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Parasitoidism of the *Sarcophaga dux* (Diptera: Sarcophagidae) on the *Mesobuthus martensii* (Scorpiones: Buthidae) and Its Implications

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ABSTRACT The Chinese scorpion, *Mesobuthus martensii* (Karsch, 1879), is a medically important arthropod, with its venom representing a rich resource for bioactive molecules. Very little is known about the natural enemies of scorpion, albeit some populations are on the verge of extinction due to human over-exploitation. In this study, we found, for the first time, that a medically and forensically important flesh fly, *Sarcophaga dux* (Thompson, 1869), can parasitize *M. martensii* in China. We identified the flesh flies by both morphology and DNA-based methods employing the mitochondrial cytochrome C oxidase I gene. Our phylogenetic analyses indicated that *S. dux* was not monophyletic with respect to *Sarcophaga aegyptica* (Salem, 1935) and *Sarcophaga harpax* (Pandellé, 1896), and was comprised of two distinct mitochondrial lineages. The flesh flies infesting the Chinese scorpion formed one of the paraphyletic lineages of *S. dux*. These lineages together with *S. aegyptica* and *S. harpax* represented a species complex with genetic distances ranging from 1.0 to 1.5%. Our findings suggested that *S. dux* was capable of larviposition nocturnally.

KEY WORDS mitochondrial DNA, nocturnal larviposition, parasitoid, phylogenetic analysis, species complex

Although envenomation by scorpion has been a scourge of human-kind since antiquity and posed a significant threat to public health in many regions around the world (Simard and Watt 1990), venoms of scorpions are a rich source of bioactive molecules which have high medical significance (Goudet et al. 2002, Rodriguez de la Vega and Possani 2005) and insecticidal potentials (Smith et al. 2013). Particularly, the Chinese scorpion, *Mesobuthus martensii* (Karsch, 1879), has been used in traditional Chinese medicine for more than 1,000 yr (Shi et al. 2007). Over the past decade, more than 70 different peptides, toxins, or homologues have been isolated from the venom of this species (Goudet et al. 2002). With the scorpion venom emerging as a rich resource for drug development in modern medicine, *M. martensii* also becomes an economically important animal, bringing considerable incomes to local residents in north China. However, over-exploitation has pushed some populations to the edge of extinction (Shi et al. 2007). Now *M. martensii* is considered vulnerable and listed in the China Species Red List (Wang and Xie 2005). Although rearing attempts have been carried out by the locals in nearly all scorpion occurring regions of China since 1990s, successful cases are, if not none, very rare. Despite of its long-acknowledged medical and economic importance, knowledge on ecology of the Chinese scorpion is still limited. In particular, little is known about its natural enemies, such as parasites or parasitoids.

Knowledge on the pest organisms parasitizing scorpion is not only beneficial to scorpion rearing practices, but will also provide first-hand information on formulating sustainable conservation programs. However, only a few species of fungi (e.g., Santana-Neto et al. 2010), nematodes (e.g., Gouge and Snyder 2005), and mites (e.g., Ibrahim and Abdel-Rahman 2011) have been described infesting scorpions. No parasitoidism of flesh flies (Diptera: Sarcophagidae) on scorpions has been formally reported, although flesh flies were often found associating with other invertebrates, such as insects, snails, earthworms, and crabs (Pape 1987; 1996). Shi (2007) observed the association of some dipteran larvae, most likely flesh fly, with a corpse of *M. martensii*. Unfortunately, the insect was not identified and the inter-relationship between fly and scorpion was not established.

Many species of flesh flies have carrion-feeding larvae and scavenging adults, occupying a great diversity of habits (Pape 1996). They contribute to nutrient cycling in ecosystems and are of substantial ecological importance (Stamper et al. 2012). Some flesh flies are also of medical and forensic significance (Pape 1996). Due to a high level of phenotypic similarity among both adult and larval stages across species and genera, species identification is notoriously difficult in this

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group. This is exemplified by lumping of about 800 species in the genus *Sarcophaga* (Piwczyński et al. 2014). DNA-based approaches are playing increasing role in biodiversity inventory of medical and forensic important insects, especially in groups that are hard to be identified by morphological means. Such approaches have been successfully employed in identifying flesh flies from many regions of the world, e.g., Australia (Meiklejohn et al. 2011; 2012), China (Guo et al. 2012; 2014), Korea (Kim et al. 2014), India (Bajpai and Tewari 2010), Malaysia (Tan et al. 2010), and Europe (Jordaens et al. 2013). In addition, DNA data not only hold high potential for reconstructing the evolutionary history of this species-rich group (Piwczyński et al. 2014), but can also provide a measurement about within-species divergence which might have consequential ecological, medical, and forensic implications.

In this study, we reported a case of parasitoidism of the Chinese scorpion *M. martensii* by a species of flesh fly for the first time. We screened a natural population of the Chinese scorpion for fly infestation, and identified the fly by both morphological and DNA-based approaches. Our results indicated that the medically and forensically important flesh fly, *Sarcophaga dux* (Thomson, 1869) was a facultative parasitoid of the Chinese scorpion. We found substantial genetic divergence in this wide spread flesh fly species, and inferred that *S. dux* was capable of larviposition nocturnally.

Materials and Methods

Scorpion Collection, Fly Rearing, and Morphological Examination. Scorpions were collected with the assistance of a portable UV-light at night from Niushou Mountain (37°48′ N, 106°01′ E), the Ningxia Hui Autonomous Region, on 1-VIII-2014. The animals obtained were kept in plastic bottles with pores (ca. 1 mm in diameter, 5–8) pierced on lids for air ventilation. Scorpions were identified according to description of Qi et al. (2004) and Shi et al. (2007).

All scorpions collected were kept in bottles and checked daily. Scorpions stayed alive were transferred into new bottles for further observation while the dead individuals were relocated to a beaker and covered with wheat bran. The beaker was sealed up with medical gauze and kept in climatic chamber at 28°C, with 75% relative humidity and natural lighting. Of the total 317 scorpions collected, 73 died out by the ninth day of collection. Maggots were observed from 54 dead scorpions. We randomly selected five infested scorpions for examination of parasitoid loads. A total of 175 larvae were collected, of which 128 were pupated, while 112 gave emergence to adult flies. All specimens were morphologically examined under a Nikon SMZ1500 stereomicroscope. Photographs were taken with a Nikon Digital Sight DS-U1 system.

Molecular Protocols and Phylogenetic Analyses. Genomic DNA for three larvae and 14 adult flesh flies were extracted using a modified phenol-chloroform extraction procedure (Zhang and Hewitt 1998). Fragments of the mitochondrial cytochrome C oxidase I gene (*mtCOI*) were PCR amplified with the primers pair: LCO1490 (GGTCAACAAATCATAAAGATATTGG, Folmer et al. 1994) and Nancy (CCCGGTAAAATTAAAATATAAACTTC, Simon et al. 1994). PCRs were carried out in volumes of 30 µl consisting of 1 × reaction buffer, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTPs, 0.3 µM of each primer, 0.6 unit of Taq DNA polymerase (Promega, Shanghai, China), and 30–50 ng of genomic DNA. The following thermal profile for PCR was applied: an initial denaturation at 94°C for 4 min was followed by 35 cycles of 30 s at 94°C, 30 s at 52°C and 30 s at 72°C, and a final extension at 72°C for 2 min. The purified PCR products were sequenced using the ABI PRISM BigDye Terminator V3.1 Cycle Sequencing Kit and sequences were resolved on an ABI 3730XL automated sequencer (Applied Biosystems, Foster City, CA, USA). All the materials (voucher specimens and DNA extracts) were deposited at the Laboratory of Molecular Ecology and Evolution, Institute of Zoology (MEE-IOZ), Chinese Academy of Sciences, Beijing. All sequences have been deposited in GenBank under accession numbers KT347323-KT347339.

Homologous sequences in GenBank were queried with BLASTn (Altschul et al. 1990) with our new sequences. We retrieved all homologous sequences with query coverage >98% and similarities >93% from GenBank (Supp Table 1 [online only]). These sequences were aligned with Clustal X 1.83 (Thompson et al. 1997), and those bearing unresolved sites and being <640 bp in alignment length were removed. Sequences of *Peckia intermutansene* and *Peckia chrysostoma* were used as outgroups based on recent large-scale molecular phylogenetic study (Piwczyński et al. 2014). We used ModelTest 3.7 (Posada and Crandall 1998) to select the appropriate model of DNA evolution according to the Akaike information criterion. Phylogenetic analyses were performed using both maximum likelihood (ML) and Bayesian methods. ML analysis was carried out using PHYML 3.0 (Guindon and Gascuel 2003). Topological robustness was assessed through 1000 bootstrapping replicates. Bayesian analysis was conducted with MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). Analyses were initiated with random starting trees and ran for 2×10^6 generations with four Markov chains employed. Trees were sampled every 200 generations. At the end of MCMC run, the average standard deviation of split frequencies were <0.01, and the average estimated sample size values were >858 and the potential scale reduction factor values approached 1.00 for all parameters. The first 25% trees were discarded as burn-in after inspections of the stationary state and the convergence of the chains with TRACER 1.5 (Rambaut and Drummond 2007). Genetic distances based on Kimura 2-parameter model (K2P distances) between phylogenetic groups were calculated with MEGA 5.05 (Tamura et al. 2011). The unique haplotypes were determined with DnaSP5.10 (Librado and Rozas 2009). Within-species relationships among haplotypes were inspected through median-joining networks (Bandelt et al. 1999) using the Network 4.6 (http://www.fluxus-engineering.com/).

Results

Morphology, Prevalence, and Life History. The morphological characteristics of both females and males were compatible with description of *S. dux* (Xue and Chao 1996; Sukontason et al. 2014). As phylogenetic analyses suggest that there are significant genetic divergence within *S. dux*, a detailed morphological description and photography of major diagnostic characters is given in Supp Materials (online only). Our observation gave a point estimate of prevalence of 17.03% (54/317) for flesh fly in the scorpion population. In the earlier infestation, scorpions looked sluggish and inactive even at disturbance. Scorpions began to die on the sixth day after collection. In all cases ($n = 5$) we examined, multiple (three to six) fly larvae were nourished in a single mesosoma of scorpion. Maggots emerged from a single opening near the genital operculum of the dead scorpions.

It should be noted that the developmental times for larvae were under room condition, whereas that for the third instars and pupae were under controlled condition with the temperature at 28°C, relative humidity of 75%, and natural lighting. Our primary goal was to rear maggots into adulthood to facilitate morphological examination and species identification. We observed a pre-pupae duration (one to three instars) of 10–13 d and pupae duration of 11–14 d. All the third instars pupated in 2–3 d after they came out from scorpion bodies. The immature growth from instars to adult emergence was 21–26 d.

Sequence Homology and Phylogenetic Analyses. A total of 17 flies (three larvae and 14 adults) were sequenced for ca. 700 bp fragments of *mtCOI* gene from both directions. No discrepancy was observed between complementary sequences. A BLAST analysis revealed that they had 100% identity to the homologous fragment of a sequence for *S. dux* (EF405937) from Malaysia (Tan et al. 2010) and 99% identity to another 31 sequences labeled as *S. dux* (Table 1). Our sequences also showed a high similarity to two sequences of *S. harpax* (JX861474 and JX861475, 99%) from Korea (Kim et al. 2014) and one sequence of *S. aegyptica* (JQ582054, 98%) from France (Jordaens et al. 2013).

Aligned matrix comprised 86 terminal units of 674 total characters, 18.5 % of which (125 sites) were parsimony-informative. According to the Akaike information criterion, GTR+I+G model was selected as the best fit model ($-\ln L = 2479.548$, AIC = 4979.097). Figure 1 shows the 50% majority consensus tree inferred from Bayesian analyses. The ML tree and the Bayesian tree were highly congruent with respect to interrelationship among major clades (species). Both analyses resolved a monophyletic major clade (ML bootstrap value/Bayesian posterior probability, 98/1.00), including all sequences of *S. dux*, two sequences of *S. harpax*, and one sequence of *S. aegyptica*. Hereafter, we refer to this major clade as the *S. dux* complex. Four subclades, *S. dux* I (73/1.00) and II (99/0.94), *S. harpax* (100/0.58), and *S. aegyptica* (94/1.00), were recognized in the *S. dux* complex in the ML or the Bayesian analyses, but relationships of the subclades were unresolved (Fig. 1 and Supp Fig. 5 [online only]). In the ML tree, subclade *S. harpax* clustered with subclade *S. aegyptica* but was virtually not supported (27), forming a sister clade to *S. dux* I (Supp Fig. 5 [online only]). *S. tibialis* is the most closely related species to the *S. dux* complex (73/1.00). The genetic distances (K2P-distances) between the major clade are larger than 5% while those within clade are normally below 1.6%. K2P distances between subclades of *S. dux* complex ranged from 1 to 1.5% (Table 2).

Table 1. Haplotypes and their frequencies, GenBank accession numbers, and geographic origin of sequences used in network analysis of *S. dux* species complex

Species/lineages	Haplotypes	Frequencies	GenBank number	Origin
S. dux I	III	7	KJ496796–799	Malaysia
			KF562109	Malaysia
			KC855284	Malaysia
			JX187398	Malaysia
	V	3	KC249713	China
			KC249714	China
			JX187399	Malaysia
	VIII	1	JX187397	Malaysia
	XIII	1	EF405939	Malaysia
	XIV	1	EF405938	Malaysia
S. dux II	I	18	EF405937	Malaysia
			KT347323-39	China
	II	1	KJ496800	Malaysia
S. aegyptica	IV	4	JQ582055	France
			JQ582056	France
			KF037974	China
			KF037975	China
	IX	1	JQ582054	France
S. harpax	VI	2	JX861475	S. Korea
			JQ350717	S. Korea
	VII	4	JX861474	S. Korea
			GQ254447	Australia
			JN964817	Australia
			JN964816	Australia
	X	1	JN964815	Australia
	XI	1	GQ254449	Australia
	XII	1	GQ254445	Australia

The detailed relationships among subclades of *S. dux* complex are shown in the median-joining network of Figure 2. This network was constructed using haplotypes (I–XIV) defined by nucleotide substitutions only thus some sequences were collapsed into the same haplotype. No reticulation occurred in network and the four subclades were separated by at least eight mutational steps. Eight base substitutions (including the two missing middle haplotypes) occurred between subclade *S. dux* I and II, eight substitutions (including one missing haplotypes) between *S. dux* II and *S. harpax*, and 11 substitutions (including two missing haplotypes) between *S. dux* II and *S. aegyptica* (Fig. 2). All the sequences of flesh fly retrieved from scorpion belong to haplotype I.

Discussion

***S. dux* is a Facultative Parasitoid of the Chinese Scorpion.** In this study, we set up an explicit host–parasitoid relationship between two medically

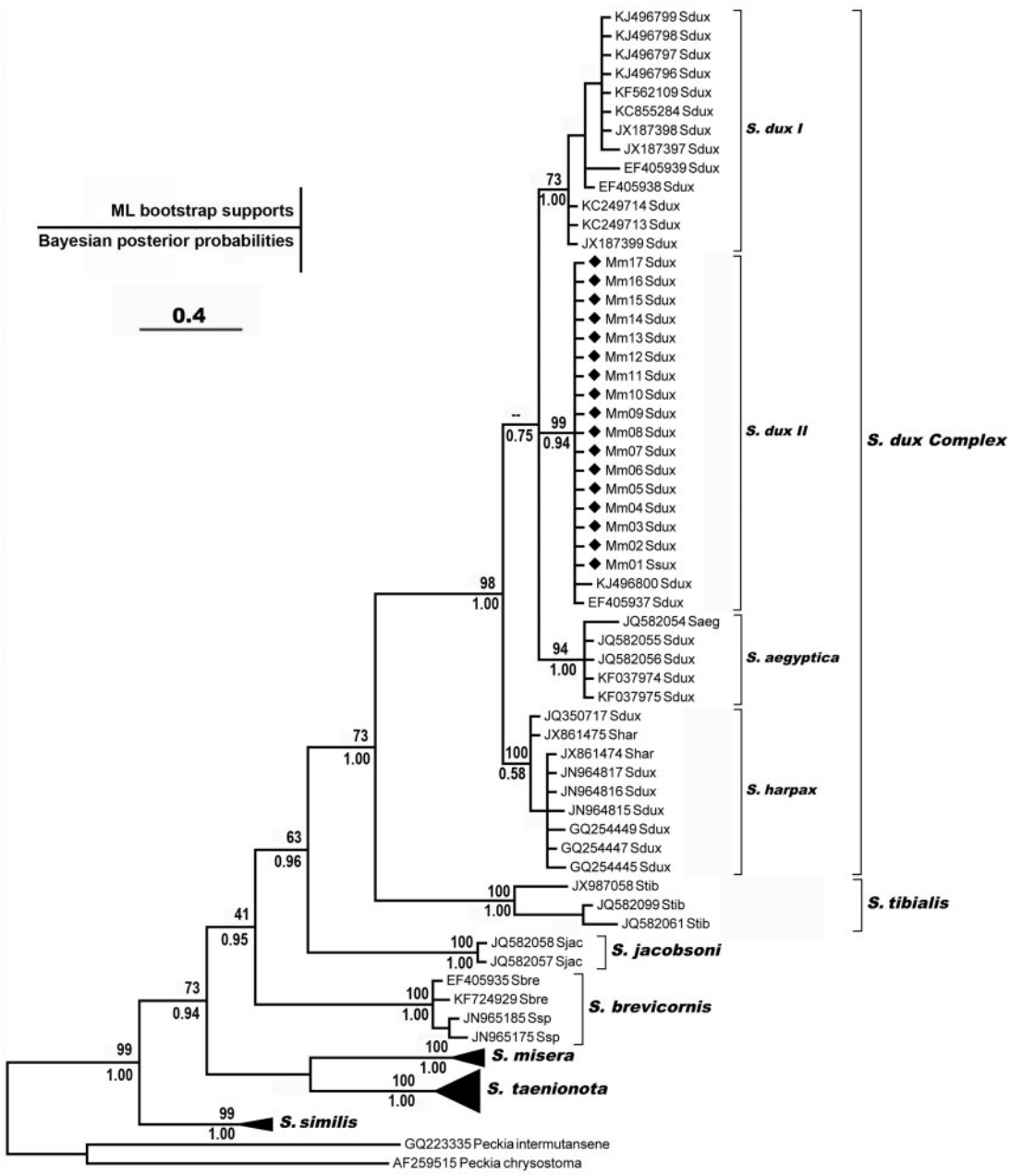

Fig. 1. The 50% majority consensus tree from Bayesian analyses of *mtCOI* sequences. Robustness of branches is indicated by Bayesian posterior probabilities along with ML bootstrapping supports from 1000 replicates. Diamond notates flesh flies from the Chinese scorpion.

important arthropods, the Chinese scorpion *M. martensii* and the flesh fly *S. dux*. Scorpion identification is rather straight forward as only two species, with sharp morphological differences, occurred in the region (Shi et al. 2007). Specimens from the same locality were examined for both mitochondrial and nuclear DNA markers in earlier phylogeographic studies (Shi et al. 2013). The population we sampled belongs to a western lineage of *M. martensii* (C.-M. Shi, unpublished data). Both analyses of *mtCOI* sequences (Figs. 1 and 2) and examination of morphological characteristics (Supp Figs. 1–4 [online only]) indicate that the flesh flies from scorpion belong to a lineage of *S. dux* (see discussion on its intraspecific genetic divergence). Our observation suggested that *S. dux* was a gregarious (three to six flies in a scorpion), koinobiont parasitoid which led a facultative life on the Chinese scorpion. *S. dux* was known as a synanthropic insect and closely associates

Table 2. Genetic distance estimates based on K2P-model between flesh fly lineages

	*S. dux*_I	*S. dux*_II	*S. aeg*	*S. har*	*S. tib*	*S. jac*	*S. bre*	*S. mis*	*S. tae*	*S. sim*
*S. dux*_I	*0.002*	0.004	0.004	0.005	0.008	0.009	0.010	0.010	0.010	0.010
*S. dux*_II	**0.010**	*0.000*	0.005	0.004	0.008	0.009	0.010	0.010	0.010	0.009
S. aeg	**0.014**	**0.015**	*0.002*	0.004	0.008	0.009	0.010	0.010	0.010	0.010
S. har	**0.015**	**0.013**	**0.015**	*0.002*	0.008	0.009	0.010	0.009	0.010	0.010
S. tib	0.056	0.053	0.056	0.057	*0.016*	0.009	0.010	0.011	0.010	0.009
S. jac	0.063	0.059	0.058	0.057	0.063	*0.000*	0.010	0.011	0.011	0.009
S. bre	0.068	0.064	0.067	0.060	0.071	0.061	*0.003*	0.010	0.010	0.009
S. mis	0.063	0.063	0.068	0.062	0.085	0.078	0.069	*0.004*	0.008	0.010
S. tae	0.063	0.063	0.068	0.066	0.076	0.074	0.063	0.050	*0.006*	0.010
S. sim	0.064	0.059	0.064	0.065	0.065	0.058	0.054	0.066	0.065	*0.004*

The means of the number of substitutions per site are shown in the lower diagonal and its standard errors above the diagonal. The italic numbers on the diagonal represent means distances within groups. The distances between lineages of *S. dux* complex are highlighted in boldfaces.

Note: *S. dux* I and II are the two mitochondrial lineages recognized in this study. Abbreviation for species names: *S. aeg*, *S. aegyptica*; *S. har*, *S. harpax*; *S. tib*, *S. tibialis*; *S. jac*, *S. jacobsoni*; *S. bre*, *S. brevicornis*; *S. mis*, *S. misera*; *S. tae*, *S. taenionata*; *S. sim*, *S. similis*.

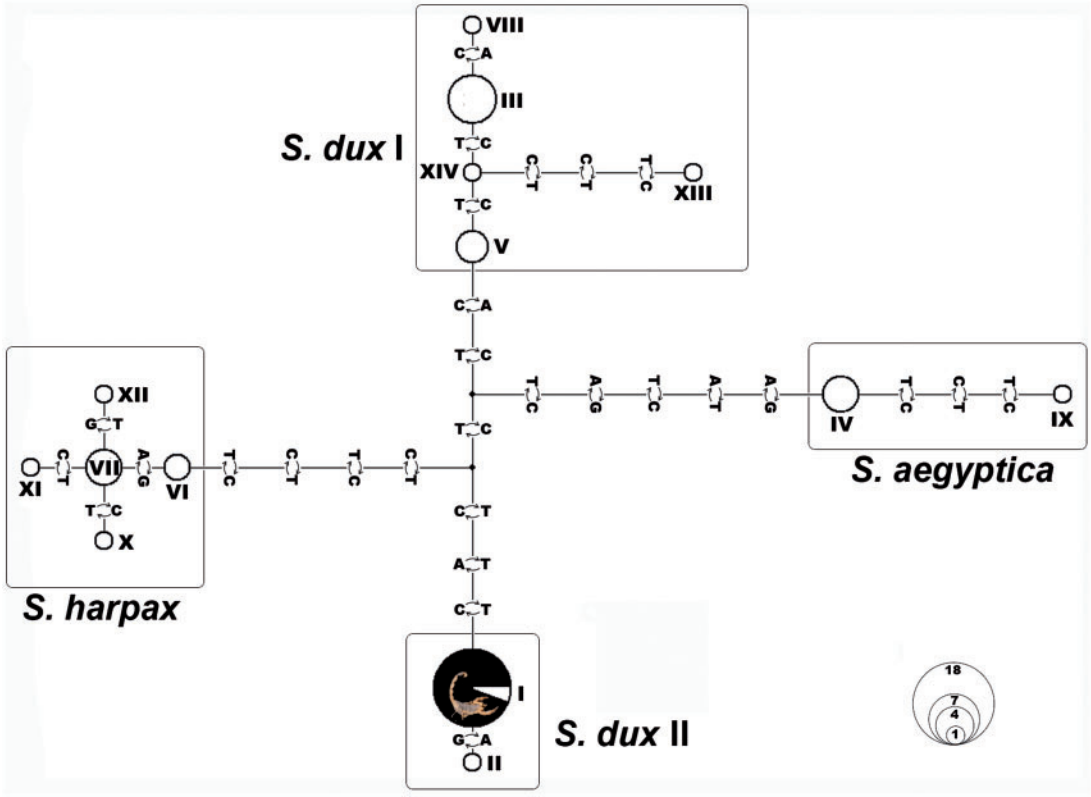

Fig. 2. Genetic divergence between lineages of *S. dux* complex shown in the media-joining networks. Branch lengths are proportional to the number of substitutions occurred as shown along the branches. Circle sizes are proportional to the number of sequences sharing the haplotypes. Sequences for flesh flies infesting the Chinese scorpion were all collapsed in the haplotype I as shown by the black proportion.

with animal and human surroundings (Sukontason et al. 2014), with larvae commonly breeding in decomposing vegetables (Xue and Zhao 1996). It was occasionally found in the dung of livestock (Chin et al. 2010) and the larvae of pine moth (Xue and Zhao 1996). Besides its sanitary impacts, *S. dux* was also of medical importance, as it is, in many parts of the world, a myiasis-producing agent (Chaiwong et al. 2014). It is also of forensic significance for their colonizing decomposed human remains (Cherix et al. 2012). Geographically, this fly was wide spread, prevailing in many parts of the Palearctic and Australasian/Oceanian regions (Pape 1996, Sukontason et al. 2014). In China, *S. dux* widely occurred in nearly all regions, from the Northeast Plain to southern Hainan Island (see Supp Materials [online only] for details).

The high prevalence (17%) of the flesh fly in the Chinese scorpion as we observed in the laboratory might be an overestimate for its occurrence under natural conditions. The high density of scorpion (>100 individuals in a 600 ml bottle) in our storage might have facilitated infestation of flesh fly, which would never occur in field. Nevertheless, our results implied that *S. dux* might represent a harmful agent to the Chinese scorpion. This should be taken into consideration in designing rearing programs. The ever increasing demand of scorpion in medicine and food market has led to extirpative collection of scorpions in China, which has pushed some populations to the edge of extinction (Shi et al. 2007). Commercial rearing was suggested to relieve this situation. However, most rearing attempts, if not all, ended up with no success. Unrealized parasitoidism may be one cause responsible to such failure. The vicious ecological consequence brought by *S. dux* to the Chinese scorpion might be more profound in natural populations. *S. dux* might directly suppress population density of scorpion which is a major predator in many ecosystems, and then provoke catastrophic ecological consequences. An impending threat would be explosive occurrences of some herbivorous pest insects. In addition, evidences show that parasites-mediated effects could not only shape host population dynamics, but also alter interspecific competition (Hudson et al. 2006). The region where scorpion was sampled represents a sympatric zone between the Chinese scorpion and another congenic species *Mesobuthus eupeus* (Koch, 1839). We also sampled *M. eupeus* nearby (ca. 20 km apart), but no flesh fly was detected. Such an asymmetric parasitizing pattern might put the Chinese scorpion at a disadvantage in interspecific competition. This issue deserves a thorough investigation in the future.

Intraspecific Genetic Divergence of *S. dux*. Our molecular phylogenetic analyses consistently revealed a strongly supported (98/1.00) clade of *S. dux* complex, which included all sequences of *S. dux*, two sequences of *S. harpax*, and one sequence of *S. aegyptica*. This clade manifested a genetic (K2P) distance of 5.6% with its most closely related species, *S. tibialis*, which corresponds to conventional interspecific divergence (Meiklejohn et al. 2012). The genetic distances (≤1.5%) between subclades recognized in *S. dux* complex appeared to be of intraspecific level according to the recent results of DNA barcoding studies (Jordaens et al. 2013). Although the divergence is shallow compared with other species, these subclades high likely represent distinct evolutionary lineages as they were well resolved in both ML and Bayesian inferences (Fig. 1). The genetic distinctness of these subclades was more clearly manifested by network analysis. The four subclades were separated by at least eight mutational steps from each other and no reticulation occurred. This result implied a long-term cessation of gene flow, and lineage sorting in isolation, and thus a clear genetic discontinuity. In addition, some subtle morphological differences have been observed (see Supp Materials [online only]) and *S. aegyptica* was initially recognized as a variety (*S. dux* var. *aegyptica*) of *S. dux*. These evidences also indicated a discontinuity on the morphological axis. Based on these observations, we speculated that either specimens whose sequences labeled as *S. dux* in subclades *S. aegyptica* and *S. harpax* were inadequately identified or those subtle morphological differences are not designating distinct species. We referred to all the four subclades collectively as *S. dux* complex. Such recognition suggested that *S. dux* was not reciprocally monophyletic with respect to *S. aegyptica* and *S. harpax*. The *mtCOI* differences between *S. dux* I and II were equivalent to that between *S. dux* II and *S. harpax* (eight substitutions, Fig. 2). Although a robust delimitation of these lineages requires further evidences from other genetic markers (especially nuclear DNA) and detailed phenotypic comparisons, our results, nevertheless, that revealed there was apparent within-species divergence in the current morphological species, *S. dux*. The wide geographic distribution across diverse climatic and ecological habitat types might be responsible for such divergence.

Ecological and Forensic implication. *S. dux* has been considered of forensic importance with the potential use for estimating the postmortem interval (Cherix et al. 2012, Sukontason et al. 2014). The accuracy of the postmortem interval estimate has been grounded in the reliable assessment of the larviposition time and the maggot developmental rate. It is widely held that flesh flies are diurnal and do not larviposit at night. On the contrary, scorpions, in general, are nocturnal predators which actively search preys at night but sit-and-wait in burrows or other shelters during daytime. In particular, the Chinese scorpion is most active 3 h after sunset (20:00–23:00) in the northwest China in summer (C.-M. Shi, personal communication). Our founding of larval parasitism of the flesh fly *S. dux* on the Chinese scorpion suggested that this fly was capable of larviposition at night. Singh and Bharti (2008) reported nocturnal larviposition levels of 20% for other species of *Sarcophaga*. Without taking this behavior into consideration, if *S. dux* was used in forensic investigation, erroneous estimates of postmortem interval could result.

We recorded the pre-pupae development of 10–13 d: 8–9 d in dead scorpions and additional 2–3 d of development of third instars before their pupating. The prepupae development time was slightly longer than that rearing at constant 28°C in the middle East (Al-Misned 2004), and roughly four times of that in north Thailand under natural ambient temperature (24–28°C) in March (Sukontason et al. 2010). The immature growth from first instar until adult emergence was 21–26 d, about two times longer than that in Malaysia (307.0 ± 3.0 h, Kumara et al. 2013). It is important to determine whether these development rate differences were caused by genetic variations of studied fly populations or by the physiological plasticity of species. Before these flies are used for forensic investigations, reliable data on their developmental rates under different environmental conditions and genetic backgrounds should be systematically collected.

Supplementary Data

Supplementary data are available at *Annals of the Entomological Society of America* online.

Acknowledgments

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