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VOUCHER SPECIMENS FOR DNA SEQUENCES OF PHYTOSEIID MITES (ACARI: MESOSTIGMATA)

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ABSTRACT — Molecular approaches are increasingly used to help in species diagnostics. These approaches have been recently and successfully applied to assess some taxonomic questions within the mite family Phytoseiidae. However, many protocols for DNA extraction of such small specimens require crushing the entire sample, precluding deposition of the carcass as a museum voucher. This study aimed to determine the efficiency of a modified Qiagen DNeasy tissue kit extraction method to both extract enough DNA for performing PCR and DNA sequencing, and recover intact the adult female specimens for identification. Two types of biological material were studied for two species: 100 % alcohol preserved females and females mounted in lactic acid for four hours. All specimens were retrieved after DNA extraction and were mounted on slides. All the structures used for species identification were visible with a phase microscope. DNA was successfully extracted from all the specimens considered and fragments of CytB mtDNA of 426 bp were amplified and corresponded to those of the two species considered. The Qiagen DNeasy tissue kit extraction protocol thus permits to retrieve, prepare and identify mites for which DNA has been successfully extracted, whatever the material considered: alcohol preserved or lactic acid treated mites. This result is of major interest for future taxonomic studies of the family Phytoseiidae, as it will allow the assignment of morphological and molecular attributes to a same specimen.

KEYWORDS — *Neoseiulus idaeus*; *Typhlodromus (Typhlodromus) exhilaratus*; barcoding; CytB mtDNA; voucher

INTRODUCTION

The family Phytoseiidae is the most studied family within the order Mesostigmata because of its major involvement in biological control programs. Currently, the most efficient predators of mite pests, especially those of families Tetranychidae and Eriophyidae, belong to this family (Kostianen and Hoy 1996; McMurtry and Croft 1997). Two thousand two

hundred and eight species dispatched in 84 genera are reported in the last world catalogue published in 2004 (Moraes *et al.* 2004) and in the last revision of the family in 2007 (Chant and McMurtry 2007).

However, a high number of synonymies has been reported (i.e., more than 10 % of the species described are synonyms) and many others are suspected (Moraes *et al.* 2004). Furthermore, morphologically cryptic species may frequently occur

in such mite groups (Magalhães *et al.* 2007). To determine species validity, molecular markers are more and more used, especially in light of barcoding approaches (Hebert *et al.* 2003, 2004; Moritz and Cicero 2004). These concepts have been recently and successfully applied to phytoseiid mites to resolve some synonymy cases (Tixier *et al.* 2006a, b; 2008; Okassa *et al.* 2009; Kanouh *et al.* 2010; Okassa *et al.* 2010; Tixier *et al.* 2010). However, the main problem that taxonomists face when working from molecular-based data is the absence of voucher specimens (Rowley *et al.* 2007). Voucher specimens are the most important currency in taxonomy for both morphological and molecular studies, and are used for diagnostic, phylogeographic and phylogenetic analyses. Unfortunately, many protocols for DNA extraction of small specimens require crushing the entire sample, precluding deposition of the carcass as a museum voucher (Whitfield and Cameron 1994). One suggested solution is to take multiple images of the specimen before crushing (De Ley *et al.* 2005). However, this cannot be easily applied to phytoseiid mites as these mites have to be mounted on slides in Hoyer's medium to be identified. For now, we do not know how this mounting medium may alter DNA. Another approach is to remove a single appendage of the specimen for DNA extraction (Starks and Peters 2002). However, this cannot be used for phytoseiid mites because of their small size (less than 500 µm in length), the low number of appendages and because some appendages bear specific characters essential for species identification. Another solution would be to use descendants from one female, in the case of parthogenetic organisms. However, mites of the family Phytoseiidae are strictly sexual species. Some recent studies have proposed non destructive DNA extraction methods for mites. Rowley *et al.* (2007) and Jeyaprakash and Hoy (2010) succeeded in extracting DNA from mites of the family Tenuipalpidae and Phytoseiidae, respectively, keeping the carcass intact for identification after the DNA extraction procedure. In these two papers, the DNA extraction method was quite complex and consisted in the use of a lysis buffer (guanidine thiocyanate [GuSCN]) followed by DNA isolation on a silica matrix. Dabert *et al.* (2008) and

Pakin and Vink (2009) succeeded in extracting DNA from mites of the family Xolalgidae (Acari: Astigmata) and from spiders, respectively, using a Qiagen DNeasy tissue extraction kit. In contrast to the methods employed by Rowley *et al.* (2007) and Jeyaprakash and Hoy (2010), the specimens (or parts of them) were recovered before the end of the DNA extraction procedure, just after the lysis step. We currently perform DNA extraction of phytoseiid mites using the Qiagen DNeasy tissue extraction kit. This latter protocol includes a lysis step and DNA isolation using a column, and has been successfully applied to extract ancient DNA of preserved dry museology material (Badhury *et al.* 2007; Espeland *et al.* 2010). The present study aimed thus to test the combination of the two methods proposed in the literature: (1) the use of a commercial qiagen DNA extraction kit (as Dabert *et al.* 2008, Pakin and Vink 2009) and (2) the recovery of the carcass after the total DNA extraction procedure (Rowley *et al.* 2007, Jeyaprakash and Hoy 2010) for use as a voucher specimen.

Two mite materials were tested: 100 % alcohol preserved females and females mounted in lactic acid. Lactic acid digests the internal tissues and clear mites so that images of the prepared material can be taken and used for partial identification (at least to the genus level) prior to DNA extraction.

MATERIAL AND METHODS

Biological material

The two species considered belong to two different sub-families: Amblyseiinae for *Neoseiulus idaeus* Denmark & Muma, and Typhlodrominae for *Typhlodromus (Typhlodromus) exhilaratus* Ragusa. *Neoseiulus idaeus* was collected in Argentina (2004) on *Solanum americanum* Miller and *T. (T.) exhilaratus* on grapevine at Restinclières (Hérault, France) in 2009. These two species were mass reared in an air-conditioned chamber (25 °C, 75 % HR, 16L/ 8D) for several months and fed on *Tetranychus urticae* Koch.

Two types of material were studied: (i) alcohol conserved specimens of *T. (T.) exhilaratus* (four females and one male) and *N. idaeus* (ten females)

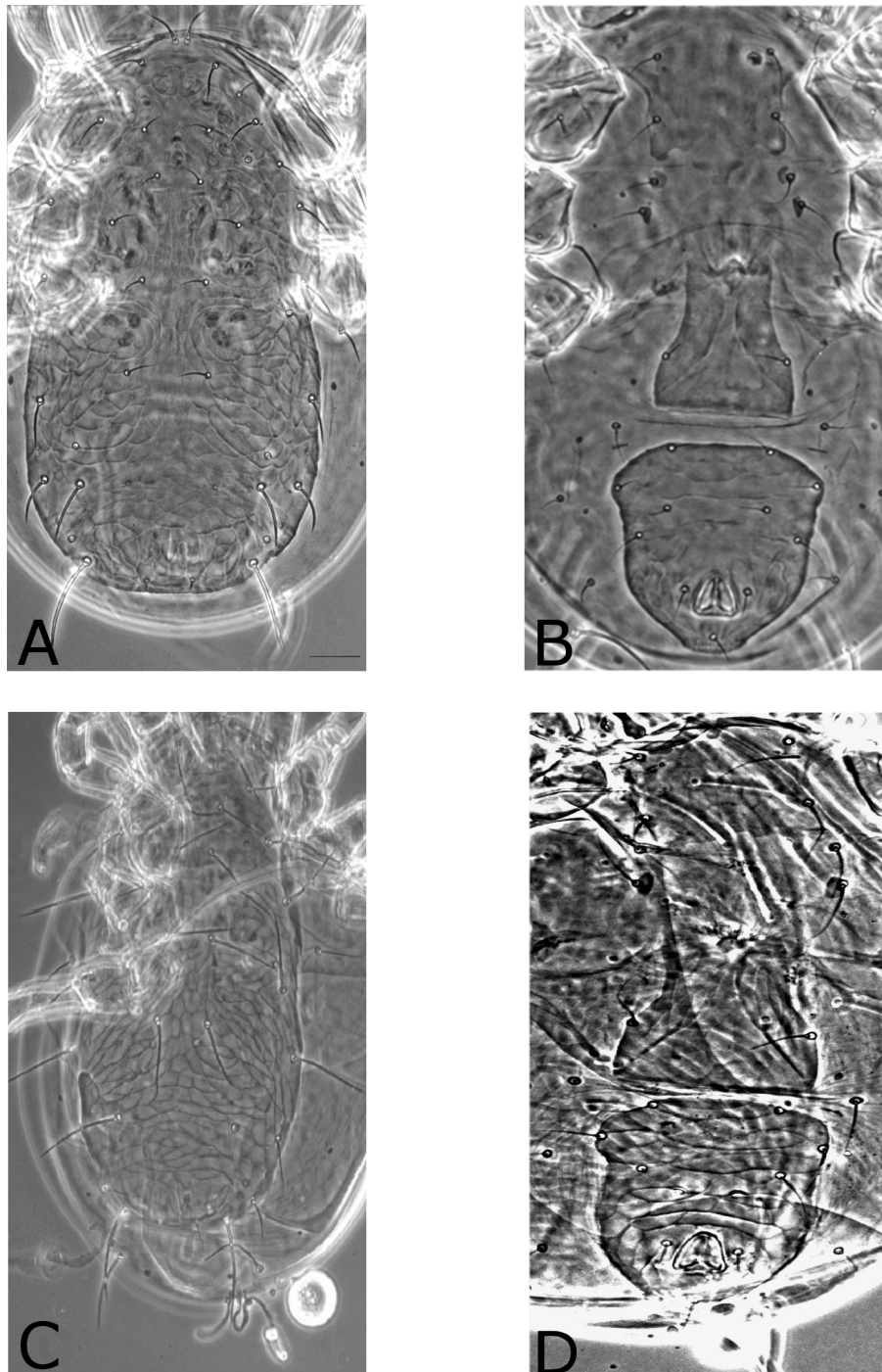


FIGURE 1: Slide-mounted females of *Typhlodromus (Typhlodromus) exhilaratus* and *Neoseiulus idaeus* after genomic DNA extraction using a Qiagen DNeasy kit. A – dorsal shield of *T. (T.) exhilaratus*; B – ventral shields of *T. (T.) exhilaratus*; C – dorsal shield of *Neoseiulus idaeus*; D – ventral shields of *Neoseiulus idaeus*.

(ii) specimens of *N. idaeus* (six females) mounted and kept in lactic acid for four hours. We choose

four hours for practical purposes (mounting in the morning and observations in the afternoon). This

duration allows sufficient time to clear mites for their identification, but longer periods are sometimes used. After four hours, the cover slip was delicately removed from the slide and the mite was collected with a fine hairbrush under a stereoscopic microscope. After observation, the mites were put in vials filled with 100 % alcohol for preservation until DNA extraction. Thus, when DNA extraction was carried out, these mites were "clean" and certainly only a trace amount of lactic acid remained associated with the specimen.

DNA extraction and carcass recovery

Total genomic DNA was individually extracted from each specimen (alcohol preserved and lactic acid treated), using a Qiagen DNeasy tissue kit (Qiagen, Hilden, Germany), according to the DNA extraction protocol "Purification of Total DNA from Animal Blood or Cells" (Spin-Column Protocol) adapted for extracting total DNA from mites, especially for the lysis phase and the volumes used. To a tube containing a single mite priorly air dried, we added lysis buffer (90 μ L Phosphate-Buffered Saline), 10 μ L Proteinase K and 100 μ L Buffer AL (Qiagen). After tubes were centrifuged for 5 min (13,000 rpm) and incubated at 56 °C for 16 h, 100 μ L of alcohol (100 %) was added. Following centrifugation during 1 min (8,000 rpm), this mix was then deposited on a DNeasy column and centrifuged for 1 min (8,000 rpm). The collection tube and its contents were thrown away and the column was kept. The DNA on the column was then washed with 250 μ L of buffer AW1 (Qiagen) which was removed by centrifuging 1min at 8,000 rpm. After that, the collection tube was thrown away and a second wash was performed with 250 μ L of buffer AW2 (Qiagen) followed by two centrifugations of 3 min each (13,000 rpm). Then, the DNA was eluted twice from the column using 50 μ L of ultra pure water and two centrifugations (1min at 8,000 rpm each).

At the end of the DNA extraction procedure, the mite carcass, located on the top of the column, usually around the edge, was collected with a fine brush using a stereoscopic microscope. Then, the mite was mounted on a slide in Hoyer's medium and observed with a phase and differential interfer-

ence contrast microscope (Leica DMLB, Leica Microsystems SAS, Rueil-Malmaison, France) (40x magnification). It was more difficult to retrieve lactic acid treated mites, because they were usually clearer than the alcohol preserved specimens and were therefore less visible on the white support (column).

Amount of DNA

The total amount of DNA was assessed using spectrophotometry (NanoDrop 8000, NanoDrop Technologies). DNA has maximal absorbance near 260 nm with an extinction coefficient of 50; whereas protein absorbs light most strongly near 280 nm. The A_{260} / A_{280} ratio therefore provides an estimate of DNA purity, where values of 1.8-2.0 suggest "clean DNA".

Marker used

A mitochondrial DNA marker (Cytochrome b mtDNA) was used. Primers for the amplification of this fragment were as follows: 5'-3' TAWRAARTATCAYTCDGGTTKRATATG and 3'-5' CCWTGAGGACAAATAWSWTT YTGAGG. These primers were obtained by Wannes Dermauw (University of Ghent, Belgium) for amplifying Cytb mtDNA of *P. persimilis* (W. Dermauw, pers. comm.). The PCR reaction was performed in a 25 μ L volume, containing 2 μ L of mite DNA (approximate concentration of 157ng/ μ L, D0: 260/280 = 2.05), 2.5 μ L (1 mM) of buffer 10X (Qiagen), 1 μ L (1.5 mM) of $MgCl_2$, 0.5 μ L dNTPs (0.05 mM for each nucleotide), 0.175 μ L (0.7 μ m) for each primer, 0.125 μ L (0.625 U) of Taq Qiagen and 18.525 μ L of water. Thermal cycling conditions were as follows: 94 °C for 3 min, followed by 35 cycles of 92 °C for 20 s, 53 °C for 1 min and 72 °C for 1 min, and an additional 5 min at 72 °C (Okassa *et al.* 2010). Amplified DNA was visualized by electrophoresis using 4 μ L of the PCR reaction on a 1.5 % agarose gel in 0.5 X TBE buffer (migration of 30 min at 100 volts).

DNA sequencing

In order to check that the PCR products obtained corresponded to phytoseiid mite DNA,

PCR products were sequenced using Dynamic ET Terminator Cycle Sequencing kit, and purified using ExoSAP-IT (Amersham Biosciences, GE Healthcare, Piscataway, NJ, USA). The sequencer used was the Megabase 1,000 apparatus (http://www.gelifesciences.com/aptrix/upp01077.nsf/content/autodna_megabase1000). DNA fragments were sequenced in both directions and were aligned and analysed with Mega 4.1 (Tamura *et al.* 2007). Accession numbers of sequences in Genbank database are shown in table 1.

RESULTS

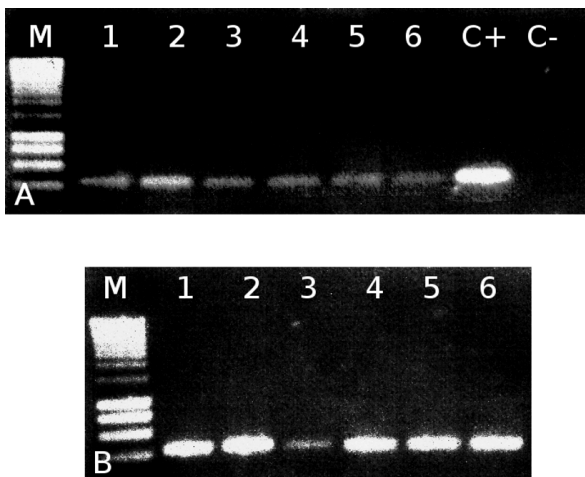


FIGURE 2: DNA bands of the expected sizes were amplified for the mitochondrial Cytb fragment from A – *Neoseiulus idaeus*, from B – from *Typhlodromus (Typhlodromus) exhilaratus*. A – Lane M shows the molecular weight marker, C indicates the water control, C+ indicates the positive control, lanes 1-6 show the PCR products of a single *N. idaeus* specimen preliminarily treated with lactic acid. Image B: Lane M shows the molecular weight marker, Lanes 1-5 show the PCR products of a single *T. (T.) exhilaratus* specimen conserved in 100% alcohol.

For the two types of mite material and the two species considered, all the specimens were retrieved on the columns after DNA extraction and all were mounted on slides. The figure 1 shows the dorsal and ventral shields of these specimens after DNA extraction. All structures used for species identification are visible; in rare cases (and only for mites treated with lactic acid) some dorsal setae were broken preventing measurements. The Qiagen kit pro-

cedure thus permits to retrieve, prepare and identify mites after DNA extraction, regardless of the material considered (alcohol or lactic acid treated mites).

Fragments of 426 base pairs (bp) were successfully amplified for all specimens (Figure 2). A BLAST search on the Genbank database and on our own DNA sequence database showed that the obtained sequences aligned with other Cytb sequences of the two species considered: *N. idaeus* and *T. (T.) exhilaratus*.

DISCUSSION

Access of voucher specimens

In agreement with results obtained by Jeyaprakash and Hoy (2010) and Rowley *et al.* (2007), the present study shows that it is possible to extract DNA from phytoseiid mite females and to preserve the carcass as a voucher specimen for the identification of morphological characters. The Qiagen DNeasy tissue kit thus seems to perform as well as the protocols proposed by these other authors. Unlike the protocols proposed by Dabert *et al.* (2008) and Pakin and Vink (2009), here, the specimens were retrieved at the end of the extraction procedure and not only after the lysis phase. The outlined method therefore provides a simple and rapid DNA extraction protocol for these mites.

Only two species were examined in the present study and DNA extraction success could vary according to mite size. However, we are quite confident that outlined method would apply equally well for other phytoseiid mite species, even smaller ones. Indeed, we have shown in a recent paper (Okassa *et al.* submitted) that the DNA extraction method we propose here allows to successfully extract DNA from eggs, larvae, protonymphs, deutonymphs and males of Phytoseiidae. Furthermore, since the development of this method, we have used it for recover voucher specimens of several Phytoseiidae species (data not shown). This approach could also be applied to other mite families; successful DNA extraction of one specimen of *T. urticae* (family Tetranychidae) was also performed using this same protocol (data not shown). The carcass of this mite was retrieved and identified, and

the DNA was successfully sequenced. However, additional investigations are required to quantify the adequacy of the present protocol for other mite families, in accordance with the required identification specificities of each group.

These results open new perspectives for Phytoseiidae taxonomy, but also for other mite families. Indeed, the accurate association of molecular and morphological attributes of a same specimen will be of great help in various taxonomic studies, including phylogenetic analyses and species identification (barcoding), particularly in the case of cryptic species (Magalhaes *et al.* 2007). Until now, Phytoseiidae specimens designated as vouchers were selected from the same series of specimens (same population, locality, and collection date) as those used for DNA extractions. These specimens were mounted on slides, identified and conserved in the mite collection. However, this method led to sev-

eral problems. When specimens of several species were found in a same sample (e.g., on the same plant in a same locality), it was impossible to determine to which species the sequenced species belonged. Thus, such samples were discarded regardless of their potential interest for specific systematic aims. Furthermore, even when only one species was present, this method limited the assessment of intraspecific variation, both for morphological and molecular analyses, as the number of specimens studied with the two approaches was obligatorily divided by two.

Acid lactic treatment and preliminary identification

The present study shows that it is also possible to extract DNA from mites preliminarily treated with lactic acid and to recover the carcass for a full mite identification afterwards. Lactic acid treatment

TABLE 1: Specimens and species considered and their accession numbers in Genbank.

Species	Stage	Specimen treatment	Accession numbers in Genbank
<i>Typhlodromus (Typhlodromus) exhilaratus</i>	female	alcohol conservation	HQ449405
<i>Typhlodromus (Typhlodromus) exhilaratus</i>	female	alcohol conservation	HQ449406
<i>Typhlodromus (Typhlodromus) exhilaratus</i>	female	alcohol conservation	HQ449407
<i>Typhlodromus (Typhlodromus) exhilaratus</i>	female	alcohol conservation	HQ449408
<i>Typhlodromus (Typhlodromus) exhilaratus</i>	male	alcohol conservation	HQ449409
<i>Neoseiulus idaeus</i>	female	alcohol conservation	HQ449410
<i>Neoseiulus idaeus</i>	female	alcohol conservation	HQ449411
<i>Neoseiulus idaeus</i>	female	alcohol conservation	HQ449412
<i>Neoseiulus idaeus</i>	female	alcohol conservation	HQ449413
<i>Neoseiulus idaeus</i>	female	alcohol conservation	HQ449414
<i>Neoseiulus idaeus</i>	female	alcohol conservation	HQ449415
<i>Neoseiulus idaeus</i>	female	alcohol conservation	HQ449416
<i>Neoseiulus idaeus</i>	female	alcohol conservation	HQ449417
<i>Neoseiulus idaeus</i>	female	alcohol conservation	HQ449418
<i>Neoseiulus idaeus</i>	female	alcohol conservation	HQ449419
<i>Neoseiulus idaeus</i>	female	lactic acid	HQ449420
<i>Neoseiulus idaeus</i>	female	lactic acid	HQ449421
<i>Neoseiulus idaeus</i>	female	lactic acid	HQ449422
<i>Neoseiulus idaeus</i>	female	lactic acid	HQ449423
<i>Neoseiulus idaeus</i>	female	lactic acid	HQ449424
<i>Neoseiulus idaeus</i>	female	lactic acid	HQ449425

clears the mite tissue, a step required to observe the structures used for species identification. After this treatment, it is possible to identify the mites (at least to the genus level) before performing DNA extractions. This is particularly important because of the relatively high cost of the Qiagen DNeasy tissue extraction kit. Preparing the mites in lactic acid enables one to observe them before DNA extraction, and therefore to select only those specimens of the species/genus of interest.

CONCLUSION

The application of recently developed molecular techniques is very important for the taxonomy of Phytoseiidae, opening new insights and facilitating in-depth studies of this group. Nevertheless, additional studies dealing with the DNA extraction of specimens from museum collections would be required to make systematic studies of phytoseiid mites even easier. Natural history collections are an invaluable source of biological data, including molecular data (Espeland *et al.* 2010). It is often easier to obtain a mite specimen from collections than to collect it in the field, particularly in remote regions of the world. Indeed, in field collections, the species expected is sometimes no longer present in the locality from where it was initially reported (due to local extinction of taxa and/or habitat loss). Phytoseiid mite collections consist in specimens mounted on slides in Hoyer's solution, a medium which contains arabic gum (30.0 g), water (50.0 ml), chloral hydrate (200.0 g) and glycerol (16.0 ml). Chloral hydrate, the main component of this medium, is a clearing agent of chitin that can alter DNA. Some preliminary tests carried out on slide-mounted mites (recent or ten years old) using the present methodology did not have positive results; PCR products were obtained, but all corresponded to human DNA (i.e., contaminations). The development of protocols that enable the extraction, amplification and sequencing of ancient DNA from these organisms are therefore required in order to be able to use museum mite collections for molecular taxonomic studies.

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