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Research Note

Identification of the forensically important beetles *Nicrophorus japonicus*, *Ptomascopus plagiatus* and *Silpha carinata* (Coleoptera: Silphidae) based on 16S rRNA gene in China

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Abstract. Sarcophagous beetles play an important role in estimating postmortem interval time (PMI) in the later stages decomposition of carcasses. However, the morphological similarity of beetles usually poses a challenge for forensic scientists within their routine work. As a supplementary to traditional morphological method, molecular genetics identification is simple and time-saving. A molecular identification method involving a 288-bp segment of the 16S ribosomal RNA (16S rRNA) gene from 15 beetles of Silphidae (Coleoptera), collected from 5 locations in 4 Chinese provinces, was evaluated. Phenogram analysis of the sequenced segments by the unweighted pairgroup method analysis (UPGMA) method showed that all specimens were properly assigned into four species with strong similarity, which indicated the possibility of separation congeneric species with the short 16S rRNA fragment. These results will be instrumental for implementation of the Chinese database of forensically relevant beetles.

Sarcophagous beetles play a pivotal role not only in estimating postmortem interval time (PMI) of dry human skeletal remains in the later stages decomposition of carcasses, but also the corruption, destruction, decomposition and posture changes of carcasses (Catts & Haskell, 1990). Thus, Coleoptera include species that could yield forensic information complementary to that obtained from Diptera (Schilthuizen *et al.*, 2011), such as Silphidae, Staphylinidae, Histeridae, and Dermestidae (Goff & Catts, 1990). The Silphidae are the first attracted group, followed by Staphylinidae and Histeridea. However, most of the research into the development of carrion-related insects has focused on maggots (fly larvae), and beetles have been neglected (Midgley & Villet, 2009), despite the fact that they are taxonomically and ecologically more diverse than Diptera (Schilthuizen *et al.*, 2011). The reason for the relative rarity of Coleoptera and many other necrobiont insect groups in the forensic literature is probably a consequence of the difficulty of identifying them from morphological characteristics (Krikken & Huijergts, 2001).

Nicrophorus japonicus, *Ptomascopus plagiatus*, and *Silpha carinata* are widespread silphid species associated with cadavers in China and are morphologically similar (Zheng & Gui, 1999). These species

have different developmental lifecycle timings and therefore, to utilize the correct developmental information, they need to be accurately identified (Guo *et al.*, 2010a). However, accurate morphologic insect identication relies on detailed examination which can require expert entomologists and are extremely difficult for almost all forensic scientists within their routine work (Saigusa *et al.*, 2005). Even for entomologist, insect checklist of one particular area should still be treated with caution, since beetles species of Silphidae are not cosmopolitan. Additionally, rearing of larvae to adult stage for accurately identifying work is a time-consuming and not always successful (Guo *et al.*, 2010b).

Mitochondrial DNA studies of Coleoptera of forensic importance have been conducted in several regions of the world (Davide *et al.*, 2001; David *et al.*, 2002; Arnoldi *et al.*, 2007), and even short fragments were proven to have discrimination power (Friedric *et al.*, 2003; Lee *et al.*, 2003; Suzuki *et al.*, 2002; Zhuang *et al.*, 2011) Some studies have indicated that the sequence of 16S rRNA accumulates mutations more rapidly than the nuclear rDNA genes (Simon *et al.*, 1994) and is potential as a discriminatory marker in identification of sarcophagous flies (Li *et al.*, 2010; Wang *et al.*, 2010). However, there are few published data on the forensically important beetles in China. The goal of this study is to determine the utility of 16S rRNA for identication of forensically important silphid species and accumulate genetic data for the database of sarcophagous beetles in China.

Fifteen dried adult silphid specimens, including three species (*N. japonicus*, *P. plagiatus* and *S. carinata*), were obtained from 5 districts of 4 provinces in China during the year 2010. Another two dried adult specimens of *Dermestes maculates* (De Geer, 1845) (Coleoptera: Dermestidae) were obtained from Datong (Shanxi) and Tianjin (Tianjin) in the year 2010. Collection of data for all specimens used in this study are listed in Table 1. All samples were collected using traps baited with animal remains (rabbit, dog, or pig). Samples were subsequently air-dried at room temperature or stored in 70% ethanol at -20°C. All adult beetles were identified using morphological keys (Lu & Wu, 2003) by entomologists in Hunan Agricultural University. The mtDNA of all samples were extracted using the CTAB method (Guo *et al.*, 2010c). A portion of 288-bp fragment of the

Table 1. Locality and reference data of specimens newly sequenced for this study

No.	Species	Accession No.	Locality and coordinates
1	*Nicrophorus japonicus*	JN112289	Chifeng, Inner Mongolia [118:38E, 43:24N]
2	(Harold, 1877)	JN112290	Chifeng, Inner Mongolia [118:38E, 43:24N]
3		JN112291	Chifeng, Inner Mongolia [118:38E, 43:24N]
4		JN112292	Chifeng, Inner Mongolia [118:38E, 43:24N]
5	*Ptomascopus plagiatus*	JN112294	Chifeng, Inner Mongolia [118:38E, 43:24N]
6	(Menetries, 1554)	JN112295	Chifeng, Inner Mongolia [118:38E, 43:24N]
7		JN112296	Chifeng, Inner Mongolia [118:38E, 43:24N]
8	*Silpha carinata*	HM051217	Chifeng, Inner Mongolia [118:38E, 43:24N]
9	(Herbst, 1783)	HM051218	Shijiazhuang, Hebei [114:26E, 38:03N]
10		HM051219	Yinchuan, Ningxia [106:16E, 38°27N]
11		HM051220	Hohhot, Inner Mongolia [111:38E, 40:48N]
12		HM051221	Hohhot, Inner Mongolia [111:38E, 40:48N]
13		JN112297	Datong, Shanxi [113:13E 40:07N]
14		JN112298	Datong, Shanxi [113:13E 40:07N]
15		JN112299	Datong, Shanxi [113:13E 40:07N]
16	*Dermestes maculates*	JF416547	Datong, Shanxi [113:13E 40:07N]
17	(De Geer, 1845)	JF416548	Tianjin, Tianjin [117:20E, 39:13N]

mitochondrial 16S rRNA gene was amplified and sequenced by using primers described by Li *et al.* (2010).

Vertical non-denaturing polyacrylamide gel electrophoresis was used to isolate PCR products, which were then purified using a QiaQuick PCR Purification Kit (Qiagen). Columns cycle sequencing was performed on both forward and reverse strands using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit by ABI PRISM 3730 (Applied Biosystems, Foster City, USA) with BigDye terminator v3.1 as the sequencing agent. Sequence chromatograms were edited, and discrepancies between forward and reverse sequences were resolved using Sequence Navigator (v1.01, Applied Biosystems, Foster City, USA). The sequences were non-protein coding and did not contain any indels, all resulting sequences in this study were aligned using Clustal W (http://www.ebi.ac.uk/Tools/msa/clustalw2/). And their accession numbers were listed in Table 1. Data analyses were conducted in MEGA4 (Tamura *et al.*, 2007). Similarities were calculated by the simple matching method, and a phenogram was constructed using the unweighted pairgroup method analysis (UPGMA) as reported in Sneath & Sokal (1973).

A total of three species of Silphidae were sequenced over 16S rRNA regions. 288-bp length fragment was successfully sequenced for all specimens. The alignments of all specimens considered in this study contained none indel. The resulting sequences were compared with the Coleoptera sequences in the NCBI web site by Blast function for distinguishing specie, and compared the results with traditional distinguishing method. The identification of Blast is well coincident with the latter. The sequences have been deposited in GenBank by Sequin (http://www.ncbi.nlm.nih.gov/Sequin/index.html). The Genbank accession numbers of all the 15 specimens are shown in Table 1.

All positions containing gaps and missing data within purpose sequences were eliminated from the dataset (Complete deletion option) at first. The set of outgroup was verified suitable for this study. Two specimens of *D. maculates* clustered together with a high supporting bootstrap of 99%, and they were clearly separated from the family Silphidae in phylogenetic tree (Figure 1).

Three key points could be summarized from the UPGMA tree graphic. Firstly, all individual sequences for a morphologically identified species clustered together with highbootstrap values (>95%) respectively. Meanwhile, separation between same species also existed in clades *S. carinata*. Secondly, sequences from Silphidae were well separated into three species without any mixture between them. Finally, in the carrion beetle clades, *N. japonicus* and *P. plagiatus* were clustered with high similarity at 98%, and separated from *S. carinata*. The divergence value between every two species within Silphidae family ranged from 0.13 to 0.33. The average of base substitutions per site for all specimens was 0.17.

The intraspecific divergence mean value was universally low in this test. The maximum one existed in clade *S. carinata* from the same cites Datong of China, which was at 0.01. The minimum mean value was 0.0, found both in clades *N. japonicus* and *P. plagiatus*. The interspecific variations between every two clades are all higher than 10%. The lowest value was found between *N. japonicus* and *P. plagiatus* at 13%. And the highest variation was located between *N. japonicus* and *S. carinata* at 33%.

The 288 bp 16S rRNA fragment was successfully sequenced for all the specimens. All beetles were rightly assigned into three species with monophyletic separation in the UPGMA tree (Fig. 1). Every group showed on the reconstructed phylogenetic tree was supported by high bootstrap value (>95%) and level of nucleotide divergence (≥13%) between groups. Output of this test demonstrated the effectiveness of partial 16S rRNA gene in identification of Silphidae. In the branch of Silphidae, *N. japonicus* grouped with *P. plagiatus* at high support rate 98%. And these two clustered with *S. carinata* together, this relationship coincided with phylogeny based on morphological characters. *Nicrophorus japonicus* and *P. plagiatus* belong to subfamily Nicrophorinae, and *S. carinata* belong to subfamily Silphinae.

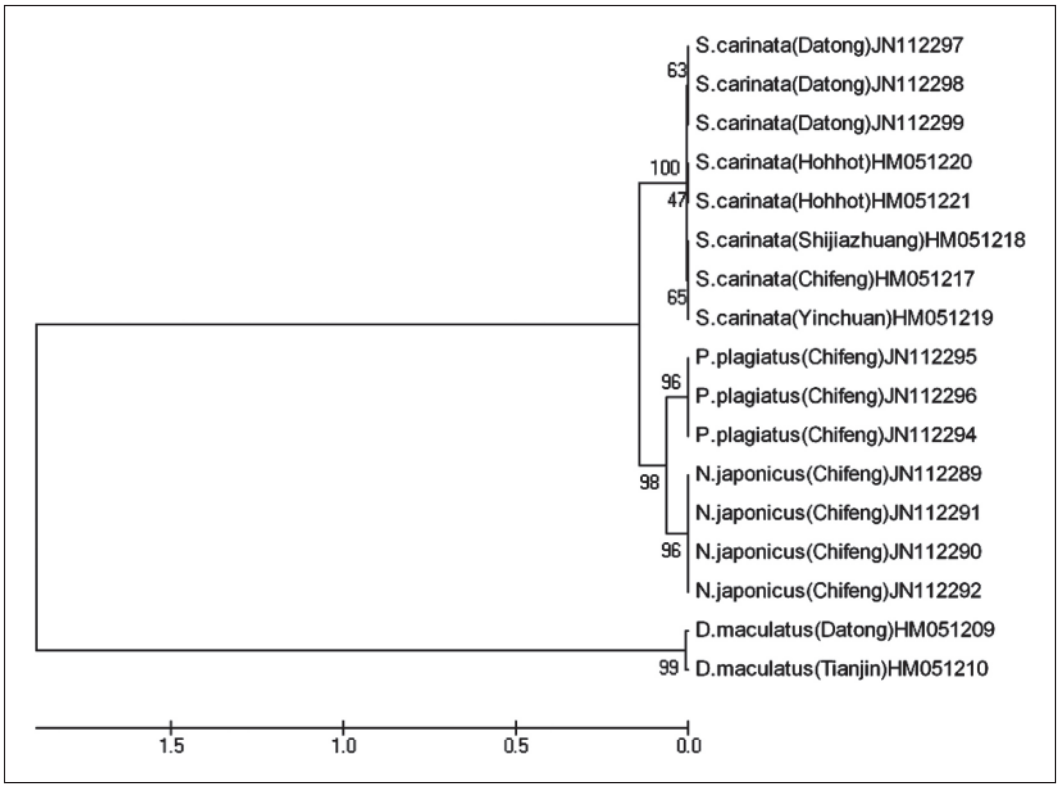

Figure 1. Phenogram from UPGMA clustering of correlation coefficients among 15 operating taxonomic units of Coleoptera in China. Morphological species identification, voucher ID and City are given in specimen label. Numbers on branches indicate the support value

The overall topological tree illustrated the taxonomic relationship between every two species, though low support value existing at high taxonomic level. Supporting rates at low taxonomic level (intraspecies) were universally high, compared with a sharp decline at subfamily level. 288-bp 16S rRNA sequence and small sample size might be insufficient for generation of deep phylogenetic information. But it is has apparently archived the main goal of the species identification of this study. Full-long sequences and larger samples were needed for more accurate results. Additionally, combined analysis (COI, COII, 16S rDNA and other genetic markers) and multidisciplinary phylogenetic computing model should be carried out in future study.

We assessed the 16S rRNA sequence as a potential marker for the identification of Silphids from China. The results indicated that the technique is as effective as morphological identification. Moreover, compared to other longer fragments, this shorter fragment is quicker, easier and more economical, which makes it in particular suitable for forensic applications. As a preliminary study of genetic identification of sarcophagid species, both the sample size and amplicon sizes were small. More samples from different locations and different regions of the COI and 16S rDNA genes need to be studied in the future.

The DNA-based method will be increasingly used in forensic entomology research and case work throughout the world. Designation of a piece of mtDNA fragment to identify and delineate the insect character is one of the key features of this method. In this study, the processes of obtaining the 288-bp 16S rRNA fragments were simple and the error of sequencing was reduced. Moreovere, the identification results of this study were comparable to the published papers using the longer fragments, which showed potentiality of the short fragments as the

discriminatory tool in identification of Silphids. The distribution and succession of Silphids from other parts of China should be studied and local database set up are strongly recommended in China.

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