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RESEARCH NOTE

DNA SAMPLING FROM BODY SWABS OF TERRESTRIAL SLUGS (GASTROPODA: PULMONATA): A SIMPLE AND NON-INVASIVE METHOD FOR MOLECULAR GENETICS APPROACHES

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Studies on molluscan conservation genetics, phylogenetics and evolution provide important data to access the diversity, populations' structure and dispersal patterns of these organisms (Barker, 2001). Current environmental changes associated to anthropogenic pressures may have several negative ecological impacts on molluscs (e.g. van der Meij, Moolenbeek & Hoeksema, 2009; Cameron, Pokryszko & Martins, 2012), involving the reduction in their habitat suitability, which is particularly damaging for terrestrial slugs requirements of persistence (e.g. Severns, 2005; Kappes, 2006). The literature on slugs is greatly biased towards those that are agricultural pests (Barker, 2002), while there is little information about benign native species such as *Geomalacus maculosus* (Platts & Speight, 1988). Therefore, it is mandatory to develop multidisciplinary studies that integrate genetic data, biological and behaviour features to understand the adaptive, evolutionary and population dynamics of these species. The improvement of non-invasive DNA-based methodologies is essential to minimize the potential harmful effects of these approaches, particularly in studies of rare or endangered molluscs. Although most of methods to collect biological materials for DNA isolation in molluscs are intrusive (e.g. Winnepenninckx, Backeljau & De Wachter, 1993; Sokolov, 2000; Pereira *et al*., 2011), some protocols have been proposed based on non-invasive sources of DNA in terrestrial and marine snails and slugs (Kawai *et al*., 2004; Armbruster, Koller & Baur, 2005; Palmer, Styan & Shearman, 2008; Régnier *et al*., 2011). All these successful strategies were developed considering the DNA isolation from foot mucus and specific procedures for sample collection. Nevertheless, the sampling protocols are time-consuming and require the handling of the individuals. The development of simple and efficient protocols to improve these characteristics can significantly enhance its field applicability. Thus, the main goal of this study is to demonstrate and report a non-invasive, rapid, efficient and cost-effective method based on DNA isolation from body swabs of terrestrial slugs. In this perspective, the hypothesis under study is that slugs' body surface epithelial cells provide an alternative good DNA source for genetic analysis.

Body swabs of *Geomalacus maculosus* ($n = 12$) and *Arion* spp. ($n = 12$) were collected in Vila Real (Northern Portugal) in their natural habitats. This procedure was accomplished by carefully scraping a sterile cotton swab against each individuals' body 10 times (Fig. 1). Swabs were directly placed in sterile 1.5 ml eppendorf tubes and stored at −20°C until DNA extraction. Samples from three different and independent specimens of *G. maculosus* and *Arion* spp. were processed following the DNA extraction methods described below.

To demonstrate the flexibility of the proposed protocol four methods of DNA isolation were tested and optimized: salting out extraction, Quick gDNA MiniPrep Kit (Zymo Research), QIAamp DNA Micro Kit (Qiagen) and DNA IQ Reference Sample Kit for Maxwell 16 (Promega). In the conventional salting out protocol, samples were incubated at 55°C for 2 h on a thermal-shaker with 300 µl of lysis buffer [10 mM Tris (pH 7.5), 400 mM NaCl, 2 mM EDTA (pH 8.0)] (pH 7.3–7.5), 15 µl of 20% sodium dodecyl sulphate (SDS) and 20 µl of 20 mg/ml proteinase K. The swabs were then removed with sterile tweezers and 50 µl of 6 M NaCl (saturated solution) was added to the extraction mixture, samples were mixed thoroughly by vortexing for 10 s, followed by centrifugation at 8000 g for 10 min to precipitate the residual cellular debris. The supernatant was transferred to a clean eppendorf tube and 500 µl of 100% ethanol was added to each sample, mixed thoroughly by vortexing for 10 s, and centrifuged at 8000 g for 5 min to pellet the DNA. The DNA pellets were washed with 250 µl of 70% ethanol, followed by centrifugation at 8000 g for 5 min. The pellets were completely air dried and resuspended in 100 µl of sterile nuclease-free water. The Quick gDNA MiniPrep Kit was used according to manufacturer's instructions, with the following optimizations: the samples were digested at 56°C for 2 h in a solution containing 500 µl of genomic lysis buffer, 15 µl of 20% sodium dodecyl sulphate (SDS) and 20 µl of 20 mg/ml proteinase K; the washing step was performed twice with 500 µl of g-DNA wash buffer, followed by recentrifugation at 10 000 g for 1 min; and DNA was collected by two sequential elutions with

50 μl of elution buffer (incubation time was extended to 15 min at room temperature). The QIAamp DNA Micro Kit was used following the standard protocol recommended by the manufacturer, performing the initial incubation for 2 h and recovering DNA samples with the same final procedure of the previous protocol. In the automated DNA extraction carried out using the Maxwell 16 System (Promega) the protocol presented in the DNA IQ Reference Sample Kit was followed, modifying only the initial incubation time (extended to 2 h). The concentration and purity of extracted DNAs were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

All DNA extraction methods previously described were optimized with simple modifications, increasing its applicability to specific biological samples constituted by mucus, proteineous fluids and epithelial cells from slugs sampling. The average yield of the DNA [DNA concentration (ng/μl) × total elution volume (μl)] extracted using the salting out (564 ± 314 ng), Zymo Research (651 ± 296 ng), Qiagen (741 ± 180 ng) and Promega (concentrations <2 ng/μl—out of nanodrop detection limit) protocols were evaluated. For best results, it is recommended the optimization of initial digestion step in all methods, adjusting the time to a minimum of 2 h and/or enhancing the digestion solution. The DNA quantities obtained by swabbing methods are generally higher than techniques based on FTA cards (Hansen *et al.*, 2007); however, the substances co-purified can be higher in DNA isolated from swabs. In fact, the average purity of the DNAs extracted in this study ($A_{260/280}$: 3.04 ± 1.64 and $A_{260/230}$: 0.27 ± 0.14) suggest the presence of co-extracted molecules. On the other hand, the direct analysis of genomic DNA on agarose gels was negative for most samples, with no evidence of high molecular weight and/or degraded DNA (data not shown). Thus, the influence of substances co-purified on estimation of DNA concentrations (described above) cannot be excluded (Hansen *et al.*, 2007).

Figure 1. Body swab method performed in the collection of biological samples from *Geomalacus maculosus* in its natural habitat.

The activity of molecular biology enzymes can be inhibited by contaminants co-purified with DNA (Bickley & Hopkins, 1999), such as the mucopolysaccharides and polyphenolic proteins present in the body fluids of mollusc species (Smith, 2010). To analyse the quality of the extracted DNAs, two nuclear and two mitochondrial markers (Table 1) were amplified by PCR, namely ribosomal DNA gene cluster (including the partial 5.8S and 28S genes, and the complete ITS2 region), the partial regions of the nuclear histone cluster 2, H4b (HIST2H4B) and two mitochondrial genes [cytochrome c oxidase subunit I (*COI*) and NADH dehydrogenase 1 (*ND1*)] with universal specific primers. All PCR amplifications were performed in a total volume of 10 μl containing 5 μl of 2× MyTaq HS Mix (Bioline), 2 pmol of each primer and 3 μl DNA (Promega protocol) or 1 μl DNA (for the other protocols) (∼1–10 ng). The amplification reactions were carried out in the thermocycler Biometra T-Gradiente, using for all fragments an initial denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 50°C for 1 min, 72°C for 30 s and a final extension at 60°C for 10 min. The PCR reactions were performed successfully in all DNA samples, which allowed the specific amplification of these fragments with different lengths (Fig. 2). The *COI* fragments of *G. maculosus* were sequenced to assess the quality of PCR products obtained using DNA samples from all optimized protocols. The PCR products were purified with Illustra ExoStar 1-Step (GE Healthcare) and bi-directionally sequenced at Stab Vida (Lisbon, Portugal). Sequencing reactions were successful, enabling the identification of two haplotypes (GenBank IDs: KF290021 and KF290022). These results confirm the feasibility and usefulness of the DNAs for subsequent application in molluscan studies integrating the analysis of molecular markers.

The collection of body swabs in terrestrial slugs proved to be a straightforward, hassle-free process in the acquisition of epithelial cells form the skin surface. In this work we demonstrated the efficiency of DNA isolation from these samples using a variety of extraction methods, including conventional protocols and commercial kits based on manual and automated workflows. The simplicity of the proposed methodology of DNA sampling, based on noninvasive field procedures (avoiding potential

Table 1. Characterization of primers utilized in the PCR amplification of nuclear and mitochondrial markers using DNA of terrestrial slugs.

Gene	Primers	Amplicon length (bp)	Reference
rDNA (5.8S and 28S)	LSU-1: 5′-CTAGCTGCGAGAATTAATGTGA-3′ LSU-3: 5′-ACTTTCCCTCACGGTACTTG-3′	∼1000	Wade *et al.* (2006)
COI	LCO1490: 5′-GGTCAACAAATCATAAAGATATTGG-3′ HCO2198: 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′	∼700	Folmer *et al.* (1994)
ND1[a]	MOL-NAD1F: 5′-CGRAARGGMCCTAACAARGTTGG-3′ MOL-NAD1R: 5′-GGRGCACGATTWGTCTCNGCTA-3′	∼500	Quinteiro *et al.* (2005)
HIST2H4B	H4F2s: 5′-TSCGIGAYAACATYCAGGGIATCAC-3′ H4F2er: 5′- CKYTTIAGIGCRTAIACCACRTCCAT-3′	∼200	Pineau *et al.* (2005)

[a]Specific primers for *Arion* spp.

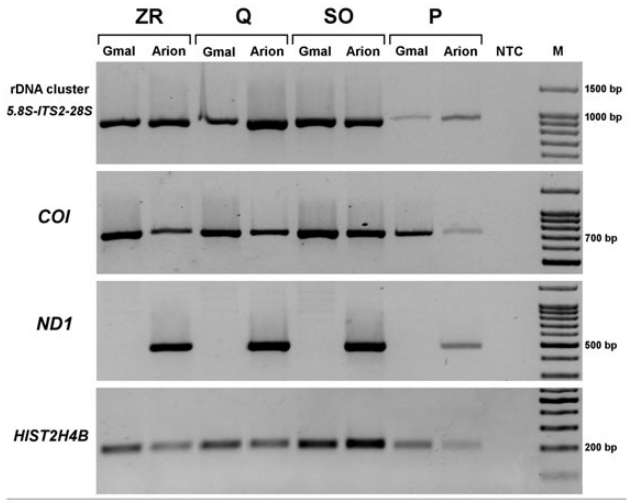

Figure 2. Agarose gel electrophoresis at 1.5% of the rDNA cluster (5.8S-ITS2-28S), *COI*, *ND1* and *HIST2H4B* fragments amplified using DNA isolated from body epithelial cells of *Geomalacus maculosus* (Gmal) and *Arion* spp. through the Quick gDNA MiniPrep Kit (ZR), QIAamp DNA Micro Kit (Q), salting out (SO), and DNA IQ Reference Sample Kit (P) optimized protocols; (NTC, nontemplate control; M, molecular marker).

damage and handling of individuals), fast material collection (samples collected in ~30 s), and practical and cost-effective field protocol (only sterile eppendorfs and cotton swabs are required in the field), makes this approach a useful and safe strategy for biological sampling in terrestrial slugs. This versatile and non-destructive technique can be an important resource in forthcoming research studies on ecology, evolution and population genetics, particularly in the case of threatened species.

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