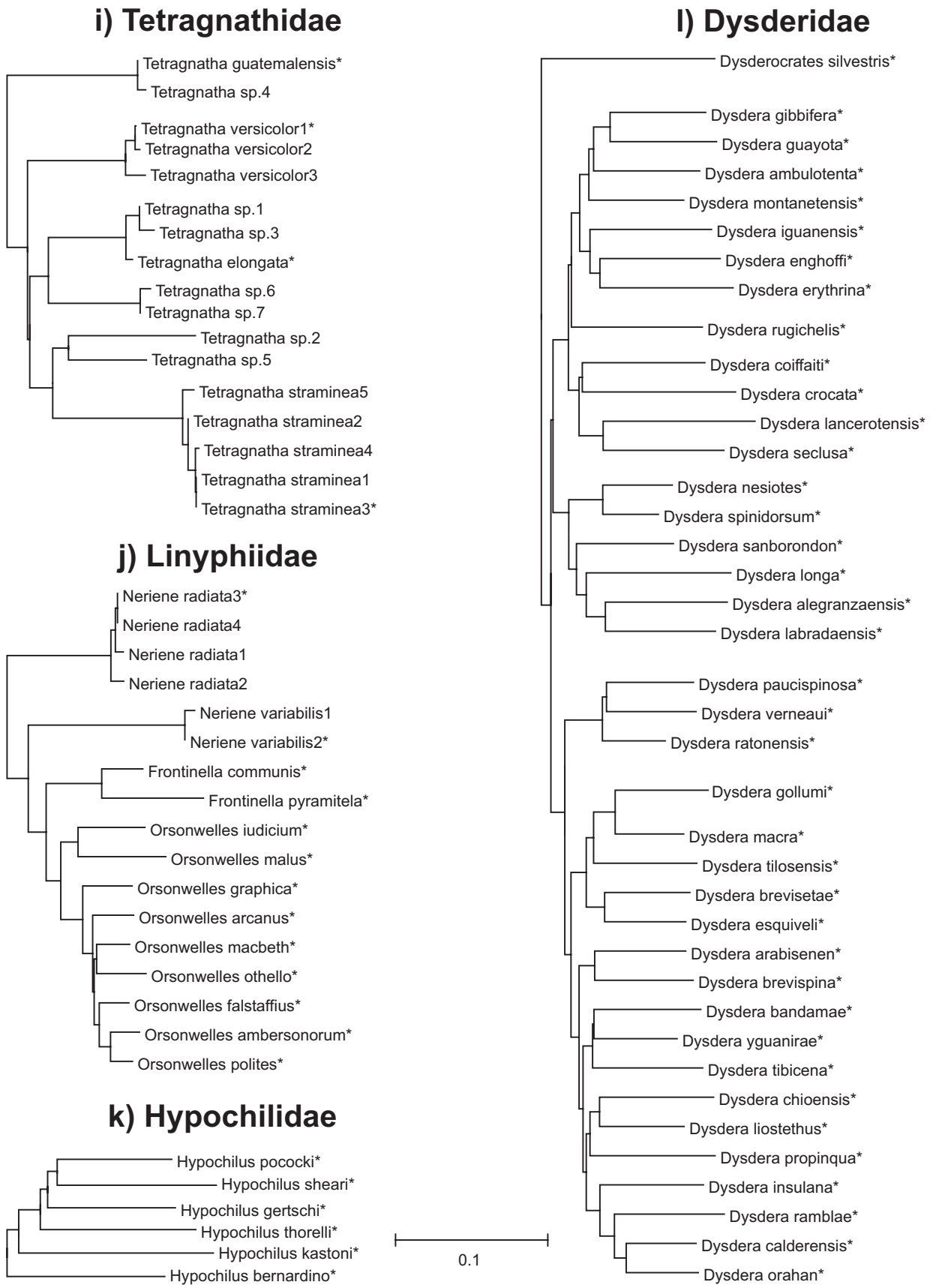


Table 1. Mean and range of intraspecific nucleotide divergences for 37 arachnid species, using Kimura's two parameter model.

Species	No. of individuals	Mean percent divergence	Range
Agelenopsis potteri (Blackwall, 1846)	2	0.7	—
Agelenopsis utahana (Chamberline and Ivie, 1933)	3	1.4	0.2–2.1
Araniella displicata (Hentz, 1847)	6	0.4	0–0.9
Argiope aurantia Lucas, 1833	2	1.1	—
Argiope trifasciata (Forskål 1775)	2	1.0	—
Cyclosa conica (Pallas, 1772)	7	0.6	0–1.4
Enoplognatha ovata (Clerck, 1757)	4	0.5	0–0.9
Eris militaris (Hentz, 1845)	3	0.9	0.4–1.3
Eustala anastera (Walckenaer, 1842)	3	0.9	0–1.4
Gnaphosa parvula Banks, 1896	2	0.5	—
Habrocestum pulex (Hentz, 1846)	2	0.3	—
Habronattus decorus (Blackwall, 1846)	2	0.4	—
Hogna helluo (Walckenaer, 1837)	2	0.3	—
Larinioides cornutus (Clerck, 1757)	3	1.0	0.8–1.2
Larinioides patagiatus (Clerck, 1757)	2	0.5	—
Larinioides sclopetarius (Clerck, 1757)	3	0.2	0.2–0.4
Latrodectus hesperus Chamberlin and Ivie, 1935	9	3.6	0–7.4
Latrodectus mactans (Fabricius, 1775)	2	0.2	—
Leucauge venusta (Walckenaer, 1842)	3	0.1	0.2
Misumenops asperatus (Hentz, 1847)	2	0.2	—
Neoscona arabesca (Walckenaer, 1842)	3	2.2	0.7–3.1
Neriene radiata (Walckenaer, 1841)	4	0.9	0–1.9
Neriene variabilis (Banks, 1892)	2	0	—
Ozyptila praticola (C.L. Koch, 1837)	3	1.3	0.7–1.8
Phidippus clarus Keyserling, 1885	3	0.5	0.2–0.8
Phidippus audax (Hentz, 1845)	2	0.4	—
Philodromus cespitum (Walckenaer, 1802)	3	1.1	0–1.9
Philodromus rufus vibrans Dondale, 1964	3	0.7	0.5–0.8
Platycryptus undatus (De Geer, 1778)	2	0	—
Salticus scenicus (Clerck, 1757)	2	0	—
Schizocosa ocreata (Emerton, 1885)	3	0.9	0.2–1.3
Tetragnatha versicolor Walckenaer, 1842	3	1.5	0.3–2.1
Tetragnatha straminea Emerton, 1884	5	0.6	0–1.4
Theridion frondeum Hentz, 1850	3	0.1	0.7–1.2
Theridion murarium Emerton, 1882	3	0.7	0.2–1.0
Tibellus oblongus (Walckenaer, 1802)	2	1.8	—
Xysticus funestus Keyserling, 1880	2	0	—

of its species in the profile, or with another species. After conducting all of the classification tests, we added all of the test sequences to the profile to allow a more detailed examination of the factors enabling successful identification. In addition, we added 49 sequences from specimens that could not be identified to a species level through morphological analysis, although they were assigned to either a genus or a family. These sequences provided a larger data set with which we could examine tree structure at higher taxonomic levels.

Results

Taxonomic assignments and tree structure

Each of the 203 species included in our neighbour-joining profile possessed a distinct *COI* sequence (Fig. 1). A detailed examination of the full neighbour-joining tree additionally revealed evidence for the clustering of taxonomically allied species (Fig. 2). For example, of the 30 genera that possessed multiple species (ranging from 2 to 37 species) in the tree, 26 formed cohesive groups. In addition, none of the split genera were embedded in other generic groupings; instead they formed distinct lineages. Also, there was a moderate level of association at the family level, with 16 out of 24 families forming cohesive assemblages. The eight split families were never partitioned into more than two groups and often represented a single genus not grouping with the rest of its family. Identification success for the test individuals was 100% as each of the 75 test individuals grouped most closely with the representative of its species in the profile.

Divergence within and between species

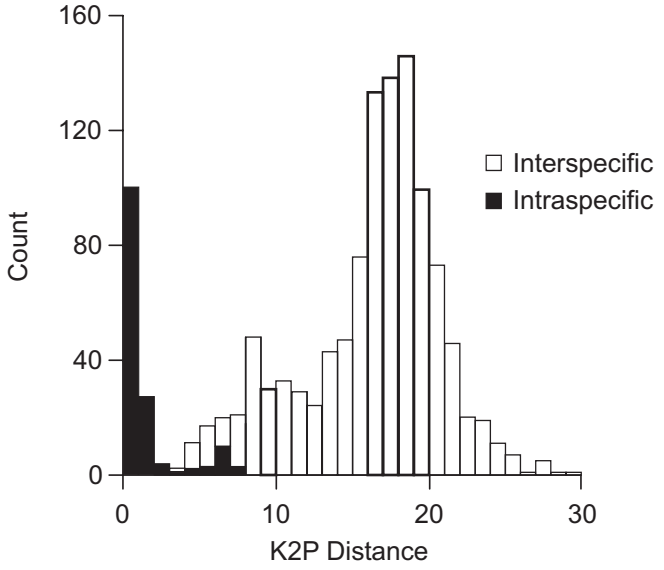
The mean percent sequence divergence between congeneric species (16.4%, SE = 0.13) was more than an order of magnitude greater than among conspecific individuals (1.4%, SE = 0.16). Mean intraspecific distances ranged from

Table 2. Mean and range of interspecific nucleotide divergences for species in 29 arachnid genera belonging to 24 different families, using Kimura's two parameter model.

Family	Genus	No. of species	Mean percent divergence	Range
Agelenidae	*Agelenopsis*	2	8.9	—
Amaurobiidae	*Coras* Simon, 1898	2	7.9	—
Araneidae	*Araneus* Clerck, 1757	2	13.4	—
Araneidae	*Argiope* Audouin, 1827	2	11	—
Araneidae	*Larinioides*	3	7.2	6.3–8.2
Clubionidae	*Clubiona* Latrielle, 1804	3	10.7	10.5–10.9
Dysderidae	*Dysdera* Latrielle, 1805	37	17.9	9.3–24.4
Gnaphosidae	*Gnaphosa*	2	6.5	—
Hypochilidae	*Hypochilus* Marx, 1888	8	23.1	9.4–29.7
Ixodidae	*Boophilus* Curtice, 1891	4	12.4	9.3–14.4
Ixodidae	*Dermacentor* C.L. Koch, 1844	4	17.4	14.2–20.5
Ixodidae	*Haemaphysalis* C.L. Koch, 1844	2	18.9	—
Ixodidae	*Hyalomma* C.L. Koch, 1844	4	16	13.4–18.6
Ixodidae	*Rhipicephalus* C.L. Koch, 1844	12	16.3	9.8–20.8
Linyphiidae	*Frontinella* F.O. Pickard-Cambridge, 1902	2	9.4	—
Linyphiidae	*Neriene*	4	18.2	10.8–23.4
Linyphiidae	*Orsonwelles* Hormiga, 2002	9	7.6	5.4–12.9
Lycosidae	*Pardosa* C.L. Koch, 1847	4	6.8	5.5–8.2
Philodromidae	*Philodromus*	3	9.2	8.0–22.7
Salticidae	*Habronattus*	2	10.4	—
Salticidae	*Phidippus*	3	8.2	8.0–8.4
Salticidae	*Sassacus* Peckham and Peckham, 1895	2	10	—
Tetragnathidae	*Nephila* Leach, 1815	3	14.7	10.5–17.3
Tetragnathidae	*Tetragnatha*	4	16.5	14.8–17.6
Tetragnathidae	*Tetranychus* Dufour, 1832	3	8.3	7.3–9.2
Theridiidae	*Latrodectus*	3	14.5	8.7–18.0
Theridiidae	*Steatoda* Sundevall, 1833	2	16.3	—
Theridiidae	*Theridion*	2	12	—
Thomisidae	*Xysticus*	9	9.9	4.8–21.2

a low of 0% in several species to a high of 3.6% in *Latrodectus hesperus* (Table 1). By contrast, mean nucleotide divergence among the 29 genera with multiple species ranged from a low of 6.5% in *Gnaphosa* to a high of 23.1% in *Hypochilus* (Table 2). There was a single case of overlap in the distribution of pairwise intra- and inter-specific distances (Fig. 3), reflecting the high pairwise distances in *L. hesperus*. Although the nine individuals of this species formed a cohesive group, two subgroups were apparent. Six specimens from Arizona and Nevada formed one subgroup, while three specimens from British Columbia formed the other. The pairwise intraspecific distances within these subgroups ranged from 0% to 1.6%, while the pairwise distances between individuals from the two subgroups of *L. hesperus* ranged from 4.9% to 7.4%.

Fig. 3. Histogram of intraspecific and interspecific (congeneric) genetic divergence across 203 arachnid species. Divergences were calculated using Kimura's two parameter (K2P) model.



Discussion

This study provides evidence that a *COI*-based identification system will be effective for identifying spider species and may also be applicable to other arachnids. This conclusion reflects the fact that there is a much lower level of sequence variation among members of a species than between closely allied species. Congeneric species pairs regularly showed marked *COI* sequence divergence, as all 1123 comparisons demonstrated at least 3% sequence divergence. In fact, the mean divergence value of 16.4% for congeneric species indicates that most pairs are separated by more than 90 diagnostic substitutions in the 600 bp of the *COI* gene

that we examined. This high level of divergence contrasted with the low intraspecific values that we observed. This dichotomy in divergences allowed success in identification tests using the neighbour-joining tree and should enable the reliable delineation of species. For example, using a 4% threshold for species diagnosis in our data set, 72 out of 75 (96%) species recognized through prior morphological study would be accurately identified. The only exceptions involved the high divergence between northern and southern populations of *L. hesperus*. However, recent pheromone and breeding studies (Kasumovic and Andrade 2004) suggest that these populations actually represent distinct species.

In a review of *COI* sequence divergences between congeneric species pairs, Hebert et al. (2003*b*) found that over 98% of animal species possess greater than 2% divergence. Since all of the congeneric species pairs examined in this study possessed at least 3% divergence, a 2% threshold for species identification would have distinguished all of the taxa we examined. We do expect that some young species pairs showing lower levels of divergence will be identified with further taxonomic sampling. However, based on our results, we expect that such cases of low divergence will be rare. Conversely, we expect that cases of high intraspecific divergence will regularly reflect the presence of a species complex, such as that in *L. hesperus*. There may be circumstances where *COI* fails to distinguish previously identified species. However, such cases should provoke both a confirmation of the initial morphological identification and the analysis of a rapidly evolving nuclear sequence to determine if mitochondrial introgression has occurred.

The greater the taxonomic representation in a *COI* profile, the more likely it will be able to discriminate between species. In our study all newly analyzed individuals were placed in the correct species, although the profile was based on only a single representative of each species. Admittedly, our profile included just 1% of the species contained within the families that we examined, but much of this diversity occurs allopatrically. Consequently, we expect that our high identification success is representative of a result that will regularly be obtained when local spider assemblages are surveyed.

Measures of genetic divergence are often used to infer species boundaries because of the strong correlation between genetic divergence and reproductive isolation (Coyne and Orr 1989; Sasa et al. 1998; Gleason and Ritchie 1998; Ferguson 2002). Although it is uncertain if this relationship arises as the consequence of cause and effect (Wu and Hollocher 1998), or as a product of a correlation with other common genetic and evolutionary factors (Templeton 1994), this correspondence has important taxonomic implications. The results of our study demonstrate that measures of genetic divergence at *COI* recognize species boundaries that closely correspond to those made through prior morphological analyses.

A DNA-based approach to species identification will not lead to the displacement of taxonomists. Thiele and Yeates (2002) make the important distinction that, unlike genes in GenBank, species are not facts but hypotheses. The scientific name does double duty as both shorthand for this hypothesis and as the handle used to collate information on the species. The validity of DNA-based species identification systems depends on establishing reference sequences from taxonomically confirmed specimens, a process requiring the cooperation of a diverse group of scientists and institutions. Moreover, further analysis of the type demonstrated here is essential to determine the ranges of intra- and inter-specific variation among different taxonomic groups. Once completed, these studies will provide the platform for a uniform, practical method of species identification, a result with broad scientific implications.

Acknowledgements

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