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A new *Rickettsia honei*-related genotype, two novel soft tick haplotypes and first records of three mite species associated with bats in Pakistan

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Abstract

Bats are well adapted to inhabit human settlements and are suitable reservoirs of a high number of vector-borne pathogens with veterinary-medical importance. Owing to these eco-epidemiological traits, the importance of studying bat ectoparasites is increasingly recognized. However, relevant molecular-phylogenetic data are missing from several countries of southern Asia, including Pakistan. In this study 11 ectoparasites, collected from bats in northern Pakistan, were analyzed morphologically and/or molecularly, phylogenetically from a taxonomic point of view. In addition, soft ticks were screened for pathogen DNA. Three mesostigmatid mite species were identified: *Steatonyssus occidentalis evansi* Micherdziński, 1980 and *Ancystropus taprobanius* Turk, 1950 from *Rousettus leschenaultii* (Desmarest 1820) and two specimens of *Spinturnix americanus* (Banks 1902) from *Pipistrellus* cf. *javanicus* (Gray 1838). Six soft tick (*Carios vespertilionis* Latreille, 1802) larvae were also removed from *Scotophilus kuhlii* Leach, 1821. Morphometric comparison revealed minor differences in comparison with *C. vespertilionis* larvae from Europe (i.e., narrower scutum and longer 4th posterolateral setae, while scutal length and shape index were not significantly different in this context). When molecularly analyzed, *C. vespertilionis* larvae from Pakistan showed the highest, 94.1% cox1 sequence identity with a sample from Kenya, while in their 16S rRNA gene these had the highest, 96.2–96.4% identity with samples from Europe and central Asia (northwestern China). These findings were confirmed in phylogenetic analyses. A further soft tick larva, collected from *R. leschenaultii* and therefore provisionally called *Argas* sp. "rousetti", yielded sequences with only 86.2% and 91% similarities in its cox1 and 16S rRNA genes, respectively, to the genetically closest species, *A. boueti.* Both *Argas* species belonged to the phylogenetic group of the subgenus *Chiropterargas*. Molecular screening of two *C. vespertilionis* larvae for a broad range of tick-borne pathogens yielded negative results. However, the larva of *Argas* sp. "rousetti" showed the presence of a spotted fever group (SFG) *Rickettsia* sp., which (based on four genetic markers) was closely related to two *Rickettsia* species reported from southeastern Asia (i.e., *R. honei* and *Rickettsia* sp. IG-1), but differed more significantly from other rickettsiae. In conclusion, the three mite species identified here are new records for their host species and for Pakistan. The present findings support that despite the observed genetic differences, *C. vespertilionis* in southern Asia (Pakistan) and Europe belong to the same species and share common ancestry with *C. vespertilionis* in east Africa (Kenya). The soft tick species, *Argas* sp. "rousetti" is most likely a not yet described species within the subgenus *Chiropterargas* Hoogstraal, 1955, because it was clearly separated from *A. boueti* Roubaud & Colas-Belcour, 1933 and *A. confusus* Hoogstraal, 1955 in the phylogenetic analysis (also taking into

account that other known species of the subgenus are unlikely to occur in Pakistan). The novel *Rickettsia* genotype from *Argas* sp. "rousetti", represents the first molecular identification of a *Rickettsia* sp. phylogenetically close to *R. honei*, and the first *Rickettsia* sp. from any bat-associated soft tick species in southern Asia.

Keywords: Chiroptera, Vespertilionidae, Pteropodidae, *Argas*, *Chiropterargas*

Introduction

Bats (Chiroptera Blumenbach 1779) are known for their widespread global occurrence, large number of species, high population densities and frequent usage of urban habitats (in particular, presence in cities, close to the human environment including buildings and stables: Dekker *et al*. 2013). Even if considered alone, these ecological and taxonomic characters justify an "above the average" epidemiological significance for bats among mammals. Furthermore, bats have a special capacity to act as reservoirs of disease agents (Brook & Dobson 2015).

In this context, bat ectoparasites represent a very important topic to study (Klimpel & Mehlhorn 2014). Among them, the number of arachnid species (mites, ticks) tends to exceed that of insects in both Europe (Frank *et al*. 2015) and the trans-Palaearctic boreal zone to the Far East (Orlova *et al*. 2017). Blood-sucking arachnids (from Parasitiformes Leach, 1815) can play a role in transmitting vector-borne pathogens not only between bats themselves, but also through the ecological interface connecting bats and domestic animals or humans. Thus, it was postulated that macronyssoid mites may transmit important viral and bacterial disease agents (causing encephalitis, hemorrhagic fever, rickettsioses and tularemia) from bats to humans (Balashov 2009; Fagre & Kading 2019). Soft ticks associated with bats may attach to and suck blood from humans (Hoogstraal 1956a; 1985; Jaenson *et al*. 1994) or domestic animals (Manzano-Román *et al*. 2012). In particular, the Old World bat soft tick, *Carios* (syn. *Argas*) *vespertilionis* Latreille, 1802 frequently infests domestic poultry in certain regions, as exemplified by Pakistan (Shah *et al*. 2006; Qamar *et al*. 2009). Such data support the veterinary-medical importance of bat ectoparasites, particularly of ticks and mites.

Recently, the phylogenetic relationships of geographically diverse isolates of *C. vespertilionis* were surveyed in the Old World, including samples from Europe, East Africa, central and southeastern Asia (Hornok *et al*. 2017a, 2017b). The presence of DNA from a great variety of vector-borne bacteria has also been demonstrated in these soft ticks (Hornok *et al*. 2019). Relevant samples were collected from three families of insectivorous bats (Rhinolophidae Gray, 1825; Vespertilionidae Gray, 1821; Miniopteridae Dobson, 1875). Taking into account that southern Asia, the genus *Scotophilus* (Leach 1821) and the family Pteropodidae Gray, 1821 (frugivorous Old World bats) were not represented in the above surveys, in this preliminary study ectoparasites were collected from a lesser Asiatic yellow bat (*Scotophilus kuhlii* Leach, 1821), a fulvous fruit bat (*Rousettus leschenaultii* [Desmarest 1820]) and a Javan pipistrelle (*Pipistrellus* cf. *javanicus* [Gray 1838]) in northern Pakistan. The species of mites were morphologically identified, whereas soft ticks were subjected to both morphometric and molecular-phylogenetic analyses.

Materials and Methods

Sample collection and morphological analyses

Mites and soft ticks were removed from three bats (*S. kuhlii*, *R. leschenaultii, P.* cf. *javanicus*) during bat monitoring in 2016, in northern Pakistan. All ectoparasites were stored in 96% ethanol. Data of sample collection are shown in Table 1.

TABLE 1. Data of sample collection.

Species and gender	Locality	Date of capture	Latitude	Longitude	Elevation	Temperature
Scotophilus kuhlii ♂	Pakistan, Khyber Pakhtunkhawa, District Dir Lower Tehsil Munda, Camp Masjid Munda	25/07/2016	N 34° 49.518	E 71° 40.950	835 m	36°C
Pipistrellus cf. *javanicus* ♀	Pakistan, Khyber Pakhtunkhawa District Bajaur Tehsil, Nawagi, Govt Degree College Nawagi	07/10/2016	N 34° 41.896	E 71° 20.345	1061 m	28.5°C
Rousettus leschenaultii ♀	Pakistan, Khyber Pakhtunkhawa District Swat Tehsil, Kabal, Kabal garden	10/10/2016	N 34° 46.880	E 72° 16.959	883 m	21.3°C

Four soft ticks (identified as *C. vespertilionis* larvae based on Petney *et al*. 2017) from *S. kuhlii* were used for morphometric analysis, during which scutal length, scutal width and the length of 4th posterolateral setae were measured, because these parameters were previously found to be the most different between specimens of *C. vespertilionis* larvae from Europe and Vietnam (Hornok *et al*. 2017a). For comparison, eight larvae of *C. vespertilionis*, collected in Hungary and in Romania in 2018 (locality data not shown) were also included. Measurements were made with a VHX-5000 digital microscope (Keyence Co., Osaka, Japan). Species and subgenus names of soft ticks are used *sensu* Mans *et al*. (2019).

Two mites collected from *R. leschenaultii* and two mites collected from *P.* cf. *javanicus* were cleared in lactic acid for morphological identification according to Rudnick (1960) (Spinturnicidae Oudemans, 1902) and Micherdzinski (1980) (Macronyssidae Oudemans, 1936).

DNA extraction and molecular taxonomic analyses

DNA was extracted from two further *C. vespertilionis* larvae, as well as from a soft tick larva collected from *R. leschenaultii* (which was severely damaged, precluding morphological identification). DNA was extracted from these larvae with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, including an overnight digestion in tissue lysis buffer and Proteinase-K at 56°C.

The cytochrome *c* oxidase subunit I (cox1) gene was chosen as the primary target for molecular analysis, on account of its suitability as a DNA-barcode sequence for tick species identification (Lv *et al*. 2014). The PCR amplifies an approx. 710 bp long fragment of the gene (Folmer *et al*. 1994), using the primers HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') and LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3'). Reaction components and conditions were modified as follows: the reaction volume of 25 µl, contained 1 U (0.2 µl) HotStarTaq Plus DNA polymerase, 2.5 µl 10× CoralLoad Reaction buffer (including 15 mM $MgCl_2$), 0.5 µl PCR nucleotide Mix (0.2 mM each), 0.5 µl (1 µM final concentration) of each primer, 15.8 µl ddH_2O and 5 µl template DNA. For amplification, an initial denaturation step at 95 °C for 5 min was followed by 40 cycles of denaturation at 94 °C for 40 s, annealing at 48 °C for 1 min and extension at 72 °C for 1 min. Final extension was performed at 72 °C for 10 min. In addition, because no cox1 PCR products were gained from *C. vespertilionis* larvae, a new internal primer pair was designed: forward (5'-TTT GGA GCA TGA TCT ATA ATA GTA-3'), reverse (5'-AAA ATA AAT GYT GGT ATA RRA TTG G-3').

Another PCR was used to amplify an approx. 460 bp fragment of the 16S rRNA gene of Ixodidae Koch, 1844 (Black & Piesman 1994), with the primers 16S+1 (5'-CTG CTC AAT GAT TTT TTA

AAT TGC TGT GG-3') and 16S-1 (5'-CCG GTC TGA ACT CAG ATC AAG T-3'). Other reaction components and cycling conditions were the same as above, except for annealing at 51°C.

Molecular screening for vector-borne pathogens

Reverse line blot hybridization assay (RLB) was performed as previously published (Schötta *et al*. 2017), in order to screen soft tick DNA extracts for a broad range of vector-borne pathogens. The oligonucleotides included group level (catch-all) probes and species-specific probes targeting eight species from Anaplasmataceae Philip, 1957, 17 species of piroplasms, eight species of borreliae and ten *Rickettsia* species (Schötta *et al*. 2017).

The citrate synthase (gltA) PCR product of the rickettsia-positive sample was sequenced. This sample was further analyzed with three PCRs: (1) an approximately 480 bp long fragment of the 17 kDa surface antigen gene of *Rickettsia* spp. was amplified with primers 17kd1 (5'-GCT CTT GCA ACT TCT ATG TT-3') and 17kd2 (5'-CAT TGT TCG TCA GGT TGG CG-3') and (2) an approximately 532 bp long fragment of the outer membrane protein A (ompA) gene of *Rickettsia* spp. was amplified with primers Rr190.70p (5'-ATG GCG AAT ATT TCT CCA AAA-3') and Rr190.602n (5'-AGT GCA GCA TTC GCT CCC CCT-3') as described previously (Hornok *et al*. 2018). In addition, the rRNA intergenic spacer (ITS) region was amplified with the primers ITS-F (5'-GAT AGG TCG GGT GTG GAA G-3') and ITS-R (5'-TCG GGA TGG GAT CGT GTG-3') (Vitorino *et al*. 2003), modified as in the latter (ompA) PCR, except denaturation at 95°C for 20 seconds and annealing at 52°C for 1 minutes.

Purification and sequencing were done by Biomi Inc. (Gödöllő, Hungary). Obtained sequences were manually edited, then aligned and compared to reference GenBank sequences by nucleotide BLASTN program (https://blast.ncbi.nlm.nih.gov). Representative sequences were submitted to GenBank (accession numbers: MK571553-4 for the cox1, MK571555-6 for the 16S rRNA and MK571557-60 for the 17kDa, ompA, ITS and gltA sequences, respectively). The MEGA model selection method was applied to choose the appropriate model for phylogenetic analyses. In the phylogenetic analyses reference sequences with high coverage (i.e. 99–100% of the region amplified here) were retrieved from GenBank and trimmed to the same length. This dataset was resampled 1,000 times to generate bootstrap values. Phylogenetic analyses were conducted with the Maximum Likelihood method, T3 and GTR models by using MEGA version 6.0.

Results

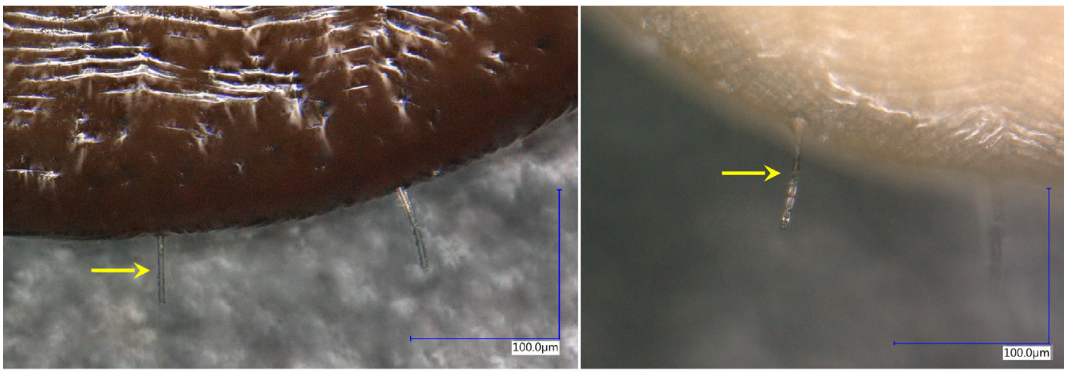
Morphological identification of bat-associated mite species

Three mesostigmatid mite species were identified. *Steatonyssus occidentalis evansi* Micherdziński, 1980 (Macronyssidae) and *Ancystropus taprobanius* Turk, 1950 (Spinturnicidae) occurred on *R. leschenaultii,* whereas two specimens of *Spinturnix americanus* (Banks, 1902) (Spinturnicidae) were collected from *Pipistrellus* cf. *javanicus*.

Morphological, molecular and phylogenetic analyses of bat-associated soft ticks

Carios vespertilionis larvae (n=6) were only collected from *S. kuhlii*. Two parameters of four of these soft ticks from Pakistan, i.e. scutal length (range: 184–192 µm, mean: 188 µm) and shape index (scutum length to width ratio, range: 1.92–2.09, mean: 1.97) were not significantly different from corresponding data of soft ticks from Europe (scutal length range 188–229 µm, mean 205.3 m; ratio range: 1.77–2.14, mean 1.93). However, *C. vespertilionis* larvae from Pakistan had significantly (P = 0.0002) narrower scutum (range: 92–97 µm, mean: 95 µm) and significantly (P = 0.007) longer 4th posterolateral setae (range: 65–79 µm, mean 71.7 µm) compared to European specimens (scutal width

range: 103–110 µm, mean: 106 µm; length of 4th posterolateral setae in the range of 52–66 µm, mean: 59.4 µm). The types of posterolateral setae were identical in the case of *C. vespertilionis* larvae from Europe and Pakistan (i.e., serrate, separated surface protrusions in the upper half: Figure 1).



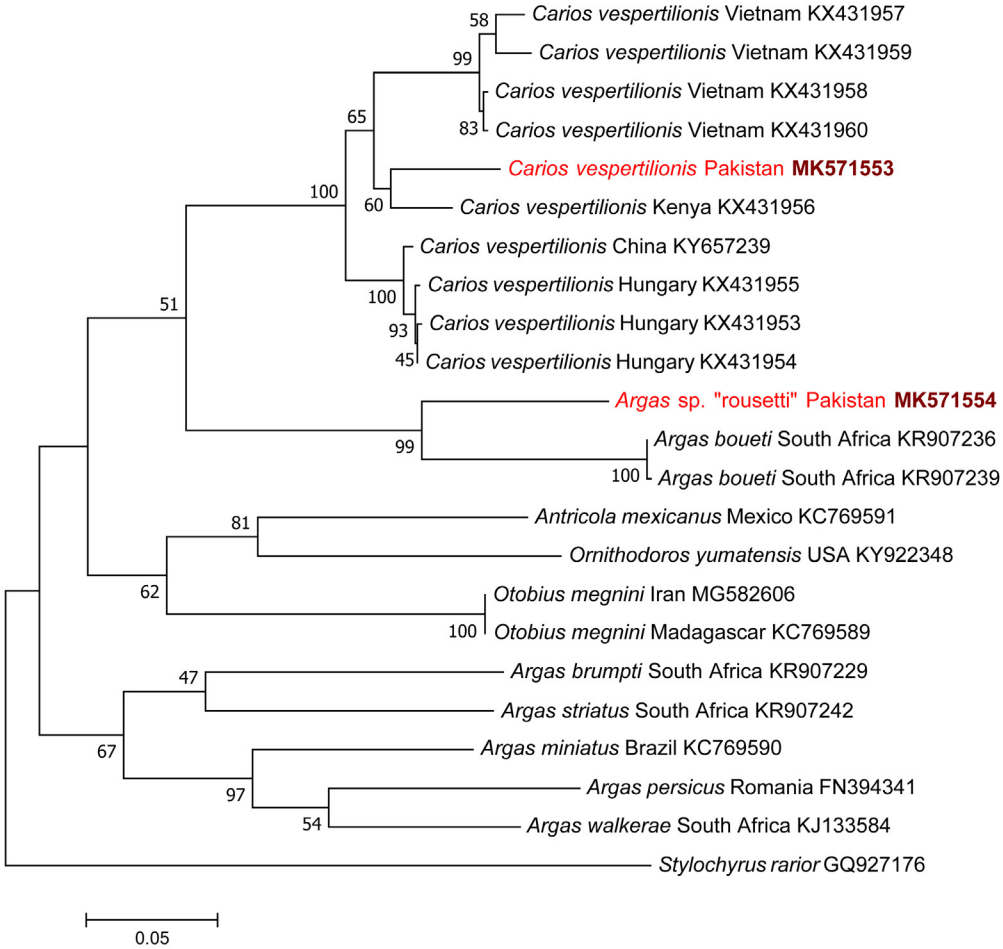


FIGURE 2. Maximum Likelihood tree of soft tick cox1 sequences, shown according to the country of origin before GenBank accession numbers. Sequences from this study are highlighted with red color. The scale-bar indicates the number of substitutions per site.

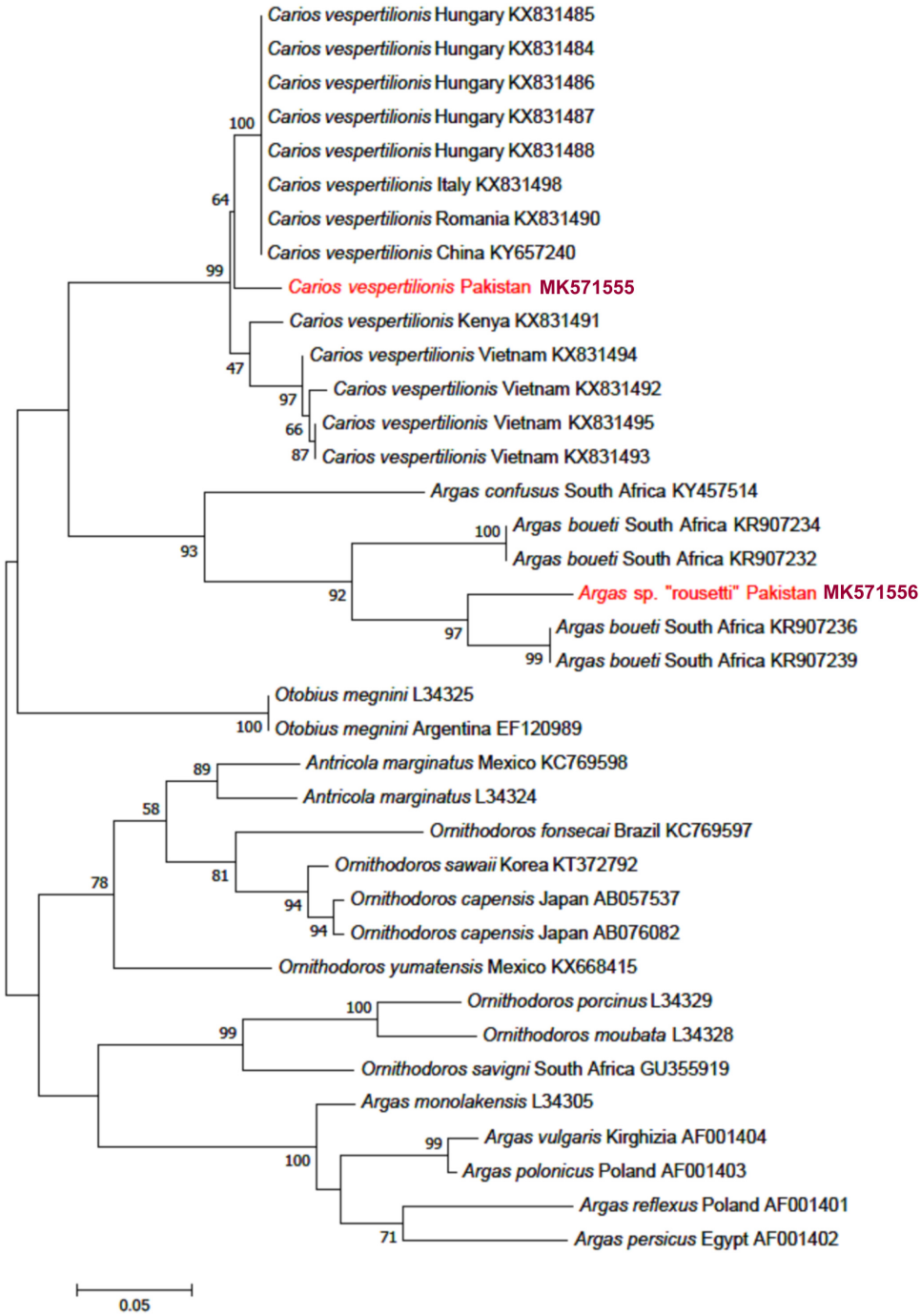


FIGURE 3. Maximum Likelihood tree of soft tick 16S rRNA sequences, shown according to the country of origin before GenBank accession numbers. Sequences from this study are highlighted with red color. The scale-bar indicates the number of substitutions per site.

The two molecularly analyzed *C. vespertilionis* larvae yielded identical sequences. In the amplified part of the cox1 gene, these samples from Pakistan showed the highest, 94.1% (591/628 bp) sequence identity with a sample from Kenya (GenBank: KX431956), and lower degrees of

identities with samples from Europe (below 92%, i.e. 578/628 bp with KX431954) or southeastern Asia, Vietnam (below 92.2%, i.e. 579/628 bp with KX431958). On the other hand, in their 16S rRNA gene, *C. vespertilionis* larvae from Pakistan had the highest, 96.2–96.4% (424/440–441 bp) identity with samples from Europe (e.g. KX831486) and central Asia (northwestern China) (KY657240), and lower degrees of identity with samples from Kenya (95%, i.e. 418/440 bp with KX831491) and southeastern Asia, Vietnam (below 94.6%, i.e. 417/441 bp with KX831495).

Results of the above sequence comparisons were well-reflected by the topologies of phylogenetic trees. Based on the cox1 gene, *C. vespertilionis* from Pakistan clustered together with *C. vespertilionis* from Kenya, and their separation from samples collected in central Asia (northwestern China) and Europe was highly (100%) supported (Figure 2). However, according to the 16S rRNA gene, *C. vespertilionis* from Pakistan clustered together with samples from Europe and central Asia (northwestern China), with strong (99%) support of their separation from other isolates (Figure 3).

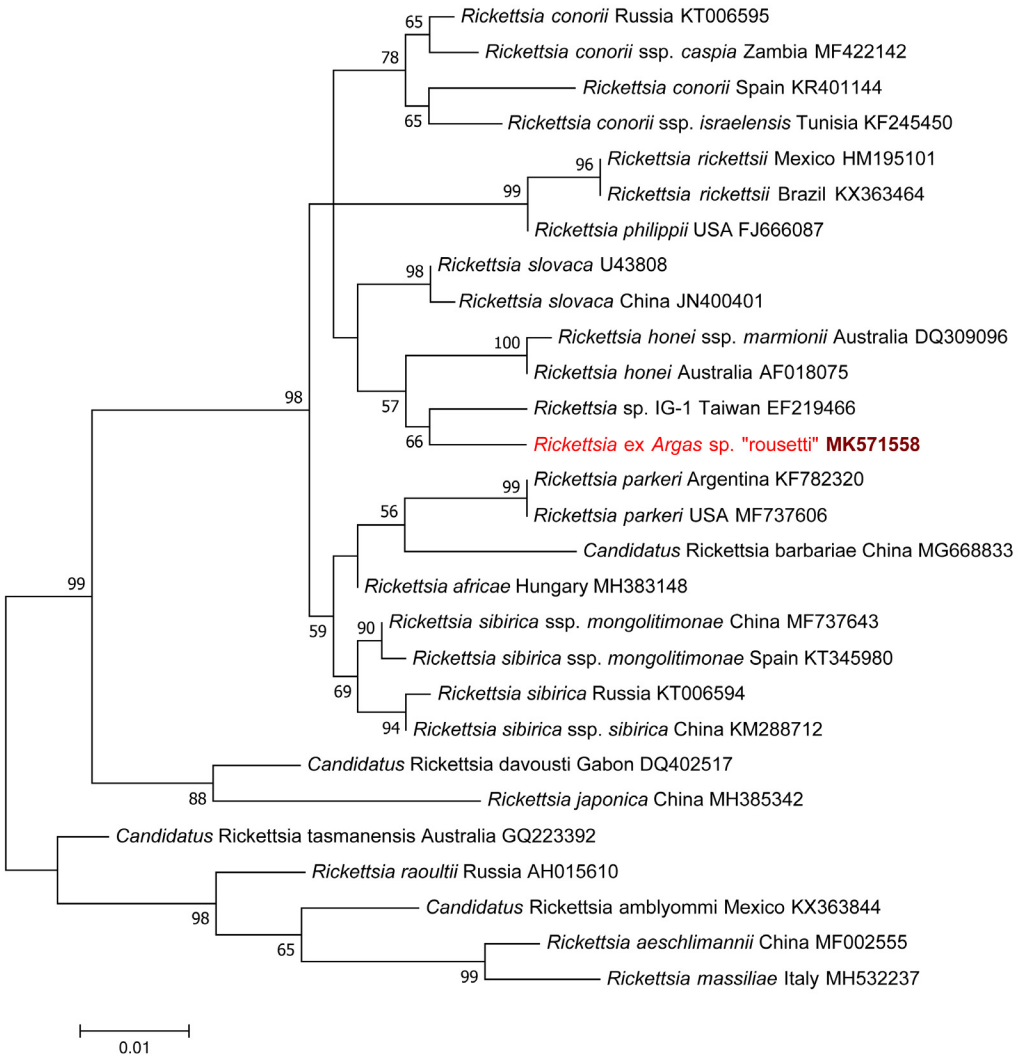


FIGURE 4. Maximum Likelihood tree of *Rickettsia* sequences, shown according to the country of origin before GenBank accession numbers. Sequences from this study are highlighted with red color. The scale-bar indicates the number of substitutions per site.

The soft tick larva collected from *R. leschenaultii* and therefore provisionally called *Argas* sp. "rousetti" yielded a cox1 sequence, which had the highest (but only 86.2%) identity (558/647 bp) with that of *A. boueti* Roubaud & Colas-Belcour, 1933 from South Africa (GenBank: KR907236). This was confirmed by the 16S rRNA gene analysis, showing the maximum (393/432 bp = 91%) identity with *A. boueti* (KR907239). Phylogenetically, *Argas* sp. "rousetti" and *A. boueti* were separated with strong (97–99%) support (Figures 2–3) but belonged to the same group formed by members of the subgenus *Chiropterargas* Hoogstraal, 1955 (including *A. confusus* Hoogstraal, 1955: Figure 3).

Molecular and phylogenetic analysis of vector-borne bacteria in bat-associated soft ticks

The RLB results of two *C. vespertilionis* larvae were negative. However, the larva of *Argas* sp. "rousetti" showed the presence of a spotted fever group (SFG) *Rickettsia* sp., which was not among those included in the species-specific probes of the test. The short gltA sequence of this rickettsia had 100% (294/294 bp) sequence identity with several SFG species, including *R. conorii* (e.g. GenBank: EU716648) and *R. honei* (e.g. GenBank: AF018074), therefore analysis of additional genetic markers was inevitable. The 17 kDa antigen gene of *Rickettsia* sp. from *Argas* sp. "rousetti" showed the highest (383/384 bp = 99.7%) sequence identity with that of *R. honei* (GenBank: AF060704). Its ompA gene was 98.3% (453/461 bp) identical to that of *Rickettsia* sp. IG-1 (GenBank: EF219466), 98.1% (452/461 bp) identical to that of *R. slovaca* (GenBank: U43808) and 97.8% (451/461 bp) identical to that of *R. honei* (GenBank: AF018075). The ITS sequence of the new genotype showed 98.6% (350/355 bp) and 98% (348/355 bp) identity with *R. mongolitimonae* (GenBank: HQ710799) and *R. slovaca* (GenBank: AY125009), respectively. The ompA phylogenetic tree reflected the close relationship of *Rickettsia* sp. from *Argas* sp. "rousetti" with *R. honei*, and their separation was poorly supported (58%: Figure 4). These species/genotypes belonged to a sister group of the clade containing *R. slovaca* and *R. conorii* (Figure 4).

Discussion

In this preliminary study ectoparasites were collected from three bat species in Pakistan. To our knowledge, all mesostigmatid mite species identified here are first records for their bat host species and for Pakistan. In the Palaearctic region, *St. occidentalis* occurs in the European-Caucasian subregion (Orlova *et al*. 2017), therefore the present finding might represent its easternmost record in Eurasia. Similarly, although spinturnicid mites were surveyed in the northern, boreal Palaearctic region, neither *Sp. americanus* nor *An. taprobanius* are known to be present (Orlova & Orlov 2015; Orlova *et al*. 2017). Considering southern-southeastern Asia, *Sp. americanus* is known to occur in Malaysia (Ahamad *et al*. 2013), whereas *An. taprobanius* was reported to have a broad distribution, including Papua New Guinea, the Philippines, Laos, Malaysia, Thailand, India, Nepal and Sri Lanka (Corpuz-Raros & Lit 2015; Amarga *et al*. 2017). Therefore, first finding of *Sp. americanus* and *An. taprobanius* in Pakistan appears to be the westernmost record of these two species in Eurasia.

The bat genus *Scotophilus* (including ten species) occurs in Sub-Saharan Africa, the Malagasy subregion and southern, southeastern Asia (Hutson *et al*. 2001). *Scotophilus* species are mentioned among the preferred hosts of *C. vespertilionis* (Hoogstraal 1985), therefore finding of *C. vespertilionis* larvae on *S. kuhlii* in the present study is not entirely new. Here, based on cox1 sequences, *C. vespertilionis* from Pakistan (representing southern Asia) was the most closely related to *C. vespertilionis* from Kenya (in east Africa), suggesting a more likely "paleogenetic connection" in this than in other directions. This may be partly explained with the co-evolution and co-dispersal

of *C. vespertilionis* with its preferred hosts, vespertilionid bats, which (together with other lineages of Yangochiroptera Koopman, 1984) most likely have an Asian-European origin dating back to approx. 50 Mya (Teeling *et al*. 2005). The presence of vespertilionid bats in the southern part of the Old World may have resulted from multiple dispersal events, implying that their conspecific populations in Madagascar (and eastern Africa) and on the drifting Indian subcontinent (today southern Asia) may have maintained the possibility of genetic exchange via vegetation rafts (acting as "stepping stones": Teeling *et al*. 2005) or islands across the broadening Indian ocean (Samonds *et al*. 2013). The common ancestry of *C. vespertilionis* in Europe and southern Asia (Pakistan) is supported by the present findings, because according to the 16 rRNA gene and/or setal morphology *C. vespertilionis* larvae from Pakistan were similar to those from Europe and central Asia (northwestern China), but differed more significantly from those in Vietnam representing southeast Asia (as reported in Hornok *et al*. 2017b).

Importantly, the level of cox1 sequence divergence between *C. vespertilionis* from Pakistan and Europe/Vietnam exceeded 7.8%, the estimated interspecific divergence between closely related hard tick species (6.1%: Lv *et al*. 2014). To exclude or to confirm the conspecificity of these soft tick larvae, selected (scutal and setal) parameters were considered based on previous work (Hornok *et al*. 2017a). Although scutal width and the length of 4th posterolateral setae were significantly different between *C. vespertilionis* larvae from Europe and Pakistan, scutal length-to-width ratio and the type of setae did not allow their distinction. The latter characters are regarded as more important to separate soft tick species based on larval morphology (Jones & Clifford 1972; Venzal *et al*. 2013). In addition, the level of 16S rRNA sequence divergence was only 3.6–3.8% between *C. vespertilionis* from Pakistan and Europe, whereas in case of different species it is expected to be above 5.4% (Lv *et al.* 2014). For example, when *Ornithodoros (Carios) rondoniensis* (Labruna, Terassini, Camargo, Brandão, Ribeiro &Estrada-Peña, 2008) was described as a new species, it had 11% 16S rRNA sequence difference from the most closely related species (Labruna *et al.* 2008).

Therefore, the present data suggest that *C. vespertilionis* in Pakistan and Europe belong to the same species, despite of the observed minor morphological differences, which should rather be interpreted as intraspecific variations between populations. Similarly, *C. vespertilionis* from Europe and Vietnam, which were genetically the most different (i.e., having up to 7.5% cox1 gene heterogeneity), were still regarded as conspecific (Hornok *et al*. 2017a). In addition, another species of the genus, *C. pusillus* Kohls, 1950 has larvae with distinct morphology (i.e. smaller size, equal palpal articles) and different geographical distribution (Bangladesh, Korea, Philippines, Malaysia) from *C. vespertilionis* (Sonenshine *et al*. 1962; Hoogstraal *et al*. 1979), therefore this species can be excluded from the explanations of the observed genetic differences.

Pteropodidae (frugivorous Old World bats) have widespread distribution in Africa, southern Asia and Australia (Almeida *et al*. 2011). Among them, *Rousettus* species are not mentioned as the typical hosts for members of the soft tick subgenus *Chiropterargas*, including *Argas boueti* (Hoogstraal 1985). On the other hand, *A. boueti* was reported from the frugivorous bat species *R. aegyptiacus* (Hoogstraal 1955). *Argas boueti* occurs in south, east and north Africa, Middle East, southern and southeastern Asia (Heisch 1951; Hoogstraal 1955; Hoogstraal 1985), but to the best of our knowledge has not been reported from Pakistan. Here, a morphologically unidentifiable soft ticks larva collected from *R. leschenaultii* (provisionally called *Argas* sp. "rousetti") yielded a cox1 sequence with the maximum, 86.2% similarity to that of *A. boueti* from South Africa, from which it clustered separately. Furthermore, *Argas* sp. "rousetti" and *A. boueti* belonged to the same phylogenetic group, represented by members of the subgenus *Chiropterargas* (including *A. confusus*). Other known species of *Chiropterargas* (for which sequences have not been available in GenBank for inclusion in the phylogenetic analysis here) are unlikely to occur in Pakistan, i.e. *A. cordiformis* (in Africa) and *A. ceylonensis* (only in Sri Lanka) (Hoogstraal *et al.* 1979). Although two

(small and large size) "races" were described within *A. boueti* (Hoogstraal 1955), both were reported from Africa. Therefore, the present results indicate the existence of a further, undescribed species within *Chiropterargas.*

Considering the novel *Rickettsia* genotype identified here in *Argas* sp. "rousetti", it was (phylo)genetically closest related to *Rickettsia* sp. IG-1 (reported previously from southeast and east Asia, i.e. Taiwan and Japan: Tsai *et al*. 2008; Fujita *et al.* 2008). At the same time, *Rickettsia* sp. ex *Argas* "rousetti" clustered together with a human pathogenic species, *R. honei*, known to occur in southeast Asia (Thailand) and Australia (Stenos *et al.* 1998; Kollars *et al.* 2001). Reports on rickettsiae identified molecularly or serologically are rare in the region of Pakistan. Although a potentially new, unnamed rickettsia (JC880) was detected in this country (Robertson *et al.* 1970, 1973), it was neither reported to be closely related to *R. honei*, nor was it associated with soft ticks or bats.

To be classified as a new *Rickettsia* species, the sequences of a new genotype should not simultaneously exceed 99.9% gltA or 98.8% ompA, and 99.2% 17 kDa identity with closely related species (Fournier *et al.* 2003, Bouyer *et al.* 2001), unlike shown here for *Rickettsia* sp. ex *Argas* "rousetti". In addition, the phylogenetic separation of the latter from *Rickettsia* sp. IG-1 (which is considered as a genetic variant of *R. honei*: Fujita et al. 2008) was poorly supported. Therefore, *Rickettsia* sp. ex *Argas* "rousetti" is considered as a new *R. honei*-related genotype, worth of further investigation. This is especially important in light of the fact that the closest relative of *Argas* sp. "rousetti" (from which this rickettsia was identified here), i.e. *A. boueti* is known to occasionally feed on humans (Hoogstraal 1956b). Taken together, this is the first molecular identification of a *Rickettsia* sp. phylogenetically close to *R. honei*, and from any bat-associated soft tick species in southern Asia.

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