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Niku-nuki: a useful method for anatomical and DNA studies on shell-bearing molluscs

HIROSHI FUKUDA[1], TAKUMA HAGA[2] & YUKI TATARA[3]

[1] *Conservation of Aquatic Biodiversity, Faculty of Agriculture, Okayama University, Tsushima-naka 1-1-1, Okayama 700-8530, Japan, E-mail: suikei1@cc.okayama-u.ac.jp*

[2] *Department of Biological Science, Graduate School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan, E-mail: haga@kahaku.go.jp*

[3] *Department of Biology, Faculty of Science, Toho University, Miyama 2-2-1, Funabashi 274-8510, Japan, E-mail: ykui@msc.biglobe.ne.jp*

Abstract

Often only one or a few individuals of rare species are collected. How do we treat them as intact voucher specimens? The shell of the whole individual in formalin or alcohol will corrode or fade. In order to dissect the soft parts, you must crack or dissolve the shell. *Niku-nuki*, a traditional method that has been used by Japanese malacologists overcomes this dilemma. It is also applicable to minute molluscs. The outline is: 1. Prepare boiling hot freshwater, a small beaker, forceps (with fine tips), a small syringe, a petri dish, and a stereomicroscope; 2. When the live animal in the beaker crawls on the bottom, pour boiling hot water over the animal, which is killed immediately. Some seconds later take the specimen out of the hot water, hold it with two fingers of one hand and hold the forceps with another hand; 3. Under the microscope, grab the foot with the forceps and pull carefully to just separate the columellar muscle from the shell; 4. Pull the foot again in a petri dish filled with cold water as under 3. With coiled gastropods, unscrew the specimen by approximately ¼ whorls. If it is difficult to move the soft parts, inject water into the aperture gently with the syringe. Repeat it several times, then you will get an empty shell and the complete soft parts. With this method, we can obtain intact shells and soft parts for multiple purposes such as conchological observation and gross anatomy. DNA can also be extracted from those soft parts because DNA is stable at high temperature. The boiled animal can be dehydrated in alcohol. We can prevent the negative effect of DNase (by heat) and magnesium (by washing in freshwater) on the DNA.

Key words: Mollusca, Gastropoda, Bivalvia, Scaphopoda, anatomy, DNA analysis, soft parts, specimen preparation

Introduction

If you are a taxonomist of shelled molluscs, sometimes you should examine a specimen of a very rare species: *e.g.*, endangered species, taxon with a narrow range, material from the locality/habitat, which is very hard to access. When you get only one individual of such a rare species that is extremely important for your study, how do you treat it? If you need to describe the species as new, you must keep the shell intact as the holotype. On the other hand, to determine the systematic position, you may need to dissect the soft parts or extract the DNA from the *same* specimen. In these cases, how is it possible to leave both shell and soft parts as an intact voucher specimen?

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If you put the whole individual directly into formalin or alcohol, the shell may corrode or fade. Moreover, we often see that the shells were dissolved or completely lost in old specimens in formalin because of acid dissolution.

In species with a thick shell and operculum, the soft parts may be rotten and not available for anatomical works, especially in specimens that were unrelaxed prior to preservation. Houbrick (1991a: 36) mentioned that most of museum specimens of *Faunus ater* (Linnaeus, 1758) (Thiaridae), which he examined, were "badly fixed due to poor penetration of the fixing fluids." He (1991b: 290) tried to crack the shells of living materials of *Terebralia palustris* (Linnaeus, 1767) and *Telescopium telescopium* (Linnaeus, 1758) (both Potamididae) "in a large vise" in order to avoid the above problem, but he complained again that "it is very difficult to remove these snails from their strong shells successfully without injuring and destroying tissues" and "damaged or injured snails secrete great quantities of mucus."

Either way, you must destroy or dissolve the shell, if you need to dissect the soft parts after fixation: then you will lose the shell. This is a dilemma. Do you give up either the shell or the soft parts? Ideally, shells are kept as dry specimens and soft parts as wet ones—*i.e.*, the soft parts are removed from their shells without damage before fixing the whole animal.

Among shell collectors, extracting the soft parts from the shell has been widely practiced to prevent damage (*e.g.*, bad smell, mold, infestation of insects) to the shell caused by the remaining rotting soft parts. Boiling in hot water is prevalent as the technique. One of the earliest accounts on this method appears to be Mawe (1825: 2): "To perform this [= removal of the animal], nothing more is requisite than to put the shell into a kettle of water, and let it heat gradually, until it boils. — After a few minutes, the shell should be taken out, and put into a bucket of cold water; the animal will then shrink, and may generally be shaken from the shell; but if it should still adhere, it may be extracted with a crooked pin or hook, great care being taken not to injure the mouth, which is commonly the most tender part."

The method has been introduced in many shell collectors' guide books (*e.g.*, Morris 1966: xi–xii; Griffith 1967: 11; Abbott 1968: 42; Coleman 1976: 89–90; Kerney & Cameron 1979: 30; Sturm *et al*. 2006: 23; Pearce 2006: 193; Poppe 2008: 59), but shell books published during the past decade tend to discourage taking live specimens. Also, this technique has been adopted mainly by shell collectors and is poorly known among younger malacologists today. The fact may reflect the growing gulf between field biologists (who might be expected to have some experience of traditional specimen preparation) and laboratory-based biologists for whom these instructions could indeed be of use.

On the other hand, Japanese malacologists have used a similar method for a long time. The method is called *niku-nuki* in Japanese (*niku* means the soft-parts and *nuki* means extracting; summarized by Geiger *et al*. 2007: 13). We here emphasize that improvement and modification of *niku-nuki*-methods have been one of the main interests of research malacologists in Japan because the method has been well-known in their community to be useful to obtain not only the clean shells but also the soft parts suitable for the anatomy and DNA analysis. Therefore, the meaning of the Japanese term *niku-nuki* is slightly different from the non-Japanese method only for shell cleaning.

When Hirase (1897) published one of the earliest Japanese textbooks for shell collecting since modern malacology started in Japan, he included a brief instruction of *niku-nuki* using hot water in a chapter about the preparation of specimens in the book. Subsequently Yagura (1922: 371) provided a more detailed malacological textbook and he wrote that "top priority is *niku-nuki*" at the beginning of

the chapter of specimen preparation. Kuroda & Habe (1949: 11–13), Kira (1959: 202–204) and Habe & Kosuge (1967: 188–190) also started the chapter about specimen preparation with the same phrase of Yagura (1922) in their books. In addition to those, several major textbooks or pictorial books of Japanese molluscs were published (*e.g.*, Habe 1958: 17–18; Azuma 1995: 233–234; Okutani 1986: 382–384; Yukita 2003: 23–25), and the detailed descriptions of *niku-nuki* occupy at least two pages of these books. General textbooks for the preparation of animal and plant specimens also include the similar descriptions in their molluscan sections (*e.g.*, Saito & Hasegawa 2003: 59–60; Ishida 2007: 83–86). Much information and many discussions about *niku-nuki* have been published in Japanese malacological journals and newsletters (*e.g.*, Taki 1929; Kosuge 1967a, b, 1969; Kojima 1972; Sorita 1973). Malacologists from Japan usually make references to these articles and benefit from the method for their research.

In this paper we introduce the procedure of *niku-nuki* in detail to the international readership and report the results for various taxa of shell-bearing molluscs. Although *niku-nuki* has been used mainly for large-sized species, it can also be applied to minute ones (less than 10 mm in shell length; Fig. 1B–D,F–K).

Case of *Ceratia nagashima* Fukuda, 2000

Ceratia nagashima (Caenogastropoda: Rissooidea: Iravadiidae; about 3 mm in shell length) was described by one of us (Fukuda 2000) based on a single specimen. The only material was collected from the proposed site for a nuclear power plant. If the construction plan were carried out, the locality would be lost to a landfill. Therefore, the species should be described immediately for discussions on conservation of the biodiversity and ecosystem of the site (Fukuda *et al*. 2000).

Although the species has some distinct characters on the shell and external head-foot, the anatomy of the soft parts (at least the reproductive system) is necessary for the determination of the generic status of the rissooideans (Ponder 1988). On the other hand, the shell should be kept intact as the type specimen without damage. This was a typical case of the dilemma that we pointed out above.

However, the shell and soft parts of the specimen were separated without any damage to both using the *niku-nuki* method. Fukuda (2000) described the species with full anatomy including the pallial cavity, buccal complex and nervous system successfully. This species has never been rediscovered since the original description.

The *niku-nuki* method

The outline of the *niku-nuki* method is rather simple. Figures 2 and 3 show examples for a large-sized gastropod [*Omphalius pfeifferi* (Philippi, 1846); Trochidae] and a bivalve (*Corbicula japonica* Prime, 1864; Corbiculidae). At first, the live individual is boiled by immersing it into hot water filled in a pan on a gas stove, which supplies high temperature (an electric heater is useful for smaller species; see below). Several minutes later, the individual is taken from the hot water, and the columellar or adductor muscle is separated from the shell using forceps. This method only works with live material. Dead animals are not suitable, because the columellar muscle and adductors can not be separated by heat.

For gastropods (Fig. 2), both the shell and soft parts are unscrewed by hand after separating the columellar muscle. After they are unscrewed several times, an intact empty shell and the complete soft parts are obtained. The shell will be dried, and the soft parts are fluid preserved.

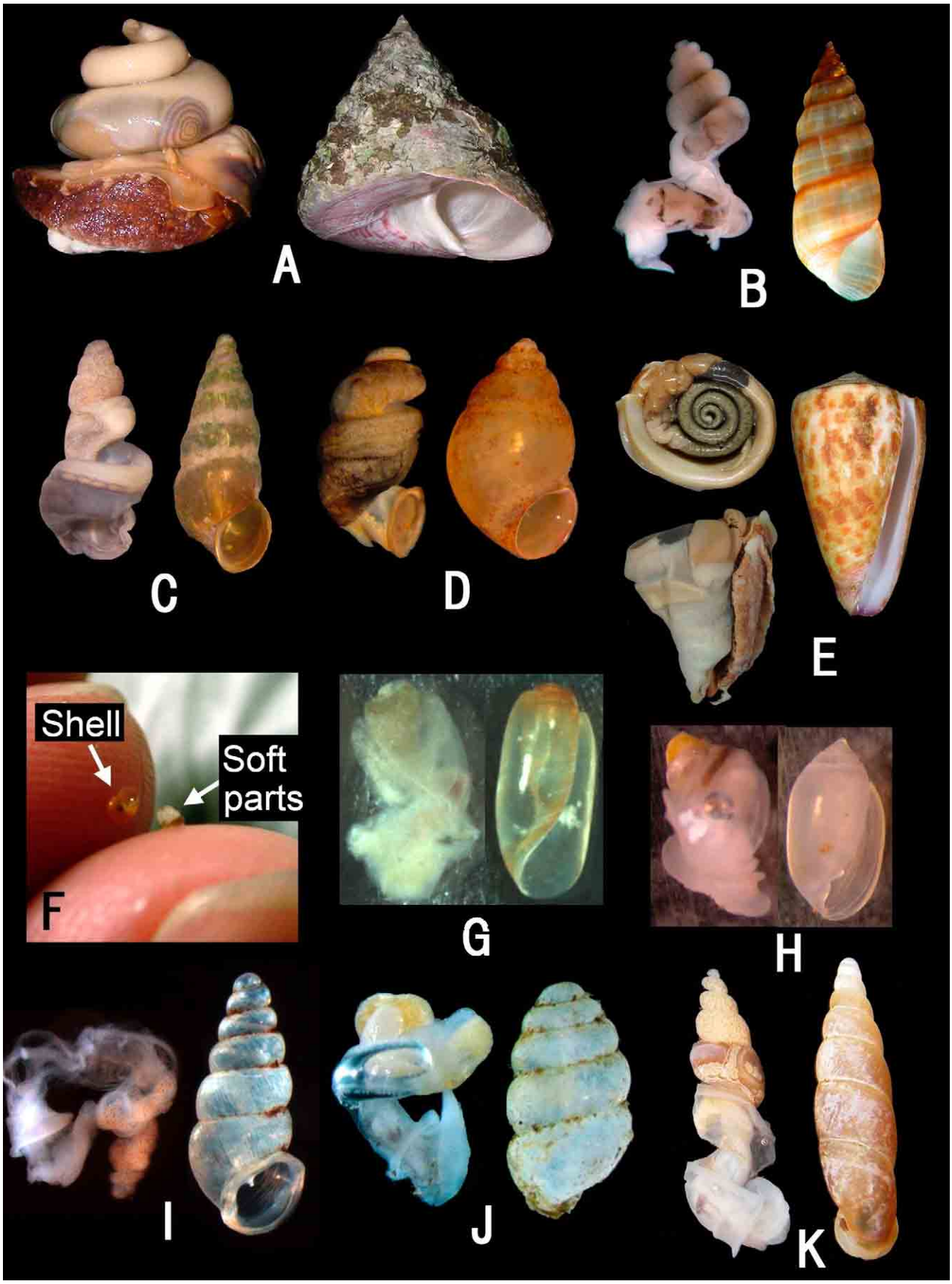

A
B
C
D
E
Shell
Soft parts
F
G
H
I
J
K

Figure 1 (previous page). Intact shells and soft-parts of various gastropods after *niku-nuki*. **A.** *Tectus conus* (Gmelin, 1791) (Trochidae). **B.** *Finella rufocincta* (A. Adams, 1861) (Scaliolidae). **C.** *Oncomelania nosophora* (Robson, 1915) (Pomatiopsidae). **D.** *Stenothyra japonica* Kuroda, 1962 (Stenothyridae). **E.** *Conus tessulatus* Born, 1778 (Conidae). **F.** *Tomura* cf. *yashima* Fukuda & Yamashita, 1997 (Cornirostridae). **G.** *Didontoglossa decoratoides* Habe, 1955 (Cylichnidae). **H.** *Microtralia acteocinoides* Kuroda & Habe, 1961 (Ellobiidae). **I.** *Carychium noduliferum* Reinhardt, 1877 (Ellobiidae). **J.** *Gastrocopta armigerella* (Reinhardt, 1877) (Pupillidae). **K.** *Hemiphaedusa pinto* (Pilsbry, 1901) (Clausiliidae).

Uncoiled gastropod (limpet) and scaphopod shells are easy to separate from the soft parts simply by boiling in hot water. We tried to do *niku-nuki* for two scaphopod species, *Graptacme buccinulum* (Gould, 1859) (Dentaliidae) and *Dischides belcheri* (Pilsbry & Sharp, 1897) (Gadilidae). Both species were easily processed using hot water of 90°C for 5 seconds.

For bivalves (Fig. 3), the method is similar to that for gastropods, but you do not need to wait for clams to open before they are boiled. At first, put a live individual in boiling water, and when the valves slightly open after several seconds, it should be taken out of the water immediately. Then insert the tip of forceps inwardly from the dorsal portions to separate the adductors. When the valves are still closed, insert a sharp blade from the ventral margin to make a narrow gape there, because the forceps should reach the adductors. Finally, both intact shells and soft parts are obtained.

A strainer is helpful to hold bivalves in boiling hot water. A specimen can be set in a strainer and put in hot water. When the valves open slightly, the specimen can be taken from the hot water immediately. If the valves do not open, a knife or other edged tool works well to assist opening the valves and draining hot water, which enters between the valves. The knife can be inserted gently and slightly to produce a gap between right and left valves. It is then twisted laterally, and the specimen is placed in hot water again.

To separate the columellar muscle from the shell, long immersion in boiling hot water is desirable. On the other hand, especially in gastropods, the visceral mass (particularly the digestive gland) becomes hard at high temperature and immovable within the shell. In this case the soft parts will break at the posterior end of the pallial cavity and the visceral mass will remain in early whorls of the shell.

Thus, the most critical point in *niku-nuki* is to find the adequate temperature and immersion period in hot water for both separating muscles and keeping the visceral mass soft. The best temperature is quite variable for different species (see below, Appendix 1).

Methods of *niku-nuki* for micromolluscs

Equipment. To boil the animal, an electric kettle with the function of heat-retention ("electric boilers and warmers" in the Americas) is convenient. We are using CH-CC10 of Zojirushi Co. Ltd. (Tokyo, Japan; 100V, 430W). Sometimes a pan on a gas stove is also needed to obtain hot water of about 100°C.

Prepare small beakers (50–200 ml), petri dishes, forceps with fine tips, and syringes of various sizes. A plastic pipette is useful, if you elongate the nozzle by heating.

Procedure for microgastropods. Figure 4 shows an example of *niku-nuki* for *Paludinellassiminea japonica* (Pilsbry, 1901) (Assimineidae; about 6 mm in shell length).

At first (Fig. 4A), a live animal is put in the beaker. Make sure the animal crawls on the bottom of beaker, for two reasons: the head-foot should remain outside the aperture after death of the animal to make it easy to pull the body out; the hot water should enter to the inside of the aperture to facilitate the separation of the columellar muscle from the shell.

Second (Fig. 4B), pour hot water from the kettle over the animal (temperature of water should be adapted for different species: see below, Appendix 1). Several seconds later, quickly pour out the water and remove the specimen from the beaker.

Under a stereomicroscope (Fig. 4C), hold the shell with two fingers of one hand [forceps should be avoided because the shell surface may be damaged and become unsuitable for observations with a scanning electron microscope (SEM)], and hold the forceps with the other hand. Then grab the foot gently with the forceps, and pull carefully to just separate the columellar muscle from the shell. One may feel when the muscle becomes free from the shell.

After the posterior half of the pallial cavity lies outside the aperture, unscrew the shell and soft parts in a petri dish filled with cold water (Fig. 4D,E). If the soft parts are hard to move, try to gently inject water into the aperture using the syringe or pipette.

Figure 2. Outline of *niku-nuki* for gastropods [example: *Omphalius pfeifferi* (Philippi, 1846), Trochidae]. **A.** Boil live individuals in hot water. **B.** Separate the columellar muscle from the shell. **C.** Unscrew the shell and soft parts. **D.** Wash and dry the shell. Fix and preserve the soft parts.

Suitable temperature and immersing period. The most suitable temperature of hot water is quite variable among species. It depends on the shell size, shape and thickness. As mentioned above, if you use water of too low temperature or boil for too short a time, you may fail to separate the columellar muscle from the shell. If you boil the animal for too long, the visceral mass will be hard and remain in the earlier whorl. Appendix 1 shows the most suitable temperature and immersion period for various gastropod taxa with some additional remarks.

The water can be cooled by blowing over the water surface. Our electric kettle (Zojirushi CH-CC10) keeps the water at 95°C. In this case, if hot water is poured into a 100 ml beaker about 1 cm deep, by blowing over it once it is cooled to approximately 90°C. Most species with small, thin, translucent shells require about 80–82°C, which can be obtained by blowing three times.

Some American and Japanese articles and books mention a method using a microwave oven for *niku-nuki* (*e.g.*, Kojima 1972; Sturm *et al.* 2006). However, the microwave oven is too radical for both the shells and soft-parts of micromolluscs. The soft-parts will always be broken and the shells sometimes crack.

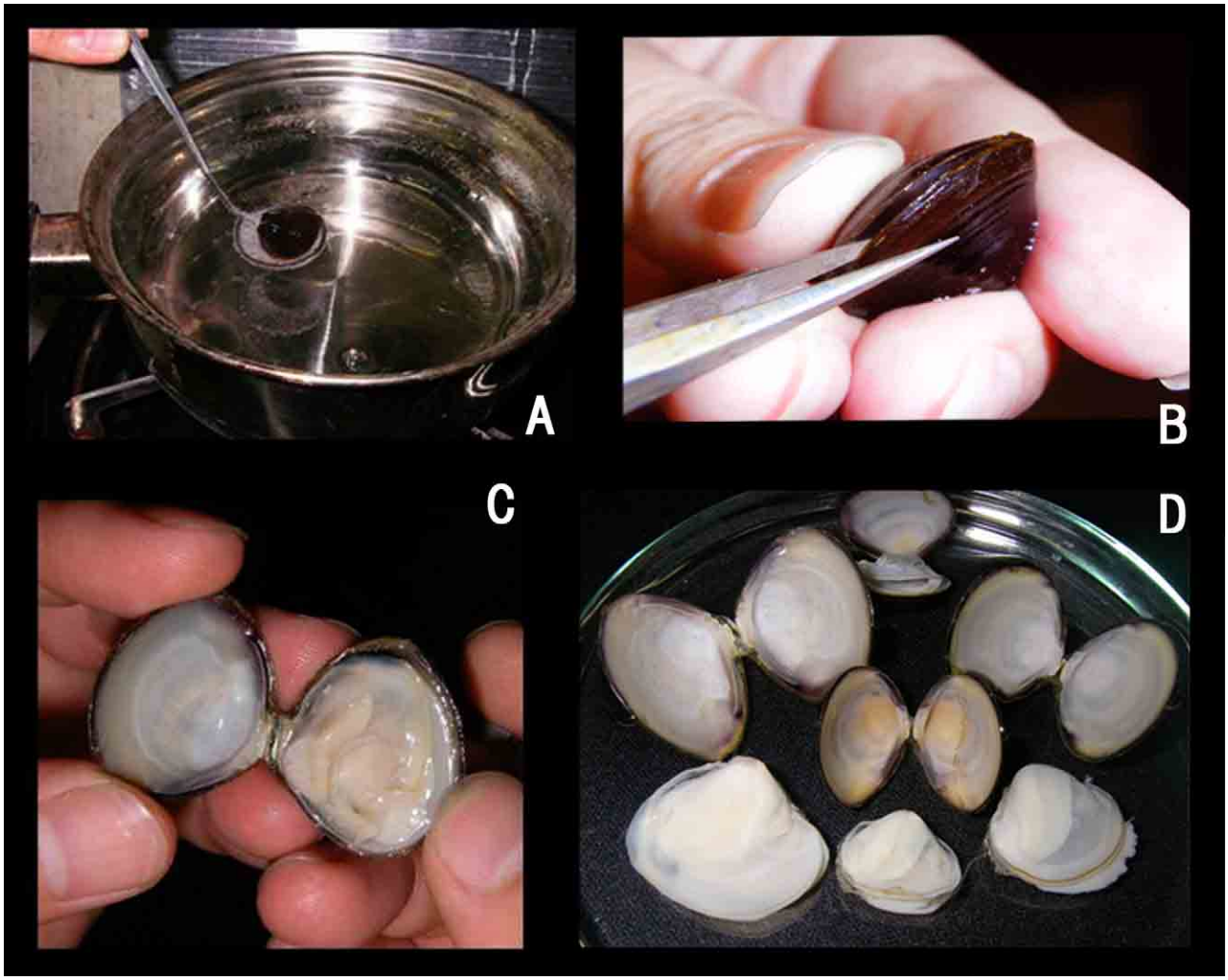
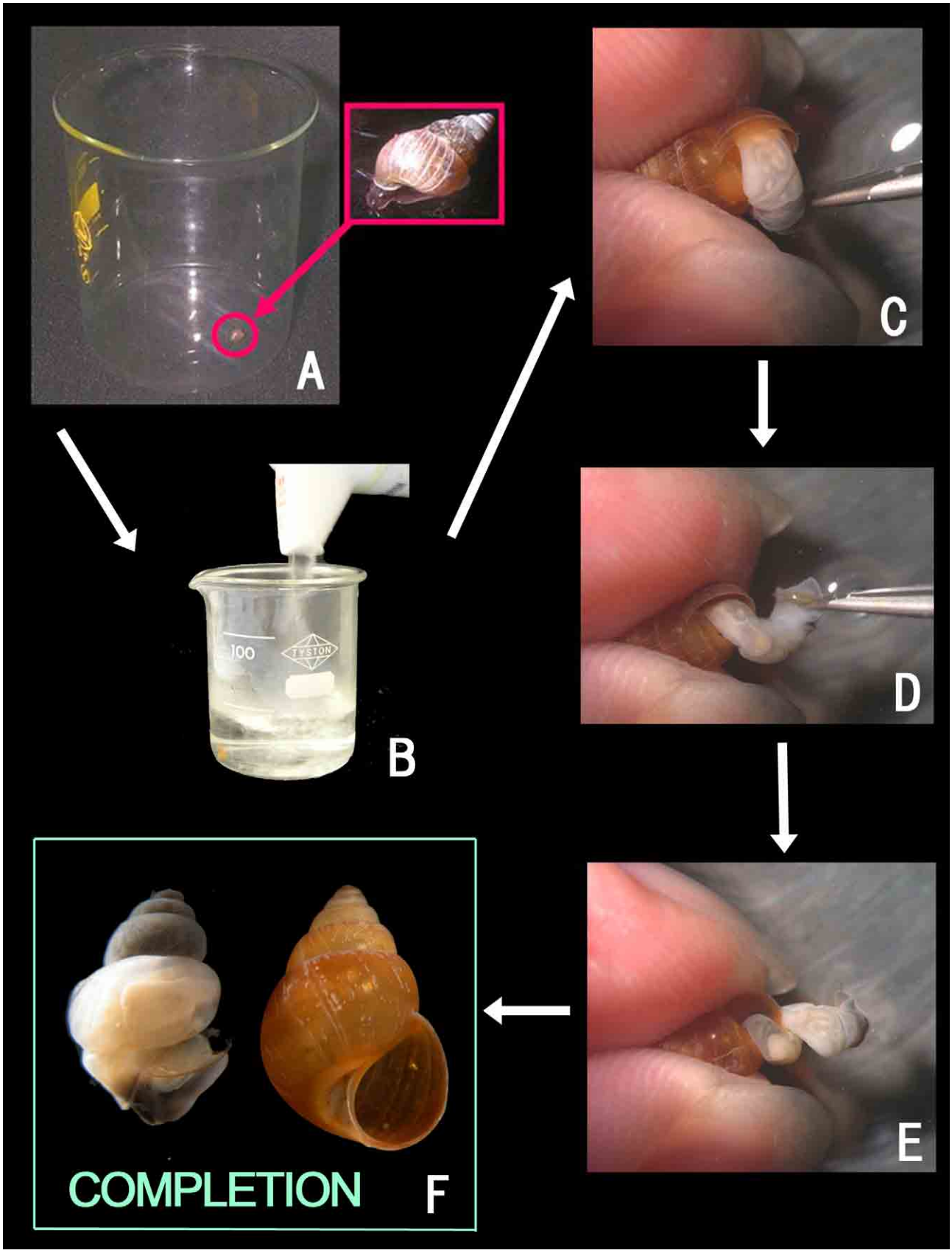


Figure 3. Outline of *niku-nuki* for bivalves (example: *Corbicula japonica* Prime, 1864, Corbiculidae). **A.** Boil a live individual in hot water. **B.** When the valves slightly open after several seconds, take it out of the water immediately. **C.** Insert the tip of forceps inwardly from the dorsal portions to separate adductors. The intact soft parts are then removed. **D.** Wash and dry the shell. Fix and preserve the soft parts.



Drilling a hole on early whorl. The soft parts of species with tall spires and many whorls are sometimes difficult to remove. For these species, drill a minute hole in the early whorl of the shell with

Figure 4 (preceding page). Procedure of *niku-nuki* for microgastropods [example: *Paludinellassiminea japonica* (Pilsbry, 1901), Assimineidae]. **A.** Put a live animal in the beaker. Make sure the animal crawls on the bottom. **B.** Pour hot water from the kettle over the animal. Several seconds later, take the specimen out of hot water. **C.** Under a microscope, hold the shell with two fingers of one hand, and hold the forceps with another hand. **D.** Grab the foot gently with the forceps, and pull carefully to just separate the columellar muscle from the shell. **E.** Unscrew the shell and soft parts in a petri dish filled with cold water. Inject water into the aperture gently using the syringe or pipette, if needed. **F.** Repeat E several times, and then intact empty shell and complete soft parts are obtained.

a very fine, solid needle (Yamasaki & Ueshima pers. comm.), just after boiling the animal (Fig. 5). Immediately after the columellar muscle is separated from the shell, inject the cold water with the pipette or syringe. The soft parts will come out from the aperture. Geiger *et al*. (2007: 8) gave a detailed description of drills and tool sharpening.

However, for some species with many whorls (*e.g.*, some terebrids, triphorids, cerithiopsids) it is quite difficult or almost impossible to remove the soft parts. We do not know the adequate method of *niku-nuki* for these species at present.

Procedure for microbivalves. For the microbivalves, the procedure mentioned in "The *niku-nuki* method" (see above) can be applied carefully under a stereomicroscope.

Advantages of *niku-nuki* for anatomical works

In our experience, boiled animals following the *niku-nuki* procedure are suitable for gross anatomy, because the transparent parts become slightly opaque by heat, and their outlines are easily detected. Especially the glandular tissue, tiny ducts, or nerves are clearly distinguishable from the background. Also, secretion of mucus from live tissue can be avoided and the problem that Houbrick (1991b; see above) complained about can be solved. As mentioned above, all anatomical drawings of *Ceratia nagashima* by Fukuda (2000) were made using a boiled animal.

Boiled animals are also useful for the SEM examination of the animal with critical point drying and freeze-drying (*e.g.*, Fukuda & Yamashita 1997 for *Tomura yashima* Fukuda & Yamashita, 1997; Cornirostridae). Furthermore, they are suitable for serial sectioning for examining the arrangements of complicated muscular tracts, chambers and glands (*e.g.*, Fukuda 1994 for *Cylindrotis quadrasi* Moellendorff, 1895; Ellobiidae), but may not suitable for fine histological examinations.

Advantages of *niku-nuki* for DNA works

Ueshima (2002) first pointed out that soft parts fixed after *niku-nuki* were available for DNA analysis, because DNA was heat stable. He also mentioned that the *niku-nuki*-treated samples could produce better results than tissues from animals that were fixed within the shell. Subsequently Kano (2008: 3) and Sekine *et al.* (2006: 230) successfully determined DNA sequences from the *niku-nuki*-treated samples for gastropods and bivalves, respectively. Pure alcohol (*e.g.*, > 95% ethanol) was used therein for dehydration and preservation after *niku-nuki*, the same as in the other preparation methods.

As Ueshima's (2002) note was written in Japanese, it is conceivable that the advantage of the *niku-nuki*-treated material for molecular studies has not been recognized outside Japan, nevertheless

Geiger *et al*. (2007: 13, based on Fukuda pers. comm.) briefly referred to it. To confirm the availability and advantage of *niku-nuki*, we conducted the following simple experiments. Experiments were duplicated to gauge the reliability.

Eight mature specimens of the trochid *Monodonta labio* (Linnaeus, 1758) were collected from intertidal boulder shores in Kitaibaraki, Ibaraki Prefecture, Japan, in May 2007. Two live individuals each were treated by four standardized procedures prior to subsequent fixation in 99% ethanol: 1) whole soft parts were extracted following *niku-nuki* protocols as mentioned here; 2) animals were anesthetized by immersion in 7.5% $MgCl_2 \bullet 6H_2O$ in freshwater solution for three hours; and 3) shells were cracked. The specimens were then immediately preserved in 99% ethanol following Ueshima (2002): animals were wiped off, preserved independently in large volumes of ethanol (more than 10 times of the volume of the soft parts), and the ethanol was replaced several times. 4) specimens were directly preserved *in situ* in 99% ethanol (more than 10 times of the volume of the whole specimens) without any special treatment, and dehydrated as mentioned above.

1 mm^3 piece of the central part of the foot tissue was dissected under a stereomicroscope after a week of preservation at room temperature, then placed in 10% Chelex 100 solution (Biorad, USA; Walsh *et al*. 1991), which was incubated following the manufacturer's procedure. After centrifugation, 1 µl of the supernatant, which contains the genomic DNA, was used in subsequent PCR amplification. Partial region of the 16S mitochondrial ribosomal gene, and the nearly complete region of the 18S nuclear ribosomal gene, were amplified with a standard polymerase chain reaction (PCR) technique. The universal primers, 16Sar (5'-CGCCTGTTTATCAAAAACAT-3') and 16Sbr (5'-CCGGTCTGAACTCAGATCACGT-3'), designed by Palumbi *et al*. (1991) were used to amplify the 16S mtRNA. The primers 18A1 (5'-CCTACCTGGTTGATCCTGCCAG-3') and 1800r (5'-ATGATCCTTCCGCAGGTTCACC-3'), designed by Steiner & Dreyer (2003), were used for 18S nRNA. PCR

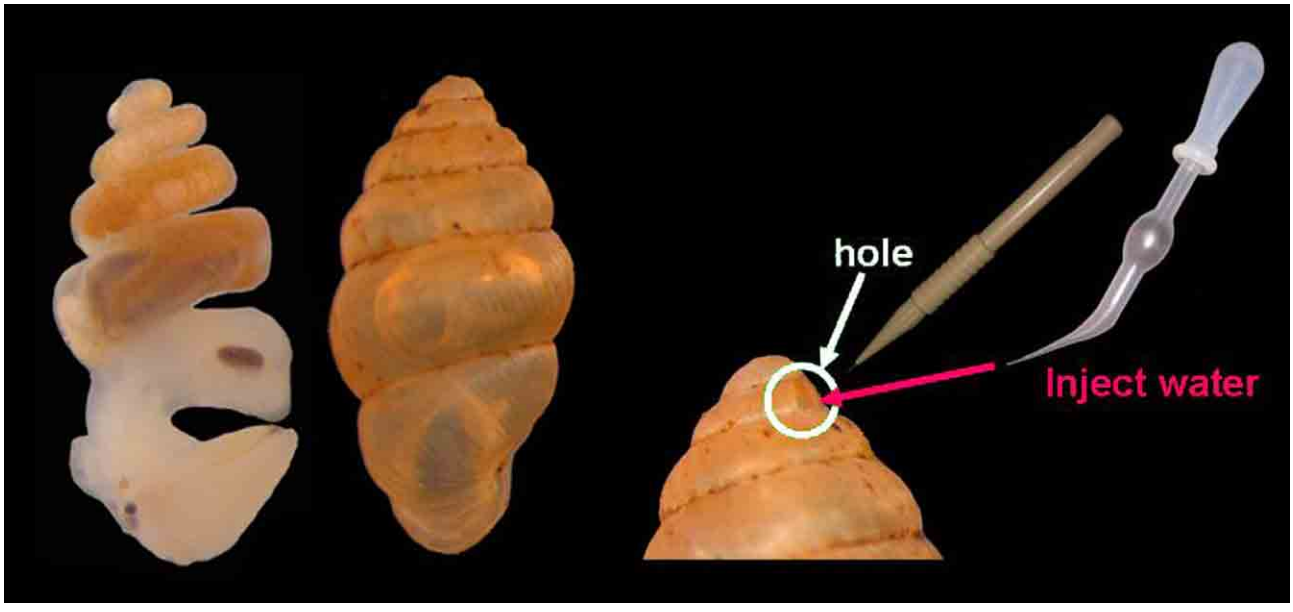


Figure 5. For species with tall spire and many whorls (example: *Diplommatina tanegashimae* Pilsbry, 1901; Diplommatinidae), drill a minute hole in the early whorl of the shell with a very fine, solid needle, just after boiling the animal. After the columellar muscle becomes free from the shell, inject the cold water with the pipette. The whole soft parts will come out from the aperture.

reactions were carried out in a total volume of 25 µl on automated thermal cyclers [1 µl of genomic DNA template (ca. 80–100 ng), 9.5 µl ddH_2O, 12.5 µl of TaKaRa Premix Ex Taq polymerase (TAKARA BIO INC, Japan), 1 µl of each primers (20 pM)]. The reaction solution for 16S was run for 30 cycles with the following parameters: an initial denaturation for 1 min at 94°C, further denaturation for 30 sec at 94°C, annealing for 30 sec at 52°C, followed by extension for 30 sec at 72°C. 18S was likewise amplified with the following parameters: an initial denaturation for 1 min at 95°C, annealing and extension for 3 min at 68°C followed by 35 running cycles. The 5 µl of PCR products were then visualized by electrophoresis on 1.2% TAE-diluted agarose gel, stained with ethidium bromide, and were finally photographed (Fig. 6).

As the result, regions of 16S were successfully amplified in all material including the *niku-nuki*-treated samples (lanes 2–9 in Fig. 6A). On the other hand, amplifications of 18S regions were only successful in the two samples treated by *niku-nuki* (lanes 4–5 in Fig. 6B) and in a single specimen from a cracked shell (lane 6 in Fig. 6B). It is noteworthy that samples fixed after anesthetization in 7.5% $MgCl_2 \bullet 6H_2O$ solution generated insufficient PCR products for sequencing (lanes 2–3 in Fig. 6B). No successful product was detected in one of the cracked shell samples (lane 7 in Fig. 6B) and from directly fixed specimens (lanes 8–9 in Fig. 6B). This result leads us to conclude that specimens treated by *niku-nuki* have excellent prospects for PCR-based molecular analysis, particularly for long sequences.

The conceivable advantage of the *niku-nuki*-treated samples for DNA sequencing-based works is as follows:

1) The negative effect of magnesium ion (Mg^{2+}), which activates DNase, can be reduced significantly with *niku-nuki* (Ueshima 2002) especially for marine species. Seawater containing Mg^{2+} generally remains on the soft parts (*e.g.*, in the pallial chamber) of the live animal *in situ*, however, *niku-nuki* with boiling freshwater can wash out the seawater.

2) *Niku-nuki* can heat deactivate the DNase because proteins will be denatured and deactivated by high temperature during boiling (Ueshima 2002). Therefore, the DNA is protected from fragmentation.

3) Quick dehydration by alcohol can be achieved by *niku-nuki* because the *niku-nuki*-treated samples require only a short period for dehydration compared to freshly fixed samples. It is evident that water quickly comes out from the tissue from the shimmering appearing around the specimen.

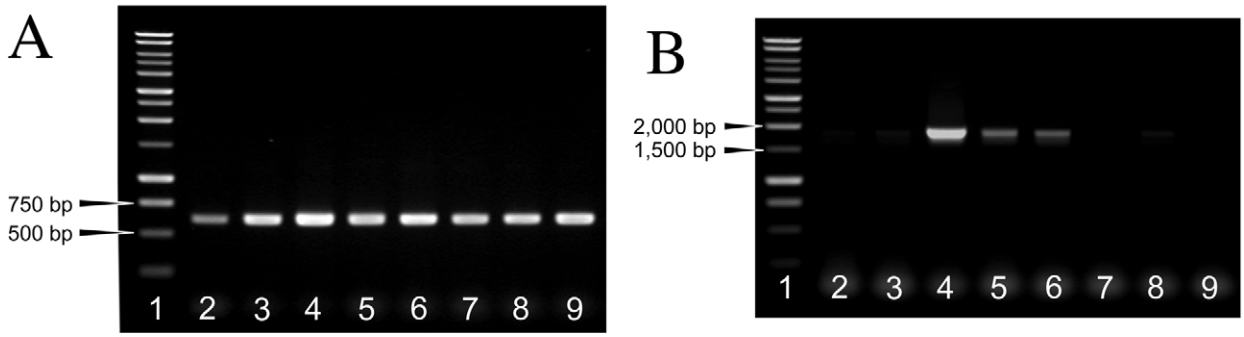


Figure 6. PCR products after the electrophoresis on 1.2 % agarose gels which document the advantage of material after *niku-nuki*. **A.** Region of 16S rRNA (ca. 650 bp). **B.** Region of 18S rRNA (ca. 1,800 bp). Lane 1 in A: 100 bp ladder, lane 1 in B: 1 kbp ladder, lanes 2–3: fixed after anesthetization in $MgCl_2$ solution, lanes 4–5: fixed after *niku-nuki*, lanes 6–7: fixed after cracking the shells, lanes 8–9: fixed *in situ* without special treatment.

4) Molluscan mucus contains mucopolysaccharides, which obstruct successful DNA amplification in PCR (Winnepenninckx *et al*. 1993). The mucus can be removed through *niku-nuki* by wiping or scratching.

As we suggest herein, *niku-nuki* is advantageous for DNA sequencing, however, quick processing is still necessary for successful preparation of samples. According to Ueshima (2002), DNase in molluscs had high activation meaning that DNA was quickly fragmentized, the soft parts treated with *niku-nuki* at relatively low temperatures [*e.g.*, 72℃ in *Hemiphaedusa pinto* (Pilsbry, 1901): see Appendix 1] should be fixed as soon as possible since the DNase may still be active.

In terms of convenience for sample preparation, alcohol dehydration and fixation are the easiest procedures for DNA analysis in comparison to frozen storage at extremely low temperature, which is widely used for DNA studies. However, the latter procedure requires a deep freezer, or even an ultra cold freezer (-80˚C); extra care must be taken to avoid sudden rises in temperature and for continuous power supply or adequate alternative backup options (generator, CO_2, liquid nitrogen). On the other hand, alcohol dehydration and fixation do not require any special storage devices and are easy to be accomplished anywhere, even in the field.

Conclusions

Niku-nuki can overcome various difficulties with respect to the preparation of shell-bearing molluscs. There is no need to fret, whether to give priority to anatomical examination, DNA work, or conchological observations when we obtain very important live specimens. We stress again that *niku-nuki* provides good results for DNA sequencing, and is a more convenient procedure than the other chemical-requiring protocols — you just use hot water and pure ethanol.

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Appendix 1.Suitable temperature and immersion times for various gastropod taxa.

Family	Species name	Length (mm)	Equipment	Temperature (°C)	Time (sec)	Difficulty	Remarks
Patellidae, Nacellidae, Lottiidae	All patellogastropod species		pan or electric kettle	> 90	10	easy	The digestive gland may be broken if the animal is boiled too long.
Haliotidae	*Haliotis* (*Sulculus*) *diversicolor* Reeve, 1846	70	pan	99	600	easy	
Fissurellidae	*Rimula cumingii* A. Adams, 1853	5	electric kettle	> 82	5	easy	
Trochidae	*Chlorostoma lischkei* Tapparone-Canefri, 1874	25	pan	99	1000	sometimes failed	Most species of *Chlorostoma*, *Omphalius* and *Tegula* are easy if boiled for a long time in a pan.
	Omphalius rusticus (Gmelin, 1791)	25	pan	99	1000	easy	
	Ginebis argenteonitens (Lischke, 1872)	40	pan	99	15	easy	
	Granata lyrata (Pilsbry, 1890)	5	electric kettle	90	10	easy	
	Monodonta labio (Linnaeus, 1758)	20	pan	99	20	sometimes failed	A syringe is needed for *Monodonta* spp.
	Tectus conus (Gmelin, 1791)	55	pan	99	1000	difficult	
	Umbonium (*Suchium*) *costatum* (Valenciennes *in* Kiener, 1838)	10	pan	99	20	easy	
Turbinidae	*Angaria neglecta* Poppe & Goto, 1993	30	pan	99	500	easy	
	Turbo (*Batillus*) *cornutus* Lightfoot, 1786	120	pan	99	800	easy	
	Turbo (*Lunella*) *coreensis* (Récluz, 1853)	20	pan	99	180	easy	
Skeneidae	*Munditiella ammonoceras* (A. Adams, 1863)	2.5	electric kettle	90	3	easy	
Neomphalidae	*Leptogyropsis inflata* Hasegawa, 1997	4	electric kettle	> 90	5	easy	
Neritidae	*Clithon retropictus* (Martens, 1879)	20	electric kettle	90	15	easy	Species with large and thick shells should be boiled for a long time. A syringe is needed for neritids with a narrow aperture.
	Nerita (*Ritena*) *plicata* Linnaeus, 1758	15	electric kettle	90	20	easy	
	Neritina (*Dostia*) *violacea* (Gmelin, 1791)	12	electric kettle	90	15	easy	
Phenacolepadidae	*Cinnalepeta pulchella* (Lischke, 1871)	10	electric kettle	90	5	easy	
Hydrocenidae	*Georissa shikokuensis* Amano, 1939	2	electric kettle	82	5	difficult	A pipette may be used after separating the columellar muscle.
Helicinidae	*Waldemaria japonica* (A. Adams, 1861)	8	electric kettle	82	5	sometimes failed	

Appendix 1 continued.

Family	Species name	Length (mm)	Equipment	Temperature (°C)	Time (sec)	Difficulty	Remarks
Cyclophoridae	*Cyclophorus herklotsi* Martens, 1861	18	electric kettle	90	15	difficult	If the columellar muscle can not be separated, use a pan and boil the animal at high temperature.
	Cyclotus (*Procyclotus*) *campanulatus* Martens, 1865	9	electric kettle	90	20	sometimes failed	For *Cyclotus*, drill a hole on the early whorl inside the umbilicus.
	Japonia barbata (Gould, 1859)	5	electric kettle	90	5	easy	
	Nakadaella micron (Pilsbry, 1900)	1	electric kettle	80	6	easy	
	Spirostoma japonicum (A. Adams, 1867)	5	electric kettle	90	10	easy	
Pupinidae	*Pupinella* (*Pupinopsis*) *rufa* (Sowerby II, 1864)	10	electric kettle	90	7	easy	
Alycaeidae	*Chamalycaeus harimensis* (Pilsbry, 1900)	2.5	electric kettle	90	5	easy	
Diplommatinidae	*Palaina* (*Cylindropalaina*) *pusilla* (Martens, 1877)	2	electric kettle	82	3	sometimes failed	Drill a hole on the early whorl and use a pipette for *Palaina* and *Diplommatina*.
	Diplommatina cassa Pilsbry, 1901	3	electric kettle	82	3	sometimes failed	
	Diplommatina labiosa Martens, 1877	4				impossible	Soft-parts are always broken.
Vivparidae	*Cipangopaludina japonica* (Martens, 1860)	50	pan	99	180	sometimes failed	The pallial cavity of viviparids is very fragile (especially in females with juveniles). Drill a hole on the early whorl and use a pipette.
	Sinotaia histrica (Gould, 1859)	30	electric kettle	90	15	sometimes failed	
Ampullariidae	*Pomacea canaliculata* (Lamarck, 1819)	50	pan	99	180	difficult	
Cerithiidae	*Cerithium kobelti* Dunker, 1877	25	pan	99	10	sometimes failed	Pull the animal after drilling a hole on the early whorl.
	Clypeomorus irrorata (Gould, 1849)	15	pan	99	10	sometimes failed	
Litiopidae	*Alaba picta* (A. Adams, 1861)	7	pan	99	5	sometimes failed	
Turritellidae	*Haustator* (*Kurosioia*) *cingulifera* (Sowerby I, 1825)	20	electric kettle	90	25	easy	Drill a hole on the early whorl and use a pipette.
Siliquariidae	*Tenagodus cumingii* (Mörch, 1860)	70	pan	> 95	15	easy	Difficult if the foot cannot be held.
Planaxidae	*Hinea fasciata* (Pease, 1868)	5	electric kettle	90	3	easy	
	Planaxis sulcatus (Born, 1778)	15	pan	99	10	easy	

Appendix 1 continued.

Family	Species name	Length (mm)	Equipment	Temperature (°C)	Time (sec)	Difficulty	Remarks
Thiaridae	*Melanoides tuberculata* (Müller, 1774)	30	electric kettle	90	> 3	easy	
	Stenomelania rufescens (Martens, 1860)	40	electric kettle	90	> 10	easy	
	Thiara scabra (Müller, 1774)	25	electric kettle	90	> 3	easy	
Batillariidae	*Batillaria multiformis* (Lischke, 1869)	35	pan	99	15	difficult	For batillariids and potamidis, before boiling, the head-foot of a living animal should be grabed tightly with forceps. Then put the animal into hot water.
Potamididae	*Cerithidea rhizophorarum* A. Adams, 1855	40	pan	99	45	difficult	
	Cerithideopsilla cingulata (Gmelin, 1791)	30	pan	99	40	sometimes failed	
	Cerithideopsilla djadjariensis (K. Martin, 1899)	35	pan	99	40	difficult	
	Telebralia palustris (Linnaeus, 1767)	100	pan	99	90	sometimes failed	
	Telescopium telescopium (Linnaeus, 1758)	100	pan	99	90	sometimes failed	
Scaliolidae	*Finella rufocincta* (A. Adams, 1861)	5	electric kettle	82	7	very difficult	
Pleuroceridae	*Semisulcospira libertina* (Gould, 1859)	35	electric kettle	90	> 10	easy	
Littorinidae	*Littoraria* (*Littorinopsis*) *scabra* (Linnaeus, 1758)	25	electric kettle	90	3	sometimes failed	
	Littorina brevicula (Philippi, 1844)	10	electric kettle	90	7	easy	
	Nodilittorina radiata (Eydoux & Souleyet, 1852)	5	electric kettle	90	3	sometimes failed	
	Peasiella habei Reid & Mak, 1998	2	electric kettle	90	2	difficult	*Peasiella* needs a pipette after separating the columellar muscle.
Rissoidae	*Alvania concinna* (A.Adams, 1861)	2.5	electric kettle	90	> 3	easy	If the animals are boiled more than 3 seconds, it always works.
	Rissoina (*Rissolina*) *costulata* (Dunker, 1860)	5	electric kettle	90	> 3	easy	
	Stosicia annulata (Dunker, 1860)	4	electric kettle	90	> 3	easy	
	Voorwindia paludinoides (Yokoyama, 1927)	2	electric kettle	90	> 3	easy	
Iravadiidae	*Ceratia nagashima* Fukuda, 2000	3.2	electric kettle	90	5	easy	
	Iravadia (*Fairbankia*) *sakaguchii* (Kuroda & Habe, 1954)	4.5	electric kettle	82	5	easy	

Appendix 1 continued.

Family	Species name	Length (mm)	Equipment	Temperature (°C)	Time (sec)	Difficulty	Remarks
	Iravadia (*Fluviocingula*) *elegantula* (A. Adams, 1863)	4	electric kettle	82	5	easy	
	Iravadia (*Iravadia*) *quadrasi* (Böttger, 1902)	3	electric kettle	90	3	easy	
Elachisinidae	*Elachisina ziczac* Fukuda & Ekawa, 1997	1.8	electric kettle	90	3	easy	
Hydrobiidae	*Hydrobia ulvae* (Pennant, 1777)	4	electric kettle	82	8–10	easy	
Bithyniidae	*Bithynia moltschanovi ussuriensis* Böttner & Ehrmann, 1927	8	electric kettle	90	3	easy	
	Parafossarulus manchouricus japonicus (Pilsbry, 1901)	10	electric kettle	90	3	easy	
Pomatiopsidae	*Blanfordia japonica bensoni* (A. Adams, 1861)	7.5	electric kettle	90	3	easy	
	Cecina manchurica A. Adams, 1861	7	electric kettle	82	5	easy	
	Fukuia kurodai Abbott & Hunter, 1949	8.5	electric kettle	90	5	easy	
	Fukuia ooyagii Minato, 1982	5	pan	99	< 2	sometimes failed	
	Oncomelania nosophora (Robson, 1915)	7	electric kettle	82	5	difficult	
Assimineidae	*Allepithema quadrasi* (Moellendorff, 1894)	3	electric kettle	90	5	difficult	
	Angustassiminea castanea (Westerlund, 1883)	4.5	electric kettle	82	5	difficult	
	Angustassiminea sp. (Japanese name: *Kinton-iro-kawa-zansho*)	3	electric kettle	82	5	sometimes failed	
	Assiminea grayana (Fleming, 1828)	5	electric kettle	90	5	easy	
	Assiminea japonica Martens, 1877	7	electric kettle	90	3	easy	
	Chalicopoma laevigata (Quadras & Moellendorff *in* Moellendorff, 1894)	6	electric kettle	90	7	easy	
	Cyclotropis bedaliensis (Rensch, 1934)	3.5	electric kettle	90	3	easy	

Appendix 1 continued.

Family	Species name	Length (mm)	Equipment	Temperature (°C)	Time (sec)	Difficulty	Remarks
	Gen. et sp. (Japanese name: *Ka-wa-tare-kawa-zansho*)	0.8	electric kettle	82	3	very difficult	
	Paludinellassiminea japonica (Pilsbry, 1901)	5.5	electric kettle	90	6	sometimes failed	
	Solenomphala debilis (Gould, 1859)	5	electric kettle	90	5	difficult	Before boilng, the head-foot of a living animal should be grabed tightly with forceps. Then pour the hot water over the animal.
	Quadrasiella mucronata Moellendorff, 1894	3.5	electric kettle	90	7	easy	
Truncatellidae	*Truncatella guerinii* A. & J. B. Villa, 1841	8	electric kettle	82	5	easy	
Stenothyridae	*Stenothyra japonica* Kuroda, 1962	5	electric kettle	82	3	easy	
Vitrinellidae	*Circulus duplicatus* (Lischke, 1872)	2.5	pan or electric kettle	> 90	3	easy	
	Pseudoliotia pulchella (Dunker, 1860)	1.5	pan or electric kettle	> 90	3	easy	
	Teinostoma lucida A. Adams, 1863	0.8	electric kettle	95	3	easy	
Caecidae	*Caecum* (*Brochina*) *glabella* (A.Adams, 1868)	2	electric kettle	82	3	sometimes failed	
Strombidae	*Strombus* (*Conomurex*) *luhuanus* Linnaeus, 1758	50	pan	90	120	sometimes failed	
Calyptraeidae	*Crepidula onyx* Sowerby I, 1824	30	pan	99	5	sometimes failed	
Xenophoridae	*Xenophora* (*Onustus*) *exutus* (Reeve, 1842)	40	pan	99	15	easy	Sturm *et al*. (2006: 27) mentioned that "removal of the body from *Xenophora* is almost impossible and boiling does not solve the problem". However, in our experience, *niku-nuki* of *X. exutus* is quite easy if the foot can be held with forceps.

Appendix 1 continued.

Family	Species name	Length (mm)	Equipment	Temperature (°C)	Time (sec)	Difficulty	Remarks
Vermetidae	*Serpulorbis imbricatus* (Dunker, 1860)	25	pan	99	30	sometimes failed	It may be impossible in some specimens with very irregularly coiled shells.
Naticidae	*Euspira fortunei* (Reeve, 1855)	40	pan	99	180	easy	
	Glossaulax didyma (Röding, 1798)	50	pan	99	180	easy	
Bursidae	*Tutufa bufo* (Röding, 1758)	140	pan	99	600	easy	
Tonnidae	*Tonna luteostoma* (Küster, 1857)	80	pan	99	300	easy	
Ranellidae	*Charonia lampas* (Linnaeus, 1758)	200	pan	99	1500	sometimes failed	
Eulimidae	*Vitreobalcis astropectenicola* (Kuroda & Habe, 1950)	5	electric kettle	82	5	difficult	Five specimens were tried and only one was successful without drilling a hole in the early whorl. If you drill a hole, it may be easier.
Muricidae	*Rapana venosa* (Valenciennes, 1846)	100	pan	99	300	easy	
	Thais (*Reishia*) *bronni* (Dunker, 1860)	40	pan	99	120	easy	A syringe may be used.
Turbinellidae	*Benthovoluta hilgendorfi* (Martens, 1897)	80	pan	99	1000	easy	
Columbellidae	*Mitrella martensi* (Lischke, 1871)	15	pan	99	10–12	difficult	The soft-parts can be removed completely, but they are always broken.
Nassariidae	*Nassarius* (*Hima*) *festivus* (Powys *in* Sowerby & Powys, 1835)	15	pan	99	1000	difficult	
	Nassarius (*Niotha*) *livescens* (Philippi, 1849)	18	pan	99	20	easy	
Buccinidae	*Buccinum isaotakii* Kira, 1959	90	pan	99	300	difficult	The columellar muscles of large-sized buccinids are hard and thus needs boiling for very long time. Try to occasionally pull the animal and boil again if the muscle cannot be separated from the shell.
	Buccinum striatissinum Sowerby, 1899	100	pan	99	500	sometimes failed	
	Japeuthria ferrea (Reeve, 1847)	35	pan	99	60	sometimes failed	
	Kelletia lischkei Kuroda, 1939	110	pan	99	250	very difficult	
	Neptunea polycostata Scarlato, 1952	150	pan	99	1000	easy	
	Siphonalia cassidariaeformis (Reeve, 1843)	40	pan	99	300	sometimes failed	
Babyloniidae	*Babylonia japonica* (Reeve, 1842)	70	pan	99	250	easy	

Appendix 1 continued.

Family	Species name	Length (mm)	Equipment	Temperature (°C)	Time (sec)	Difficulty	Remarks
Melongenidae	*Hemifusus tuba* (Gmelin, 1891)	150	pan	99	500	easy	
Fasciolariidae	*Fusinus perplexus* (A. Adams, 1864)	110	pan	99	180	sometimes failed	
	Latirulus nagasakiensis (Smith, 1880)	40	pan	99	180	very difficult	
Volutidae	*Fulgoraria* (*Nipponomelon*) *prevostiana* (Crosse, 1878)	100	pan	99	> 30	sometimes failed	
Olividae	*Oliva elegans* Lamarck, 1811	40	electric kettle	90	15	easy	
Conidae	*Conus* (*Lithoconus*) *tessulatus* Born, 1778	50	pan	99	120	easy	The foot is narrow and not easy to grab. The animal may be removed with a pin.
Cornirostridae	*Cornirostra* sp.	0.8	electric kettle	> 90	> 1	easy	
	Tomura yashima Fukuda & Yamashita, 1997	0.8	electric kettle	> 90	> 1	easy	
Valvatidae	*Cincinna piscinalis* japonica (Martens, 1877)	4	electric kettle	90	3	easy	
	Valvata hokkaidoensis Miyaji, 1935	1.2	electric kettle	90	3	easy	
Pyramidellidae	*Odostomia sperabilis* Hedley, 1909	2	electric kettle	82	5	easy	
	Orinella ebarana (Yokoyama, 1927)	5	electric kettle	90	6	sometimes failed	Drill a hole on the early whorl and use a pipette.
	Paramormula scrobiculata (Yokoyama, 1922)	5	electric kettle	82	5	easy	
	"*Sayella*" sp. (Japanese name: *Nukarumi-kuchi-kire*)	3.5	electric kettle	> 90	3	easy	
Cylichnidae	*Didontoglossa decoratoides* Habe, 1955	3	electric kettle	78	3	easy	
Retusidae	*Retusa* (*Decorifer*) *insignis* (Pilsbry, 1904)	4	electric kettle	82	5	easy	
Diaphanidae	*Diaphana* sp.	1	electric kettle	> 90	3	easy	
Amphiboridae	*Lactiforis takii* (Kuroda, 1928)	7	electric kettle	90	3	easy	
Siphonariidae	*Siphonaria* (*Sacculosiphonaria*) *japonica* (Donovan, 1824)	15	electric kettle	90	19	easy	

Appendix 1 continued.

Family	Species name	Length (mm)	Equipment	Temperature (°C)	Time (sec)	Difficulty	Remarks
Ellobiidae	*Carychium noduliferum* Reinhardt, 1877	2	electric kettle	82	3	sometimes failed	Ellobiids have animals with a large visceral mass with no spiral. Thus the animal may be broken if the foot is simply pulled. A syringe or pipette should be used.
	Ellobium chinense (Pfeiffer, 1855)	35	pan	99	15	easy	
	Melampus nuxeastaneus Kuroda, 1949	12	electric kettle	90	7	very difficult	*Melampus* spp. need a hole on the early whorls.
	Microtaralia acteocinoides Kuroda & Habe, 1961	3	electric kettle	78	5	easy	
	Myosotella myotosis (Draparnaud, 1801)	9	electric kettle	82	3	sometimes failed	
Lymnaeidae	*Lymnaea* (*Galba*) aff. *truncatula* (Müller, 1774) (Japanese name: *Koshi-daka-hime-mono-ara-gai*)	4	electric kettle	90	3	easy	
	Lymnaea (*Radix*) *japonica* Jay, 1857	15	electric kettle	90	3	easy	
Physidae	*Physella acuta* (Draparnaud, 1805)	10	electric kettle	90	3	easy	
Planorbidae	*Amerianna carinata* (H. Adams, 1861)	7	electric kettle	82	3	easy	
	Camptoceras hirasei Walker, 1919	8	electric kettle	90	3	easy	
	Culmenella prashadi (Clench, 1931)	5	electric kettle	90	3	easy	
	Gyraulus chinensis Dunker, 1854	1.5	electric kettle	82	3	easy	
	Hippeutis cantori (Benson, 1850)	2	electric kettle	82	3	easy	
	Indoplanorbis exustus (Deshayes, 1832)	6	electric kettle	90	3	easy	
	Polypylis hemisphaerula (Benson, 1842)	2	electric kettle	78	5	very difficult	The soft-parts of *Polypylis* will often be broken because of the folds on the inner wall of the shell.
Ancylidae	*Laevapex nipponicus* (Taki, 1960)	2	electric kettle	> 78	3	easy	
Succineidae	*Oxyloma hirasei* (Pilsbry, 1891)	10	electric kettle	90	3	easy	
	Succinea lauta Gould, 1859	20	electric kettle	90	3	easy	

Appendix 1 continued.

Family	Species name	Length (mm)	Equipment	Temperature (°C)	Time (sec)	Difficulty	Remarks
Achatinellidae	*Tornatellides boeningi* (Schmacker & Böttger, 1891)	3	electric kettle	82	3	easy	
Cionellidae	*Cochlicopa lubrica* (Müller, 1774)	6	electric kettle	82	3	easy	
Pupillidae	*Gastrocopta* (*Sinalbinula*) *armigerella* (Reinhardt, 1877)	2	electric kettle	78	6	very difficult	
Strobilopsidae	*Eostrobilops nipponica reikoae* Matsumura & Minato, 1998	1.7	electric kettle	82	3	very difficult	
Acathinulidae	*Parazoogennetes* (*Salpingoma*) *japonicum* (Pilsbry, 1902)	2	electric kettle	78	3	easy	
Valloniidae	*Vallonia tenera* Reinhardt, 1877	1	electric kettle	82	3	sometimes failed	
Enidae	*Mirus andersonianus* (Moellendorff, 1885)	22	electric kettle	82	3	easy	
Clausiliidae	*Euphaedusa tau* (Böttger, 1877)	12	electric kettle	90	3	easy	Small clausiliid species with many whorls are sometimes difficult. In these species, drill a hole on the early whorl.
	Hemiphaedusa (*Hemizaptyx*) *pinto* (Pilsbry, 1901)	9	electric kettle	72	2	sometimes failed	
	Hemiphaedusa (*Hemizaptyx*) *stimpsoni* (A. Adams, 1868)	12	electric kettle	78	3	sometimes failed	
	Phaedusa (*Stereophaedusa*) *japonica* (Crosse, 1871)	30	electric kettle	78	5	easy	
	Zaptychopsis buschi (Pfeiffer, 1846)	18	electric kettle	82	3	sometimes failed	
Achatinidae	*Achatina* (*Lissachatina*) *fulica* Bowdich, 1822	120	pan	99	120	easy	
Subulinidae	*Allopeas clavulinum kyotoense* (Pilsbry & Hirase, 1904)	10	electric kettle	82	3	easy	
	Paropeas achatinaceum (Pfeiffer, 1846)	10	electric kettle	82	3	easy	
Streptaxidae	*Sinoennea iwakawa* (Pilsbry, 1900)	3	electric kettle	82	3	sometimes failed	Always drill a hole on the early whorl.
Punctidae	*Punctum* spp.	1.5	electric kettle	82	3	easy	
Helicodiscidae	*Helicodiscus* (*Hebetodiscus*) *singleyanus inermis* (Baker, 1929)	1	electric kettle	82	3	easy	

Appendix 1 continued.

Family	Species name	Length (mm)	Equipment	Temperature (°C)	Time (sec)	Difficulty	Remarks
Discidae	*Discus pauper* (Gould, 1859)	3	electric kettle	82	3	easy	
Zonitidae	*Hawaiia minuscula* (Binney, 1840)	1.3	electric kettle	90	3	sometimes failed	
	Zonitoides (*Zonitellus*) *arboreus* (Say, 1817)	2.2	electric kettle	82	5	easy	
Helicarionidae	*Bekkochlamys kagaensis* (Pilsbry & Hirase, 1902)	6	electric kettle	90	5	difficult	The pallial cavity is fragile in most helicarionids. If the animal is likely to be broken, drill a hole on the early whorl.
	Ceratochlamys ceratodes (Gude, 1900)	1.2	electric kettle	82	3	sometimes failed	
	Discoconulus sinapidium (Reinhardt, 1877)	1	electric kettle	82	3	very difficult	
	Parakariella spp.	2	electric kettle	82	3	difficult	
	Parasitala reinhardti (Pilsbry, 1900)	3	electric kettle	82	3	easy	
Camaenidae	*Satsuma myomphala* (Martens, 1865)	25	pan	99	60	very difficult	
	Satsuma japonica (Pfeiffer, 1847)	17	electric kettle	90	15	very difficult	
Helicidae	*Cepaea nemolaris* (Linnaeus, 1758)	18	electric kettle	90	> 30	easy	
	Eobania vermiculata (Müller, 1774)	18	pan	99	20	easy	
	Helix aspersa Linnaeus, 1758	28	electric kettle	90	20	easy	
Bradybaenidae	*Acusta despecta sieboldiana* (Pfeiffer, 1850)	20	electric kettle	90	30	easy	
	Aegista (*Aegista*) *tokyoensis* Sorita, 1980	5	electric kettle	90	5	sometimes failed	
	Aegista (*Plectotropis*) *conomphala* (Pilsbry & Hirase, 1903)	7	electric kettle	90	15	easy	
	Bradybaena similaris (Férussac, 1831)	10	electric kettle	90	3	easy	
	Euhadra peliomphala (Pfeiffer, 1850)	21	electric kettle	90	20	easy	
	Euhadra quaesita (Deshayes, 1850)	25	electric kettle	90	40	easy	Try to pull the animal once and boil again if the muscle cannot be separated from the shell.
	Phaeohelix submandrina (Pilsbry, 1890)	20	pan	99	12	easy	
	Trishoplita commoda (A. Adams, 1868)	5	electric kettle	90	5	easy	