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Identification of forensically significant beetles (Coleoptera: Staphylinoidae) based on COI gene in China

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Abstract: Accurate and fast identification of insect species is an initial and crucial step of using entomological evidence in forensic cases. Beetles of Coleoptera usually take large parts of taxonomic composition of the carrion-arthropod community and have important meaning for the estimation of postmortem interval (PMI). Traditional morphological taxonomy of beetles poses a great challenge for forensic investigators without entomological background. A cytochrome oxidase subunits one (COI) sequence on mitochondrial DNA with 816-bp length was studied for molecular identification of forensic significant beetles. Six beetle species in superfamily Staphylinoidae collected from four locations in China were sampled. All species were successfully separated with high support value, which indicated this partial COI fragment was sufficient for distinguishing these beetles.

Further step utilization of COI fragment in identification of forensically important beetles was also discussed.

Key Words: forensic entomology; coleoptera; staphylinoidae; specie identification; cytochrome oxidase subunits one

Forensic entomology is the study of insects and other arthropods, being used to solve litigation in civil and criminal cases. The value of forensic entomology for postmortem interval (PMI) in practical forensic work has been well demonstrated in many case studies [1-3], research articles [4-6] and books [7,8] by entomologists and forensic investigators. For studies of PMI, flies (Diptera) and beetles (Coleoptera) both fall into the most important groups among a great number of insects species related with carrion. But most researches have focused on flies, and beetles (Coleoptera) have been at under-emphasized. This situation should attribute to the assumption that Diptera locate corpses faster, and thus give a more accurate estimate of minimum PMI [9]. It is believed that Diptera had a peak during the initial stage, and the Coleoptera during the final decay process [10].

Actually, the duration of early instars on carrion is generally longer in Coleoptera than that of Diptera [9]. And the former usually takes a large parts of taxonomic composition of the carrion-arthropod community [10,11], which makes it especially important for the utility of PMI in forensic case.

The potential of beetles in investigation of forensic case and research work has been discussed in a few articles published recently, usually including beetle species from family Staphylinidae (rove beetles) [2,12,13], Silphidae (carrion beetles) [13-15]. These two families belong to the superfamily Staphylinoidae and bear morphological similarity [16]. Adults and larvae of these two families are common insect groups infesting on carrion. They feed on decayed carrion, flies maggot, or both [17,18].

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Such feeding behavior would evidently change carrion-insect fauna composition and outlook of carrion [8]. For the further utility of theses carrion correlated beetles in forensic entomology, accurate and fast species identification is needed. But morphological taxonomy of these beetles, especially for subspecies level and immature instars, poses a difficult problem for forensic investigators without entomological background. Even for entomologist, insect checklist of one particular area should still be treated with caution, since beetles species of Staphylinidae and Silphidae are not cosmopolitan.

Additionally, rearing of larvae to adult stage for accurately identifying work is a time-consuming and risk bearing (fail to keep insect alive) procedure.

DNA-based analysis has particular advantages in species distinguishing work of forensic important insects. Molecular analysis can achieve the goal of species identification and can be compared with morphological studies. Meanwhile, it provides information about phylogenetic development or evolutionary history [19]. Currently, mitochondrial DNA (mtDNA) has played a leading role in insect genomic studies [20]. Mitochondrial DNA (mtDNA) has been widely used for analyses of metazoan phylogenetic relationships at various axonomic levels, because of the lack of intermolecular recombination, its maternal inheritance, the relatively rapid evolutionary rate, and the large numbers of copies [19,21].

Cytochrome oxidase subunits I (COI) gene of different lengths and regions has been studied, and several short fragments were proven to have sufficient discrimination power, which makes it in particular suitable for species identification of Diptera [20,22,23]. Studies in arthropods showed that this gene evolve at an appropriate speed for reconstruction of insect phylogeny at different taxonomical levels [24-27]. However, to the best of our knowledge, few studies on genetic identification of beetles based on COI have been published yet. The present study aimed at exploring the utility of COI gene and discussing its usage in forensic important beetle species identification. Six species of Staphylinidae and Silphidae were identified and sampled. And for the distribution of staphylinid and silphid beetles are not worldwide, the results of this study would contribute to the construction of location-specific database of forensic related insects.

Material and methods

Specimens

Beetles specimens in this study were all collected on corpse-baited traps (rabbit and pig) during the years 2009 to 2010. Collecting area included four districts in three provinces of China. All specimens were firstly treated by air-drying method at room temperature. Then they were transferred into refrigerator as soon as possible for insuring its usability for both morphological and molecular study. Species identification was carefully performed by traditional morphological characters at laboratory condition. Eight specimens of three species respectively belong to Aleochara curtula, Creophilus maxillosus, and Platydracus sp. in family Staphylinidae. Others were Ptomascopus plagiatus, Thanatophilus sinuatus, Nicrophorus japonicus in family Silphidae. Details of collecting locations and results of identifying work were listed in Table 1.

DNA extraction

18 specimens of six species were sampled. Prior to DNA extraction, the abdomina of the investigated specimens were removed to prevent contamination by DNA of parasites or food remains and to confirm specific identification by examination of genitalia [24]. Muscle tissue of specimens forebodies were used for DNA extraction. The mtDNA of all samples were extracted using the CTAB method [28]. The remaining portions of voucher specimens were kept in refrigerator for future reference.

PCR process

The PCR reaction volume was 25µl, containing 1-5µl (20-40ng) of template DNA, 12.5µl 2×GoTaq® Green Master Mix (Promerga, Madison, WI, USA) (4µl dNTP (1mmol/ml), 1.0u Taq polymerase, 2.5µl 10×buffer (Mg2+1.5mmol/l)), 0.25-2.5µl each primer (10µM), Nuclease-Free.

Table 1. location and GenBank accession number of all specimens utilized in this study.

Species	Accession No.	Collecting locality	NO.
Silphidae			
Nicrophorus japonicus (Harold, 1877)	JN086491	Chifeng, Inner Mongolia (42°.26′N, 18°.89′E)	1
	JN086492	Chifeng, Inner Mongolia (42°.26′N, 18°.89′E)	2
	JN086493	Chifeng, Inner Mongolia (42°.26′N, 18°.89′E)	3
	JN086494	Chifeng, Inner Mongolia (42°.26′N, 18°.89′E)	4
Ptomascopus plagiatus (Menetries, 1554)	JN086495	Chifeng, Inner Mongolia (42°.26′N, 18°.89′E)	5
	JN086496	Chifeng, Inner Mongolia (42°.26′N, 18°.89′E)	6
	JN086497	Chifeng, Inner Mongolia (42°.26′N, 18°.89′E)	7
Thanatophilus sinuatus Fabrieius, 1775	JN086498	Qiqihaer, Heilongjiang (123° 47′N, 58° 20′E)	8
	JN086499	Qiqihaer, Heilongjiang (123° 47′N, 58° 20′E)	9
	JN086500	Qiqihaer, Heilongjiang (123° 47′N, 58° 20′E)	10
Staphylinidae			
*Creophilus maxillos*us (Linnaeus, 1758)	JN086502	Fuzhou, Fujian (25°.18′N, 118°.08′E)	11
	JN086501	Qiqihaer, Heilongjiang (123° 47′N, 58° 20′E)	12
	JN086503	Suihua, Heilongjiang (127° 46′N, 0° 38′E)	13
Aleochara curtula (Goeze, 1777)	JN086504	Qiqihaer, Heilongjiang (123° 47′N, 58° 20′E)	14
Platydracus sp.	JN086506	Qiqihaer, Heilongjiang (123° 47′N, 58° 20′E)	15
	JN086507	Qiqihaer, Heilongjiang (123° 47′N, 58° 20′E)	16
	JN086508	Qiqihaer, Heilongjiang (123° 47′N, 58° 20′E)	17
	JN086505	Suihua, Heilongjiang (127° 46′N, 0° 38′E)	18
Leiodidae			
Agathidium atrum (Paykull, 1798)	DQ155802	Genbank	19
Agathidium seminulum (Linnaeus, 1758)	DQ156011	Genbank	20

Water added to a total volume of 25 µl.

The following primers were used:

– C 1 - J - 2 1 8 3 5 ‘ - C A A C A T T T A T T T T G A T T T T T T T G G - 3 ’ , – T L 2 - N - 3 0 1 4 5 ‘ -TCAATTGCACTAATCTGCCATATTA-3’, according to Wells and Sperling [23].

A Perkin-Elmer 9600 thermocycler was used. For COII and 16S rRNA the following conditions for touch-down PCR were used: initial step at 94°C (4 min), continued for 32 cycles of 94°C (30s) and 55°C (45s for mtDNA annealing) and 72°C (30s). An elongation of PCR products by 72°C for 5min completed the reaction.

PCR products were purified using the QiaQuick PCR Purification Kit (Qiagen) following the manufacturer’s protocol. Columns cycle sequencing on both forward and reverse strands was analyzed using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit by ABI PRISM 3730 (Applied Biosystems) with Big Dye terminator v3.1 as sequencing agent. Results of sequences have been submitted to GenBank by Sequin (http://www.ncbi.nlm.nih.Gov/equin/index.html) and the accession numbers were listed in Table 1.

Sequencing analysis and phylogenetic tree construction

Based on the fact that sequences were conservation and without any indels, all resultant sequences studied here were aligned using Clustal W software package (http://www.clustal.org/download/current/). Obtained nucleotide sequences were compared with previously reported sequences

of Coleoptera on the NCBI web site via the BLAST function (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for verifying accurate of traditional morphological identification.

Before the phylogenetic analysis, Agathidium atrum (Paykull, 1798) and Agathidium seminulum (Linnaeus, 1758) in family Leiodidae were determined as outgroup. Leiodiae is sister group of Staphylinidae and Silphidae in superfamily Staphylinoidae. 820-bp length COI sequences of these two species were gained from NCBI Nucleotide Database (http://www.ncbi.nlm.nih.gov/nuccore). Accession number of A. seminulum is DQ156018 and A. atrum is DQ155802.

Phylogenetic analyses were conducted in MEGA4 by Neighbor-joining method [29]. Bootstrapping (n=1000) was carried out using MEGA too. Outgroup and purpose species were put together for phylogenetic tree reconstruction. The neighbor-joining (NJ) tree was based on COI gene sequence data analyzed with Kimura's two-parameter model. The consensus tree was computed, and the bootstrapped version resulted in the same tree as gained using neighbor-joining method.

Results

Alignment of sequences and Specimens identification

A total of six species in two families were sequenced over COI regions. 816-bp length fragment was successfully sequenced for all specimens. The alignments of all specimens considered in this study contained none indel and revealed 313 variable positions on 816-bp analyzed.

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/equin/index.html). The Genbank accession numbers of all the 18 specimens are shown in Table 1.

Phylogenetic tree construction

All positions containing gaps and missing data within purpose sequences were eliminated from the dataset (Complete deletion option) at first. The evolutionary history was inferred using the Neighbor-Joining method [30]. The optimal tree was shown with the sum of branch length (1.00569142). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood (ML) method [31] and were in the units of the number of base substitutions per site.

The set of outgroup was verified suitable for this study. A. seminulum and A. atrum clustered together with a high supporting bootstrap of 99%, and they were clearly separated from the family Silphidae, Staphylinidae in phylogenetic tree (Figure 1).

Three key points could be summarized from the N-J tree graphic. Firstly, all individual sequences for a morphologically identified species clustered together with 100% similarities respectively. Meanwhile, separation between same species also existed in clades T. sinuatus, C. maxilllosus and Platydracus sp. Secondly, sequences from Staphylinidae and Silphidae were well separated into these two families without any mixture between them. Finally, in the carrion beetle clades, N. japonicus and P. plagiatus were clustered with high similarity at 95%, and separated from T. sinuatus with lower percentage at 46%. An analogous situation can be detected in the rove beetles clades. C. maxilllosus and Platydracus sp. grouped with good support value at 88%, but companied by A. curtula with low similarity (55%).

Intraspecific and interspecific variation

The divergence value between every two specimens within Celeoptera family was listed in Table 2. Pairwise divergence between species was ranged from 0 to 0.3511. The average of base substitutions per site for all specimens was 0.247. The intraspecific divergence mean value was universally low in this test. The maximum one existed in clade C. maxillosus from the same cites Qiqihaer of China, which was at 0.0151 (Table 3).

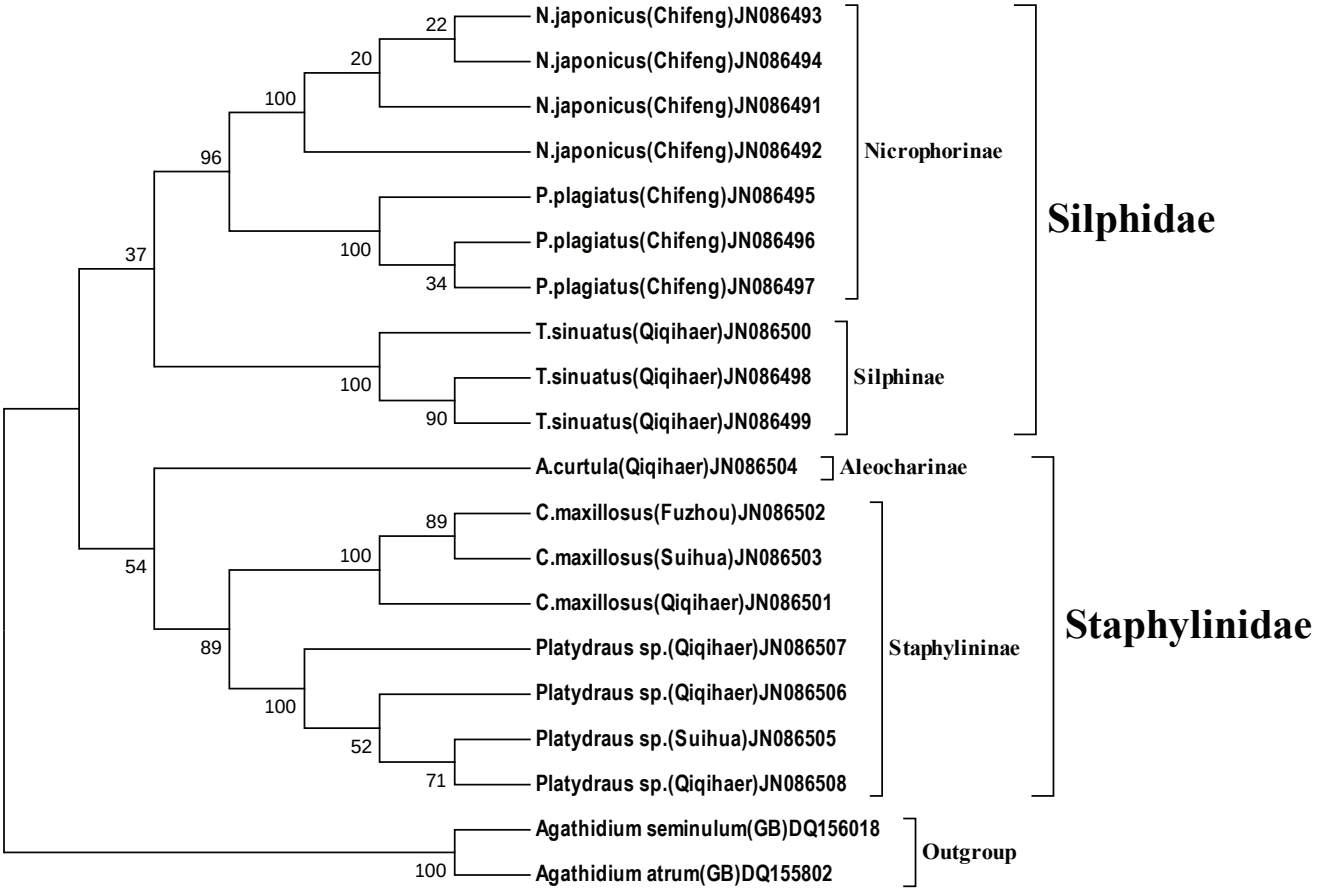

Figure 1. N-J tree displayed evolutionary relationships of 20 taxa operating taxonomic units of Coleoptera based on 816-bp region of the COI gene. Numbers on branches indicate the support value.

Table 2. Pirwise distances for the analyzed regions of 816-bp length of COI

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
[1]																		
[2]	0.0000																	
[3]	0.0000	0.0000																
[4]	0.0000	0.0000	0.0000															
[5]	0.1630	0.1630	0.1630	0.1630														
[6]	0.1630	0.1630	0.1630	0.1630	0.0000													
[7]	0.1630	0.1630	0.1630	0.1630	0.0000	0.0000												
[8]	0.2515	0.2515	0.2515	0.2515	0.2109	0.2109	0.2109											
[9]	0.2495	0.2495	0.2495	0.2495	0.2090	0.2090	0.2090	0.0012										
[10]	0.2455	0.2455	0.2455	0.2455	0.2071	0.2071	0.2071	0.0037	0.0025									
[11]	0.2621	0.2621	0.2621	0.2621	0.2331	0.2331	0.2331	0.2430	0.2450	0.2470								
[12]	0.2467	0.2467	0.2467	0.2467	0.2294	0.2294	0.2294	0.2447	0.2467	0.2487	0.0201							
[13]	0.2446	0.2446	0.2446	0.2446	0.2274	0.2274	0.2274	0.2426	0.2446	0.2466	0.0227	0.0025						
[14]	0.2179	0.2179	0.2179	0.2179	0.1840	0.1840	0.1840	0.2486	0.2466	0.2445	0.2058	0.2029	0.2035					
[15]	0.3511	0.3511	0.3511	0.3511	0.3403	0.3403	0.3403	0.3165	0.3143	0.3186	0.2677	0.2501	0.2509	0.2727				
[16]	0.3460	0.3460	0.3460	0.3460	0.3310	0.3310	0.3310	0.2956	0.2935	0.2977	0.2480	0.2312	0.2319	0.2548	0.0138			
[17]	0.3441	0.3441	0.3441	0.3441	0.3291	0.3291	0.3291	0.3088	0.3066	0.3109	0.2641	0.2468	0.2475	0.2671	0.0074	0.0163		
[18]	0.3507	0.3507	0.3507	0.3507	0.3400	0.3400	0.3400	0.3100	0.3079	0.3122	0.2628	0.2455	0.2462	0.2698	0.0087	0.0100	0.0125	

†The number in this Table is corresponding with Table 1.

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 16%. The lowest value was found between N. japonicus and P. plagiatus at 16.30%. And the highest variation was located between N. japonicus and Platydracus sp. at 34.80% (Table 3). In addition, Platydracus sp. displayed high variation value (>24%) with all others clades in this test.

Table 3. Calculated intraspecific and interspecific variations expressed as a percentage of the 816 bp COIdata.

No.	Species	NO	Mean[a] (%)	Interspecific variations mean (%)					
1	*Nicrophorus japonicus*	4	0	-					
2	*Ptomascopus plagiatus*	3	0	16.3	-				
3	*Thanatophilus sinuatus*	3	0.25	24.9	20.9	-			
4	*Creophilus maxillosus*	3	1.51	25.1	23.0	24.5	-		
5	*Aleochara curtula*	1	-	21.8	18.4	24.6	20.4	-	
6	*Platydracus* sp.	4	1.15	34.8	33.5	30.8	24.9	26.6	-

a mean= within group means

Discussion

Beetles, like families Silphidae, Staphylinidae, and Histeridae, were used in succession-based PMI estimations [13,32] and as area indicators [10]. In this present paper, sarcosaphagous beetles specimens were all obtained from families Silphidae and Staphylinidae. Both belong to the superfamily Staphylinoidae. Silphids were the first group attracted to carrions, followed by staphylinids and other beetles [33]. Species identification and phylogenetic work of each family were reported utilizing partial COI sequences [24,34,35]. Entomological evidence must be evaluated on a regional scale and the creation of local databases with referred ecological data for insect identification is strongly recommended [36,37]. This study evaluated the suitability of the 816 bp COI fragment for identification of these sarcosaphagous beetles, under experimental conditions prior to application in Chinese criminal investigations. The loci of 18 specimens were sequenced and deposited in GenBank to expand local databases.

The 816 bp COI fragment was successfully sequenced for all the specimens. All beetles were rightly assigned into six species with monophyletic separation in the N-J tree (Fig. 1). Every group showed on the reconstructed phylogenetic tree was supported by high bootstrap value (100%) and level of nucleotide divergence (>17%) between groups. Output of this test well demonstrated the effectiveness of partial COI gene in identification of Silphidae and Staphylinidae. In the branch of Silphidae, N. japonicus grouped with P. plagiatus at high support rate 97%. Although these two clustered with T. sinuatus together at a relatively low rate 37%, this relationship coincided with phylogeny based on morphological characters. N. japonicus and P. plagiatus belong to subfamily Nicrophorinae, and T. sinuatus belong to subfamily Silphinae. The Silphidae is composed of sister groups Nicrophorinae and Silphinae. The sister groups Silphidae and Staphylinidae were successfully separated into two major clusters.

The overall topological tree well illustrated the taxonomic relationship between every two species, though low support value existing at high taxonomic level. And it was coincident with the morphological classification which being shown in Figure 1. Supporting rates at low taxonomic level (genus and intraspecies) were universally high, compared with a sharp decline at subfamily level.

816-bp COI 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. And it could not be neglected that this result provided a valuable potential utilization of COI for deep research in Coleoptera phylogenetic analysis. Full-long sequences and larger samples were needed for more accurate results. Additionally, combined analysis (COI, COII, 16S rDNA and other genetic mark) and multidisciplinary phylogenetic computing model should be carried out in future study.

Data about regional variability could be evidence to infer the geographical origin of forensically important insect species. Number of collecting locations and sample size were very limit in this study, including 19 specimens from four cities. But it could also detect some clues of regional variability.

As it shown on the NJ-tree script, three individuals (from one same city) in group of T. sinuatus were separated into two clades with high support rate at 91%. C. maxillosus from Fuzhou and Suihuawere were clustered together with support rate at 92%, diverged from individual from Qiqihaer. Better results and revelation of intraspecific variation between different locations should be gained from wider area investigation in China.

Conclusion

Potential utility of COI region in identifying work of forensic important beetles in superfamily Staphylinoidae was studied. This 816-bp fragment of COI had sufficient discrimination power for Staphylinoidae species identification. For the future utilization of forensic related beetles as a tool in forensic investigations, new studies and exploration should be performed using full-length sequence of COI of large size beetles samples from a wider range of China.

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