MOLECULAR ECOLOGY
RESOURCES
Molecular Ecology Resources (2010) **10**, 942–945 doi: 10.1111/j.1755-0998.2010.02839.x

DNA BARCODING

Coupling non-destructive DNA extraction and voucher retrieval for small soft-bodied Arthropods in a high-throughput context: the example of Collembola

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Abstract

Here, we describe a simple method adapted for high-throughput protocols allowing voucher specimen recovery for Collembola and by extension for other soft-bodied small arthropods. A standard extraction protocol was tested to examine the effects of lysis duration (1, 2, 4, 12 h) on DNA concentration, amplification success and specimen condition. Good quality DNA was obtained after 1 h of lysis, while voucher condition was fine for up to 12 h. The lysis step substantially shortens the clearing process necessary for morphological examination.

Keywords: Collembola, DNA barcoding, high-throughput, non-destructive DNA extraction, voucher retrieval

Received 28 June 2009; revision received 24 January 2010; accepted 31 January 2010

With the rapid development of DNA barcoding, large-scale projects are emerging in all major animal taxa, involving both the building of comprehensive sequence libraries for the chosen gene region (Hebert *et al.* 2003) and the creation of corresponding specimen reference collections of the voucher specimens that were analysed. In these projects, requiring strong taxonomic foundations, voucher collections are crucial as they allow subsequent verification of identifications via morphological analysis.

Misidentifications of the specimen that was analysed are common in existing genetic databases such as GenBank and EMBL, but as most sequences are not linked to a voucher specimen, error correction is impossible. Furthermore, cases of misidentification sometimes lead to the propagation of errors into new accessions (Harris 2003; Vilgalys 2003; Bidartondo 2008). The need for vouchers is particularly critical for small organisms such as Collembola that require several steps to gain a reliable species identification: specimens must be cleared in potassium hydroxide and lactic acid before slide mounting (Massoud 1967), but DNA extraction must occur before such preparation.

Until now, destructive DNA extraction protocols were used for Collembola, and the small size of specimens forced the use of whole specimens (Fanciulli *et al.* 1991; Frati *et al.* 2001; Simonsen & Christensen 2001; D'Haese 2002; Carapelli *et al.* 2005; Xiong *et al.* 2008). The creation of a reference collection of voucher specimens was hence impossible, especially in a high-throughput context involving automated extraction protocols. Yet the retention of voucher specimens is essential to build a valid barcode library for these organisms.

Methods of non-destructive extraction have been developed in other groups (Rohland *et al.* 2004; Favret 2005; Rowley *et al.* 2007; Hunter *et al.* 2008), but none allow voucher retrieval in a high-throughput context, which is a key requirement for DNA barcoding. The vast number of species to be barcoded precludes the use of classical workflows, necessitating new approaches.

In this work, we describe an adaptation of the automated high-throughput DNA protocol of Ivanova *et al.* (2006) modified to ensure the recovery of voucher specimens of Collembola. We evaluated the effect of lysis exposure time on the condition of vouchers and on the concentration of extracted DNA. We also report the

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Table 1 DNA concentration and number of sequences obtained for the different lysis durations

Time (H)	12	4	2	1
N	24	24	24	23
Mean DNA concentration (ng/μL) ± SE	10.2 ± 0.8	10.7 ± 1.2	9.9 ± 1.2	11 ± 1.4
Number of sequences	20	22	21	21

successful use of this modified protocol in different Collembola orders.

Ninety-five individuals of *Parisotoma notabilis* with lengths ranging from 0.5 to 1.0 mm were used for analysis. The specimens were placed into a 96-well plate in 50 μL of 95% ethanol. Before lysis, most of the ethanol was evaporated in an incubator at 65 °C for 20 min, but the retention of some residual ethanol was critical. Complete dryness damages the morphological characters and leads specimens to 'fly' out of the wells because of static electricity. Fifty microlitre of lysis buffer (described in Ivanova *et al*. 2006) was added to each well with the plate divided into four groups exposed to varying lysis intervals (1, 2, 4, 12 h) to test the effects on DNA concentration and condition of the voucher specimen.

Following lysis, specimens were spun down at 1000 rpm for 1 min. The lysis buffer was then transferred to a 96-well GHP Pall plate with a pore size of 0.45 μm (Pall Corporation, Ann Harbor, USA) and filtered into a new 96-well plate via 2 min of centrifugation at 3000 rpm.

After the lysis step, those vouchers that had gone through the tips of the pipette during the buffer transfer remained in the filter plate, while the others stayed in the original lysis plate. All of the vouchers from the filter plate (44 of 95) were transferred back into the original lysis plate, and 95% ethanol was added to each well to prevent desiccation. Vouchers were then prepared on slides for morphological examination.

Meanwhile, the lysis plate containing the filtered lysis buffer was placed into the Cytomat APL (Beckman-Coulter Inc., Fullerton, USA) for automatic DNA extraction following the standard high-throughput protocol of the Canadian Center for DNA Barcoding (CCDB)

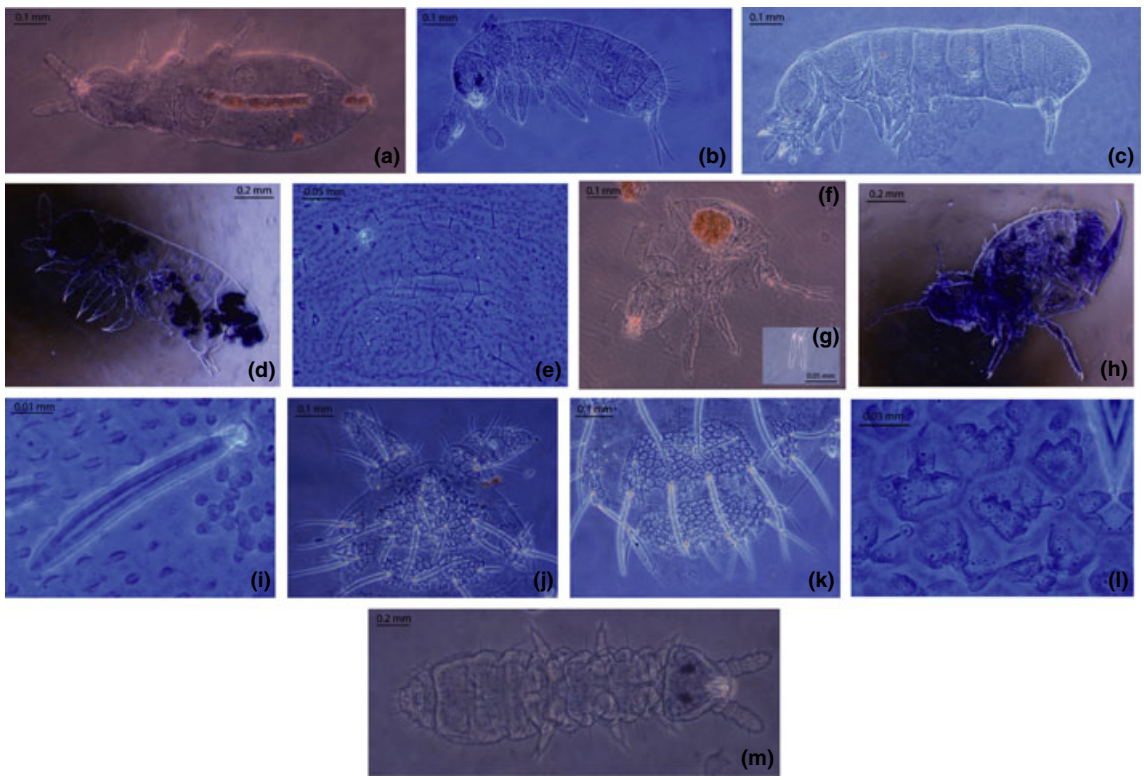

Fig. 1 Microscopic slides of processed specimens from different species (a) *Xenylla yucatana* - ventral view; (b) *Desoria sp.* – lateral view; (c) *Folsomia sp.* - lateral view; (d) *Hypogastrura sp.* – lateral view; (e) *Pronura sp.* – genital plates; (f) *Neelus murinus* – lateral view; (g) *Neelus murinus* – mucron; (h) *Lipothrix lubbocki* – lateral view; (i) *Monobella grassei* – macrochaeta on the Thorax III; (j) *Monobella grassei* – head tubercules and chaeta; (k) *Monobella grassei* – tubercules and chaeta on the abdominal segment; (l) *Monobella grassei* – tegument detail; (m) *Xenylla sp.* – dorsal view.

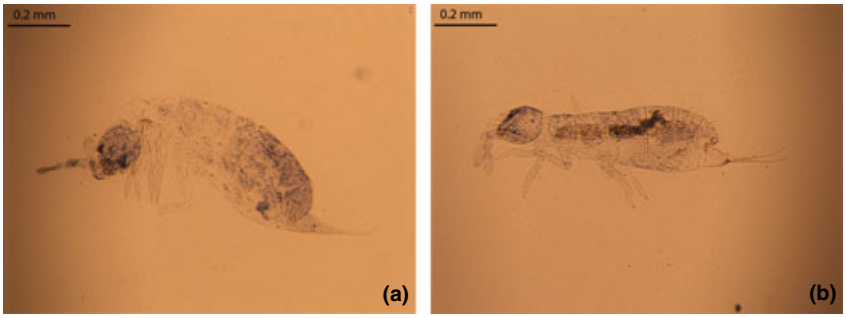

Fig. 2 Photos of microscopic preparations of specimens lysed for (a) 1H, (b) 12H.

(Ivanova *et al.* 2006), consisting of a silica-based extraction method performed by a Biomek FX liquid handling station (Beckman-Coulter Inc., Fullerton, USA).

To compare DNA concentrations obtained after the different lysis durations, the UV absorption of each DNA extract was measured, using a NanoDrop spectrophotometer ND-8000 (NanoDrop products, Wilmington, USA). Mean values for the different durations of lysis were compared using pairwise t-tests performed with SYSTAT 12 (Systat Software, Inc., Chicago, USA) (Table 1).

For each extract, DNA barcodes were generated for the 5′ region of the mitochondrial COI gene following the CCDB protocol for PCR and sequencing (detailed in Hajibabaei *et al.* 2005).

Microscopic examination of the specimens revealed that all diagnostic characters were intact after extraction including the presence and the position of chaeta, the tegument granulation and structures. Although this test of the protocol focused on a single species, *Parisotoma notabilis*, the same procedure was employed successfully for varied groups of Collembola (Fig. 1). *Parisotoma notabilis* was targeted for analysis because of its thin integument and fragile chaetae; other species from the Poduromorpha, Entomobryomorpha, Symphypleona and Neelipleona orders performed even better (Fig. 1). No difference in specimen condition was observed across the different lysis exposure times (Fig. 2). The centrifugation step did flatten some specimens, but they recovered their original shape in the potassium hydroxide and lactic acid stages required for clearing. Interestingly, the lysis process reduced the time needed for clearing of the specimens by approximately 50%.

The DNA concentrations obtained after the varied durations of lysis were not significantly different (Table 1), and sequencing success was similar, proving that good quality DNA can be obtained from a 1-h lysis (Table S1-supplementary material).

Without this voucher retrieval process added to the conventional high-throughput DNA extraction protocols (Ivanova *et al.* 2006), specimens are picked up by the Biomek FX liquid handling robot with the lysis buffer, and they are dried on the glass fibre during the residual ethanol evaporation step before DNA elution. For arthropods with a soft cuticle, this period of desiccation completely destroys the specimen, preventing morphological examination. By contrast, the simple modifications described here provide a solution for all small soft-bodied Arthropods in a high-throughput context, enabling retention of the voucher specimens needed to validate barcode reference libraries.

Acknowledgements

This work was supported by grants to PDNH from NSERC and from Genome Canada through the Ontario Genomics Institute. We thank Natalia Ivanova for technical advice.

References

Bidartondo MI (2008) Preserving accuracy in GenBank. *Science*, **319**, 1616.

Carapelli A, Frati F, Fanciulli PP, Nardi F, Dallai R (2005) Assessing species boundaries and evolutionary relationships in a group of south-western European species of *Isotomurus* (Collembola, Isotomidae) using allozyme data. *Zoologica Scripta*, **34**, 71–79.

D'Haese CA (2002) Were the first springtails semi-aquatic? A phylogenetic approach by means of 28S rDNA and optimization alignment. *Proceedings of the Royal Society of London B*, **269**, 1143–1151.

Fanciulli PP, Frati F, Dallai R, Rusek J (1991) High genetic-divergence among populations of *Tetrodontophora bielanensis* (Insecta, Collembola) in Europe. *Revue d'Ecologie et de Biologie du Sol*, **28**, 165–173.

Favret C (2005) A new non-destructive DNA extraction and specimen clearing technique for aphids (Hemiptera). *Proceedings of the Entomological Society of Washington*, **107**, 469–470.

Frati F, Spinsanti G, Dallai R (2001) Genetic variation of mtCOII gene sequences in the collembolan *Isotoma klovstadi* from Victoria Land, Antarctica: evidence for population differentiation. *Polar Biology*, **24**, 934–940.

Hajibabaei M, DeWaard JR, Ivanova NV *et al.* (2005) Critical factors for assembling a high volume of DNA barcodes. *Philosophical Transactions of the Royal Society B-Biological Sciences*, **360**, 1959–1967.

Harris DJ (2003) Can you bank on GenBank? *Trends in Ecology & Evolution*, **18**, 317–319.

Hebert PDN, Cywinska A, Ball SL, DeWaard JR (2003) Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **270**, 313–321.

Hunter SJ, Goodall TI, Walsh KA, Owen R, Day JC (2008) Non-destructive DNA extraction from blackflies (Diptera : Simuliidae): retaining voucher specimens for DNA barcoding projects. *Molecular Ecology Resources*, **8**, 56–61.

Ivanova NV, Dewaard JR, Hebert PDN (2006) An inexpensive, automation-friendly protocol for recovering high-quality DNA. *Molecular Ecology Notes*, **6**, 998–1002.

Massoud Z (1967) *Monographie des Neanuridae, Collemboles Poduromorphes a pièces buccales modifiées, Biologie de l'Amérique Australe*, Volume III, Centre National de la Recherche Scientifique, Paris.

Rohland N, Siedel H, Hofreiter M (2004) Nondestructive DNA extraction method for mitochondrial DNA analyses of museum specimens. *BioTechniques*, **36**, 814–821.

Rowley DL, Coddington JA, Gates MW *et al.* (2007) Vouchering DNA-barcoded specimens: test of a nondestructive extraction protocol for terrestrial arthropods. *Molecular Ecology Notes*, **7**, 915–924.

Simonsen V, Christensen PG (2001) Clonal and genetic variation in three collembolan species revealed by isozymes and randomly amplified polymorphic DNA. *Pedobiologia*, **45**, 161–173.

Vilgalys R (2003) Taxonomic misidentification in public DNA databases. *New Phytologist*, **160**, 4–5.

Xiong Y, Gao Y, Yin WY, Luan YX (2008) Molecular phylogeny of Collembola inferred from ribosomal RNA genes. *Molecular Phylogenetics and Evolution*, **49**, 728–735.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Pairwise t-tests comparing mean DNA concentration between the different extraction times (test of the 'not equal' hypothesis). Values above the diagonal are P-values (all non-significant) while t-statistics are shown below the diagonal.

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