

Optimized drowning procedures for pulmonate land snails allowing subsequent DNA analysis and anatomical dissections

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Abstract

We present a method for drowning landsnails from temperate climate that is suitable for large-scale studies that include anatomical, morphological and DNA-based methods. The snails were drowned for 30 min at room temperature and subsequently incubated in 37 °C water for 30 min. The water was then drained and the snails stored in 80% ethanol.

Key words: land snails, DNA extraction, anatomical analyses

Zusammenfassung

Wir präsentieren eine Methode zur Präparation von Landschnecken aus gemäßigtem Klima, die im Zuge einer groß angelegten Studie sowohl eine anatomische, als auch eine morphologische und genetische Untersuchung von möglichst vielen Individuen ermöglicht. Die Schnecken werden 30 min bei Raumtemperatur und anschließend 30 min bei 37 °C in Wasser inkubiert. Anschließend wird das Wasser abgeleert und die Schnecken in 80% Alkohol gelagert.

Introduction

Land snails in scientific collections that are intended for analyses of the soft body (e.g., anatomical dissection) are stored as whole individuals preserved in ethanol or formalin after they were killed by drowning. The classical procedure is drowning in boiled water for 12 to 48 hours to obtain well-relaxed soft bodies, which facilitates dissection (PIECHOCKI 1975). Also incubating 40-50 min in water up to 70 °C was proposed by BRATCHIK (1976). However, previously, drowning was abandoned as a method for killing snails for DNA extractions. In their study of large slugs (*Arion lusitanicus*), SCHANDER & HAGNELL (2003) stated that "prolonged drowning does have an acute effect on the possibility of obtaining DNA suitable for PCR. Already after one hour of drowning, the amount had decreased noticeably, and after six hours it was no longer possible to identify any distinct bands of PCR product". In the same study, drowning combined with cooling of the sample did not improve the results.

In the course of an extensive phylogeographic analysis of different Alpine land snail taxa (genera *Orcula*, *Trochulus* and *Cylindrus*), we wanted to obtain data on shell mor-

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phology, DNA analyses, as well as anatomical characters from each individual. However, leaving specimens after their death in water for up to 48 hours is not optimal for further DNA analyses. This is because, immediately after the specimen dies, the DNA starts to degrade until the specimen is either stored in ethanol or frozen (KRUCKENHAUSER & HARING 2010). At the same time, a well-stretched soft body that remains the shell undamaged is required for the anatomical preparation. Especially when handling many thousands of individuals, these requirements are a logistic and methodological challenge that should not to be underestimated. All these snails are smaller than 15 mm with respect to shell height or diameter. We developed a modified method of drowning, considering the fact that the snails die more quickly at higher temperature (BRATCHIK 1976). Hence the procedure allows successful PCR amplification from specimens with stretched soft bodies.

Methods and Results

We tested several methods of the drowning procedure with respect to their effect on DNA quality and stretching of the body. After collection and determination, snails of one taxon and from one collection locality were pooled in a 50 ml glass bottle with screw closure. The bottle was then filled to the top with water. The glass bottle did not contain more snails than one fourth of the bottle's volume. The following methods were tested, whereby both water directly from the tap and distilled water were tried for each approach: 1) Adding preheated (50 °C) water, 10 min incubation. 2) Incubation for 30 min at room temperature in water, incubation for 15 min (50 °C) or up to 45 min (37 °C). Subsequently, the water was drained and the glass filled with 80% ethanol. After fixation with 80% ethanol, the snails were stored at 4 °C. For DNA extraction we individualised them, cut off a tiny piece of the foot and stored the tissue as well as the remaining snail in 80% ethanol in separate 1.5 ml microcentrifuge tubes. DNA was extracted with four different extraction methods: DNAeasy blood and tissue kit (QUIAGEN), DNeasy Plant Mini Kit (QUIAGEN), First-DNA all-tissue Kit (GEN-IAL), and a standard phenol/chloroform extraction protocol.

Of all drowned specimens, selected individuals were used for DNA analyses (2450 to date). All of them, irrespective of the drowning method, contained DNA of sufficient quality for our purposes as measured by successful PCR amplification of mitochondrial DNA (standard fragment size: 705 bp, maximal size: 2.5 kb), nuclear DNA (maximal size 1.1 kb) as well as microsatellite loci (maximal fragment sizes of 290 bp; only in *C. obtusus*). Concerning the different methods of extraction, all protocols and kits proved useful and yielded high-quality DNA. Thus, there seems to be no inhibiting effect of mucopolysaccharides of the snails, which has been reported by different authors and are summarized in SKUJIENE & SOROKA (2003). Since, these methods proved to be successful for DNA analyses; we neither tested the DNA quality after incubation longer than 45 min nor after incubation at higher temperature than 50 °C. But we presume that there is some tolerance of both parameters with respect to the resulting DNA quality. This makes the method also applicable apart from laboratory conditions, as long as a thermometer and a heater are available.

It was very difficult to clearly identify the influence of the treatment of the snails on the percentage of individuals with well stretched bodies. None of the procedures tested so

far guaranteed an extended soft body. Moreover, our experience shows that the posture in which a snail dies differs among the tested taxa. The death of an individual was tested by taking it out of the glass bottle and irritating the soft body with a needle. Whereas nearly all specimens of the genus *Trochulus* died with an extended soft body, this was less frequently the case for *C. obtusus* and only rarely so for the different taxa of *Orcula*. We assume that these differences are caused by physiological disparities of the three taxa. The following approach, however, seems to increase the percentage of snails with extended soft body: Early in the morning the snails are removed from the refrigerator, where they were stored after taxonomic identification. Several drops of water are added to increase humidity and the glasses are allowed to warm up to room temperature. As soon as the snails are active, water (at room temperature) is poured on them. Of the preparation procedures mentioned above, method 2 with 37 °C seems to work best for achieving extended soft bodies.

DNA analyses were successfully performed also on selected individuals of larger snails treated with the same methods (e.g., *Euomphalia strigella*, *Helicodonta obvoluta*, *Chilostoma achates*, *Arianta arbustorum*, *Cepaea hortensis*, *Helicigona lapicida*, *Monacha cantiana*). The only difference with those specimens was that warmer water (50 °C) was used to shorten the time until the snails die. It also has to be stated that the required temperature is supposed to depend on the natural temperature optimum of each snail species.

In summary, drowning the snails for 30 min at room temperature and subsequent incubation in 37 °C water for 30 min is a suitable method for preparing snails from temperate climate for large-scale studies that include anatomical, morphological and DNA-based methods.

Acknowledgements

We want to thank Elisabeth Haring, Wilhelm Pinsker, Anatoly Schileyko and two anonymous reviewers for fruitful comments on the manuscript, as well as Michael Stachowitsch for English editing.

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