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Identification of necrophagous fly species from 12 different cities in China using ISSR and SCAR markers

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ABSTRACT

Objective: To identify necrophagous fly speies from different regions in China using inter simple sequenc repeat (ISSR) and sequence-characterized amplified region (SCAR) melocular markers and to analyze their gene difference and genetic relationship. **Methods:** Five carrion fly species were collected from 12 cities and regions in China, including *Musca domestica (M. domestica)*, *Lucilia sericata (L. sericata)*, *Chrysomya megacephala (C. megacephala)*, *Helicophagella melanura (H. melanura)*, Boethcherisca peregrina, and they were studied using ISSR and SCAR markers. **Results:** Eight ISSR primers were used for amplification of 121 samples. 679 clear and stable bands were identified, of which 516 bands were polymorphic. Several species-specific ISSR fragment were cloned and sequenced as an initial effort to derive the SCAR markers. Using *M. domestica* SCAR specific primers, SCAR-PCR amplification was performed for 8 *M. domestca* population sample DNA from different regions in China as well as *L. sericata*, *C. megacephala*, *H. melanura* and Lucillia cupirina. The result showed only *M. domestica* produced specificalty 600 bp fragment, but *L. sericata*, *C. megacephala*, *H. melanura* and *Lucillia cupirina* did not produce the same specific fragment. Clustering analysis showed clustering of most flies of *M. domestica*, *C. megacephala* and *L. sericata*. *M. domestica* samples from different regions in China yielded different banding patterns. **Conclusions:** Application of ISSR-PCR and SCAR markers to identify necrophagous fly species from 12 cities and regions in China is first reported. ISSR-PCR and SCAR markers provide a quick reliable molecular marker technique for the identification of different species of necrophagous fly.

1. Introduction

Although the great majority of forensic DNA analyses are aimed at human identity testing, a growing number of applications have been proposed for identifying other species. For a forensic entomologist, identifying an insect specimen is typically an important early step in an investigation[1,2]. Traditional morphological keys are unavailable or difficult to use for many immature stages of these insects or even adult specimens such as some female Sarcophagidae. DNA-based approaches appear promising due to the its durability and stability[3-5]. The techniques can also overcome the difficulties associated with the morphological identification of damaged specimens and the lack of the diagnostic characters. In addition, the molecular techniques potentially provide a more rapid identification as DNA can be extracted from any stage and subjected to further analysis immediately[6]. The molecular techniques, such as random amplified polymorphic DNA (RAPD)[7], restriction fragment length polymorphism (RFLP)[8], DNA single strand conformation polymorphism (SSCP)[9], sequencing the 28S rRNA gene[10], and COI gene of mitochondrial DNA[11]. Simple sequence repeat (SSR)[12] and inter simple sequence repeat (ISSR)[13] have been applied to forensic insect identification. All these methods have their own advantages and disadvantages.

ISSR employs a single PCR primer which bind directly to microsatellites, such as (CA)n which are abundant in eukaryotic genomes[13,14]. Since sequences of microsatellites are conserved over wide ranges of organisms, ISSR-PCR can use universal primers which do not need to be adapted to individual species as in microsatellite PCR. In ISSR-PCR, the stretches of DNA between adjacent microsatellite elements are amplified[13,15].

In China, the most abundant carrion fly species are: *Lucilia sericata (L. sericata)*, *Aldrichina grahami*, *Chrysomya megacephala (C. megacephala)*, *Parasarcophaga*

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crassipalpis and *Musca domestica (M. domestica)*. We intend to use the ISSR marker to analyze these five insects from 12 different cities in China. Amplification by PCR using ISSR primers that target SSR motifs can generate additional polymorphism which may result in a much more complex profile. In the amplified products, some fragments exist only in particular species, which ascertain the use of these proprietary bands as the indication makers. As an initial effort for developing reliable and reproducible markers, we also performed the conversion of ISSR fragments into the sequence-characterized amplified region (SCAR) markers, resulting in species-specific tools that can potentially be applied for molecular identification.

The objective of this study is to test the possibility and potential of the ISSR markers in investigating the inter- and intra-specific variation in some carrion fly populations and also the analysis of the DNA polymorphism among the necrophagous fly species from different cities in China using ISSR.

2. Material and methods

2.1. Fly samples

In this study, major forensically important carrion fly species were selected, namely *M. domestica*, *L. sericata*, *C. megacephala*, *Helicophagella melanura (H. melanura)*, *S. peregrina*, *Boettcherisca peregrina*, *Lucilia cupirina*. There specimens came from Beijing (Xihongmen village, Daxing), Shengzheng (Futian), Guangzhou (Tonghe), Yangjiang (Pinggang village), Changchun (Guilin road, Chaoyang), Jilin (Douguo village), Chongqing (Gaotanyan, Shapingba), Guangzhou (Shiqiao village, Panyu), HubeiYichang (Longquan village, Yiling), Nanjing (Raohuamen village, Qixia) and Wuhan (Jiangan, Hankou)(Figure 1). The specimens came from Guangzhou, Changchun, Jilin were captured on died pigs. The speciems came from natural captures entrapped by entrails of fish in other cities and regions of China. All specimens were identified by morphological method. The adult flies were used for DNA extraction.

Figure 1. Cities and regions of China for fly specimens procured for the research analysis.

2.2. DNA extraction

Adult fly sample were washed with distilled water and ground in liquid nitrogen. The material was transferred to a plastic centrifuge tube and extraction buffer (10% SDS; 0.5 mol/L Tris, pH 8.0; 1 mol/L EDTA pH 8.0; 5 mol/L NaCl) was added and incubated at 55 ℃ for 1h , followed by the addition of proteinase K to a final concentration of 100 μg/mL and a further incubation at 42 ℃ for 3h. RNase was added to a final concentration of 200 μg/mL followed by incubation at 37 ℃ overnight and centrifuged the supernatant at 12 000 g. Supernatant were extracted with phenol, phenol-chloroform/isoamyl alcohol. DNA was dissolved in TE and stored at 4 ℃.

2.3. Primer screening

We designed and synthesized 22 primers based on a reported by He and Huang[13]. Since not all ISSR primers was not appropriate to all species and the reaction conditions were different for different primers, it was necessary to screen the effective primers and optimize the reaction conditions of PCR. To confirm several PCR parameters, including the concentrations of DNA template, primer, $MgCl_2$, and dNTPs, the optimum annealing temperature and number of cycles, we carried out the preliminary experiment on *M. domestica*, *L. sericata*, *C. megacephala*, *H. melanura* from Guangzhou city in China using 22 primers. Eight primers which gave bright distinguishable and polymorphic bands were selected for further study (Table 1).

Table 1
The primer of ISSR and its denaturation temperature.

Primer number	Sequence	Denaturation temperature
01	(AC)7	46 ℃
02	(CAC)4GC	45 ℃
03	(GAG)6GC	50 ℃
04	(AC)6GG	41 ℃
05	(CTC)4GC	48 ℃
06	(AG)8AT	54 ℃
07	(AG)8TC	54 ℃
08	(CT)8AC	54 ℃

2.4. ISSR amplification and gel electrophoresis

The ISSR PCR reactions were performed in a final volume of 25 μL containing 10-100 ng of template DNA, 10 pM of each primer, 0.25 mM each of dNTP, 1× reaction buffer [200 mM Tri-HCl, 100 mM KCl and 20 mM $MgCl_2$, 100 mM $(NH_4)SO_4$], and 1 unit of Taq DNA polymerase (TaKaRa Inc.). The PCR reaction consisted of an initial denaturation of 3 min at 94 ℃ and 35 cycles of 30 second at 94 ℃, 1 min at T_m, and 1 min at 72 ℃, with a final extension step at 72 ℃ for 10min. The ISSR amplification products were resolved in 1.5% horizontal agarose gels in 1× TBE buffer (40 mM Tris-acetate, 1 mM EDTA pH 8.0) with a voltage of 100 V for 40 min and stained with ethidium bromide. A 100 bp DNA

marker (TaKaRa Inc.) was used as a molecular standard. The ISSR profiles were visualised under UV light.

2.5. Data analysis

Data were analyzed with the pooled products of the three amplification reactions bands observed in each lane were compared with all the other lane of the same gel and only reproducible bands were scored as present (1) or absent (0). The proportion of bands that were shared between any of the two varieties screened averaged over loci (SSRs) and primers (ISSR-PCR) were used as the measure of similarity (S) and genetic distance (D) were calculated as follows: $S=2Nxy/Nx+Ny$ and $D=1-S$, Where Nxy is number of commom bands of sample X and sample Y. Cluster analysis was based on distance matrices by using the unweighted pair group method analysis (UPGMA) program in WINBOOT software[16].

2.6. Conversion of specific ISSR fragment into SCAR markers

After electrophoretic separation of the ISSR fragment, the selected bands were excised from the gel. DNA was isolated using the agarose gel DNA purification kit (Omega, Inc.) and ligated to pMD18-T vector (Taka Ra.inc.). The clones were sequenced in an automatic DNA sequencer (ABI PRISM 3730 DNA analyzer) by invitro. Specific primers were designed from the sequence of the specific DNA fragments using oligo 6.0 and synthesized by Sangon company of Shanghai. SCAR-PCR amplification was performed with the same reaction mixture (containing 0.2 μmol/L of the upper and lower primer) in the same thermal, cycler as described above, with an initial denaturation of 5 min at 94 ℃ and 35 cycles of 1 min at 94 ℃, 45 s at 65 ℃, 30 s at 72 ℃, and a final extention at 72 ℃ for 5 min. Amplification products were resolved electrophoretically in 1.5% TBE agarose gels.

3. Results

3.1. ISSR amplification

The amplification was performed with selected ISSR primers with carrion flies from 12 cities and different regions. Of the 22 primers tested, eight was selected. These were: (AC)7, (CAC)4GC, (GAG)6GC, (AC)6GG, (CTC) 4GC, (AG)AT, (AG) TC, (CT)8AC, and were referred to as ISSR01, ISSR02, ISSR03, ISSR04, ISSR05, ISSR06, ISSR07 and ISSR08, respectively. The primers showed rich and highly polymorphic fingerprints for necrophagous flies from different regions in China. Total number of amplifications performed was 121 yielding 679 clear and stable bands (of an average of 5.6 band per sample), of which 516 bands were polymorphic. The results indicated that *M. domestica, L. sericata, C. megacephala, H. melanura*, *Boethcherisca peregrina* from different regions in China had different band patterns.

3.2. Identification of species

Amplification with 8 primers showed that *M. domestica, L. sericata, C. megacephala, H. melanura* and *S. peregrina* from different regions in China produced the completely different band patterns. *M. domestica* from different regions in China presented the polymorphic genetic band patterns of geographic population in species. Especially the ISSR primer ISSR01[(AC)7], ISSR02[(CAC)4GC], ISSR03[(GAG)6GC], ISSR04[(AC)6GG], ISSR06[(AG)8AT] in identification of carrion fly species from different regions in China generated very good ploymorphic band patterns (Figures 2-4).

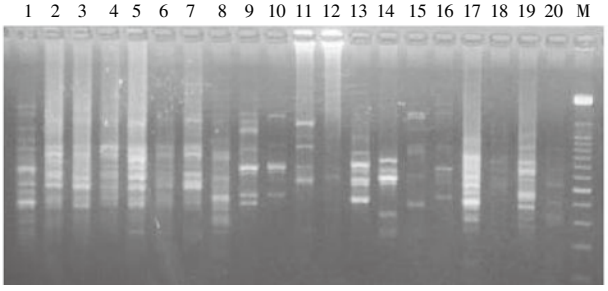

Figure 2. Amplification of genetic DNA in four necrophagous fly species from different areas in China using primer ISSR(01).
1: *M. domestica* (Shenzhen), 2: *M. domestica* (Guangzhou), 3: *M. domestica* (culture), 4: *M. domestica* (Yangjiang), 5: *M. domestica* (Nanjing) 6: *M. domestica* (Changchun), 7: *M. domestica* (Yichang), 8: *M. domestica* (Panyu), 9: *M. domestica* (Beijing), 10: *C. megacephata* (Jilin),11: *C. megacephata* (Wuhan), 12: *C. megacephata* (Yichang), 13: *L. sericata* (Yangjiang), 14: *L. sericata* (Shenzhen), 15: *L. sericata* (Beijing), 16: *L. sericata* (Jilin), 17: *Boettcherisca peregrina* (Guangzhou), 18: *H. melanura* (Chongqing), 19: *H. melanura* (Changchun), 20: *Boettcherisca peregrina* (Jilin).

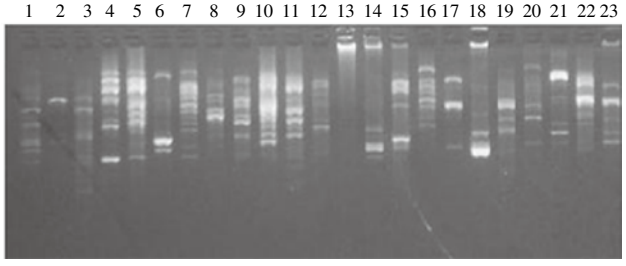

Figure 3. Amplification of genetic DNA in four necrophagous fly species from different areas in China using primer ISSR(02).
1: *M. domestica* (Shenzhen), 2: *M. domestica* (Guangzhou), 3: *M. domestica* (Yangjiang),4: *M. domestica* (culture), 5: *M. domestica* (Changchun) 6: *M. domestica* (Beijing),7: *M. domestica* (Jilin), 8: *M. domestica* (Chongqin), 9: *M. domestica* (Panyu), 10: *M. domestica* (Yichang), 11: *M. domestica* (Nanjing), 12: *C. megacephata* (Beijing), 13: *C. megacephata* (Guangzhou), 14: *C. megacephata* (Yangjiang), 15: *C. megacephata* (Wuhan),16: *C. megacephata* (Jilin), 17: *C. megacephata* (Chongqin), 18: *C. megacephata* (Yichang),19: *L. sericata* (Yangjiang), 20: *L. sericata* (Jilin), 21: *L. sericata* (Beijing), 22: *L. sericata* (Changchun), 23: *L. sericata* (Shenzhen), 24: *L. sericata* (Chongqing).

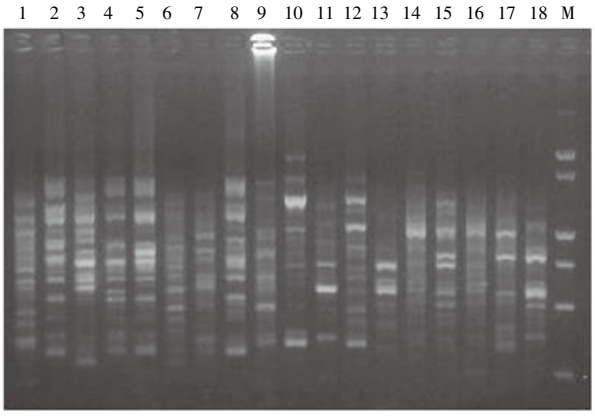

Figure 4. Amplification of genetic DNA in four necrophagous fly species from different area in China using primer ISSR(03).
1: *M. domestica* (Shenzhen), 2: *M. domestica* (culture), 3: *M. domestica* (Yangjiang), 4: *M. domestica* (Yichang), 5: *M. domestica* (Nanjing), 6: *M. domestica* (Changchun), 7: *M. domestica* (Panyu), 8: *M. domestica* (Guangzhou), 9: *C. megacephata* (Yichnag),10: *C. megacephata* (Jilin), 11: *L. sericata* (Beijing), 12: *L. sericata* (Jilin), 13: *L. sericata* (Shenzhen), 14: *L. sericata* (Chongqing), 15: *Boettcherisca peregrina* (Guangzhou), 16: *H.melanura* (Chongqing) , 17: *H. melanura* (Changchun), 18: *Boettcherisca peregrina* (Panyu).

3.3. Species-specific marker

Species-specific ISSR fragment was identified among necrophagous fly species from 12 cities and regions in China using 8 ISSR primers. Amplification with 8 primers showed that specific ISSR fragment of *M. domestica* was 240 bp, 360 bp, 400 bp, 500 bp, 580 bp, 610 bp, 630 bp, 700 bp, 1 000 bp, 1 500 bp, respectively; specific ISSR fragment of *C. megacephala* was 370 bp, 430 bp, 800 bp, 1 100 bp, respectively; specific ISSR fragment of *L. sericata* was 500 bp, 580 bp, 600 bp, 680 bp, respectively; specific ISSR fragment of *H. melanura* was 320 bp, 340 bp, 370 bp, 420 bp, 520 bp, 540 bp, 560 bp, 580 bp, 770 bp, respectively; specific ISSR fragment of Boettcherisca peregrina was 320 bp, 340 bp, 370 bp, 420 bp, 520 bp, 540 bp, 560 bp, 580 bp, 770 bp, respectively. All of the ISSR bands reproduced in at least three independent experiments and several species-specific ISSR fragment were cloned and sequenced as an initial effort to derive SCAR markers.

Using *M. domestica* SCAR specific primers, SCAR-PCR amplification was carried out for 8 *M. domestica* population sample DNA from different regions in China and *L. sericata*, *C. megacephala*, *H. melanura* and *Lucillia cupirina*. The result showed only *M. domestica* produced a specific 600 bp fragment and not by other species. The validity of species-specific SCAR markers is obvious (Figure 5).

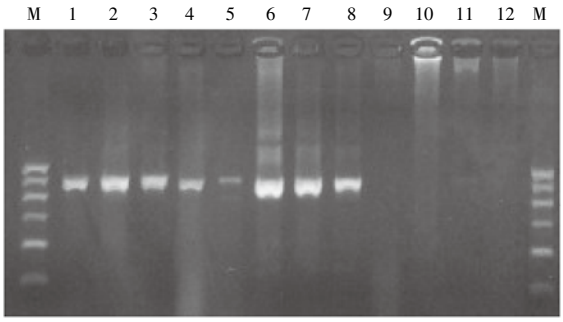

Figure 5. Reliability test of SCAR marker for *M. domestica* in different *M. domestica* population and other fly population and from different regions in China.
1: *M. domestica* (Beijing), 2: *M. domestica* (Jilin), 3: *M. domestica* (Chang chun), 4: *M. domestica* (Chengdu), 5: *M. domestica* (Nanjing), 6: *M. domestica* (Guangzhou),7: *M. domestica* (Jingzhou), 8: *M. domestica* (Culture), 9: *C. megacephata* (Guangzhou),10: *L. sericata* (Guangzhou), 11: *H. melanura* (Guangzhou),12: *Lucilia cupirina* (Guangzhou), M: 100 bp DNA marker.

3.4. Cluster analysis

The identification of necrophagous fly species used 8 specific ISSR primers, and the result of UPGMA analysis among the most abundant carrion fly species from 12 cities and regions in China the following clustering pattern was demonstrated: *M. domestica* from 10 different regions in China clustered together in one tree and the *M. domestica* from different regions in China had been divided into four groups at different levels. *M. domestica* from Guangzhou, Yichang, Nanjing was clustered into one group and from Jilin and Chongqing was clustered into another group. *M. domestica* from Guangzhou culture fly, Guangzhou Panyu and Changchun was clustered into one group. *M. domestica* from Shenzhen, Guangdong Yangjiang and Beijing was clustered into one group. Most of the *C. megacephala* from different regions in China was clustered into one tree. Most of *L. sericata* from different regions in China was clustered into one tree (Figure 6).

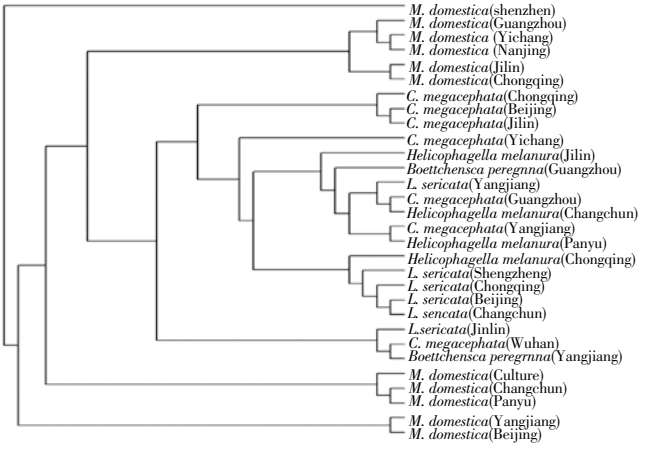

Figure 6. Dendrograms derived from an unweighted pair group method analysis (UPGMA) with cluster analysis based on ISSR markers among different necrophagous fly species.

4. Discussion

In this study, we demonstrated that ISSR markers are promising for the molecular identification of different species of necrophagous fly and with the advantages of high accuracy, rapidity and convenience, to perform ISSR could offer a quick and reliable alternation in practice. He and Huang (2007) identified five necrophagous fly species from Shanghai in China using ISSR demonstrated ISSR markers are promising for molecular identification of the fly related to the forensic applications in court events. In this work, eight ISSR primers were selected from the 22 ISSR primers to identify analysis of necrophagous fly species from different regions in China. The result indicated that *M. domestica*, *L. sericuta*, *C. megacephala*, *H. melanura* and *S. peregrina* from different regions in China had produced the completely different band patterns. *M. domestica* from different regions in China presented the polymorphic genetic band patterns of geographic population in species. The 8 ISSR primers provided a useful tool in discriminating species and geographic population of flies.

SCAR is a genomic fragment localized in a single genetically defined locus that can be amplified by PCR using a pair of specific primers. Compared to ISSR markers, SCARs are less sensitive to reaction conditions, allowing for a high reliability and reproducibility among distinct laboratories using different brands of reagents and equipments[17]. Therefore, the robust characteristics of SCARs make them an appropriate diagnostic tool for practical applications. In order to obtain a reliable PCR-based marker, we identified several species-species ISSR fragment in carrion fly species, cloned and sequenced as conversion of ISSR marker into SCARs for the five necrophagous flies. Using *M. domestica* SCAR specific primers in performing, SCAR-PCR amplification of 8 *M. domestica* populations from different regions in China and other species including *L. sericata* , *C. mgacephala* , *H. melanura* and *Lucillia cupirina*, and the validity and reliability of species-specific SCAR markers is proved. These markers were tested using several individuals from different species which confirmed their universal characters. This work represents a remarkable advance in fly diagnosis, but it was restricted to one single marker per species, with all of the amplicons having similar sizes. The generation of a higher number of SCARs with different amplicon sizes will permit the development of multiplex PCR assays in the near future. It may allow the complete diagnosis attained in a single-tube reaction or with DNA bars identification of the necrophagous fly species, especially in large scale surveys.

In summary, we have identified the promising marker of ISSR and SCAR. To date, it is the first report of identification of *M. domestica* populations from different cities and regions in China using ISSR and SCAR markers, which reveals great difference in genetic diversity and gene types of geographic populations of *M. domestica* from different cities and regions in China. It also reveals that there is difference in genome of *M. domestica* from different regions in China.

Conflict of interest statement

We declare that we have no conflict of interest.

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References

[1]Amendt J, Krettek R, Zehner R. Forensic entomology. *Naturwissenschaften* 2004; 9151-65.
[2]Benecke M. A brief history of forensic entomology. *Forensic Sci Int* 2001; **120**: 2-14.
[3]Well JD, Sperling FAH. DNA-based identification of forensically important Chrysomyinae (Diptera: Calliphoridae). *Forensic Sci Int* 2001; **120**: 110-5.
[4]Wells JD, Williams DW. Validation of a DNA-based method for identifying Chrysomyinae (Diptera: Calliphoridae) used in a death investigation. *Int J Legal Med* 2007; **121**: 1-8.
[5]Zehner R, Amendt J, Schutt S, Sauer J, Krettek R, Povolny D. Genetic identification of forensically important flesh flies (Diptera: Sarcophagidae). *Int J Legal Med* 2004; **118**: 245-7.
[6]Nybom H. Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. *Mol Ecol* 2002; **13**: 1143-55.
[7]Benecke M. Random amplified polymorphic DNA (RAPD) typing of necrophageous insects (Diptera: Coleoptera) in criminal forensic studies: validation and use in practice. *Forensic Sci Int* 1998; **98**: 157-68.
[8]Schroeder H, Klotzbach H, Elias S, Augustin C, Pueschel K. Use of PCR-RFLP for differentiation of calliphoric larvae (Diptera Calliphoridae) on human corpses. *Forensic Sci Int* 2003; **132**: 76-81.
[9]Marquez JG, Krafsur ES. Mitochondrial diversity evaluated by the single strand conformation polymorphism method in African and North American house flies (*Musca domestica* L.). *Insect Mol Biol* 2003; **12**: 99-106.
[10]Stevens J, Wall R. Genetic relationships between blowflies (Calliphoridae) of forensic importance. *Forensic Sci Int* 2001; **120**: 116-23.
[11]Harvey ML, Dadour IR, Gaudieri S. Mitochondrial DNA cytochrome oxidase I gene: potential for distinction between immature stages of some forensically important fly species (Diptera) in Western Australia. *Forensic Sci Int* 2003; **131**:134-9.
[12]Florin AB, Gyllenstrand N. Isolation and characterization of polymorphic microsatellite markers in the blowflies *Lucilia illustris* and *Lucilia sericata*. *Mol Ecol Notes* 2002; **2**: 113-6.
[13]Lin H, Wang SB, Miao XX, Wu H, Huang YP. Identification of necrophagous fly species using ISSR and SCAR markers. *Forensic Sci Int* 2007; **168**: 148-53.
[14]Martins M, Tenreiro R, Oliveira MM. Genetic relatedness of *Portuguese almond* cultivars assessed by RAPD and ISSR markers. *Plant Res Int* 2003; **22**: 71-8.
[15]Luoue C, Legal L, Staudter H, Gers C, Wink M. ISSR (Inter Simple Sequence Repeats) as genetic markers in Noctuids (Lepidoptera). *Hereditas* 2002; **136**: 251-3.
[16]Nagaraju J, Kathirvel M, Kumar RR, Siddiq EA, Hasnain SE. Genetic analysis of traditional and evolved Basmati and non-Basmati rice varieties by using fluorescence-based ISSR-PCR and SSR markers. *Proc Natl Acad Sci USA* 2000; **99**: 5836-41.
[17]Fernandez S, Costa AC, Katsuyama AM, Madeira AMBN, Gruber A. A survey of the inter- and intraspecific RAPD markers of *Eimeria* spp. of the domestic fowl and the development of reliable diagnostic tools. *Parasitol Res* 2003; **89**: 437-45.