

In a relationship: sister species in mixed colonies, with a description of new *Chikunia* species (Theridiidae)

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Group-living behaviour is rare in spiders but has evolved repeatedly, yielding several species, some showing cooperation among close kin, and others living in colonies where each female builds its own web and is territorial. The most frequent origins of group living are seen in the cobweb spiders (Theridiidae) that commonly build three-dimensional webs and show extensive maternal care, both putatively pre-adaptive traits to spider sociality. A very unusual behaviour was recently discovered in the theridiid genus *Chikunia*, where two distinct but related species occur in mixed-species colonies with potentially indiscriminate brood care. These mixed colonies consist of *Chikunia nigra* and a newly discovered species. Here, we describe the new species, *Chikunia bilde* sp. nov., and summarize the unique biology of this species pair. We also place the origin of mixed-species group living in a phylogenetic context, firmly confirming the placement of *Chikunia* within the clade (lost colulus clade) previously characterized as containing a concentration of independent origins of group living. The two *Chikunia* studied here are sister species, representing a rare case of close genetic and behavioural interspecific relationship. We conclude that the loss of aggression that accompanies group living and social behaviour in cobweb spiders might help to explain the origin of mixed-species colonies.

ADDITIONAL KEYWORDS: behaviour, phylogenetics – phylogenetics – phylogenetics, sociality – taxonomy, theridiid phylogeny.

INTRODUCTION

Group-living spiders are typically categorized as either social or colonial. Social spiders are non-territorial, and group members cooperate in all tasks within the colony, whereas colonial spiders aggressively defend territories within the colony and lack cooperation in colony tasks (Lubin & Bilde, 2007; Bilde & Lubin, 2011). Social spiders have evolved in family groups through extended maternal care that may include maternal

feeding of offspring by regurgitation or sharing of prey with offspring, and through the loss of a pre-mating dispersal stage (Avilés, 1997; Lubin & Bilde, 2007; Viera & Agnarsson, 2017). In contrast, close kinship and maternal care are thought to be of little importance to the evolution of colonial spiders (Johannesen *et al.*, 2012). Colonial spiders are likened to foraging flocks of birds, benefitting in groups from increased success in the capture of prey, irrespective of genetic relatedness between group members (Rypstra, 1979; Uetz, 1989).

Although sociality is very rare phylogenetically in spiders, it has evolved repeatedly with a concentration of origins in derived cobweb spiders (Theridiidae) (Avilés, 1997; Agnarsson *et al.*, 2006; Bilde & Lubin, 2011; Yip & Rayor, 2014; Viera & Agnarsson, 2017).

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Within Theridiidae, nearly all subsocial (temporarily social, displaying cooperation only amongst juveniles) and social (permanently social and cooperatively breeding) species belong to the sister subfamilies Theridiinae and Anelosiminae (Liu *et al.*, 2016). Together, these form the 'lost colulus clade', which is ancestrally reconstructed to display extensive maternal care and construct three-dimensional webs (Agnarsson, 2004), both putative pre-adaptations to sociality in spiders (Avilés, 1997; Bilde & Lubin, 2011; Yip & Rayor, 2014). In both subsocial and social spider species of this clade, cooperative groups are made up of close kin, typically a single matriline (Avilés, 1997; Agnarsson *et al.*, 2010, 2013; Avilés & Guevara, 2017; Settepani *et al.*, 2017; Viera & Agnarsson, 2017).

Recently, a new mixed-species spider system was discovered on Bali, Indonesia, where two theridiid species from the genus *Chikunia* were found to live together in colonies (Grinsted *et al.*, 2012). This system is intriguing not only because mixed-species spider associations are rare, but also because both *Chikunia* species were found to display extended maternal care and possible indiscriminate care of young (pre-adaptations to sociality) while being territorial and lacking cooperation amongst adult group members (i.e. being colonial). Hence, this spider system appears to combine traits from two distinctly different types of group living and warrants further study. The two species involved are the known species *Chikunia nigra* and a newly discovered species of *Chikunia* described herein.

The aims of this paper were to describe *Chikunia bilde* sp. nov., redescribe *C. nigra*, place the origin of this mixed-species group living in a phylogenetic context and summarize the biology of this unique mixed-species system. In the field, we measured nearest neighbour distances between females with a brood (egg sacs or young) within a large, dense, mixed-species colony to ask the following question: do females show a higher tolerance and closer nearest neighbour distance for females of their own species compared with the other species?

MATERIAL AND METHODS

STUDY ORGANISMS

Chikunia is a genus of small, often dark or orange spiders (Fig. 1), belonging to the cobweb subfamily Theridiinae (Liu *et al.*, 2016). Only two species are currently placed in this genus: *C. nigra*, known from Asia (from China, India and Sri Lanka to Taiwan and Indonesia), and *Chikunia albipes*, also from Asia. The new species we describe here, *Chikunia bilde* sp. nov., distributed in Indonesia, Singapore and Malaysia, and possibly more widely, is thus the third species in this genus. *Chikunia* and relatives, such as *Chrysso*,

Helvibis and *Theridula*, are well known for displaying extensive maternal care (Fig. 1), with some being classified as subsocial (Coddington & Agnarsson, 2006; Yip & Rayor, 2014). *Chikunia* and relatives build simple three-dimensional webs with sticky silk distributed throughout the web (Fig. 2).

Mixed-species colonies of ≥ 100 individuals of *C. nigra* and *C. bilde* were found in small trees and on garden plants near the lakes Beretan and Buyan in central northern Bali (Fig. 3). Extended maternal care is provided to spiderlings up to about instar IV and includes guarding of the egg sac and feeding hatchlings by catching prey and sharing it with them. Females of both species have been found readily to accept foreign egg sacs and young (Grinsted *et al.*, 2012; see also Samuk & Avilés, 2013); hence, they potentially provide indiscriminate brood care to their own and, possibly, the other species. Both species can be found living solitarily, in colonies containing only their own species or in mixed-species colonies (L.G., personal observations). Adult colony members maintain their own territories (including their own capture web) within the group, can behave aggressively towards intruders, and adults do not cooperate in prey capture, feeding or brood care (Grinsted *et al.*, 2012).

PHYLOGENETIC METHODOLOGY

Sequences for two mitochondrial genes, cytochrome c oxidase subunit I (*COI*) and ribosomal RNA 16S (16S), and three nuclear genes, ribosomal RNAs 18S (18S) and 28S (28S) and histone (*H3*), were obtained from the comprehensive phylogenetic matrix for Theridiidae from Liu *et al.* (2016). These included a dense sampling of 'lost colulus clade' members and all sequenced Theridiinae. We also sequenced 12 additional specimens of *Chikunia* from Malaysia and Bali, adding to the prior *Chikunia* sequences from Bali, India and Sri Lanka from Grinsted *et al.* (2012) and Liu *et al.* (2016). DNA was extracted from two to four legs of each specimen using Qiagen DNeasy Tissue Kit (Qiagen, CA, USA). For the new specimens, we sequenced fragments of *COI*, and ITS2, using primers and protocols as described by Agnarsson (2010) and Agnarsson *et al.* (2013). Amplified fragments were sequenced in both directions by Genewiz LCC and then assembled and proofread using the Chromaseq module (Maddison & Maddison, 2018) in Mesquite (Maddison & Maddison, 2011) using PHRED (Green & Ewing, 2002) and PHRAP (Green, 1999). Sequences were submitted to Genbank (accession numbers MH256142-MH256215, MH256384-MH256489).

We aligned all sequences using MAFFT (Kazutaka *et al.*, 2005) using the EMBL-EBI online portal. For all analyses, gaps and ambiguous bases were treated as missing data.

For the final matrix (Supporting Information), the appropriate models for the Bayesian analysis were

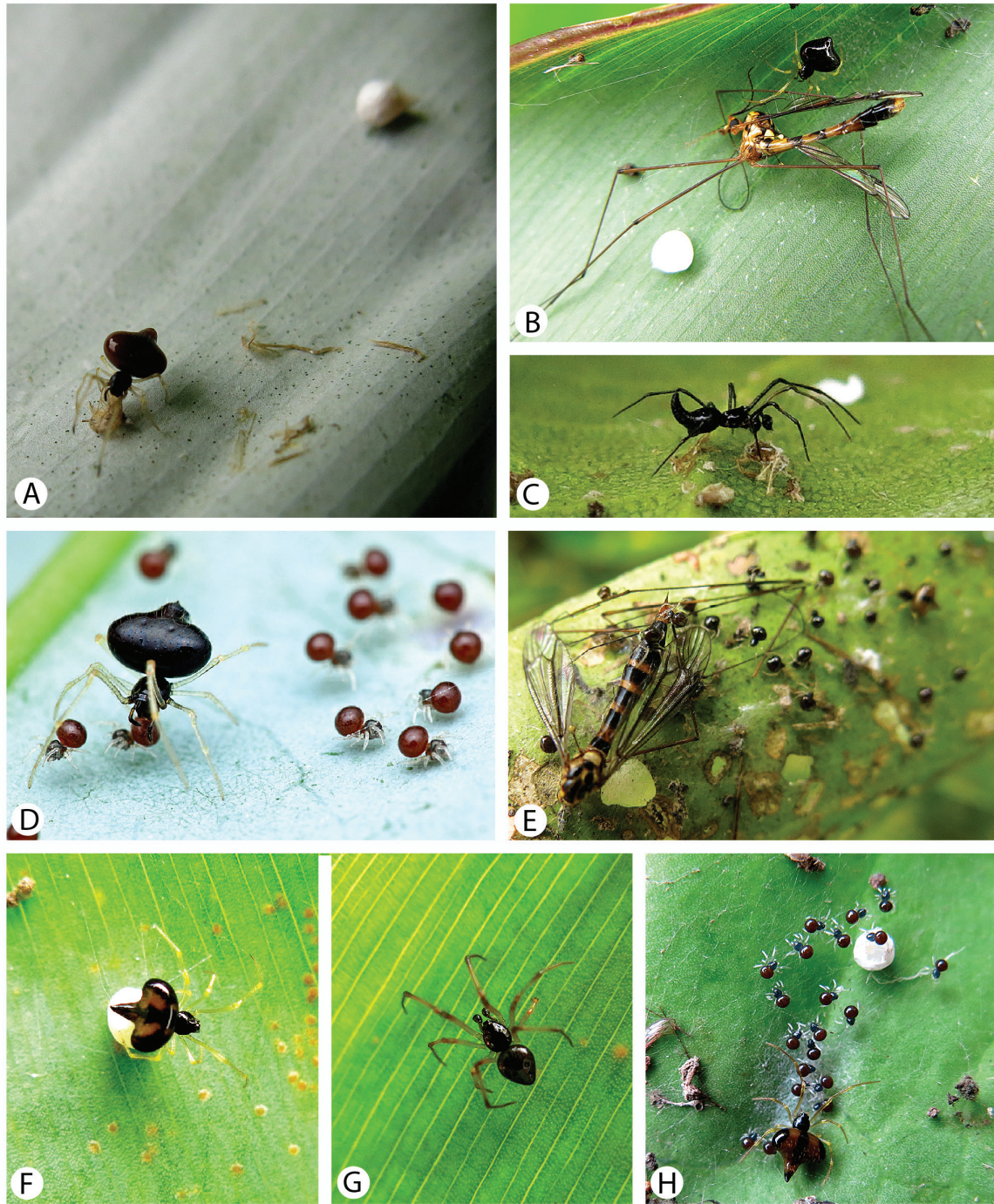


Figure 1. Field photographs of *Chikunia nigra* (A–C) and *Chikunia bilde* sp. nov. (D–H), from Bali (other than D). A, female with egg sac. B, female with large tipulid prey. C, male. D, female from Singapore with spiderlings (photograph by Melvyn Yeo, used with authorization). E, female and spiderlings with large tipulid prey. F, female with egg sac; note light brown-orange markings on abdomen. G, male. H, female and spiderlings with egg sac.

selected with PartitionFinder (Lanfear *et al.*, 2012). The data were analysed using Bayesian inference with MrBayes v.3.2.3 on XSEDE (v.3.2.3) (Huelsenbeck & Ronquist, 2001). The Markov chain Monte Carlo search

for each matrix ran with four chains for 50 000 000 generations, sampling the Markov chain every 1000 generations, and the sample points of the first 12 500 000 generations were discarded as 'burn-in',



Figure 2. Examples of *Chikunia* spp. webs.

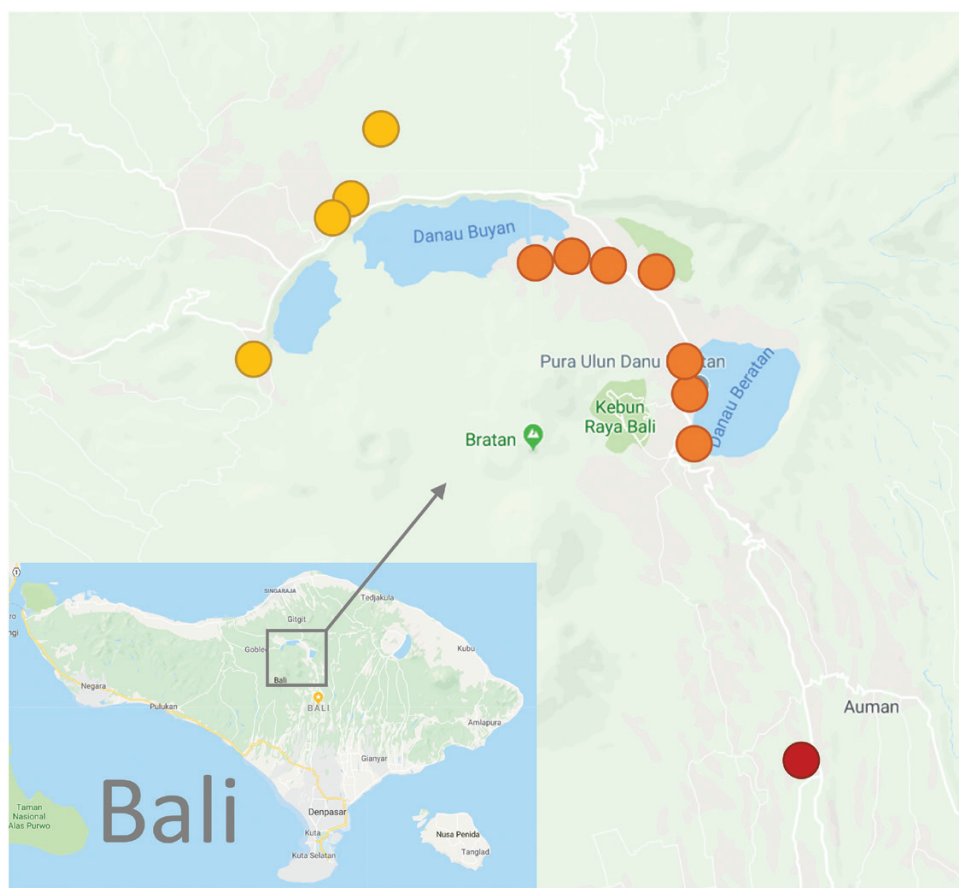


Figure 3. Map of localities on Bali where *Chikunia* spp. were found. Red circles indicate the occurrence of *Chikunia nigra*, yellow circles indicate occurrences of *Chikunia bilde* sp. nov., and orange circles denote occurrences of both species at the location.

after which the chains had reached approximate stationarity as determined by analysis in Tracer.

The ages were estimated using BEAST v.1.8 under a relaxed clock model (Drummond & Rambaut, 2007). We used calibration points as in the most recent Theridiidae phylogeny (Liu *et al.*, 2016) of which the current matrix is a subset; see (Liu *et al.*, 2016) for justification.

The BEAST analysis was done with same partitions and models as implemented in the MrBayes analysis. The analysis was run for 50 000 000 generations with the Yule tree prior. The results were checked in Tracer v.1.5 (Rambaut *et al.*, 2018) to determine burn-in and to check for stationarity.

PHOTOGRAPHIC DOCUMENTATION

Adult female and male representative specimens of both *Chikunia* species were photographically documented using the Visionary Digital BK Laboratory System. Spiders and dissected genitalia were positioned using Germ-X hand sanitizer (63% ethanol) and then covered in 95% ethanol to reduce glare. Images were taken using a Canon 5D camera and 65 mm macro zoom lens. Multiple image slices were taken of each specimen using the focus stacking program Helicon Focus v.5.3. Each final image was compiled using 15–30 image slices for whole specimens and four to ten slices for individual genitalia. Epigyna were prepared in potassium hydroxide and deionized water solutions for clearer imaging. Male palps were submerged in potassium hydroxide and then expanded in deionized distilled water. Images were edited in Adobe Photoshop CS6 to adjust lighting and sharpness, to eliminate background blemishes and to place scale bars. Species plates were constructed using Adobe Illustrator CS6.

FIELD OBSERVATIONS

The largest and densest colony discovered in the field consisted of ≥ 100 individuals of both *Chikunia* species occupying every single leaf of a couple of small banana trees located next to a pigsty. For each female with brood (egg sac or young), we measured the distance (in centimetres) to its nearest neighbouring female with brood ($N = 78$) and identified the species of both females. To ask whether females tended to have a same-species neighbour, we performed a χ^2 test on each species. Observed values were numbers of same- vs. different-species neighbours, and expected values were numbers of same- vs. different-species females present in the colony. To investigate the level of tolerance to other females, we asked whether the two species differed in the distance they kept to

their nearest neighbour and whether the distance to the nearest neighbour was affected by the species of the neighbour. To ask these questions, we built a generalized linear model, with distance to nearest neighbour as the response variable and focal female species, same- vs. different-species neighbour and an interaction term between the two factors as predictor variables. The model had a Gaussian error structure and conformed to the assumptions of normally distributed and homogeneous residuals. All tests were performed in R (R Core Team, 2016).

RESULTS

PHYLOGENETICS

As expected, the phylogeny in broad strokes recaptures the findings of Liu *et al.* (2016), on which it is based. It placed *Chikunia* within the ‘*Chrysso* group’ of the subfamily Theridiinae, members of the more inclusive ‘lost colulus clade’, in which group living and sociality are concentrated (Fig. 4). The phylogeny strongly supports the monophyly of *Chikunia*, each of its three species, and the sister relationship between *C. nigra* and *C. bilde* (Fig. 4). A dating analysis suggests that these species diverged ~ 8 Mya (Fig. 5), with old divergence reflected in both *COI* and the nuclear ITS2. Within both *C. nigra* and *C. bilde*, we find relatively deep divergences dating back 3–4 Mya, and in neither species are samples from Bali monophyletic (Figs 4, 5). However, this intraspecific divergence is reflected only in the mitochondrial DNA, whereas an analysis of the rapidly evolving ITS2 marker shows almost no variation within each species (Fig. 4, inset).

TAXONOMY

THERIDIIDAE SUNDEVALL, 1833

CHIKUNIA YOSHIDA, 2009

Chikunia Yoshida, 2009: 378; type species *C. alba* Yoshida, 2009 by original designation.

CHIKUNIA NIGRA (O. PICKARD-CAMBRIDGE, 1880)

Originally, *Argyrodes nigra* O. Pickard-Cambridge, 1880. Later placed in *Theridion* by Simon (1905), *Theridula* by Saito (1935) and *Chrysso* by Levi (1962). Transferred here by Grinsted *et al.* (2012).

Material examined

Types from Sri Lanka (Thwaites’s Ceylon collection), not examined. Multiple male and female specimens from northern central Bali, near lake Buyan,

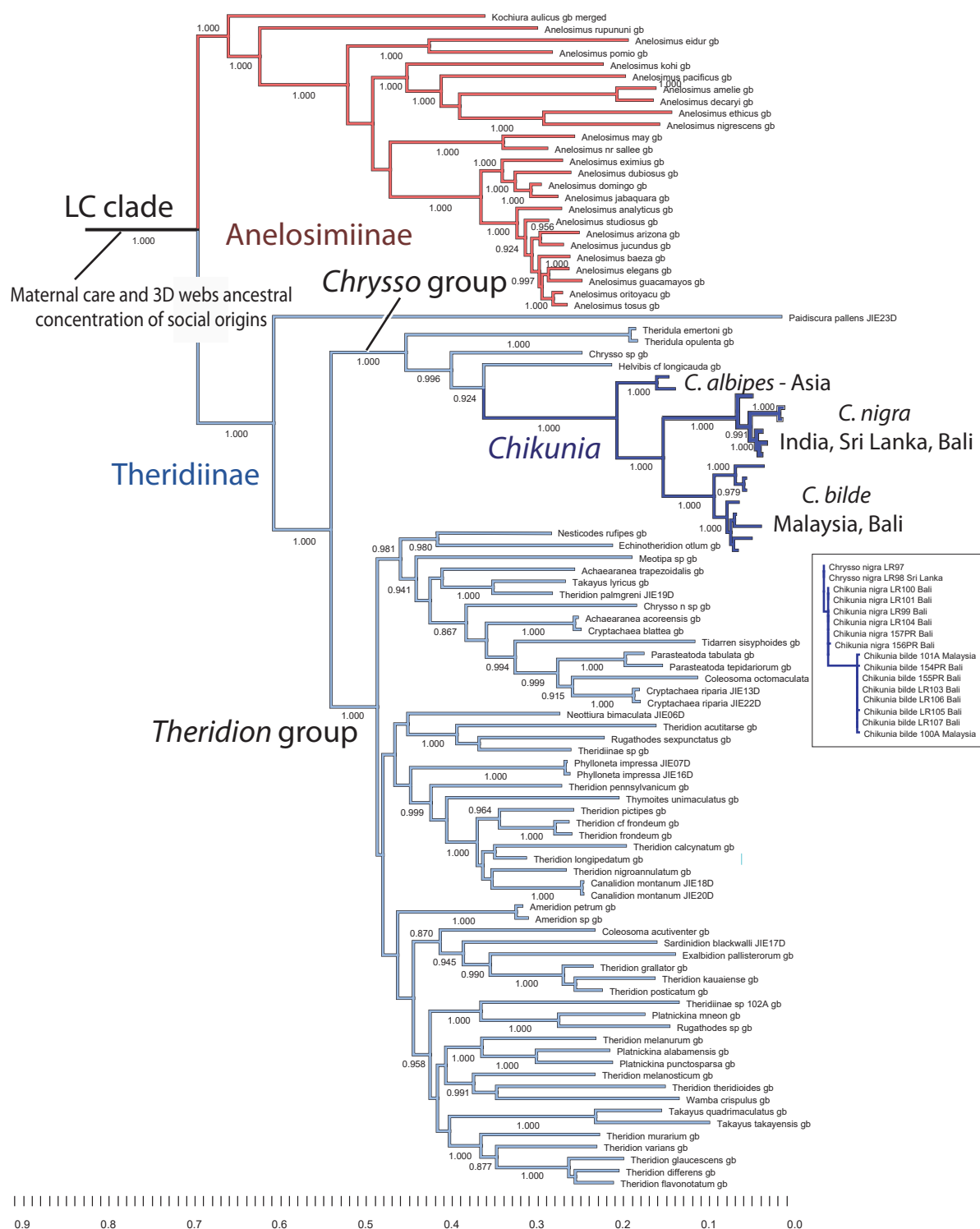


Figure 4. Phylogenetic placement and structure of *Chikunia*. *Chikunia* belongs to the Chryso group of Theridiinae, within the lost colulus (LC) clade, marked by social pre-adaptations and multiple origins of cooperative behaviour. The phylogeny supports the sister relationship between *Chikunia nigra* and *Chikunia bilde* sp. nov. Deep genetic structure indicated within each species is reflecting only mitochondrial DNA, whereas the rapidly evolving nuclear DNA ITS2 marker supports only the deep split between the species but shows almost no variation within either (inset). scale below tree indicates number of expected changes.

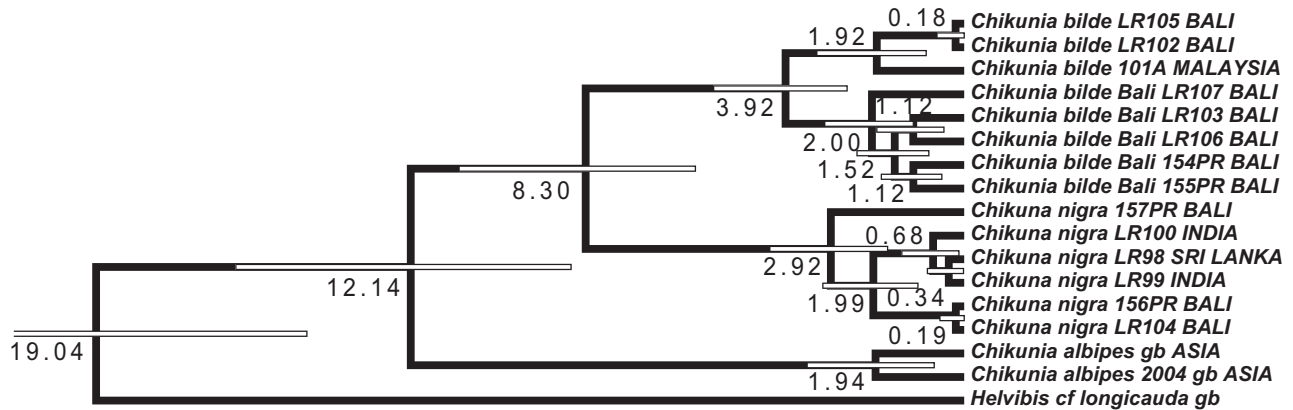


Figure 5. Dated phylogeny of *Chikunia*, indicating divergence between the focal species ~8 Mya.

approximately 8.243693°S, 115.104070°E to 8.282064°S, 115.164817°E (Fig. 3), collected in July 2017. Five specimens from Sri Lanka and India (see Grinsted *et al.*, 2012) were also re-examined.

Diagnosis

Chikunia nigra differs from other *Chikunia* species in the long, tapering male abdomen, conformation of male palp (Figs 6J, 7J, 8) with very long embolus and large conductor, and in the epigynum with oval spermathecae and simple non-spiralling copulatory ducts. Furthermore, *C. nigra* females are distinct by having both pedipalps and tarsus of leg I black or very dark brown in colour (Fig. 6A, C), in contrast to the remaining leg segments, which are pale white or yellow in colour.

Redescription

Female (from Bali, Indonesia): Legs I–IV. Total length 3.54 mm. Cephalothorax 1.11 mm long, 0.91 mm wide, 0.71 mm high, medium to dark brown with tan markings laterally. Chelicerae are dark brown in colour. Sternum 0.69 mm long, 0.56 mm wide, extending halfway through coxae IV, medium brown. Abdomen 2.43 mm long, 2.55 mm wide, 1.77 mm, subtriangular in shape and tapering towards posterior (Fig. 6A–C). Wide dark brown patch and scattered spots occur dorsally along abdomen (Fig. 6A–C). Eyes approximately equal in size anterior lateral eyes (ALE) 0.06 mm, anterior median eyes (AME) 0.08 mm in diameter. All eyes within one eye diameter apart from each other excluding the anterior median, which are 0.12 mm apart. Leg I femur 1.70 mm, patella 0.40 mm, tibia 1.0 mm, metatarsus 1.10 mm and tarsus 0.61 mm. All legs tan in colour; tarsus of leg I dark brown (Fig. 6A,

C). Leg formula 1423. Epigynum simple, translucent cuticle, with tiny copulatory openings, leading to oval spermathecae (Fig. 6D–F).

Male (from Bali, Indonesia): Total length 2.32 mm. Cephalothorax 0.83 mm long, 0.71 mm wide, 0.53 mm high, dark brown to black in colour with faint medium brown broad patches dorsally, relatively slender and tapering towards end (Fig. 6G–I). Sternum 0.60 mm long, 0.47 mm wide, dark brown. Abdomen 1.49 mm long, 0.81 mm wide, 0.72 mm high, black in colour. Eyes approximately equal in size, ALE 0.07 mm, AME 0.08 mm in diameter. All eyes within one eye diameter apart from each other excluding the anterior median, which are 0.12 mm apart. Leg I femur 1.60 mm, patella 0.31 mm, tibia 0.95 mm, metatarsus 0.74 mm and tarsus 0.46 mm. All legs dark brown with white coxae and white coloration where trochanter and femur meet. Leg formula 1423. Male palps with prominent and long embolus, spiral covering outer edge of tegulum, distally supported by a large conductor.

Variation: Female total length 2.01–3.54 mm, femur I 1.11–1.70 mm; male total length 2.32–3.20 mm, femur I 1.37–1.98 mm. Female abdomen shape and coloration vary greatly (Fig. 9A–I). Colours range from pale orange to reddish orange, amber, various shades of brown, greenish grey and black. Some abdomens are uniformly coloured, whereas others have a non-distinct, mottled black pattern of varying size in the centre of the abdomen. Abdomen shape varies from having rather large abdominal humps and a short, pointy rear end (abdomen wider than long, similar to *C. bilde*) to a more slender look with smaller abdominal humps and a longer, tapering rear end (abdomen longer than wide).

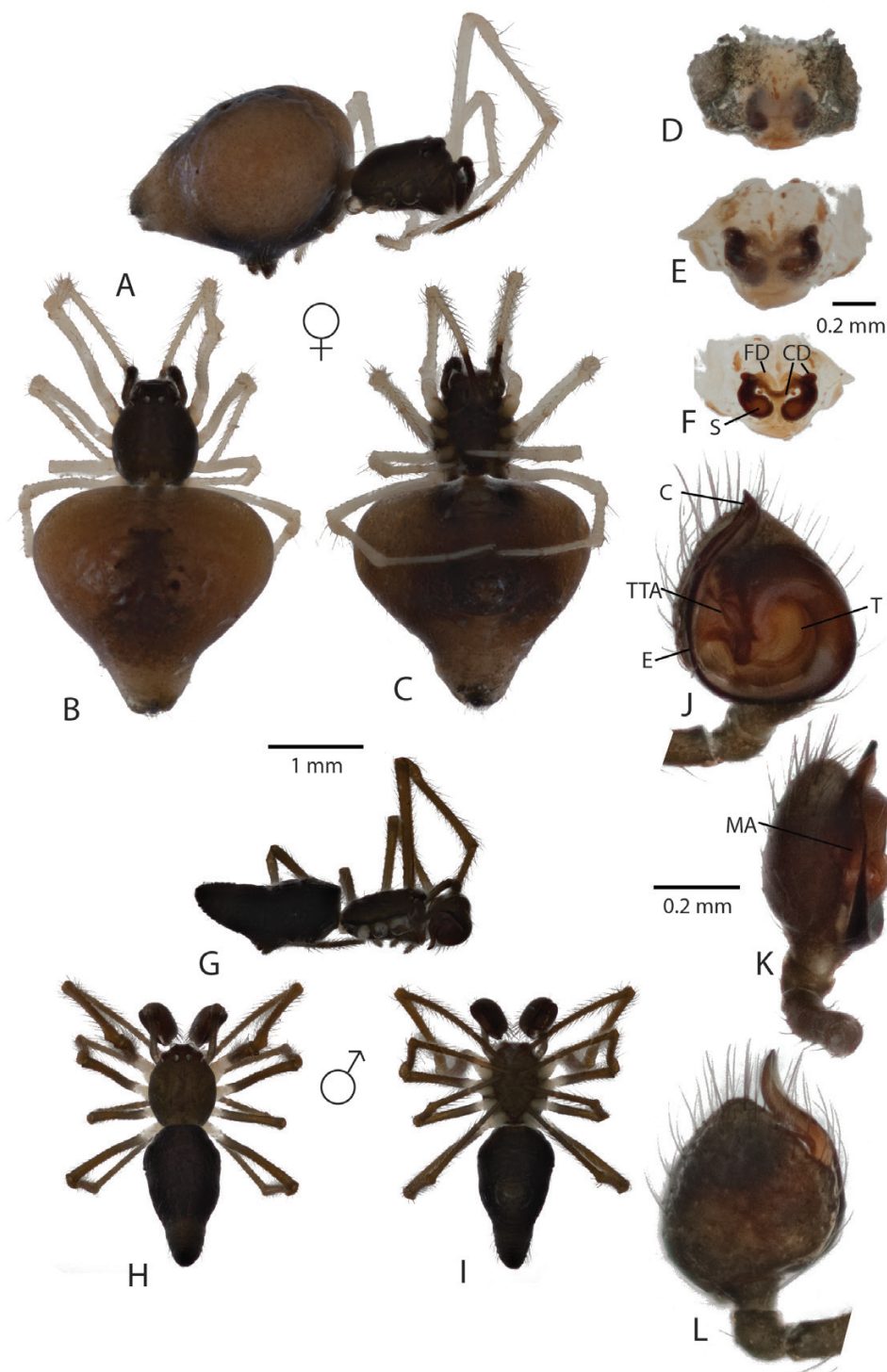


Figure 6. *Chikunia nigra*. A–C, female habitus. A, lateral. B, dorsal. C, ventral. D–F, epigynum. D, ventral. E, dorsal. F, dorsal after digestion. G–I, male habitus. G, lateral. H, dorsal. I, ventral. J–L male pedipalp. J, ventral. K, lateral. L, dorsal. Abbreviations: C, conductor; CD, copulatory ducts; E, embolus; FD, fertilization ducts; MA, median apophysis; S, spermathecae; T, tegulum; TTA, theridiid tegular apophysis.

CHIKUNIA BILDE SMITH, AGNARSSON & GRINSTED
SP. NOV.

Chikunia nigra (misidentified).

Type material

Male holotype from northern central Bali, near Lake Beretan, -8.271211, 115.165842, collected in July 2017, legit L. Grinsted, in the Smithsonian MNH, Washington DC.

Material examined

Multiple additional male and female specimens from type locality and nearby (-8.271211, 115.165842 to -8.282525, 115.164386; Fig. 3), collected in July 2017, legit L. Grinsted. Two females from Malaysia, Pahang Tanah Rata, 4.46°N, 101.40°E, 1500 m, 21–22 May 2005, legit I. Agnarsson.

Etymology

The species epithet honours Professor Trine Bilde, Aarhus University, for her significant scientific contributions to the field of behavioural ecology and social evolution in spiders and for her inspiring role as a PhD supervisor to L. Grinsted. The discovery of *C. bilde* by L. Grinsted in 2010 was a direct result of Professor Bilde's approval and encouragement of an exploratory expedition to Bali. Professor Bilde is a strong female role model of high scientific integrity.

Diagnosis (Smith *et al.*, 2019: 345)

Chikunia bilde differs from *Chikunia nigra* in the blunt terminus of the male abdomen (Smith *et al.*, 2019: fig. 7H, I) and in the male leg colouration that gives legs a stripy appearance (yellow-brown stripes; Fig. 1G). Furthermore, *C. bilde* differs in the conformation of male palp with embolus covering the tegulum almost entirely, and short and transparent conductor (Smith *et al.*, 2019: figs 7J, 8), and in having a conspicuous epigynum with oval to round spermathecae and spiralling copulatory ducts (Smith *et al.*, 2019: fig. 7A–L). In habitus, *C. bilde* females tend to have larger bulbous abdominal humps than *C. nigra*. Furthermore, *C. bilde* females differ from *C. nigra* by lacking the dark brown or black tarsus of leg I.

Description

Female: Total length 2.83 mm. Cephalothorax 1.13 mm long, 1.01 mm wide, 0.61 mm high, dark brown with faint tan patches stretching across the midsection. Chelicerae shift from tan in colour to a medium brown as they transition outward from the body (Fig. 7B). Sternum 0.71 mm long, 0.56 mm wide, dark brown and

extending halfway through coxae IV. Abdomen 1.88 mm long, 2.42 mm wide, 1.59 mm high, subtriangular with large bulbous humps on both laterals (Fig. 7A, B). Large dark brown-coloured patches laterally, a dark brown patch present at the posterior of the abdomen, and a dark brown streak running dorsally in a groove between the two humps (Fig. 7A–C). Abdomen tapers distinctly behind humps. Eyes approximately equal in size, ALE 0.09 mm, AME 0.10 mm in diameter. All eyes within one eye diameter apart from each other excluding the anterior median, which are 0.10 mm apart. Leg I femur 1.93 mm, patella 0.37 mm, tibia 1.32 mm, metatarsus 1.20 mm and tarsus 0.81 mm. All legs tan in colour. Leg formula 1423.

Male: Total length 2.34 mm. Cephalothorax 1.06 mm long, 0.86 mm wide, 0.61 mm high, black in colour. Sternum 0.63 mm long, 0.58 mm wide, dark brown in colour extending to past coxae IV. Abdomen 1.29 mm long, 1.01 mm wide, 0.88 mm high, dark brown in colour with brown speckled pattern covering entire abdomen, oval in shape, and lacking any humps or ridges; narrows slightly to an abruptly blunt posterior (Fig. 7G–I). Eyes approximately equal in size, ALE 0.08 mm, AME 0.09 mm in diameter. All eyes within one eye diameter apart from each other excluding the anterior median, which are 0.10 mm apart. Leg I femur 2.17 mm, patella 0.35 mm, tibia 1.39 mm, metatarsus 1.37 mm and tarsus 0.75 mm. Legs gradually darken moving from the coxae all the way to the tarsus. Coxae are white in colour. Femora fade from white to tan to dark brown where they meet the patella. Metatarsus and tarsus both medium brown in colour. Leg formula 1423.

Variation: Female total length 2.64–3.18 mm, femur I 0.94–1.48 mm; male total length 2.09–2.78 mm, femur I 1.62–2.19 mm. Female abdomen coloration varies greatly (Fig. 9J–O). Colours range from bright yellow to orange, amber, various shades of brown and black. Black abdomens are uniformly coloured, whereas all other colour variants have a clearly defined black or dark brown pattern running dorsally. Additionally, the very tip of the abdomen can be black or dark brown, and often there is a dark brown or black patch, of varying size, on the tip of each of the abdominal humps.

FIELD OBSERVATIONS

Within a dense mixed-species colony, nearest neighbouring females with a brood were of the same species significantly more often than would be expected by chance (Figs. 10, 11). This was true for both *C. nigra* and *C. bilde* (Pearson's χ^2 test with Yates' continuity correction: *C. nigra* $\chi^2 = 4.52$, d.f. = 1, $P = 0.034$; *C. bilde* $\chi^2 = 4.45$, d.f. = 1, $P = 0.035$). Within the colony, we

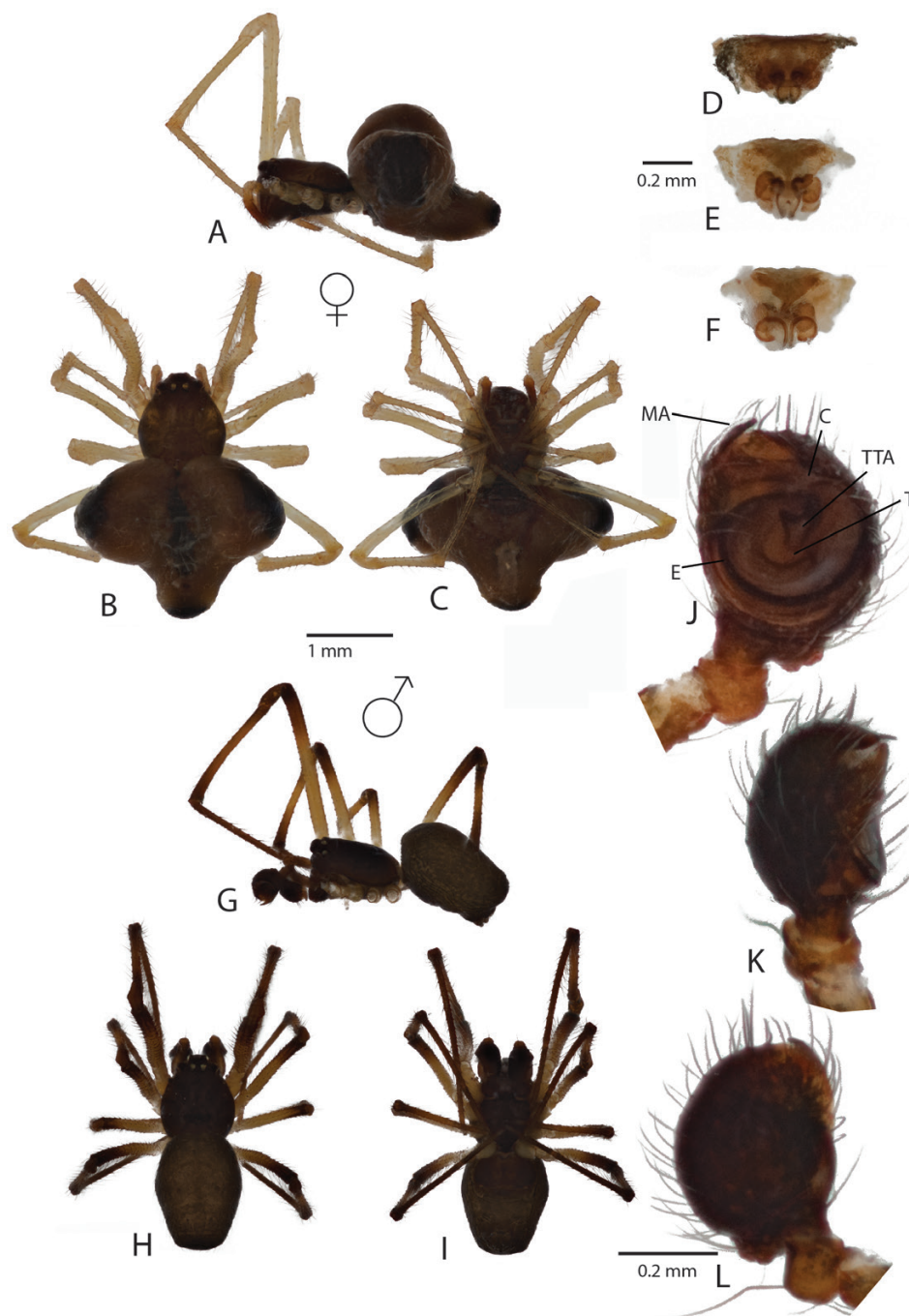


Figure 7. *Chikunia bilde* sp. nov. A–C, female habitus. A, lateral. B, dorsal. C, ventral. D–F, epigynum. D, ventral. E, dorsal. F, dorsal after digestion. G–I, male habitus. G, lateral. H, dorsal. I, ventral. J–L, male pedipalp. J, ventral. K, lateral. L, dorsal. Abbreviations: C, conductor; CD, copulatory ducts; E, embolus; FD, fertilization ducts; MA, median apophysis; S, spermathecae; T, tegulum; TTA, theridiid tegular apophysis.

recorded 48 *C. nigra*, of which 39 had a same-species nearest neighbour, and 30 *C. bilde*, of which 19 had a same-species nearest neighbour.

Distances to nearest neighbours ranged from 1 to 14 cm, with both mean and median = 7.0 cm. There

was no significant difference between species in the distance they kept to their nearest neighbour (Fig. 11; t -value = 0.32, P = 0.75), nor was there a significant difference in distances kept to nearest same-species vs. different-species neighbour (Fig. 11,

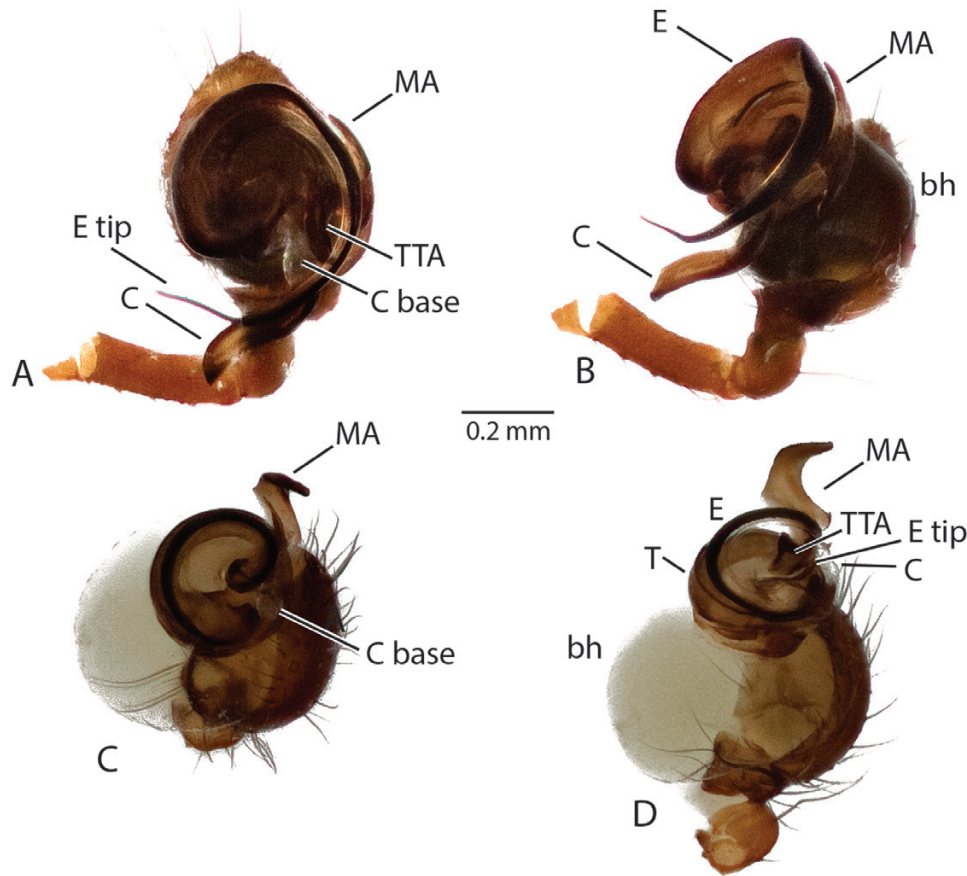


Figure 8. Expanded male pedipalps. A, B, *Chikunia nigra*. A, ventral. B, ectal. C, D, *Chikunia bilde* sp. nov. C, ventral view of embolus. D, ectal. Note differences in size and length of embolus, the large and extended conductor of *C. nigra* vs. the small and transparent conductor of *C. bilde* sp. nov., and the large and finger-like median apophysis of *C. bilde* sp. nov. Abbreviations: bh, basal hematodocha; C, conductor; C base, conductor base; E, embolus; E tip, embolus tip; MA, median apophysis; T, tegulum; TTA, theridiid tegular apophysis.

t -value = -0.99 , $P = 0.33$; the interaction term between the two factors was also not significant: t -value = -0.34 , $P = 0.73$).

DISCUSSION

We examined two facultatively group-living *Chikunia* species and provide a description of *C. bilde*, a redescription of *C. nigra*, and a phylogeny showing the placement of the two species as sister species within their genus and Family. We also show deep splits in mitochondrial DNA genotypes both within and between species, whereas nuclear DNA genotypes support a split only between species and find little genetic structuring within species. Finally, we present behavioural data from a dense, mixed-species colony in the field. These data show that although females with a brood maintain similar physical distances from

females of both species, they tend to keep same-species females as their closest neighbour.

GROUP LIVING, SOCIALITY AND COLONIALITY

We argue that the dense groups of mixed-species *Chikunia* spiders can be considered spider colonies in accordance with Grinsted *et al.* (2012), rather than simple aggregations of solitary spiders, for multiple reasons. First, the definition of a colonial spider is very broad (Bilde & Lubin, 2011; Avilés & Guevara, 2017). Most spiders described as colonial are web builders that attach their individual webs together and gain foraging benefits in doing so. However, several spider species that do not build webs for prey capture are considered colonial. For example, several species of salticids live in mixed-species colonial groups in Africa (Jackson *et al.*, 2008). Their nests are attached together with silk, but they forage and breed solitarily. Hence, individual prey capture webs do not need to be attached

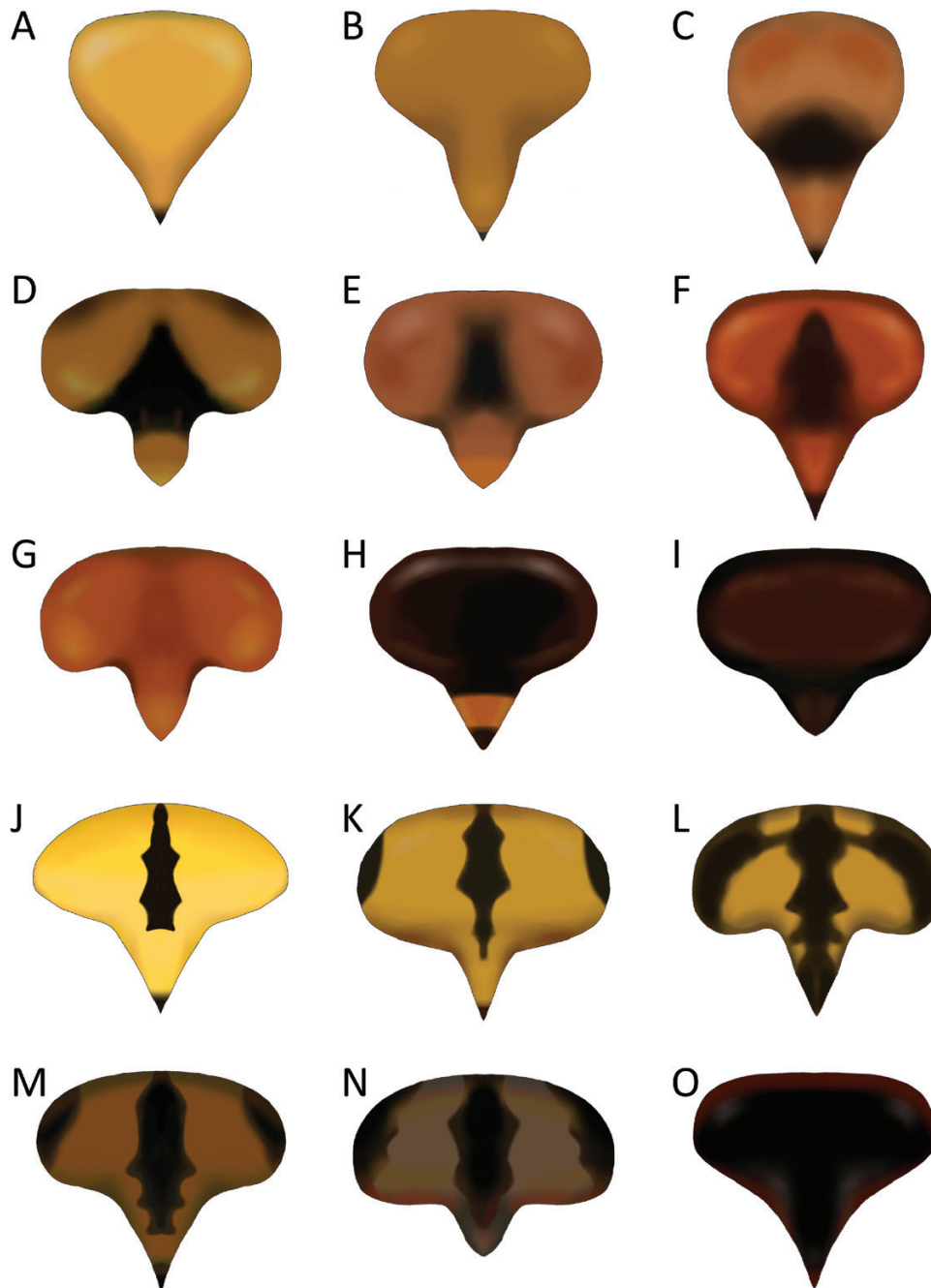


Figure 9. Illustrations of female abdomens of *Chikunia nigra* (A–I) and *Chikunia bilde* sp. nov. (J–O), exemplifying some of the variation in shape, patterns and coloration found within natural populations.

for spiders to be colonial. Second, what is common for almost all colonial species is that they can occur and breed in high-density aggregations, often around a high concentration of prey, but can also live and breed solitarily. In other words, they tend to be facultatively group living. Colonial spiders have been likened to foraging flocks of birds that hunt and feed alone, but squabble over prey (Rypstra, 1979). Third, in colonial

spiders there is very little interaction amongst adult group members (as opposed to social spiders that are highly cooperative), and if one crosses the territory of another, there may be aggressive disputes. However, as opposed to typically solitary spiders, tolerance towards neighbours is high and cannibalism low. Fourth, colonial spiders have very clumped distributions; in an area with lots of seemingly appropriate habitat, most

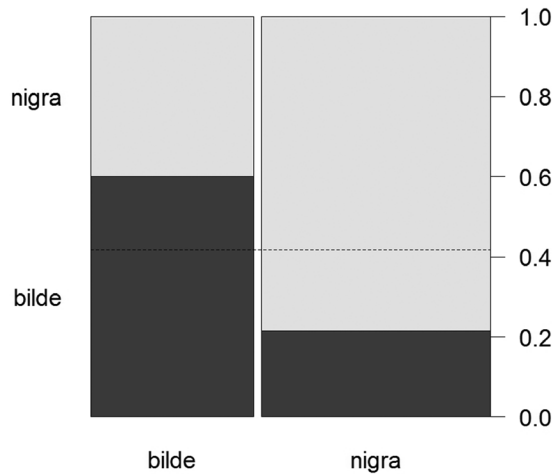


Figure 10. Proportion of nearest neighbouring females with brood of same vs. different species ($N = 78$) in a large, dense colony in the field. The stippled line indicates the proportion of each species observed within the colony and therefore represents the values expected if females were choosing their neighbours randomly.

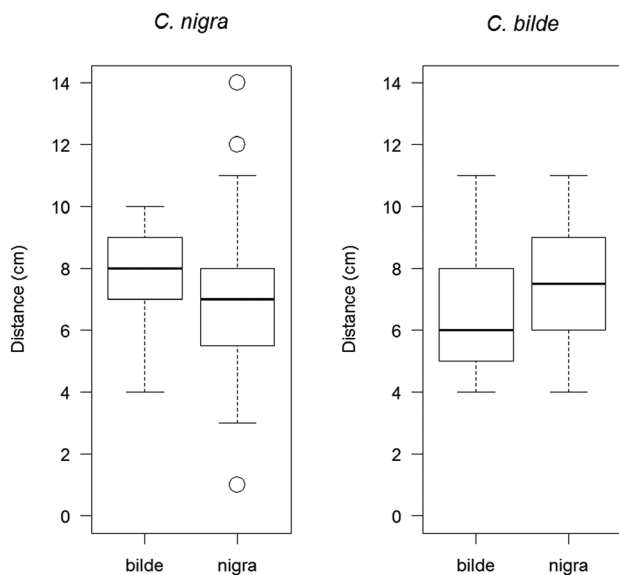


Figure 11. Boxplot depicting the distances to nearest female with brood of either species for *Chikunia nigra* and *Chikunia bilde* sp. nov. within a dense, mixed-species colony in the field.

of it is unoccupied, but when the spiders do occur, they aggregate in high concentration (Lubin & Bilde, 2007; Bilde & Lubin, 2011). This is exactly what we see with the *Chikunia* spiders. *Chikunia* spiders were found on a broad range of trees and smaller plants (especially avocado trees, guava and banana trees and a range of ornamental garden plants and small palms, such as hibiscus and dracaena) and although these habitats

were vastly abundant, spiders clustered within a select few of the plants in the area (Fig. 12). They also defend their individual territories while still maintaining extraordinarily high tolerance towards neighbours and avoiding cannibalism, exactly as other colonial spiders do (Grinsted *et al.*, 2012). However, this recently discovered mixed-species system is mostly unexplored, and little is known of the interactions between group members and whether they benefit from grouping. For example, do individuals cooperatively defend against predators and parasites? Do individuals gain foraging benefits by building individual webs next to each other? Until we identify specific cooperative behaviours and fitness benefits associated with group living, other classifications might be justified, such as both species being solitary with exceptionally high levels of intra- and interspecific tolerance and a tendency to form dense aggregations.

PHYLOGENETICS AND HYPOTHETICAL ORIGIN OF MIXED-SPECIES COLONIES

Group living is phylogenetically rare in spiders (Avilés, 1997), as is the tight sympatry of closely related species (Agnarsson *et al.*, 2016), especially with direct interaction among them (Jackson, 1986). Thus, it was surprising to discover tight relationships between two species both genetically (Fig. 4) and behaviourally (Figs. 10, 11), in the theridiid genus *Chikunia*. In this sympatric relationship, two sister species occur in mixed-species colonies, with potentially indiscriminate brood care within and between species (Grinsted *et al.*, 2012) and apparently near-identical ecological niches and maternal care behaviours. We have no obvious indication of hybridization between the two species, hence they appear to be genetically isolated (Fig. 5). Lack of gene flow in close sympatry provides a strong support for these being separate species. Our morphological examinations suggest that despite great similarities in the overall appearances of the two species (body size, shape and coloration), their reproductive organs differ substantially; hence, they are likely to maintain reproductive isolation between species.

The phylogenetic placement of the two *Chikunia* spiders as sister species, nested within the lost colulus clade (Fig. 4), is interesting for two key reasons. First, origins of group living and social behaviour are concentrated within cobweb spiders (Theridiidae) that ancestrally build three-dimensional webs and show extensive maternal care, both putatively pre-adaptive traits to spider sociality. These traits demarcate the lost colulus clade. Second, remarkably, this clade also contains the best-known example of extensive sympatry among multiple closely related spider species (including sibling species) in the subsocial Malagasy

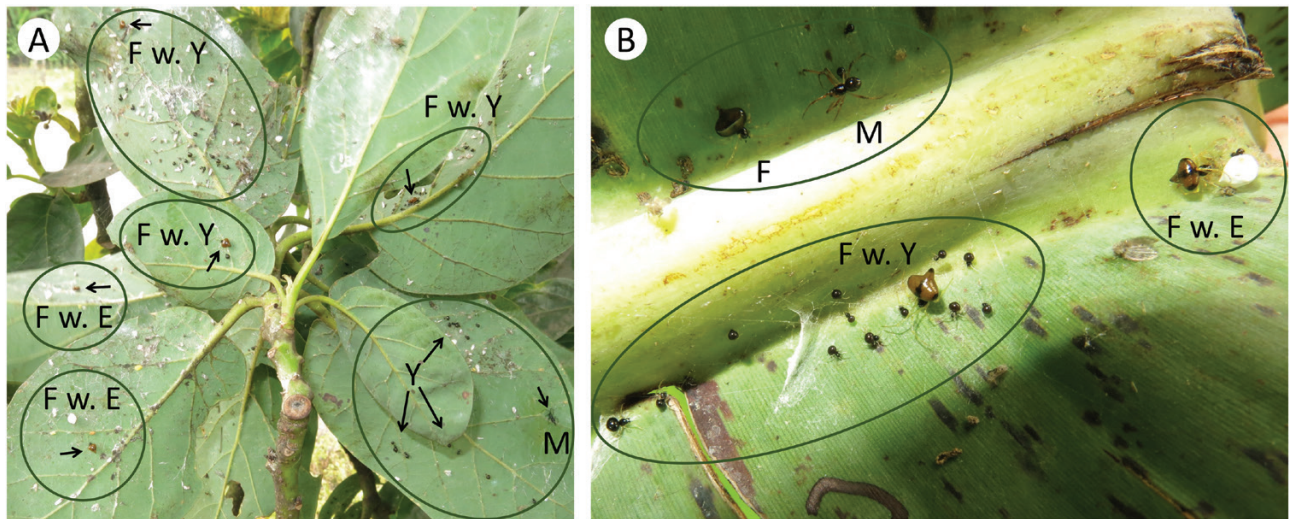


Figure 12. Mixed-species *Chikunia* colonies in the field. A, the male and most of the females in this photograph are *Chikunia nigra*. Each female maintains her territory on a single leaf on an avocado tree. B, in this photograph all specimens are *Chikunia bilde* sp. nov. Each female occupies a small territory on a banana leaf. Each banana leaf on the tree contained ≥ 10 territories of females and their brood. Abbreviations: F, female; F w. E, female with egg sac; F w. Y, female with young; M, male.

Anelosimus (Agnarsson *et al.*, 2015, 2016). However, unlike *Chikunia*, sympatric sibling *Anelosimus* species never share colonies.

It has been hypothesized that loss of aggression in the lost colulus clade, during an ancestral and extensive maternal care period, pre-dates the origin of sociality. A high level of tolerance of conspecifics is seen in many *Anelosimus* species (Kullmann, 1972) and many others, and among species in both *Anelosimus* and in *Stegodyphus* (L.G., personal observation; Bilde & Lubin, 2011). In many such species, aggression towards other species living in the web is also dramatically reduced, which has resulted in extensive kleptoparasitic loads in some species' colonies (Cangialosi, 1990). We hypothesize that this reduced aggression, and the close relationships between the two species discussed here, might have facilitated interspecific tolerance within colonies. Females do not cooperate with each other within colonies and keep their own territories, which they sometimes defend aggressively (Grinsted *et al.*, 2012). However, they do tolerate other females of both species within only a few centimetres of themselves and their brood, and we show that there is no difference between the distances they keep from their own vs. the other species (Fig. 11). We also show that females tend to have same-species nearest neighbours. In general, our findings suggest a benefit of having a close neighbour. The clustering of same-species neighbours within mixed-species colonies might be the result of various mechanisms. A passive mechanism could be natal philopatry, whereby female

offspring have limited dispersal and settle down close to their maternal nest. An active mechanism would imply that females are capable of recognizing conspecific females and actively seek out these as neighbours. Although we cannot conclude which mechanism brings about same-species clustering within colonies, the discordance between results from mitochondrial and nuclear markers discussed in the next subsection strongly supports natal philopatry and limited female dispersal.

DISCORDANCE BETWEEN MITOCHONDRIAL AND NUCLEAR DNA

It is notable that mitochondrial and nuclear markers are highly concordant in recovering deep and old divergences among the three *Chikunia* species (Figs 4, 5); however, they are discordant in genetic structure within our two focal species (Fig. 4). In particular, mitochondrial DNA recovers deep and potentially old divergences within both species where the local Bali assemblages of each species are non-monophyletic. This could potentially indicate multiple colonizations of Bali and a lack of panmixia within both *C. nigra* and *C. bilde*. This scenario would be highly surprising given the extremely small geographical range of the Bali colonies studied here (Fig. 3). In striking contrast, there is almost no intraspecific variation in the nuclear DNA markers explored here, not even in the rapidly evolving ITS2 (Fig. 4, inset), except for a single nucleotide difference between Indian/Sri Lankan vs. Bali specimens of *C. nigra*. This pattern is

consistent with a more intuitive scenario of a single colonization of Bali and intraspecific panmixia within the geographically tiny research area.

What then could explain the contrast between the two sets of markers? The simplest explanation, we speculate, would be extreme site fidelity of females/matrilines, where they stay in their maternal colony or disperse only very short distances, possibly with their close kin. In this scenario, we would expect males to disperse among colonies/areas to secure gene flow and thus avoid inbreeding, leading to deep mitochondrial but limited nuclear DNA genetic structuring. Interestingly, social spiders lack a pre-mating dispersal stage for both males and females and therefore have extraordinarily high levels of inbreeding (Settepani *et al.*, 2017), whereas both sexes in colonial spiders are expected to maintain relatively high levels of dispersal (Johannessen *et al.*, 2012). The results from our molecular study indicate that females might have severely limited dispersal in these two *Chikunia* species. This is yet another trait showing features from both the colonial spider system and the social spider system, making the mixed-species *Chikunia* system unique and intriguing. Detailed population genetics studies are currently underway (L.G., unpublished) to test the currently speculative hypotheses presented here.

CONCLUSIONS

In sum, we describe a new spider species, *C. bilde*, that is found in mixed non-cooperative colonies with its sister species, *C. nigra*. Mixed-species groups are seen in various organisms, but this is the first record of colonial groups made up of two species, which are each other's closest relative, in spiders, and we could not find similar examples from other arthropods. These colonies are also unique in combining elements of spider coloniality and spider sociality. As in colonial spiders, each individual builds its own web, and direct cooperation seems absent. However, as in social species, these spiders show extensive (and possibly indiscriminate) maternal care. Our genetic analyses indicate that these close relatives do not interbreed in sympatry, hence these societies are clearly made up of two different biological species. Further genetic analyses will reveal the dispersal patterns of both species between colonies and between populations and help to shed light on how these curious mixed-species associations are formed.

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REFERENCES

- Agnarsson I. 2004.** Morphological phylogeny of cobweb spiders and their relatives (Araneae, Araneoidea, Theridiidae). *Zoological Journal of the Linnean Society* **141**: 447–626.
- Agnarsson I. 2010.** The utility of ITS2 in spider phylogenetics: notes on prior work and an example from *Anelosimus*. *Journal of Arachnology* **38**: 377–382.
- Agnarsson I, Avilés L, Coddington JA, Maddison WP. 2006.** Sociality in theridiid spiders: repeated origins of an evolutionary dead end. *Evolution* **60**: 2342–2351.
- Agnarsson I, Avilés L, Maddison WP. 2013.** Loss of genetic variability in social spiders: genetic and phylogenetic consequences of population subdivision and inbreeding. *Journal of Evolutionary Biology* **26**: 27–37.
- Agnarsson I, Gotelli NJ, Agostini D, Kuntner M. 2016.** Limited role of character displacement in the coexistence of congeneric *Anelosimus* spiders in a Madagascan montane forest. *Ecography* **39**: 743–753.
- Agnarsson I, Jencik B, Veve G, Hanitriniaina S, Agostini D, Goh S, Pruitt J, Kuntner K. 2015.** Systematics of the Madagascar *Anelosimus* spiders: remarkable local richness and endemism, and dual colonization from the Americas. *Zookeys* **509**: 13–52.
- Agnarsson I, Maddison WP, Avilés L. 2010.** Complete separation along matrilineal lines in a social spider metapopulation inferred from hypervariable mitochondrial DNA region. *Molecular Ecology* **19**: 3052–3063.
- Avilés L. 1997.** Causes and consequences of cooperation and permanent-sociality in spiders. In: Choe JC, Crespi BJ, eds. *The evolution of social insects and Arachnids*. Cambridge: Cambridge University Press, 476–498.
- Avilés L, Guevara J. 2017.** Sociality in spiders. In: Rubenstein DR, Abbot P. eds. *Comparative social evolution*. Cambridge: Cambridge University Press, 188–223.
- Bilde T, Lubin Y. 2011.** Group living in spiders: cooperative breeding and coloniality. In: Herberstein ME, ed. *Spider behavior flexibility and versatility*. Cambridge: Cambridge University Press, 275–306.

- Cangialosi KR. 1990.** Kleptoparasitism in colonies of the social spider *Anelosimus eximius* (Araneae, Theridiidae). *Acta Zoologica Fennica* **190**: 51–54.
- Coddington JA, Agnarsson I. 2006.** Subsociality in *Helvibis thorelli* Keyserling 1884 (Araneae, Theridiidae, Theridiinae) from French Guiana. *Journal of Arachnology* **34**: 642–645.
- Drummond AJ, Rambaut A. 2007.** BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* **7**: 214.
- Green P. 1999.** PHRAP. Version 0.990329. Available at: <http://phrap.org/>
- Green P, Ewing B. 2002.** PHRED. Version 0.020425c. Available at: <http://phrap.org/>
- Grinsted L, Agnarsson I, Bilde T. 2012.** Subsocial behaviour and brood adoption in mixed-species colonies of two theridiid spiders. *Die Naturwissenschaften* **99**: 1021–1030.
- Huelsenbeck JP, Ronquist F. 2001.** MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**: 754–755.
- Jackson RR. 1986.** Communal jumping spiders (Araneae: Salticidae) from Kenya: interspecific nest complexes, cohabitation with web-building spiders, and intraspecific interactions. *New Zealand Journal of Zoology* **13**: 13–26.
- Jackson RR, Ximena JN, Salm K. 2008.** The natural history of *Myrmarachne melanotarsa*, a social ant-mimicking jumping spider. *New Zealand Journal of Zoology* **35**: 225–235.
- Johannessen J, Wennmann JT, Lubin Y. 2012.** Dispersal behaviour and colony structure in a colonial spider. *Behavioral Ecology and Sociobiology* **66**: 1387–1398.
- Kazutaka K, Kei-ichi K, Hiroyuki T, Takashi M. 2005.** MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Research* **33**: 511–518.
- Kullmann E. 1972.** Evolution of social behavior in spiders (Araneae, Eresidae and Theridiidae). *American Zoologist* **12**: 419–426.
- Lanfear R, Calcott B, Ho SY, Guindon S. 2012.** PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution* **29**: 1695–1701.
- Levi HW. 1962.** More American spiders of the genus *Chrysso* (Araneae, Theridiidae). *Psyche, Cambridge* **69**: 209–237.
- Liu J, May-Collado LJ, Pekár S, Agnarsson I. 2016.** A revised and dated phylogeny of cobweb spiders (Araneae, Araneioidea, Theridiidae): a predatory Cretaceous lineage diversifying in the era of the ants (Hymenoptera, Formicidae). *Molecular Phylogenetics and Evolution* **94**: 658–675.
- Lubin Y, Bilde T. 2007.** The evolution of sociality in spiders. *Advances in the study of behavior*, Vol. **37**. San Diego: Elsevier Academic Press Inc., 83–145.
- Maddison DR, Maddison WP. 2018.** Chromaseq: a Mesquite package for analyzing sequence chromatograms. Version 1.31. <http://chromaseq.mesquiteproject.org>
- Maddison WP, Maddison DR. 2011.** Mesquite: a modular system for evolutionary analysis. Version 2.75 (build 566). Available at: <http://mesquiteproject.org>
- R Core Team. 2016.** R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing.
- Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA. 2018.** Posterior summarisation in Bayesian phylogenetics using Tracer 1.7. *Systematic Biology* **67**: 901–904.
- Rypstra AL. 1979.** Foraging flocks of spiders: a study of aggregate behavior in *Cyrtophora citricola* Forskål (Araneae, Araneidae) in West Africa. *Behavioral Ecology and Sociobiology* **5**: 291–300.
- Saitō S. 1935.** Further notes on spiders from southern Saghalin, with descriptions of three new species. *Annotationes Zoologicae Japonenses* **15**: 58–61.
- Samuk K, Avilés L. 2013.** Indiscriminate care of offspring predates the evolution of sociality in alloparenting social spiders. *Behavioral Ecology and Sociobiology* **67**: 1275–1284.
- Settepani V, Schou MF, Greve M, Grinsted L, Bechsgaard J, Bilde T. 2017.** Evolution of sociality in spiders leads to depleted genomic diversity at both population and species levels. *Molecular Ecology* **26**: 4197–4210.
- Simon E. 1905.** Arachnides de Java, recueillis par le Prof. K. Kraepelin en 1904. *Mitteilungen aus dem Naturhistorischen Museum in Hamburg* **22**: 49–73.
- Uetz GW. 1989.** The “ricochet effect” and prey capture in colonial spiders. *Oecologia* **81**: 154–159.
- Viera C, Agnarsson I. 2017.** Parental care and sociality. In: Viera C, Gonzaga MO, eds. *Behavior and ecology of neotropical spiders - contributions of studies from the neotropical region*. Cambridge: Springer, 351–381.
- Yip EC, Rayor LS. 2014.** Maternal care and subsocial behaviour in spiders. *Biological Reviews of the Cambridge Philosophical Society* **89**: 427–449.