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# Brief report
# ISSR (Inter Simple Sequence Repeats) as genetic markers in Noctuids (Lepidoptera)

C. LUQUE[1,2], L. LEGAL[1,2], H. STAUDTER[2], C. GERS[1] and M. WINK[2]

[1] *Laboratoire d'Écologie Terrestre, CNRS (UMR 5552)/Université Paul Sabatier, Bat IVR3, 118 route de Narbonne, F-31062 Toulouse, France. E-mail: legal@cict.fr*
[2] *Institut für Pharmazeutische Biologie, Universität Heidelberg, Heidelberg, Germany*

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Microsatellites among Lepidoptera are poorly known. We have shown that some ISSR amplifications are possible and demonstrate their applicability in studying intra- and inter-specific variation in some Noctuid populations.

The Lepidoptera are sensitive to habitat and climatic change (HARPER et al. 2000). We analyse Noctuid populations in natural and disturbed habitats in the Pyrenées (France). We have attempted to find a versatile method that can be used in species determination, and, to demonstrate small genetic differences, in the nuclear genome of moths.

Compared to other groups of animals relatively few microsatellite studies have been carried out with Lepidoptera (NÈVE and MEGLÉCZ 2000). Only few studies involve ISSR (REDDY et al. 1999). One reason was that it has been assumed that microsatellites are lacking or are very rare in Lepidoptera. However, MEGLÉCZ and SOLIGNAC (1998) and HARPER et al. (2000) have successfully localised $(CA)_n$ repeats in insect microsatellites.

We found that: (i) $(CA)_n$ primer gives the most informative profiles; (ii) DNA profiles between species differ substantially; (iii) comparison of ISSR profiles can be successfully applied to study intra-specific variation.

## MATERIALS AND METHODS

ISSR-PCR of nuclear DNA is a PCR method to map the nuclear genome and to discover rearrangements. ISSR usually produces a genomic fingerprint that is similar to that generated by AFLP-PCR. ISSR employs a single PCR primer only. These PCR primers bind directly to microsatellites, such as $(CA)_n$, which are abundant in eukaryotic genomes. 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 stretches of DNA between adjacent microsatellite elements are amplified. Since we use a single PCR primer only, a necessary prerequisite is the inversion of a microsatellite motive in the neighbourhood (up to 2000 bp distance) of an existing microsatellite element.

Noctuids from six species (*Lycophotia porphyrea*—as outgroup, *Diarsia brunnea* and four species belonging to another close genus, *Xestia*: *X. baja*, *X. c-nigrum*, *X. ditrapezium*, and *X. rhomboidea*) were used in this study. All came from natural or exploited forests of the Pyrenées (France) (1500 m above sea level). Moths were caught in light traps, killed by KCN and stored at $-20$°C with negligible effects on yield and quality of DNA.

Half an abdomen was cut from dead and frozen moths and incubated overnight at 50°C in 700 µl of lysis buffer (10 mM Tris, pH 7.5, 25 mM EDTA, 75 mM NaCl) with 1 mg of Proteinase K (Boehringer, Mannheim) and 35 µl of 20 % SDS, or in guanidinium thiocyanate buffer (4 M guanidinium thiocyanate, 0.1 M Tris-HCl pH 7.5), 1 % β-mercaptoethanol, followed by a standard phenol–chloroform protein extraction. DNA was precipitated with 800 µl of cold isopropanol, centrifuged, washed, dried and resuspended in TE buffer (SAMBROOK et al. 1989; SWATSCHEK et al. 1994).

For amplifications, 300 ng of total DNA was used as a template, plus 10 pmol primer 5′-$(CA)_{10}$, 0.1 mM of dGTP, dCTP, and dTTP, 0.075 mM dATP, 1 µCi [α-$^{33}$P]-dATP, 2.5 µL of 10 × amplification buffer (100 mM Tris-HCl, pH 8.5, 500 mM KCl 5 % Triton X-100, 15 mM $MgCl_2$,) and 0.15 units Taq polymerase (Pharmacia Biotech, Freiburg) in a total volume of 25 µL. After an initial denaturation (4 min at 94°C), 40 cycles of 45 s at 94°C, 45 s at 40°C, and 45 s at 72°C were performed on a Biometra thermocycler; then at 72°C for 20 min, followed by 4°C for storage. After mixing with a specific blue marker (95 % formamide, 20 mM EDTA, 0.05 % bromphenol blue, 0.05 % xylene cyanol FF) and denaturation at 90°C for 5 min, PCR products were electrophoretically separated on a high resolution Sequagel matrix at 65 W for 4 h (size 45 × 30 cm). The separation of ISSR fragments on such gel can enhance the resolu-

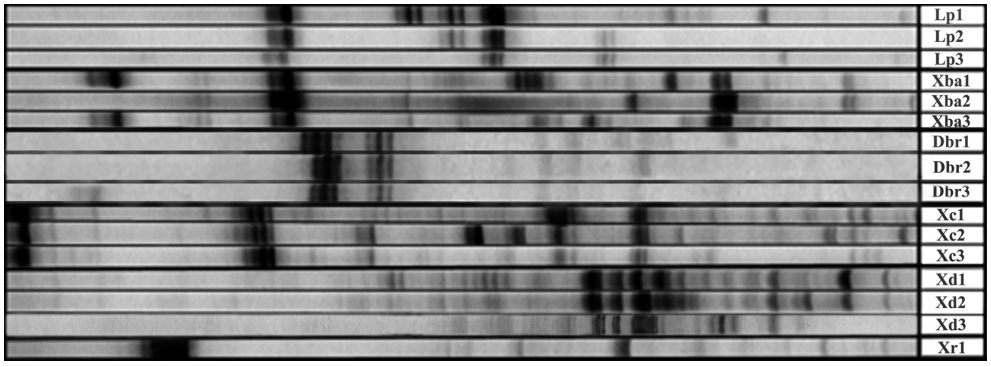


**Fig. 1.** ISSR Autoradiogram of various species of Noctuidae using $(CA)_{10}$ primers. Lp = *Lycophotia porphyrea*; Xba = *Xestia baja*; Dbr = *Diarsia brunnea*; Xc = *Xestia c-nigrum*; Xd = *Xestia ditrapezium*; Xr = *Xestia rhomboidea*.

tion of ISSR bands (ARCADE et al. 2000). After drying, the gel was exposed to an X-ray film (Kodak BIOMAX MR) overnight at room temperature and developted (Kodak).

## RESULTS AND DISCUSSION

Several primers were employed in order to see whether informative genomic fingerprints could be generated. Best results were obtained with the $(CA)_{10}$ primer (Fig. 1), whereas $(CTGT)_4$, $(GC)_{10}$, $(TCC)_5$, $(CT)_8$, $(AG)_{12}$, $(GATA)_4$, $(GTG)_5$ failed to produce polymorphic PCR products. PCR primers $(GACA)_4$, $(GGAT)_4$, and $(CT)_4(CA)_5$ showed some variability but were inferior to $(CA)_{10}$ primer. This observation supports the finding of MEGLÉCZ and SOLIGNAC (1998) and HARPER et al. (2000) that $(CA)_n$ is the main elements of microsatellites in Lepidoptera. Therefore, we use them in this work in order to study these Noctuidae.

In our first experiments, conditions for ISSR with $(CA)_{10}$ primer were not optimal. In particular, if differing amounts of DNA were loaded, electrophoretic resolution was impaired. In order to obtain comparable and reliable results, the same DNA concentrations should be used for all samples. 300 ng DNA per sample was found to be optimal.

Some results are enhanced in Fig. 1. The aim of this study is to adapt technically ISSR-PCR (inter simple sequence repeat-PCR) for these Noctuids and to do an exploratory study to see if this technique is available to understand and analyse genetic structures of Noctuid populations. Actually, for such studies in populations, NAGARAJU et al. (2001) reported that ISSR-PCR has the best cost-benefit ratio compared with RFLP and is more reliable and repeatable than RAPD.

Fig. 1 unequivocally shows that the DNA profiles between species differ substantially. Some specific PCR products may be useful as a diagnostic marker to separate species with similar morphology. *X. c-nigrum* is easy to determine and *L. porphyrea* is from another sub-family of Noctuidae (used here as an "outgroup"). Morphologically, *D. brunnea* is close to *X. baja*, *X. ditrapezium* and *X. rhomboidea* (more especially, confusions are possible between females). In Fig. 1, it is shown that the four species have different ISSR patterns.

ISSR-PCR was already known as a very effective method to understand intra-specific and genetic structure of populations (FANG and ROOSE 1997; GE and SUN 1999; ZHOU et al. 1999; NAGARAJU et al. 2001), to sex determine (WINK et al. 1998), to generate species-specific genomic fingerprints (GUPTA et al. 1994; ZIETKIEWICS et al. 1994; HUANG and SUN 2000) and to detect hybridisations (WINK et al. 2000).

Among species, a polymorphism can be detected that allows discrimination between individuals. Further studies using the $(CA)_{10}$ primer will be done to study the full range of intra-specific variation and genetic structure of Noctuid populations in the Pyrenées, in particular to characterise populations of *D. brunnea*. In this work, we have shown that ISSR-PCR methods are suitable to study intra- and inter-specific variation in this group of insects.

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