Original Article

Discrimination of *Paederus fuscipes* and *Paederus littoralis* by mtDNA-COI PCR-RFLP

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Abstract

Background: Linear dermatitis is endemic in Iran where most cases occur in the Caspian Sea coast and Fars province. The disease is caused by beetles of the genus *Paederus* which are active from early spring to beginning of autumn although its incidence rises from May to August. The classic taxonomy of *Paederus* spp. is based on the male genitalia that is very complex and needs expertise. In this study, we report a DNA-based method to discriminate *Paederus fuscipes* and *Paederus littoralis* (=syn: *P. lenkoranus*, *P. ilsae*).

Methods: Type specimens were collected from north and south of Iran. Molecular typing of the species was performed using restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR)-amplified fragments of mtDNA-COI.

Results: Sequence analyses of the data obtained in this study showed significant DNA polymorphisms. There were 89 substitutions between COI sequences of the two species. The mtDNA-COI fragment comprises several useful species-specific restriction sites comprising *Hae*III that could result in distinctively different species-specific PCR–RFLP profiles. The *Hae*III enzyme cuts the 872 bp PCR amplicon of *P. littoralis* into 737 and 100 bp and two small nonvisible bands whereas it does not cut *P. fuscipes* amplicon into fragments.

Conclusion: This study demonstrates that molecular typing is useful method and allows one to differentiate between two species and is recommended for discrimination of other *Paederus* species, which morphologically are indistinguishable or very difficult to be distinguished.

Keywords: *Paederus*, Linear dermatitis, mtDNA- COI, PCR-RFLP, Molecular typing

Introduction

The genus *Paederus* Fabricius (Staphylinidae: Coleoptera) is represented by 622 species worldwide and 85 species and subspecies in the Palaearctic region. The hemolymph of some species within the genus *Paederus* is nuisance as, once released, it causes linear dermatitis and conjunctivitis in humans. The symptoms are due to a toxic amide substance, which has been named Pederin (Pavan and Bo 1953) and makes up approximately 0.025% of an insect's weight (for *P. fuscipes*). Most cases of linear dermatitis in Iran occur in the Caspian Sea shoreline and Fars in south of Iran (Nikbakhtzadeh and Tirgari 2008).

Systematics of *Paederus* beetles at species

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level is rather difficult and is based on the morphology of male primary and secondary sexual characters (Coiffait 1982). This makes them difficult to identify and limits their study and management. Consequently, this has made a very complicated history for *Paederus* taxonomy and has changed it dramatically and some species are treated as synonyms of each other and or downgraded to a single subspecies/species (Nikbakhtzadeh et al. 2012 and references herein). For example *P. lenkoranus* Scheerpeltz (Scheerpeltz 1957) *P. littoralis ilsae* (Bernhauer 1932) and *P. ilsae* (Coiffait 1982) have been recently considered synonymous (Nikbakhtzade 2012). *Paederus fuscipes fuscipes* Curtis (1826) was formerly known by the synonymous names of *P. iliensis* Coiffait (1970) and *P. kalalovae* Roubal (1932). These older species are downgraded to a single subspecies of *P. fuscipes* in the current systematics of Staphylinidae.

Previous studies on the fauna, geographical distribution, ecology and medical importance of *Paederus* beetles in Iran revealed presence of 14 *Paederus* species or subspecies in the country (Nikbakhtzadeh et al. 2012). However, due to difficulties in *Paederus* species discrimination, different lists of species have been reported for an identical region. In north of Iran, for example, Janbaksh and Ardalan (1977) reported three species of *P. fuscipes*, *P. pietschmanni* (synonym of *P. mesopotamicus*), and *P. spectabilis* in Mazandaran province at the Caspian Sea coast. Majidi-Shad et al. (1989) reported three species of *P. fuscipes*, *P. riparius*, and *P. littoralis* from the same region**.** Later on, *P. fuscipes*, *P. kalalovae* and *P. balcanicus* were reported from the province by Nikbakhtzadeh and Tirgari (2008). Finally, Nikbakhtzadeh et al. (2012) reported three species of *P. fuscipes*, *P. balachowskyi* (synonym of *P. mesopotamicus*), and *P. balcanicus* from the same area. In southern part, Nikbakhtzadeh (2002, 2008) reported *P. ilsae* and *P. iliensis* Coiffait from Fars Province. In 2012, *P. littoralis ilsae* Bernhauer (=syn: *P. lenkoranus* Scheerpeltz, *P. ilsae*) and *P. fuscipes fuscipes* Curtis (=syn: *P. iliensis* Coiffait, *P. kalalovae* Roubal) were reported from that area.

Recent advances in DNA-based technology have made a wide range of molecular characteristics and markers available for taxonomic and systematic studies of insects. One region of the insect genome including beetles that has received particular attention is the mitochondrial DNA (mtDNA). Mitochondrial DNA with a fast mutation rate has significant variation in sequences between species. A 658 bp region (the Folmer region) of the mitochondrial cytochrome c oxidase subunit I (COI) gene was proposed as a potential barcode (Hebert et al. 2003).

The mtDNA genes have many advantages including a relatively fast mutation rate, easy to use and known PCR primers. The discrepancy and inconsistency in the number of species in Iran plus difficulties in systematics of *Paederus* species encouraged us to test sequence variation of mtDNA-COI, to introduce a molecular marker to discriminate *P. fuscipes* and *P. littoralis*, the two sympatric and common species in the Mediterranean basin including southern part of Iran.

Materials and Methods

Paederus specimens and morphological identification

Adult specimens were collected by aspirator on rice plants and weeds in early morning or afternoon and under clays at hot hours of day time. Collection of *P. fuscipes* and *P. littoralis* were performed mostly in rice fields in various locations of Mazanderan and Fars Province during the growing season from May to August 2011. The specimens were preserved in 70% ethanol and were sent to the insect molecular biology laboratory of the School of Public Health, Tehran Univer-

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sity of Medical Sciences, Iran for molecular characterization. The pictorial key of Coiffait (1982) was used to identify the specimens to genus level. The specimens were then identified to species level based on the habits and morphology of male primary and secondary sexual characters by the Turkish expert, S. Anlaş.

DNA extraction

Paederus specimen representative different populations of both species were selected for DNA analysis. Total genomic DNA was extracted from total body of individual samples using DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The specimens were frozen prior to DNA extraction and then grinded in the Kit supplied buffer and extraction followed according to the manufacturer's directions.

DNA amplification

The COI (Cytochrome Oxidase subunit 1) region of mtDNA gene (mitochondrial) was amplified with the primer pair c1-j-2183 (5′-caacatttattttgatttttttgg-3′) and tl-2-n-3014 (5′-ccattgcatctgccatatta-3′). These primers have already been introduced by Simon et al. (1994) and used for some Colleopteran. PCR amplification reaction conditions were: 5 µl 10× PCR-Buffer, 120 µM of each dNTPs, 50 pmol of each primer, 2 µl (about 100 ng) of template DNA, and 2.5 U of *Taq* polymerase (Sinaclone, Iran) in a 25 µl reaction volume. PCR amplification was performed with an Eppendorf thermal cycler (Germany). The cycling parameters were: 2 min initial denaturation at 94 °C followed by 5 cycles of 30 sec at 94 °C, annealing at 45 °C for 40 sec and extension at 72 °C for 1 min and 35 cycles of 94 °C at 30 sec, annealing at 51 °C for 40 sec and extension at 72 °C for 1 min. The final extension step was 72 °C for 10 min (www.dnabarcodes2011.org). Double distilled water was used as negative and well-characterized DNA samples were used as positive controls.

Sequencing and PCR-RFLP

The PCR products of COI fragment were purified from gels by using a gel purification kit, and subjected to sequencing. Sequencing was performed using an ABI 3730 sequencer machine by Bioneer (South Korea). The resultant sequences were checked to correct ambiguities. Homologies with the available sequence data in GenBank was checked by using basic local alignment search tool (BLAST) analysis software (www.ncbi.nlm.nih.gov/BLAST). The COI sequences obtained in this study was checked to obtain its physical map and to select restriction enzymes by using the Nebcutter program (Vincze et al. 2003). Restriction enzymes were selected based on their positions (beginning, middle, and last part of PCR product), profiles, costs, and availabilities in the market. Digestion of PCR products was performed in 25 µL of a solution containing 15 µL of PCR product mixed with 2.5 µL of enzyme buffers and 5 units of *Hae*III restriction enzyme overlaid with two drops of mineral oil. The mixture was incubated at the temperature recommended by the enzyme supplier. An aliquot (14 µL) of the digestion product was mixed with 6 µL of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol), loaded on to a 1% agarose gel, and subjected to electrophoresis. Gels were stained with ethidium bromide (2 mg/mL) and the RFLP profiles were visualized under ultraviolet light.

Results

Species identification and PCR-RFLP

In this study, a total of 154 adult *Paederus* specimens were collected from the two Iranian provinces. The *P. littoralis* specimens and a subset of the morphologically-identified *P. fuscipes* were subjected to mtDNA-

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COI PCR amplification followed by PCR-direct sequencing. Sequencing was performed for both strands and the consensus data were submitted to GenBank with accession numbers (ANs): KF724713, KF724714, KM07 1620, KM071621, KM071622, KM071623, KM071624, KM071625, KM071626, KM07 1627, KM071628 and KM071629. There were minor intra-species sequence variations between individuals of both species; however, there were considerable DNA polymorphisms between the two species. Subsequent sequence analysis confirmed identical 872 bp PCR amplicons for both species. An alignment of the 805 bp of the COI region from these sequences is shown in Fig. 1. The amount of sequence similarity between the two species was 88% and 98% at DNA and amino acid level respectively. Subsequently, a BLAST search on the sequences revealed that there were corresponding COI sequences for *P. fuscipes* and *P. littoralis* species in Genbank respectively with 96% and 98% maximum identity. Sequence analysis of the COI fragments of both species revealed a number restriction sites for discrimination of the species. Among them, an appropriate restriction site for *Hae*III (GG↓CC) gave two distinct profiles of 737/100/23/12 bp fragments for *P. littoralis* and an intact 872 bp for *P. fuscipes* (Fig. 2). Of course, for *P. littoralis*, only the two 737 and 100 bp bands were visible because the small ones (23 and 12 bp) were not visible in the gel.

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P.fuscipes     TTACCTGGATTTGGAATAATTTCTCATATTATCTCTTACAGAAGTGGAAAACAAGAAACT   60
P.littoralis   TTACCAGGATTTGGAATAATTTCTCATATCATTTCTTACAGAAGAGGGAAACAAGAAACT   60
               ***** ********************** ** *********** ** ************

P.fuscipes     TTTGGAGCAATTGGGATAATTTATGCTATGCTTGCAATTGGTTTATTAGGTTTTATTGTA   120
P.littoralis   TTTGGGGCAATAGGAATAATTTATGCTATATTAGCAATTGGTTTATTAGGTTTTATTGTT   120
               ***** ***** ** **************  * *************************

P.fuscipes     TGAGCTCATCATATATTTACTGTCGGAATGGACATTGATACTCGAGCTTACTTTACATCA   180
P.littoralis   TGAGCCCATCATATATTTACAGTAGGTATAGATATTGATACACGAGCTTATTTTACCTCA   180
               ***** ************** ** ** ** ** ******** ******** ***** ***

P.fuscipes     GCCACAATAGTAATTGCTGTTCCAACTGGAATTAAGGTTTTTAGATGAATAGGAACAATT   240
P.littoralis   GCAACTATAGTAATTGCTGTACCTACAGGAATTAAAGTATTTAGTTGAATAGCAACAATT   240
               ** ** ************** ** ** ******** ** ***** ******* *******

P.fuscipes     TATGGTGGAAATTTAAATTTTAGCCCACCAATAATCTGAAGTTTAGGGTTTGTATTTTTA   300
P.littoralis   TATGGAGGAAATTTAAATTTTAGACCCCCAATAATTTGAAGATTAGGTTTTGTATTTTTA   300
               ***** ***************** ** ******** ***** ***** ************

P.fuscipes     TTTACTGTCGGAGGATTAACTGGAGTAATTTTAGCTAATTCATCAATTGATATTGTATTA   360
P.littoralis   TTTACTGTAGGAGGATTAACAGGAGTGATTTTAGCTAATTCATCAATTGATATTGTTTTA   360
               ******** *********** ***** ****************************** ***

P.fuscipes     CATGACACATATTATGTTGTAGCTCATTTTCATTATGTCTTATCAATAGGGGCAGTATTT   420
P.littoralis   CATGATACTTACTATGTAGTAGCTCACTTTCACTATGTTTTATCAATAGGAGCTGTTTTT   420
               ***** ** ** ***** ******** ***** ***** ************ ** ** ***

P.fuscipes     GCTATTATAGCAGGGTTAGTACAATGATACCCAATATTTATTGGGTTAATATTAAACGAA   480
P.littoralis   GCTATTATAGCAGGATTAGTGCAATGATTTCCAATATTCATTGGATTAATATTAAATGAA   480
               ************** ***** *******  ******** ***** *********** ***

P.fuscipes     AAATATTTAAAAATTCAATTTTTAATTATATTTATTGGGGTAAATTTAACTTTTTTCCCT   540
P.littoralis   AAATACTTAAAAATCCAATTTTTAATTATATTTATTGGGGTAAATTTAACATTTTTTCCT   540
               ***** ******** ************************************ ***** ***

P.fuscipes     CAACATTTTTTAGGTTTATCAGGAATACCACGTCGATATTCAGATTACCCAGATGCTTAC   600
P.littoralis   CAACATTTTTTAGGATTATCAGGAATACCTCGTCGATACTCAGATTACCCTGATGCTTAT   600
               ************** ************** ********* *********** ********

P.fuscipes     ACAATATGAAATGTAATTTCATCTATTGGATCAATAATTTCATTTATTGGAATTATATTC   660
P.littoralis   ACAATATGGAACGTAATTTCATCTATTGGATCAATAATTTCATTTATTGGAATTATATTC   660
               ******** ** ********************************************

P.fuscipes     TTTTTATGAATTATTTGAGAAAGATTTATTTCAATACGAAAAATTATTGGAGCTCCAATC   720
P.littoralis   TTTTTATGAATTATTTGAGAAAGATTTATTTCTATACGAAAAATTATTGGGGCCCCAATT   720
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Fig. 1. Continued…

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P.fuscipes      CCACCAACAGCATTAGAATGAATGCATTCATACCCCCCATCAGAACACACCTATTCTGAG   780
P.littoralis    CCCCCAACTGCTTTAGAATGGATACATTCTTATCCACCCTCAGAGCATACTTACTCAGAA   780
                ** ***** ** ******** ** ***** ** ** ** ***** ** ** ** ** **

P.fuscipes      TTGCCTTTTATAACAATTAAGTTCT                                     805
P.littoralis    TTACCTTTTATAACAATTAAATTCT                                     805
                ** ***************** ****
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Fig. 1. Alignment of 752 bp of mtDNA-COI sequences of *Paederus fuscipes* and *Paederus littoralis*. Stars show the conserved position and gaps or dots indicate substitutions. The *Hae*III restriction site (GGCC) for *P. littoralis* is shown at position of 705–708.

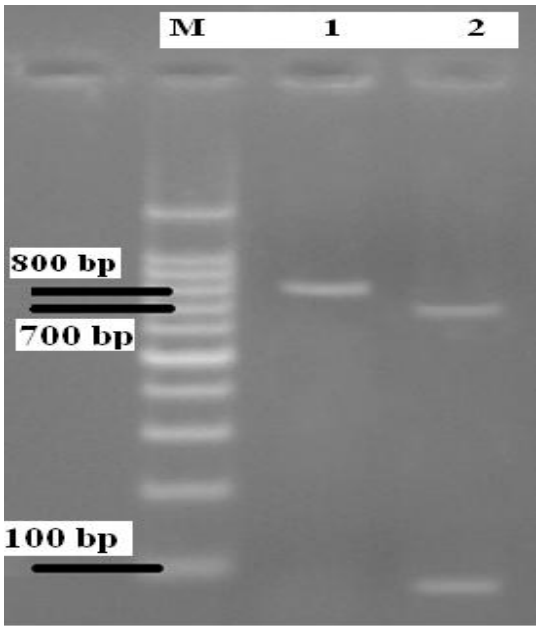

Fig. 2. Digestion profiles of mtDNA-COI PCR products (872 bp) with *HaeIII* in *Paederus fuscipes* and *Paederus littoralis*. Lane 1 (872 bp) for *P. fuscipes* and lane 2 (737 and 100 bp) for *P. littoralis*, M: 100 bp molecular weight marker (SinaClon, Iran).

Discussion

To our knowledge, this is the first molecular investigation aiming at discrimination of *Paederus fuscipes* and *Paederus littoralis* by mtDNA-COI in literature. According to the present results, *P. fuscipes* and *P. littoralis* specimens can be easily distinguished by mtDNA-COI PCR-Restriction fragment length polymorphism (RFLP) using *Hae*III. This assay could end up the discrepancy, inconsistency, and difficulties present in systematics of *Paederus* species, allowing studies regarding their distribution, biology, and behavior to proceed, as well as better understanding of their role in linear dermatitis and antiviral and antitumor activities of the pederin presents in their hemolymph.

Correct species identification is crucial in entomological surveys and application of control measures for the species that are morphologically indistinguishable or difficult to be distinguished. Targeting correct species is particularly important where more than one species live sympatric which is the case for *P. littoralis* and *P. fucipes* in south of Iran (Nikbakhtzadeh et al. 2012). *Paederus littoralis* has been collected from different parts of Fars province (Nikbakhtzadeh et al. 2012). On the other hand, *P. fuscipes* has a worldwide geographical distribution and has been reported from different countries in Africa, Asia and Europe (Coiffait 1982, Smetana 2004). This species is reported from central and southern Iran and is the most frequent species in north of Iran (Janbakhsh and Ardalan 1977, Tirgari and Nikbakhtzadeh 2002, Nikbakhtzadeh and Tirgari 2008, Anlas and Newton 2010, Nikbakhtzadeh et al. 2012).

Nowadays many researchers have been focused on medicinal insects such as *Paederus* species as well as horseflies, blister beetles and American cockroaches that have been well known due to their effects against various pathogens such as viruses and bacteria as well as diseases such as thrombosis and cancer (Richter et al. 1997, Witczak et al. 2012, Mosey et al. 2012). Pederin of *Paede-*

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rus species has been used in cancer studies in recent years. Pederin and its common substructure accounts for similar antiviral and antitumor activities, cytotoxicity and disruption of DNA metabolism that are mainly based on inhibition of eukaryotic protein biosynthesis (Piel 2002). The production of pederin relies on the activities of endosymbiont bacteria (*Pseudomonas* species) within *Paederus* (Kellner 2002, Piel et al. 2004). The manufacture of pederin is largely confined to adult female beetles which protects the beetles against predators (Kellner and Dettner 1996, Kellner 2002). Larvae and males only store pederin acquired maternally (i.e., through eggs) or by ingestion (Piel 2002).

The species commonly causing linear dermatitis are beetles of *P. fuscipes* in Asia (Anlaş and Çevik 2008). Also amount of pederin and its related compounds might be variable in different species (Kellner and Dettner 1996). Therefore correct species identification is essential to study the biological effect of the molecule.

In this study we used the COI gene of mtDNA genome, which is a known powerful molecular marker for molecular identification of various organisms. However, in addition to COI, other interest marker such as *ITS2* (*rDNA*), *wingless*, *Topoisomerase I*, and *28S* might be useful in order to develop a molecular key for discrimination of *Paederus* species. There are considerable reports on using COI gene in species diagnosis, population genetics, and systematics of beetles in the literature (e.g. Andreev et al. 1998, Gallego and Galián 2001, Becerra 2004, Chatzimanolis et al. 2010, Germain et al. 2013). Also there are a few available COI sequence entries of *P. littoralis*, *P. ruficollis*, *P. riparius*, *P. moesopotamicus*, and *P. fuscipes* in Genbank database. Species discrimination can be achieved by using RFLP profiles on PCR products or designing species specific primers to produce species specific product. However, PCR-RFLP method is reasonably cheap, fast, and user friendly and has been used frequently in many laboratories involving species identification including insects and the microbes they transmit (Clark et al. 2001, Mukabana et al. 2002, Armstrong and Ball 2005, Oshaghi et al 2006a and 2006b, Greenstone 2006, Oshaghi et al 2008, 2009, 2010, Kato et al. 2010, Oshaghi et al 2011).

Conclusion

Further molecular studies using type or morphologically well-known species are now required to verify the species composition of *Paederus* in Iran and other countries in Europe, Asia, and Africa. By performing molecular typing of *Paederus* species in the future, we expect that the *Paederus* fauna of Iran and other countries will be identified more accurately.

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