SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

The Value of PCR-RFLP Molecular Markers for the Differentiation of Immature Stages of Two Necrophagous Flies (Diptera: Calliphoridae) of Potential Forensic Importance

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O Valor de Marcadores Moleculares do Tipo PCR-RFLP Para a Diferenciação de Estágios Imaturos de Duas Moscas Necrófagas (Diptera: Calliphoridae) de Potencial Importância Forense

RESUMO - A identificação de insetos envolvidos na decomposição de corpos é de suma importância para a estimativa do intervalo pós-morte (IPM) na ciência forense, particularmente quando o IPM baseia-se no ciclo de vida de insetos necrófagos. Entretanto, a identificação de algumas espécies de insetos, especialmente em seus estágios imaturos, pode ser complicada por muitos fatores, mesmo para taxonomistas bem treinados. Espécies do mesmo gênero como *Hemilucilia segmentaria* (Fabricius) e *Hemilucilia semidiaphana* (Rondani) (Diptera: Calliphoridae) são morfológica e comportamentalmente muito similares, mas diferem em suas taxas de crescimento e maturação. Essas espécies são abundantes em florestas, exclusivamente necrófagas e, portanto, de potencial importância médico-legal para a estimativa do IPM em eventos criminais que possam ocorrer nessas áreas. Neste estudo, avaliou-se a utilidade da reação em cadeia da polimerase, associada ao polimorfismo baseado no comprimento do fragmento de restrição (PCR-RFLP), na diferenciação dessas duas espécies. Duas regiões específicas do DNA mitocondrial, a subunidade I da Citocromo oxidase (COI) e a região controle (RC) foram amplificadas por PCR e digeridas usando endonucleases de restrição. Os padrões de clivagem gerados pelas endonucleases *Dra*I e *Ssp*I foram apropriados para diferenciar as duas espécies de *Hemilucilia*. Esse método pode ser útil no trabalho do entomologista forense na estimativa do IPM por fornecer rápida identificação, além disso, pode ser aplicado a qualquer estágio de vida dos insetos, incluindo imaturos, e em qualquer condição de preservação (mortos ou vivos).

PALAVRAS-CHAVE: Entomologia forense, DNA mitocondrial, *Hemilucilia segmentaria, Hemilucilia semidiaphana*

ABSTRACT - The identification of insect species involved in corpses decomposition is of particular importance in estimating the post-mortem interval (PMI) in forensic science, since the PMI is based on information about the life cycle of necrophagous insects. However, the identification of some insect species, especially in their immature stages, may be complicated by many factors, even for experienced taxonomists. Species of the same genus such as *Hemilucilia segmentaria* (Fabricius) and *H. semidiaphana* (Rondani) (Diptera: Calliphoridae) are morphologically and behaviorally very similar, but differ in their growth and maturation rates. These species are abundant in forests, exclusively necrophagous and, therefore, are of potential medicolegal importance for estimating the PMI in criminal events that would take place in those areas. In this study, we assessed the usefulness of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) in the differentiation of these two species. Two specific regions of mitochondrial DNA, the Cytochrome oxidase subunit I (COI) and the control region (CR), were amplified by PCR and digested using restriction endonucleases. The cleavage patterns generated by the endonucleases *Dra*I and *Ssp*I were suitable for differentiating the two *Hemilucilia* species. This method can be helpful for the forensic entomologist in estimating the PMI because it provides a fast identification, also making possible the use of the insect at any life stage, including immature specimens, regardless of the conditions of preservation (dead or live specimens).

KEY WORDS: Forensic entomology, mitochondrial DNA, *Hemilucilia segmentaria, Hemilucilia semidiaphana*

In recent years, the study of necrophagous insects in forensic entomology has provided useful information for criminal investigations (Nuorteva 1977, Smith 1986, Catts & Goff 1992). In particular, careful analysis of the insects and arthropods on cadavers allows to determine the time or post-mortem interval (PMI), site and mode or cause of death, as well as possible displacement of the cadaver. With such analysis, it is also possible to link suspects with the crime scene and to investigate the presence of toxic substances in the body (Catts & Haskell 1990).

For an accurate estimate of the PMI, correct identification of the insects associated with decomposition is essential, as is the knowledge of their life cycle and their biological and ecological characteristics (Erzinçlioglu 1983, Marchenko 2001). The insects that occur during cadaver decomposition are more difficult to identify to the species level as immature, especially early instars. The main reasons are the intraspecific variation and the inconspicous morphological differences among several species, and this fact makes the fast and accurate identification of the species difficult, even for a well-trained taxonomist (Liu & Greenberg 1989). Other factors may hamper identification, including immature specimens that may have to be reared to the adult stage for a more accurate identification, usually a time-consuming process; the collecting of only dead or damaged specimens that may lack diagnostic characters and prevent identification of the species (Harvey *et al.* 2003).

Recent surveys of necrophagous dipterans associated with animal carcasses and human corpses in the region of Campinas, São Paulo State, Southeastern Brazil, have identified several species of Calliphoridae that are involved in cadaver decomposition (Souza & Linhares 1997, Carvalho *et al.* 2000, Carvalho & Linhares 2001). The Calliphoridae is represented in Brazil by 36 endemic species belonging to 14 genera (Mello 2003), including the genus *Hemilucilia*.

The genus *Hemilucilia* contains six species, and four of them are found in Brazil but only two, *Hemilucilia segmentaria* (Fabricius) and *H. semidiaphana* (Rondani), have been collected in the area of this study. A third species *H. benoisti* (Séguy) was reported by Séguy (1925) occuring in São Paulo State, but has never been collected ever since. *H. segmentaria* and *H. semidiaphana* are found in Central and South America and are asynanthropic, because they are very abundant in natural forest environments, but occur in reduced number or are completely absent in urban areas. They are morphologically and behaviorally very similar, and use the same resources, but differ in their growth and maturation rates. A previous work done in the same area showed that the developmental time of *H. segmentaria* varied from 13 to 15 days, whereas in *H. semidiaphana* it lasted up to 20 days at 18-22°C (Carvalho & Linhares 2001). They were the most abundant fly species reared from pig carcasses in wooded areas of Southeastern Brazil (Carvalho *et al.* 2000, Carvalho & Linhares 2001) and are exclusively necrophagous, thus being of potential medico-legal importance because they are able to colonize human remains. However, data on arthropod fauna colonization of human cadavers in Brazilian forests is non-existent.

Some studies have used the morphology and the behavior aspects to identify insect species in the immature stages (Greenberg & Szyska 1984, Liu & Greenberg 1989, Amorim & Ribeiro 2001). More recently, techniques of molecular biology have been used to identify and differentiate insect species that are helpful in estimating the PMI (Sperling *et al.* 1994, Schroeder *et al.* 2003, Zehner *et al.* 2004).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis has been used to identify closely related species of forensic importance from different life stages (Sperling *et al.* 1994, Malgorn & Coquoz 1999). It is a fast, easy, low cost technique for routine diagnostic purposes (Litjens *et al.* 2001). In PCR-RFLP analysis, the detection of polymorphic patterns between individuals is based on differences in the sizes of the restriction fragments obtained from the amplified DNA region generated by a specific endonuclease or a multiple set of restriction enzymes.

Mitochondrial DNA (mtDNA) has been used as a suitable molecular marker because of the simple and uniform organization of the genome, the lack of recombination, and the high rate of nucleotide substitutions. In addition, the ability to retrieve genetic information efficiently from damaged or poorly preserved samples also facilitates the use of mtDNA markers in forensic investigations (Otranto & Stevens 2002).

In view of the difficulties associated with identification by morphological characteristics, in this study we used molecular markers of mtDNA obtained by PCR-RFLP to differentiate *H. segmentaria* and *H. semidiaphana*. These markers can be helpful to the forensic entomologist in estimating the PMI, since it provides a quick identification method which may be crucial for the conclusion of a criminal investigation, in addition to making the use of any life stage of the insects possible and regardless of the state of preservation (dead or live specimens).

Materials and Methods

Samples. For each *Hemilucilia* species, we analyzed 45 adults from São Paulo State, Brazil, including three different localities: Campinas, Jundiaí and Mogi Guaçu. Fifteen out of 45 adults of *H. semidiaphana* from Manaus (Amazonas State) were also collected and analyzed in this work. These flies were collected in the field from 2000 to 2002, using pig carcasses as baits. Only adult individuals were used in order to assure more accuracy in the identification process.

The specimens were identified morphologically based on Dear (1985) and then preserved at -20ºC and in absolute ethanol. The species were stored separately and were labeled according to their geographic region of origin.

DNA Extraction. Total genomic DNA of the whole insect was extracted using phenol-chloroform extraction and ethanol precipitation as described by Infante & Azeredo-Espin (1995). The DNA pellets were dried at room temperature and resuspended in 200 µl of sterile 1xTE buffer (1mM Tris-HCl, 0.1 mM EDTA, pH 7.0). All the extracted DNA samples were stored at -20ºC for further analysis by PCR-RFLP.

PCR Amplification. The Cytochrome oxidase subunit I (COI)

and control (CR) regions were analyzed. The COI region was amplified using the universal primers C1-J-2183 (5'-CAACATTTATTTTGATTTTTTGG-3') and TL2-N-3014 (5'-TCCAATGCACTAATCTGCCATATTA-3') (Simon *et al.* 1994) from the insect mtDNA oligonucleotide set, and the CR was amplified using the primers TI-N-4 (5'-ATTTACCCTATCAAGGTAA-3') (Simon *et al.* 1994) and CMEG-AR (5'-AATCCAGTTAAGAATATCAT-3') (Lessinger *et al.* 2004).

The amplifications were done in 25-µl reaction volumes containing 10xPCR buffer, 0.2 mM of dNTPs, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 1.25 units of *Taq* DNA polymerase (Invitrogen) and 2-4 µl of extracted DNA. For the COI region, the temperature cycling included an initial denaturing step of 3 min at 94ºC followed by 34 cycles of 1 min at 94ºC, an annealing step of 1 min at 42ºC and an extension step of 2 min at 72ºC. The last cycle included an extended elongation step of 7 min at 72ºC. For amplification of CR, an initial denaturing step of 4 min at 94ºC was followed by 34 cycles of 1 min at 94ºC, 1 min at 45ºC and a 2-min extension at 60ºC. The last cycle included an extended elongation step of 10 min at 60ºC. A lower extension temperature was used because of the high A+T composition of this region (Lessinger & Azeredo-Espin 2000).

The PCR products were separated by electrophoresis on 1% agarose gels (Sigma, USA) in 1xTAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.0) at 80V, stained with ethidium bromide (EtBr) and photographed using a Polaroid system. The sizes of the amplified fragments were estimated by comparison with a ÖX174/*Hae* III and a 1kb DNA Ladder used as the molecular weight standard.

Digestion with Restriction Endonucleases (RFLP). The following restriction enzymes were used to digest the PCR products: *Dra*I, *Eco*RV, *Ssp*I and *Taq*I. A 3-µl aliquot of each PCR product was digested in a 15-µl reaction mixture according to the manufacturer's instructions (Invitrogen). Following incubation at 37ºC for 4h, the digested products were separated by electrophoresis on 2% agarose gels at 60V, stained with EtBr, and on non-denaturing 10% polyacrylamide gels at 90V, where the bands were visualized by silver staining. Agarose gels were used for screening the restriction profiles for all *H. segmentaria* and *H. semidiaphana* samples. Just a few individuals were screened on polyacrylamide gels in order to provide a better resolution of fragments with less than 200bp. The size of the restriction fragments was estimated graphically using a Kodak® Electrophoresis Documentation and Analysis System (EDAS 290) by comparison with a ÖX174/*Hae* III and a 1kb DNA Ladder molecular weight standard.

Results

Amplified products of approximately 880 bp were recovered from the COI region of both *Hemilucilia* species. For the CR, products of approximately 560 bp and 450 bp were amplified for *H. segmentaria* and *H. semidiaphana*, respectively (Fig. 1), providing a preliminary marker for species differentiation, since this character (size of the CR-PCR product) was conserved within each species.

The amplified sequences were initially analyzed with four restriction endonucleases to identify potential diagnostic sites. Among the restriction profiles of the PCR products, CR digestion with *Dra*I and *Ssp*I and COI digestion with *Ssp*I produced efficient diagnostic restriction patterns for the unambiguous differentiation of these two *Hemilucilia* species (Table 1 and Fig. 2). The other PCR products were not used for further analysis because they had no diagnostic value.

The preliminary characterization of *Eco*RV restriction sites revealed the occurrence of polymorphic patterns in the COI region (A and B, see Table 1) and no occurrence of restriction sites in the CR of both *Hemilucilia* species, which resulted in ambiguous taxonomic identification. Despite of the presence of two restriction sites for *Taq*I in the COI region, both *Hemilucilia* species shared the same monomorphic pattern, which resulted in lack of discrimination. No sites for *Taq*I were found in the CR of these species.

Discussion

The molecular identification of immature or adult flies of closely related species has played a key role in forensic entomology, in the last ten years. As shown here, PCR-RFLP markers successfully differentiated adults of *H. segmentaria* and *H. semidiaphana*, two morphologically related calliphorid species of potential forensic importance in Brazil, which coexist in the same area and are not easily distinguished by morphological criteria in their immature stages, though it is possible to separate these species from others that breed in carrion. This experiment was carried through with adult individuals but the technique can be used with the immature ones.

The size of the CR in insects varies considerably in different taxa and even between closely related taxa (Zhang

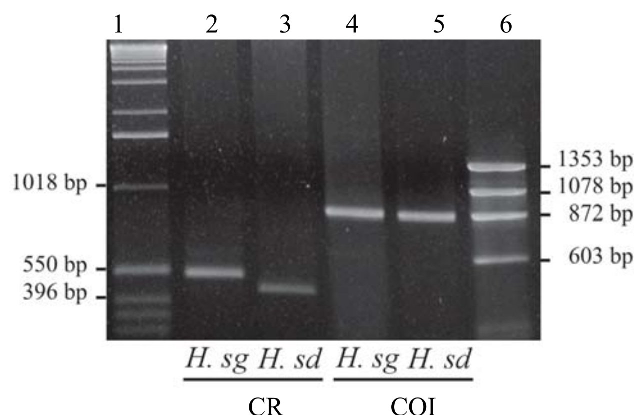

Figure 1. Control region (CR) and COI amplification products of *Hemilucilia segmentaria* (*Hsg*) and *H. semidiaphana* (*Hsd*). Columns: 1, 1kb DNA Ladder molecular size standard; 2-3, CR amplicons of *Hsg* and *Hsd*, respectively; 4-5: COI amplicons of *Hsg* and *Hsd*, respectively; 6: ÖX174/*Hae* III molecular size standard.

Table 1. Characterization of the restriction sites in *Hemilucilia segmentaria* and *H. semidiaphana*.

mtDNA regions	Restriction enzymes	*H. segmentaria* (n = 45)	*H. semidiaphana* (n = 60)
COI	*Dra*I [1]	Not digested	Not digested
	*Eco*RV [1]	520 / 360 (A) [3] Not digested (B)	520 / 360 (A) Not digested (B)
	*Ssp*I [2]	550 / 330	Not digested
	*Taq*I [1]	420 / 230 / 190	420 / 230 / 190
CR	*Dra*I [2]	370 / 100 / 90	Not digested
	*Eco*RV [1]	Not digested	Not digested
	*Ssp*I [2]	410 / 85 / 65	290 / 100 / 65

[1]These enzymes were tested in only six individuals of each species during an initial screening of potential diagnostic restriction sites; [2]indicate diagnostic PCR-RFLP patterns useful for species differentiation; [3](A) and (B) denote polymorphic patterns for *Eco*RV.

& Hewitt 1997). Although size variation in the CR amplicons provides a preliminary diagnosis for the differentiation of both *Hemilucilia* species, we cannot recommend the method without the digestion of the PCR products as an adequate means to distinguish the species because more individuals may be needed to confirm the degree of intraspecific variation for each species. According to data from other necrophagous flies, the intraspecific variation occurs but does not exceed 1% (Wallman & Donnellan 2001, Wells & Sperling 2001). Particularly when using PCR or PCR-RFLP for attempts at species identification (Sperling *et al*. 1994, Schroeder *et al.* 2003) intraspecific variation has to be taken into account. Single mutation events could alter a restriction site or a locus e.g., thus generating the possibility of false exclusions (Zehner *et al*. 1998).

Compared to the intraspecific variation, the interspecific differences are large enough to allow an unambiguous association of a species to a certain molecular characteristics (Sperling *et al*. 1994). The growing usefulness of the CR as a source of species-specific markers in PCR-RFLP for the identification of *Cochliomyia hominivorax* and *C. macellaria* in Brazil (Litjens *et al.* 2001) and of species of Culicidae in Argentina (Duenas *et al.* 2002) has been demonstrated. Because the product sizes obtained using CR primers were identical within species and consistently different between species, the CR marker shows reliability as a marker.

As expected for mitochondrial coding genes, the COI amplicon had a conserved size in both *Hemilucilia* species (880 bp), a finding that fit in with the structural model of COI genes in insects (Lunt *et al.* 1996). COI has proven to be particularly suitable as a molecular marker for taxonomic and evolutionary studies in insects (Lunt *et al.* 1996, Caterino *et al*. 2000). Indeed, PCR-RFLP analysis of COI has provided diagnostic markers for the identification of species in many different groups (Simon *et al.* 1993).

In this study, both the COI and CR PCR-RFLP allowed accurate differentiation between adults of these two *Hemilucilia* species, whether through independent analysis (digestion of one specific region) or combined analysis (multiple digestions in both regions). Comparisons of digested *versus* non-digested patterns (COI/*Ssp*I and CR/*Dra*I) can also be applied for this purpose. If the specimens are well preserved, the locus COI can be used to differentiate the two species, but if the specimens are not well preserved, the CR could provide an adequate differentiating element because of better recovering of short PCR products from poorly preserved samples. In addition to that, amplification of CR amplicons may represent a more reliable strategy for forensic analysis since *Ssp*I digest the CR amplicon in both *Hemilucilia* species, thus providing a better differentiation marker.

The restriction profiles for all *H. segmentaria* and *H. semidiaphana* samples were screened using agarose gels, though the polyacrylamide gels provide better resolution for small fragments. This doesn't impair its use as a means of differentiation because the larger fragments are easily distinguishable in any system and there is only a small loss of resolution for fragments smaller than 200 bp. This decision was made in order to make available a low-cost method that could be easily used by forensic technicians in a laboratory routine.

The recommended diagnostic restriction patterns here were monomorphic for all samples from two Brazilian populations (São Paulo and Amazonas) that were analyzed. The conservation of PCR-RFLP markers among the analyzed individuals indicates that this approach provides a reliable means of species differentiation.

The results of this work confirm the usefulness of molecular markers in differentiating and identifying flies of forensic importance and represent one more step toward a more detailed investigation of the molecular identification of flies. Such approaches could prove useful in the routine analysis of endemic Diptera species of forensic importance in Brazil. However, for specimens from Central America or other regions of South America, further studies are needed.

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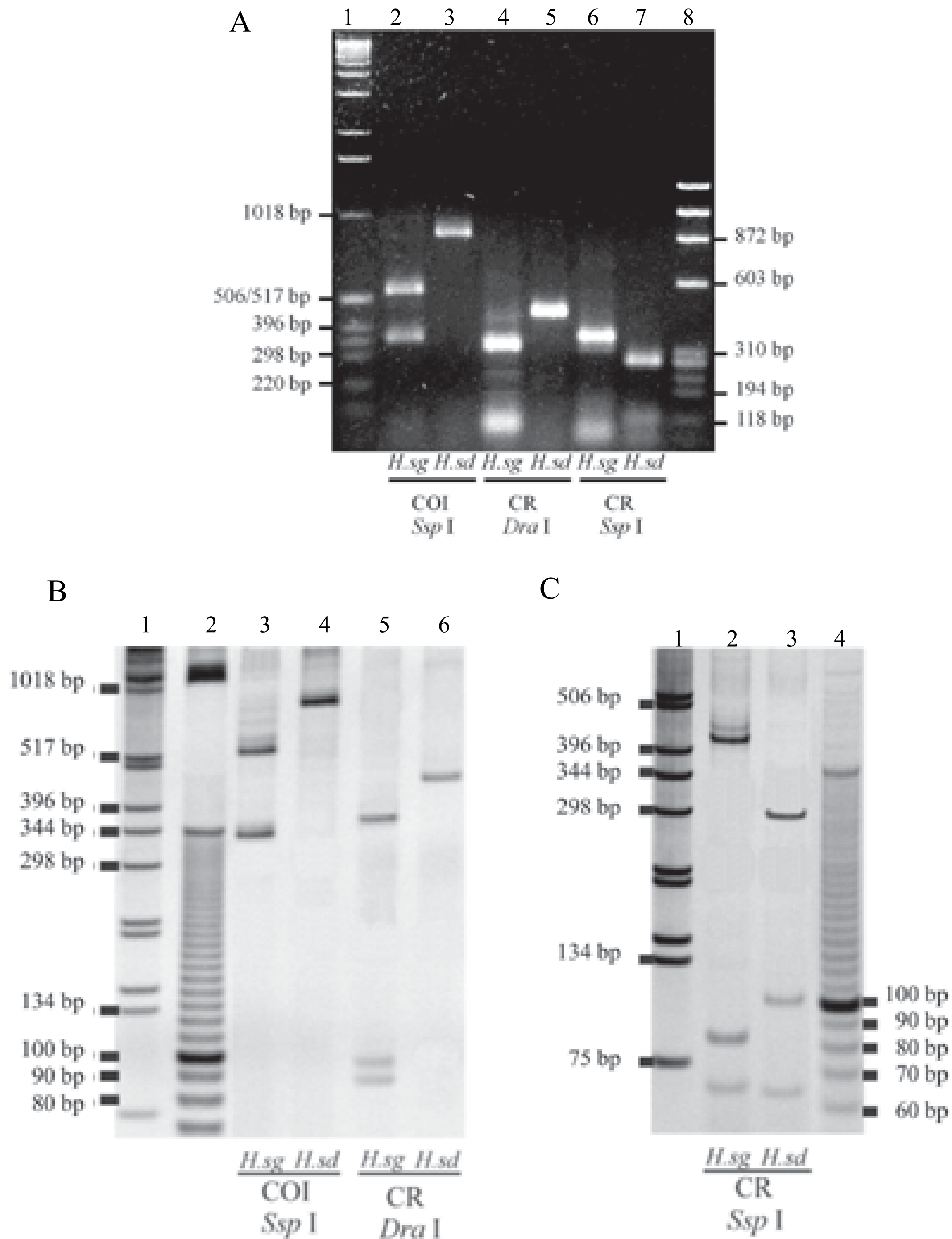

Figure 2. Diagnostic PCR-RFLP patterns of COI and control region (CR) amplicons for *Hemilucilia segmentaria* (*Hsg*) and *H. semidiaphana* (*Hsd*) in *A* 2% agarose and in *B* and *C* 10% acrilamide gels. In *A*: Columns: 1, 1kb DNA Ladder molecular size standard; 2-3, digestion of the COI region using the enzyme *Ssp*I for *Hsg* and *Hsd,* respectively; 4-7, digestion of the CR for *Hsg* and *Hsd,* respectively; 4-5: enzyme *Dra*I; 6-7: enzyme *Ssp*I; 8: ÖX174/*Hae* III molecular size standard. In *B*: Columns: 1, 1kb DNA Ladder molecular size standard; 2, 10bp DNA Ladder molecular size standard; 3-4, digestion of the COI region using the enzyme *Ssp*I for *Hsg* and *Hsd,* respectively; 5-6, digestion of the CR using the enzyme *Dra*I for *Hsg* and *Hsd,* respectively. In *C*: Columns: 1, 1kb DNA Ladder molecular size standard; 2-3, digestion of the CR using the enzyme *Ssp*I for *Hsg* and *Hsd,* respectively; 4, 10bp DNA Ladder molecular size standard.

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