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METHODS AND RESOURCES

A size and taxonomic assessment of non-lethal DNA sampling of gastropods using Flinders Technology Associates (FTA) cards

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Abstract
Sampling the DNA of rare animal species should have minimal impacts on individual health. This can be accomplished through non-lethal/non-invasive sampling. Few of these methods have been developed for invertebrates, including the Mollusca, which are in global decline. Tissue clipping the foot is a common non-lethal method for gastropods. However, it causes permanent damage and is inappropriate for smaller snails. This study used Flinders Technology Associates (FTA) cards to sample DNA from snail mucus for species of different sizes and habitat types, and across evolutionarily distant lineages. In a survival assay, the death rate of individuals sampled with FTA cards (12.1%) was greater than in the controls (3.7%), but the difference was not significant. Of 224 individuals representing 27 snail species (17 Hawaiian native, ten non-native) sampled using both FTA cards and tissue clipping, 80.4% of FTA samples and 91.6% of tissue samples amplified for COI, a significant difference. COI sequencing success did not differ significantly between the two methods. For individuals that failed to produce a COI sequence, an attempt was made to sequence 16S. For 16S, amplification and sequencing rates did not differ significantly between FTA and tissue samples. Habitat type and shell size did not affect FTA sampling success. Phylogenetically basal taxa exhibited lower success rates, but this may have been because of difficulty in sampling operculate taxa, and not because of identity. These results indicate that the FTA sampling is a viable non-lethal alternative to tissue clipping and can be used for diverse gastropods.

Keywords Conservation genetics · Non-invasive sampling · Terrestrial snails · Aquatic snails · DNA sequencing

Introduction

Genetics plays a prominent role in conservation biology, particularly for investigating and developing solutions to problems of inbreeding and taxonomic uncertainty (Frankham 1995; Kohn et al. 2006; Avise 2012; Frankham et al. 2014). Conservation biology studies aim to sample with minimal impacts on the individual or population, particularly when working with rare or endangered species (Morin et al. 1993; Taberlet and Luikart 1999; Riddle et al. 2003; Lefort et al. 2014). For genetic studies, this can be accomplished through non-lethal or non-invasive DNA sampling. The term "non-invasive sampling" has been widely applied to any sampling method that does not kill the animal (Morin and Woodruff 1996). Following Lefort et al. (2014), methods that make some contact with the animal, even if it is not perceptibly harmful (e.g. swabbing with a cotton swab), are considered here as "non-lethal" and distinguished from methods that avoid contact with the animal entirely and are therefore truly "non-invasive" (e.g. sampling animal products such as feces).

Non-lethal methods have been developed extensively for vertebrates (reviewed by Morin and Woodruff 1996; Lefort et al. 2014). However, the small size of most invertebrates makes it difficult to remove body parts non-lethally. When populations are large, whole animals are usually sacrificed to obtain DNA (Hamm et al. 2010). But if a population is small, removing the number of individuals needed to obtain statistical significance for analyses may compromise the population's viability (Hamm et al. 2010). Non-lethal sampling is critical in these cases. But despite invertebrates

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accounting for over 95% of all animal diversity (Cardoso et al. 2011; Böhm et al. 2012), far fewer resources have been invested in their conservation and study than in studies of vertebrates (Cardoso et al. 2011). This includes research on non-lethal sampling methods (Harvey 2005; Régnier et al. 2011).

Molluscs typify the challenges of invertebrate conservation. Mollusca is the second most diverse animal phylum after Arthropoda in terms of recognized species (Ponder and Lindberg 2008; Cowie et al. 2022). Because of the impacts of habitat loss and invasive species, molluscs account for almost 40% of all animal extinctions recorded on the IUCN Red List (IUCN 2022) yet the real number of known mollusc extinctions is in fact much higher (Régnier et al. 2009, 2015a, b; Cowie et al. 2017, 2022). Despite being in global decline, most mollusc groups are severely understudied (Gaston and May 1992; Lydeard et al. 2004; Régnier et al. 2009; Collen et al. 2012). This includes Gastropoda, which includes at least 72,000 recognized extant species (MolluscaBase ed. 2023) but features some of the most critically threatened animal taxa in the world (Lydeard et al. 2004; Régnier et al. 2009, 2015b). For example, Pacific Islands are land snail biodiversity hotspots, but there have been mass extinctions in many groups (e.g. Solem et al. 1990; Cowie 1992, 2001; Régnier et al. 2009, 2015a, b; Sartori et al. 2012; Richling and Bouchet 2013). Conservation efforts have been insufficient because of inadequate knowledge of species diversity, distributions and biology; these inadequacies must be addressed to better conserve the remaining species (Solem 1990; Cowie 2004; Lydeard et al. 2004; Richling and Bouchet 2013; Yeung and Hayes 2018; Cowie et al. 2022).

There has been interest in developing non-lethal sampling methods for rare snails, but studies to date have only tested a few species. Non-lethal methods for gastropods have included sampling the periostracum (the organic outermost layer of the shell), swabbing mucus from the foot (Kawai et al. 2004; Armbruster et al. 2005; Morinha et al. 2014), and removing a small amount of tissue from the foot using a razor (tissue clipping) (e.g. Thacker and Hadfield 2000; Anderson 2007; Haskell and Pan 2010). The first two methods have been used for relatively large species ranging in size from *Arianta arbustorum* (17–25 mm greatest shell width, Baminger 1997; Armbruster et al. 2005) to *Geomalacus maculosus* (70–80 mm length, Morinha et al. 2014). However, tissue clipping is the most commonly used method, and is often used for endangered species such as those of the Hawaiian genus *Achatinella* (average shell length ~ 19 mm, U.S. Fish and Wildlife Service 1992; Thacker and Hadfield 2000; Holland and Hadfield 2002; Price et al. 2021). While all of these methods have obtained amplifiable DNA without causing long-term health effects in sampled individuals (Armbruster et al. 2005; Kawai et al. 2004; Haskell and Pan 2010; Morinha et al. 2014), they may not be suitable for smaller species. Mucus swabbing may not collect enough DNA for amplification, and periostracum and tissue sampling may result in permanent damage, extreme stress, or death. Tissue sampling may be especially injurious since it removes tissue from the soft body of the animal, which does not regenerate.

A promising non-lethal and less invasive alternative to tissue clipping is to use mucus collected on Whatman Flinders Technology Associates (FTA) cards. While mucus swabbing relies on the presence of detached blood and epithelial cells in the mucus (Kawai et al. 2004; Morinha et al. 2014), FTA cards have buffers that lyse cells, denature proteins, and protect DNA from oxidation, ultraviolet light, and nuclease digestion (Harvey 2005; Régnier et al. 2011; Whatman 2011). This may make FTA cards more appropriate than mucus swabbing for sampling smaller species with less mucus, and correspondingly fewer total free cells (although it is unclear which method is more invasive). Nucleic acids from this non-lethal method have been successfully amplified from several snail species with adult shell width as small as 3–5 mm (*Hiona radians*, Régnier et al. 2011) and as large as ~ 32 mm (*Cornu aspersum*, Ansart and Vernon 2004; Régnier et al. 2011).

With FTA cards, samples can be collected with minimal trauma and without removing individuals from the field, which is optimal for rare or endangered species. In this study FTA cards were tested as a non-lethal sampling method for gastropods collected from the Hawaiian Islands. There are over 750 recognized species of native Hawaiian land snails in ten families (Cowie et al. 1995). An estimated 70% are already extinct and many of the remaining taxa are in decline (Solem et al. 1990; Lydeard et al. 2004; Régnier et al. 2015b; Yeung and Hayes 2018). Hawaii also has more than 40 established non-native snail species (Cowie et al. 2008; Hayes et al. 2012). The 27 species (17 Hawaiian native and ten non-native) sampled in this study represent both rare and abundant taxa across phylogenetically diverse snail families and a range of sizes and habitat types (freshwater, terrestrial, and marine). Contrasting habitat types were chosen because variation in mucus composition has been linked to environmental conditions (Davies and Hawkins 1998), and could affect DNA sampling efficiency. Extremely rare Hawaiian endemic snails (*Kaala subrutila* and multiple species of Amastridae) were chosen as examples of how conservation efforts for critically endangered land snails could benefit from a non-lethal sampling option. Also, non-native *Succinea tenella* and two Hawaiian *Catinella* species (Succineidae) were sampled to represent a globally distributed family (Patterson 1971; Pilsbry 1948; Rundell et al. 2004),

with endangered species on multiple island chains and continents (IUCN 2022).

The amplification and sequencing results from tissue-based samples were compared with those from FTA card collected samples. It was expected that FTA cards would not cause mortality or long-term health effects, and would produce amplification products and sequences with similar success to tissue sampling. It was also hypothesized that taxonomic identity and habitat would not impact the success of FTA sampling. Logistic regression analyses were used to investigate how size affects obtaining a sequence using an FTA collected DNA sample, with a positive correlation between size and success predicted.

Materials and methods

Individual collection

Species were chosen based on availability in Hawaii, but also to sample broadly across the snail phylogenetic tree (Table 1; Fig. 1). Species represented both native and

Table 1 Death rates for FTA sampling and BPBM numbers

Species	Family	Native or Invasive	Habitat type	BPBM Number	N	Dead in ≤ 7 days
Planorbella duryi	Planorbidae	Invasive	Freshwater	278103	10	0
Physella acuta	Physidae	Invasive	Freshwater	278106	15	9**(60.0%)
Tarebia granifera	Thiaridae	Invasive	Freshwater	278107	15	0
Pseudosuccinea columella	Lymnaiedae	Invasive	Freshwater	278105	4	2**(50.0%)
Bradybaena similaris	Bradybaenidae	Invasive	Freshwater	278095 278100 278101	15	0
Lacteoluna selenina	Sagdidae	Invasive	Terrestrial	278097 278098 278104	10	0
Succinea tenella	Succineidae	Invasive	Terrestrial	278113	15	2 (13.3%)
Allopeas clavulinum	Subulinidae	Invasive	Terrestrial	278099 278102 278109	15	0
Deroceras laeve	Agriolimicidae	Invasive	Terrestrial	278110	11	2 (18.2%)
Amastra spirizona	Amastridae	Native	Terrestrial	278118 278122	12	0
Amastra micans	Amastridae	Native	Terrestrial	278116	6	0
Amastra rubens	Amastridae	Native	Terrestrial	278117	6	0
Amastra intermedia	Amastridae	Native	Terrestrial	278115	6	0
Amastra cylindrica	Amastridae	Native	Terrestrial	278119	6	0
Leptachatina crystallina	Amastridae	Native	Terrestrial	278114	6	0
Leptachatina cerealis	Amastridae	Native	Terrestrial	278120	6	0
Laminella sanguinea	Amastridae	Native	Terrestrial	278121	6	0
Philonesia sp.	Helicarionidae	Native	Terrestrial	278124	3	1**(33.3%)
Philonesia waiheensis	Helicarionidae	Native	Terrestrial	278123	5	0
Philonesia hartmanni	Helicarionidae	Native	Terrestrial	278126	3	0
Kaala subrutila	Helicarionidae	Native	Terrestrial	278125	1	0
Auriculella uniplicata	Achatinellidae	Native	Terrestrial	278127	3	2*(66.6%)
Auriculella turritella	Achatinellidae	Native	Terrestrial	278128 278132 278133 278134	15	9 (60.0%)
Pleuropoma laciniosa	Helicinidae	Native	Terrestrial	278082	6	0
Catinella rotundata	Succineidae	Native	Terrestrial	278111	6	0
Catinella baldwini	Succineidae	Native	Terrestrial	278112 278129 278130 278131	8	0
Nerita picea	Neritidae	Native	Terrestrial	278108	10	0
TOTAL					**224**	**27 (12.1%)**

*Some deaths probably due to handling or housing problems

**All deaths probably due to handling or housing problems

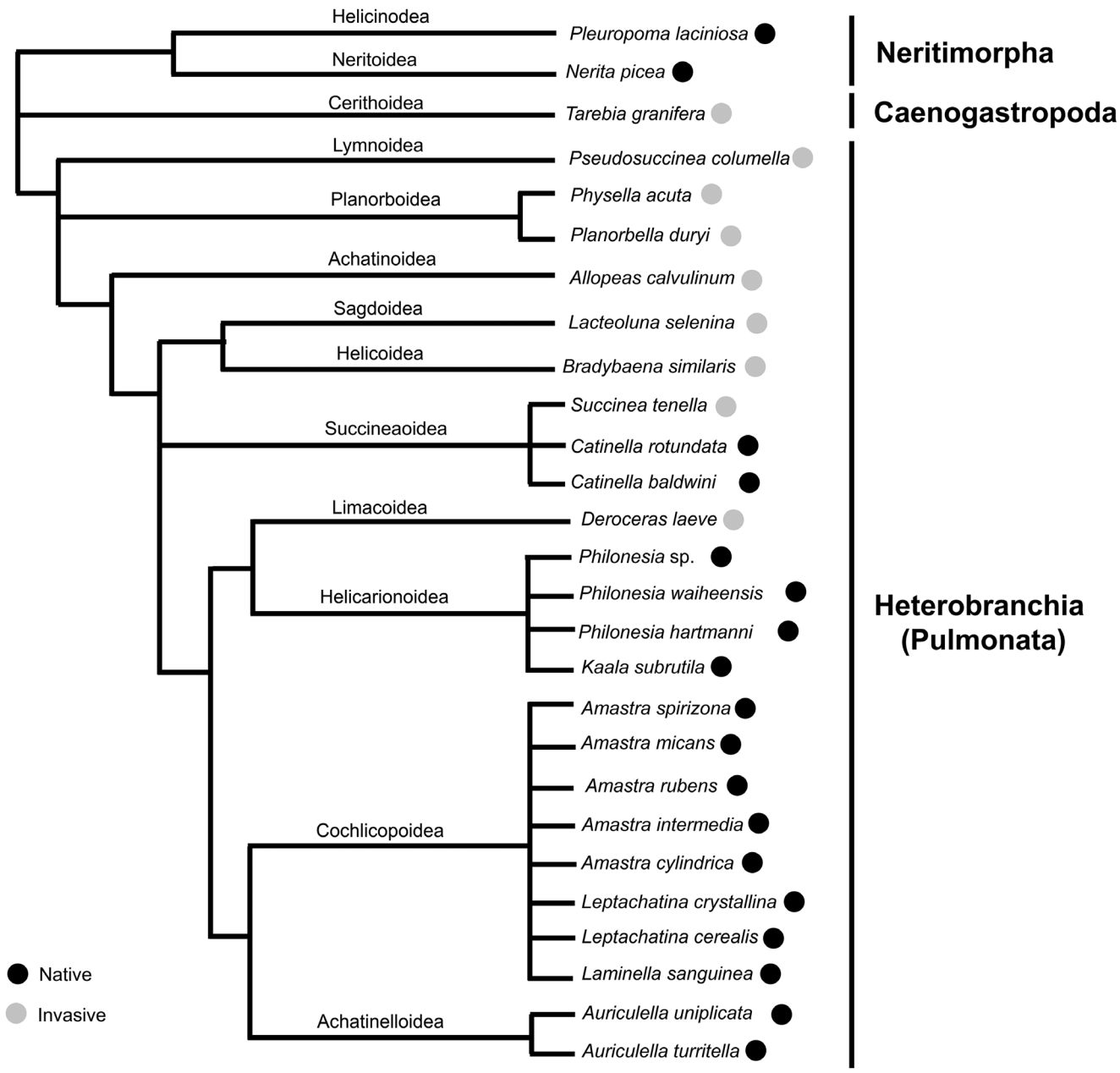

Fig. 1 Phylogeny of sampled species (using superfamily classifications of Bouchet et al. 2017 and phylogeny of Aktipis et al. 2008) indicating phylogenetic diversity of lineages

non-native species across a range of sizes from marine, freshwater, and terrestrial habitats (Table 1; Fig. 2). Taxa were identified to the lowest taxonomic level possible (Table 1), and identities verified against DNA databases using BLAST searches (Boratyn et al. 2013). The targeted sample size for each abundant, non-native species was 15, and the sample size for native species was up to ten depending on availability (Table 1). All individuals of native species were collected with appropriate permits and as part of a large-scale Hawaiian land snail biodiversity inventory project, and were not collected for the sole purpose of use in this study. All individuals have been deposited in the Bishop Museum malacology collection in Honolulu (BPBM 278,082, 278,095–278,124; Table 1).

DNA sample collection

Whatman FTA Micro Cards (Fig. 2) were used to collect mucus (including DNA) from live individuals. Before sampling, each snail was submerged in sterile double distilled water (ddH20) for 20 s. It is possible that this step introduces osmotic stress to the snail, particularly non-aquatic species.

Fig. 2 A Whatman FTA micro card. The sampling area is within the circle. Image taken from Fisher Scientific

However, this was done to prevent desiccation upon contact with the salts of the FTA card sampling area. It also aimed to reduce the chances of sequencing trace DNA from other animals in the snail's shared environment, as the COI and 16S test genes of this study are universal markers. The snail was then allowed to crawl on the card within the sampling area circle for 20 s and observed for any behavior suggesting adverse reaction to sampling (e.g. withdrawing the foot into the shell). The snail was then removed and submerged again in sterile ddH_20 for 20 s to remove potentially harmful buffer residue. As a control for effects of card contact versus exposure to FTA buffers, four species (freshwater *Physella acuta*, terrestrial *Auriculella* sp. and *Succinea tenella*, and marine *Nerita picea*) were exposed to an untreated area of an FTA card (Fig. 2) for 20 s (Table 2). These individuals were only used as controls for the survival assay, and were not processed for molecular assessment. Following the survivorship monitoring period post-FTA sampling, individuals were preserved and tissue samples for DNA extraction were taken.

Survivorship post-FTA sampling

After FTA exposure or contact with an untreated portion of the card, all snails were kept in containers with water, food, and vegetation (habitat). Housing conditions were customized for each taxon (Table 3). Invasive species were kept in an open lab area reserved for snail rearing, and all native species, except amastrids and the littoral marine species *Nerita picea*, were kept in an environmental chamber set to conditions simulating light and temperature cycles of the forest habitat from which they were collected. Amastrid species were part of captive breeding colonies maintained at the Bishop Museum (Honolulu), and housed according to protocols developed there. *Nerita picea* were kept in the same open lab area as the invasive species but in a container with seawater. All aquatic species were supplied with water from their collection sites. Vegetation consisted of plants the snails were found on, in, or around (Table 3). *Nerita picea* were given rocks they were found on instead of vegetation.

Individuals were monitored over seven days for health. This consisted of checking that they returned to the normal behaviors observed pre-sampling, such as crawling and protracting the antennae; recording reproductive events (egg laying or live birth); and recording deaths. To keep track of individuals, snails were painted with a unique dot pattern using paint markers, or if they were too small to be painted or likely to lose paint dots as a result of water exposure, they were housed separately as individuals (Table 3). Paint tagging has been found to have no impacts on life history or survival (Henry and Jarne 2007). After seven days or upon their death, all snails except amastrids (see below) were euthanized with the heatshock method (submerging the animal in boiling water for several seconds, Fukuda et al. 2008) and fixed in 95% ethanol. After fixation, a small piece of foot tissue (~0.25 mg) was removed from each individual for DNA extraction.

The FTA sampling protocol for amastrids was the same as for all other species. *Amastra* spp. were sampled alive using the tissue-clipping method, as they are large enough to use this method without inflicting long-term negative impacts. But most *Leptachatina* individuals were too small to collect tissue from, so tissue samples were taken from only a few live individuals of each species (Table 4). In addition, only one individual per amastrid species was euthanized (and deposited in the Bishop Museum as a voucher), as they are part of a conservation captive breeding colony.

The death rate of *Physella acuta* individuals after FTA sampling was unexpectedly high, so another experiment was designed to investigate whether this was because of housing or sampling problems. In contrast to the original experiment (water volume of 33 ml and a leaf clipping for each separately housed snail), fifteen *Physella acuta* individuals sampled with FTA cards were kept in a large single container with 1000 ml of collection site water (~66 ml/snail) and a whole *Nasturtium officinale* plant. Another 15 individuals sampled with an untreated portion of the card were kept in another container under the same conditions to isolate any effects of contact alone (Table 3). Individuals were monitored for 7 days for health in the same manner as described above, and the number of deaths recorded. At point of death or at the end of the monitoring period, snails

Table 2 Housing conditions of each species

Species	Housing Type	Water Volume	Vegetation[1]	Light cycle[2]	Temperature[2]	Individual Tracking
Planorbella duryi	Fresh water	33 ml	*Egeria densa* (clipping)	24 h (L)	24 °C	Separation
Physella acuta (FTA experiment)	Fresh water	33 ml	*Nasturium officinale* (clipping)	24 h (L)	24 °C	Separation
Physella acuta (Housing experiment)	Fresh water	66 ml	*Nasturium officinale* (whole plant)	24 h (L)	24 °C	N/A
Tarebia granifera	Fresh water	33 ml	*Nasturium officinale* (clipping)	24 h (L)	24 °C	Separation
Pseudosuccinea columella	Fresh water	33 ml	*Egeria densa* (clipping)	24 h (L)	24 °C	Separation
Bradybaena similaris	Leaf litter	N/A	Garden litter mix	24 h (L)	24 °C	Paint marker
Lacteoluna selenina	Leaf litter	N/A	Garden litter mix	24 h (L)	24 °C	Paint marker
Succinea tenella	Leaf litter	N/A	*Nasturium officinale* (clipping)	24 h (L)	24 °C	Paint marker
Allopeas clavulinum	Leaf litter	N/A	Garden litter mix	24 h (L)	24 °C	Paint marker
Deroceras laeve	Leaf litter	N/A	Native litter mix	24 h (L)	24 °C	Separation
Amastra spirizona	Leaf litter	N/A	*Pepturus albidus* litter	24 h (D)	21.7-28.9 °C	Paint marker
Amastra micans	Leaf litter	N/A	*Pepturus albidus* litter	24 h (D)	21.7-28.9 °C	Paint marker
Amastra rubens	Leaf litter	N/A	*Pepturus albidus* litter	24 h (D)	21.7-28.9 °C	Paint marker
Amastra intermedia	Leaf litter	N/A	*Pepturus albidus* litter	24 h (D)	21.7-28.9 °C	Paint marker
Amastra cylindrica	Leaf litter	N/A	*Pepturus albidus* litter	24 h (D)	21.7-28.9 °C	Paint marker
Leptachatina crystallina	Leaf litter	N/A	*Pepturus albidus* litter	24 h (D)	21.7-28.9 °C	Paint marker
Leptachatina cerealis	Leaf litter	N/A	*Pepturus albidus* litter	24 h (D)	21.7-28.9 °C	Paint marker
Laminella sanguinea	Leaf litter	N/A	*Pepturus albidus* litter	24 h (D)	21.7-28.9 °C	Paint marker
Philonesia sp.	Live plants	N/A	*Metrosideros polymorpha* (clipping)	12 h (L) 12 h (D)	20 °C (L) 18 °C (D)	Paint marker
Philonesia waiheensis	Live plants	N/A	*Metrosideros polymorpha* (clipping)	12 h (L) 12 h (D)	20 °C (L) 18 °C (D)	Separation
Philonesia hartmanni	Live plants	N/A	*Metrosideros polymorpha* (clipping)	12 h (L) 12 h (D)	20 °C (L) 18 °C (D)	Separation
Kaala subrutila	Live plants	N/A	*Metrosideros polymorpha* (clipping)	12 h (L) 12 h (D)	20 °C (L) 18 °C (D)	N/A
Auriculella uniplicata	Live plants	N/A	*Metrosideros polymorpha* (clipping)	12 h (L) 12 h (D)	20 °C (L) 18 °C (D)	Paint marker
Auriculella turritella	Live plants	N/A	*Metrosideros polymorpha* (clipping)	12 h (L) 12 h (D)	20 °C (L) 18 °C (D)	Paint marker
Pleuropoma laciniosa	Leaf litter	N/A	Native litter mix	12 h (L) 12 h (D)	20 °C (L) 18 °C (D)	Separation
Catinella rotundata .	Live plants	N/A	*Metrosideros polymorpha* (clipping)	12 h (L) 12 h (D)	20 °C (L) 18 °C (D)	Paint marker
Catinella baldwini	Live plants	N/A	*Metrosideros polymorpha* (clipping)	12 h (L) 12 h (D)	20 °C (L) 18 °C (D)	Paint marker
Nerita picea	Seawater	100 ml	N/A (rocks)	24 h (L)	24 °C	Paint marker

[1]*Egeria densa* is an aquarium plant, *Nasturtium officinale* is watercress, garden litter mix was predominantly ornamental hibiscus (*Hibiscus rosea*) and banana (*Musa acuminata*), *Metrosideros polymorpha* (ohia) and *Pipturus albidus* (mamaki) are native Hawaiian shrubs, and native litter mix was predominantly *Metrosideros polymorpha* and *Broussaisia arguta* (native Hawaiian hydrangea)

[2] L=Light, D = Dark

were heatshocked, preserved and deposited in the Bishop Museum collection.

Extraction, amplification and sequencing

Total cell DNA was extracted from tissue samples using a Machery-Nagel Nucleospin tissue kit following the manufacturer's protocol, with modifications to optimize snail extractions (185 µL instead of 180 µL buffer TI and 15 µL instead of 25 µL Proteinase K solution for the lysing step, 500 µL instead of 600 µL Buffer B5 for second washing of the silica membrane, and 60 µL instead of 100 µL Buffer BE for elution; refer to Machery-Nagel 2014). Total DNA from FTA cards was extracted using a 1 cm^2 sample circle area excised with a sterile razor, and extracted following the same protocol as used for the tissue samples, but with reduced vortexing to prevent shearing of lower DNA yields. Extracted DNA was eluted in sterile de-ionized water.

A fragment of the mitochondrial COI gene was amplified and sequenced for all templates (FTA and tissue derived)

Table 3 Control death rates from card contact without FTA buffers

Species	Habitat	Number sampled	Number dead in ≤ 7 days
*Physella acuta** (Housing exp.)	Freshwater invasive	15	1
Succinea tenella	Terrestrial invasive	15	0
Auriculella sp. †	Terrestrial native	14	1
Nerita picea	Marine native	10	0
	TOTAL	**54**	**2**

*Additional 15 individuals were sampled with FTA cards for a separate housing control experiment

† Significantly fewer deaths than individuals exposed to FTA buffers

using the primers of Folmer et al. (1994). Because the mitochondrial ribosomal 16S gene fragment amplifies more reliably than COI, it was used to check those samples that failed to amplify for COI, using primers 16San and 16S2 (Garey et al. 1998). All amplifications were done in 25 µl reactions containing 1.5-3 µl template DNA and final concentrations of 1 U Mango Taq (Bioline), 1 X Mango Buffer (Bioline), 2.5 mM $MgCl_2$, 200 µM dNTPs, 0.4 mg/µl BSA, 0.5% DMSO, and 0.2 µM of each primer. Cycling parameters for both markers consisted of an initial denaturation at 95 °C (5 min), annealing at 45 °C (60s), and extension at 72 °C (45s), followed by 34 cycles of 95 °C (20s), 48 °C (30s), and 72 °C (45s). Amplification was terminated after a final extension of 72 °C (5 min) and a 4 °C soak (30 min). Amplified products were run on a 2% agarose gel. Those that produced a strong, single band were scored as successful.

Amplified products were purified using the Machery-Nagel Nucleospin Extract II kit according to the manufacturer's instructions. They were then sequenced in both forward and reverse directions on an Applied Biosystems 3730XL Genetic Analyzer at the University of Hawaii Genomics Core facility. Sequences were assembled into contigs in Geneious, version 7.0 (Drummond et al. 2014). To confirm that FTA card sample sequences were identical to the tissue sample sequences, contig assemblies were created using the sequences of FTA and tissue samples, and visually checked for ambiguities or disagreement between sequences. Taxon and locus identities were verified using the nucleotide BLAST algorithm (Boratyn et al. 2013)

Table 4 Shell dimensions of sampled individuals

Species	N	Avg height (mm)	Std dev (mm)	Height min (mm)	Height max (mm)	Avg greatest width (mm)	Std dev (mm)	Greatest width min (mm)	Greatest width max (mm)
Planorbella duryi	10	4.56	0.78	3.73	6.25	5.91	1.57	4.45	8.67
Physella acuta	15	5.08	0.60	4.14	6.19	10.12	1.19	7.47	11.90
Tarebia granifera	15	8.04	1.22	6.60	10.27	16.67	2.94	16.72	22.54
Pseudosuccinea columella	4	2.75	0.63	2.20	3.45	6.58	0.62	5.95	7.19
Bradybaena similaris	15	8.95	2.65	3.39	12.27	13.19	4.00	5.02	17.95
Lacteoluna selenina	10	2.25	0.34	2.03	2.83	4.13	0.22	4.72	4.88
Succinea tenella	15	3.80	0.56	2.94	4.90	8.11	1.04	6.25	9.93
Allopeas clavulinum	15	10.56	1.18	8.73	12.14	3.49	0.30	3.01	3.94
Deroceras laeve	11	2.82	1.18	1.18	4.67	7.22	3.39	2.62	12.54
Amastra spirizona†	12	20.22	N/A	N/A	N/A	10.9	N/A	N/A	N/A
Amastra micans†	6	15.22	N/A	N/A	N/A	8.81	N/A	N/A	N/A
Amastra rubens†	6	14.79	N/A	N/A	N/A	8.76	N/A	N/A	N/A
Amastra intermedia†	6	10.71	N/A	N/A	N/A	8.45	N/A	N/A	N/A
Amastra cylindrica†	6	19.02	N/A	N/A	N/A	10.34	N/A	N/A	N/A
Leptachatina crystallina†	6	6.75	N/A	N/A	N/A	3.15	N/A	N/A	N/A
Leptachatina cerealis	6	8.57	N/A	N/A	N/A	3.63	N/A	N/A	N/A
Laminella sanguinea†	6	12.90	N/A	N/A	N/A	7.74	N/A	N/A	N/A
Philonesia sp.	3	3.34	1.83	1.25	4.67	5.87	3.48	1.89	8.29
Philonesia waiheensis	5	6.16	1.54	5.21	8.24	10.93	3.10	7.04	15.44
Philonesia hartmanni	3	3.70	0.73	2.97	4.42	6.24	1.49	4.64	7.58
Kaala subrutila†	1	6.71				11.19			
Auriculella uniplicata	3	8.17	0.28	7.86	8.41	4.10	0.21	4.56	4.97
Auriculella turritella	15	5.96	1.54	3.61	8.03	3.18	0.69	2.18	3.76
Pleuropoma laciniosa	6	2.47	0.32	1.95	2.27	3.34	0.48	2.55	3.18
Catinella rotundata	6	3.70	0.75	2.86	4.70	8.06	0.87	6.72	9.24
Catinella baldwini	8	4.66	1.27	2.98	7.33	10.16	2.67	6.37	14.80
Nerita picea	10	6.15	0.76	4.38	7.11	11.14	0.97	9.00	12.47

† Only one individual sampled

available on GenBank and by comparison to the DNA database maintained as part of the overall project of which this study is part. If a species did not already have sequences on GenBank or in the project database, a match to the correct family or genus was taken as a positive match. Amino acid translations were used to verify an open reading frame for all COI sequences. DNA from control individuals were not sequenced.

Morphological data collection

Shell height and width were measured for all individuals. To take measurements, individuals were photographed using a camera mounted on a stereoscope and connected to a computer running EOS Utility Version 2.13.0 software. Individuals were photographed with a millimeter scale bar in apical, umbilical, apertural, and side views. Dimensions were measured using the ruler tool with appropriate scaling in Photoshop CS5. Measurements were taken three times for precision and accuracy, with the median value recorded for the purpose of analysis. Measurement values and basic statistics are reported for each species in Table 4.

Statistical analyses

Statistical tests were chosen according to data parameters and criteria as defined by McDonald (2014) and performed in JMP Pro 11 (SAS Institute Inc. 1989–2015). Two-sample z-tests for proportions were used to test for significant differences in death rate of FTA sampled individuals and control individuals that had contact only with an untreated portion of the FTA card. Significant differences in success rates for amplification and sequencing of both COI and 16S between FTA samples and tissue samples were determined for each taxon (Fisher's exact tests) and for pooled data of all species (two-sample z-tests for proportions). Significance levels were set to $\alpha = 0.05$. Two-sample z-tests and Fisher's exact tests were used because all datasets had two nominal variables (FTA vs. control or FTA vs. tissue) and independent observations. A Bonferroni correction was used to correct for multiple testing.

Logistic regression was used to analyze the relationship between shell size and the probability of successfully obtaining a COI sequence for both FTA card samples and tissue samples. The logistic regression model related continuous data (shell dimension measurements for individual specimens) to categorical data (yes or no for success in obtaining a sequence) with a best-fit model line (McDonald 2014). Negative fit lines corresponded to a decreasing probability of failure (a "no" outcome for success) as the size of the individual increased, and positive fit lines corresponded to an increasing probability of failure as the size of the individual increased. The two dimensions analyzed were shell height and greatest width. Due to an emergent pattern of lower success with operculate species (species with a shell seal for the aperture), a binary logistic regression was further performed to test for significant differences in COI amplification between operculate species and non-operculate specimens. The target variable was set to yes/no for COI amplification success. Significance levels of both regression tests were set to $\alpha = 0.05$.

Results

Behavioral response to FTA card sampling

All snails retracted the foot or withdrew within the shell when they contacted the FTA card. Some produced mucus bubbles and released copious amounts of mucus in reaction to contact with the FTA card, particularly *Bradybaena similaris*. Within 24 h of sampling, however, most individuals returned to states exhibited prior to exposure (i.e. crawling with the foot and antennae extended). The exception was several individuals of *Auriculella turitella* that remained withdrawn for two days. A single *Philonesia* sp. individual gave birth to two live offspring and *Physella acuta* individuals housed together laid eggs within the seven-day monitoring period. Operculate species (*Pleuropoma laciniosa, Tarebia granifera*, and *Nerita picea*) would quickly withdraw the foot and seal the aperture, preventing the sample from being taken. Some individuals required several attempts, in which they were allowed to rest and re-extend the foot between attempts. Other individuals, particularly operculate species, would not crawl on the card for a full 20 s of sampling before retracting.

Survivorship post FTA sampling

Of 114 native and 110 non-native individuals belonging to 27 species sampled using FTA cards, 27 individuals died within the seven-day post exposure monitoring period (Table 3). Of the 27 deaths, 12 were native terrestrial species: one *Philonesia* sp., two *Auriculella uniplicata*, and nine *Auriculella turritella*. The remaining 15 deaths were non-native taxa. Eleven were freshwater species: two *Pseudosuccinea columella* and nine *Physella acuta*, and four were terrestrial species, two *Succinea tenella* and two *Deroceras laeve* (Table 3). These deaths represent a 12.1% death rate overall for FTA card sampling.

In the control experiments (individuals sampled with the untreated portion of the card), two out of 54 (3.7%) died: one *Physella acuta* and one *Auriculella* sp. (Table 4). For individual species comparisons, the only taxon that had a

significantly lower death rate for the control versus FTA card sampling was *Auriculella* sp. versus *A. uniplicata* and *A. turritella* tested with the FTA cards (11/18 for FTA, 1/14 for untreated card control; Fisher's exact test, $p = 0.0028$). For the control experiment that was also a housing control experiment for *Physella acuta*, no snails died in the FTA sampled container, and one individual from the non-FTA sampled container died. The 12.1% FTA death rate was higher than the 3.7% control death rate, but not significantly (two-sample z-test, $p = 0.072$).

Amplification and sequencing using FTA card samples

Of the 224 snails sampled with FTA cards, 180 (80.0%) amplified for COI. Of these 180 amplifications, 171 (95.0%) resulted in clean, readable forward and reverse sequences that could be unambiguously assembled into contigs. For the 49 samples that either did not amplify or could not be sequenced for COI, 22 (44.9%) successfully amplified for 16S, and 17 of these 16S amplifications (77.2%) were sequenced successfully. Tissue samples were taken from 214 snails. Tissue could not be taken from eight *Leptachatina* individuals because they were too small, and the two smallest *Deroceras laeve* individuals, which decayed quickly after death. Of these, 196 (91.6%) amplified for COI and 184 of these amplifications (93.9%) were sequenced successfully for COI. For the remaining 30 tissue samples that did not amplify or sequence for COI, 11 (36.6%) successfully amplified for 16S and 10 of these 16S amplifications (90.9%) were successfully sequenced (Table 5). For pooled data of all 27 species, the difference between FTA and tissue sampling methods was significant for COI amplification, but not for COI sequencing, 16S amplification, or 16S sequencing (2-sample z-tests: COI amplification, $p < 0.001$; COI sequencing, $p = 0.638$; 16S amplification, $p = 0.472$; 16S sequencing, $p = 0.337$).

For each species there was no significant difference between FTA and tissue sampling success (Table 5), except for one case. Tissue sampling was significantly more successful than FTA sampling in producing a COI sequence for the native marine species *Nerita picea* (1/10 for FTA and 9/10 for tissue; Fisher's exact test, $p < 0.001$), an operculate species. Noting the overall lower but statistically insignificant sequencing success of the other two operculate species (invasive freshwater *Tarebia granifera* with 6/15 for FTA and 15/15 for tissue; and terrestrial native *Pleuropoma laciniosa* with 0/6 for FTA and 5/6 for tissue), presence/absence of the operculum was tested as factor for FTA success. Pooled data of these operculate species had a significantly lower COI sequencing rate from FTA cards than pooled data of non-operculate species (all other species) (binary logistic regression, $p < 0.001$).

For all individuals that yielded COI or 16S sequence data for both FTA card and tissue methods, each individual's FTA and tissue nucleotide sequences were identical. BLAST searches indicated that all sequences correctly matched our identifications of the sampled individuals. All COI sequences translated to amino-acid sequences without stop codons, and amino acid sequence identities were the same for FTA and tissue samples.

Species identity, habitat, and size as factors in FTA sampling

Except for *Nerita picea*, no species had significantly greater amplification and sequencing success for tissue sampled DNA versus FTA sampled DNA (Table 5). Both are in Neritimorpha and are the most basal of the species tested (Fig. 1). No caenogastropod, heterobranch, or freshwater species had significantly greater amplification or sequencing success for either marker using tissue sampled DNA versus FTA sampled DNA.

Since there were no significant differences in success of 16 S amplification or 16 S sequencing for FTA and tissue sampling, individual size was only investigated for its ability to impact the likelihood of obtaining a COI sequence. Species sampled were diverse in size, ranging from ~2–20 mm in greatest shell width (Fig. 3; Table 4). The logistic regression analyses evaluating whether height or greatest width correlates with success for obtaining a COI sequence resulted in a negative correlation for shell height but a positive correlation for greatest width for both FTA and tissue methods (Figs. 4 and 5). The lines of fit were also similar between FTA card and tissue samples (Figs. 4 and 5). However, no models were significant except that for shell height of tissue-sampled individuals (FTA height, $p = 0.084$; FTA greatest width, $p = 0.173$; tissue height, $p = 0.007$; tissue greatest width $p = 0.075$).

Discussion

In this study, invasiveness refers to any impact on the snail's health or physical being, long term or short term. Tissue clipping causes permanent physical damage to a living individual by removing tissue from the foot, but FTA sampling causes no known lasting physical changes, so it is the less invasive option of the two. Based on the behavioral response of sampled individuals, the FTA method also appears to cause no permanent health effects. While snails initially seemed distressed from contact with the FTA buffers, this was temporary. However, as long-term health

Table 5 PCR amplification and sequencing success for FTA samples and tissue samples

Species	FTA COI amp	FTA COI seq	FTA 16 S amp	FTA 16 S seq	Tissue COI amp	Tissue COI seq	Tiss 16 S amp	Tiss 16 S seq
Planorbella duryi	10/10 (100.0%)	10/10 (100.0%)	N/A	N/A	10/10 (100.0%)	10/10 (100.0%)	N/A (0.0%)	N/A
Physella acuta	14/15 (93.3%)	14/14 (100.0%)	1/1 (100.0%)	0/1 (0.0%)	13/15 (80.0%)	13/13 (100.0%)	0/2 (0.0%)	N/A
Tarebia granifera	6/15 (40.0%)	6/6 (100.0%)	2/9 (22.2%)	0/2 (0.0%)	12/15 (73.3%)	12/12 (100.0%)	0/3 (0.0%)	N/A
Pseudosuccinea columella	3/4 (75.0%)	3/3 (100.0%)	1/1 (100.0%)	1/1 (100.0%)	4/4 (100.0%)	2/4 (50.0%)	0/2 (0.0%)	N/A
Bradybaena similaris	15/15 (100.0%)	15/15 (100.0%)	N/A	N/A	15/15 (100.0%)	15/15 (100.0%)	N/A	N/A
Lacteoluna selenina	8/10 (80.0%)	7/8 (87.5%)	2/3 (66.7%)	2/2 (100.0%)	10/10 (100.0%)	10/10 (100.0%)	N/A	N/A
Succinea tenella	15/15 (100.0%)	14/15 (93.3%)	1/1 (100.0%)	1/1 (100.0%)	15/15 (100.0%)	12/15 (80.0%)	0/3 (0.0%)	N/A
Allopeas clavulinum	13/15 (86.7%)	13/13 (100.0%)	0/2 (0.0%)	N/A	14/15 (93.3%)	14/14 (100.0%)	1/1 (100.0%)	1/1 (100.0%)
Deroceras laeve	11/11 (100.0%)	11/11 (100.0%)	N/A	N/A	9/9* (100.0%)	6/9* (66.7%)	0/3 (0.0%)	N/A
Amastra spirizona	12/12 (100.0%)	11/12 (91.7%)	0/1 (0.0%)	N/A	12/12 (100.0%)	12/12 (100.0%)	N/A	N/A
Amastra micans	6/6 (100.0%)	5/6 (83.3%)	0/1 (0.0%)	N/A	6/6 (100.0%)	6/6 (100.0%)	N/A	N/A
Amastra rubens	6/6 (100.0%)	4/6 (66.7%)	0/2 (0.0%)	N/A	6/6 (100.0%)	5/6 (83.3%)	0/1 (0.0%)	N/A
Amastra intermedia	4/6 (66.7%)	4/4 (100.0%)	0/2 (0.0%)	N/A	6/6 100.0%	6/6 100%	N/A	N/A
Amastra cylindrica	6/6 (100.0%)	6/6 (100.0%)	N/A	N/A	6/6 (100.0%)	6/6 (100.0%)	N/A	N/A
Leptachatina crystallina	6/6 (100.0%)	4/6 (80.0%)	1/2 (50.0%)	1/1 (100.0%)	1/1* (100.0%)	1/1* (100.0%)	N/A	N/A
Leptachatina cerealis	5/6 (83.3%)	5/5 (100.0%)	1/1 (100.0%)	0/1 (0.0%)	3/3* (100.0%)	3/3* (100.0%)	N/A	N/A
Laminella sanguinea	6/6 (100.0%)	6/6 (100.0%)	N/A	N/A	6/6 (100.0%)	6/6 (100.0%)	N/A	N/A
Philonesia sp.	2/3 (66.6%)	2/2 (100.0%)	1/1 (100.0%)	1/1 (100.0%)	1/3 (33.3%)	1/1 (100.0%)	2/2 (100.0%)	1/2 (50.0%)
Philonesia waiheensis	2/5 (40.0%)	1/2 (50.0%)	2/4 (50.0%)	2/2 (100.0%)	3/5 (60.0%)	1/3 (33.3%)	4/4 (100.0%)	4/4 (100.0%)
Philonesia hartmanni	1/3 (33.3%)	1/1 (100.0%)	0/2 (0.0%)	N/A	2/3 (66.6%)	2/2 (100.0%)	N/A	N/A
Kaala subrutila	1/1 (100.0%)	1/1 (100.0%)	N/A	N/A	1/1 (100.0%)	1/1 (100.0%)	N/A	N/A
Auricuella uniplicata	3/3 (100.0%)	3/3 (100.0%)	N/A	N/A	3/3 (100.0%)	3/3 (100.0%)	N/A	N/A
Auriculella turritella	14/15 (93.3%)	14/14 (100.0%)	0/1 (0.0%)	N/A	15/15 (100%)	15/15 (100%)	N/A	N/A
Pleuropoma laciniosa	0/6 (0.0%)	N/A	0/1 (0.0%)	N/A	5/6 (83.3%)	5/5 (100.0%)	0/1 (0.0%)	N/A
Catinella rotundata	6/6 (100.0%)	6/6 (100.0%)	N/A	N/A	6/6 (100.0%)	6/6 (100.0%)	N/A	N/A
Catinella baldwini	4/8 (50.0%)	4/4 (100.0%)	2/4 (50.0%)	1/2 (50.0%)	2/8 (25.0%)	0/8 (0.0%)	4/8 (50.0%)	4/4 (100.0%)
Nerita picea†	1/10 (10.0%)	1/1 (100.0%)	8/10 (80%)	8/8 (100%)	10/10 (100.0%)	10/10 (100.0%)	N/A	N/A
TOTAL†	**180/224 (80.4%)**	**171/180 (95.0%)**	**22/49 (44.9%)**	**17/22 (77.3%)**	**196/214 (91.6%)**	**184/196 (93.9%)**	**11/30 (36.6%)**	**10/11 (90.9%)**

*Tissue sample number does not match FTA sample number because of unavailability of individuals for tissue sample collection

†Significantly greater success in obtaining COI sequence data using the tissue sample method versus the FTA method (Fisher's exact test for individual taxon, 2-sample Z test for pooled total; $\alpha = 0.05$)

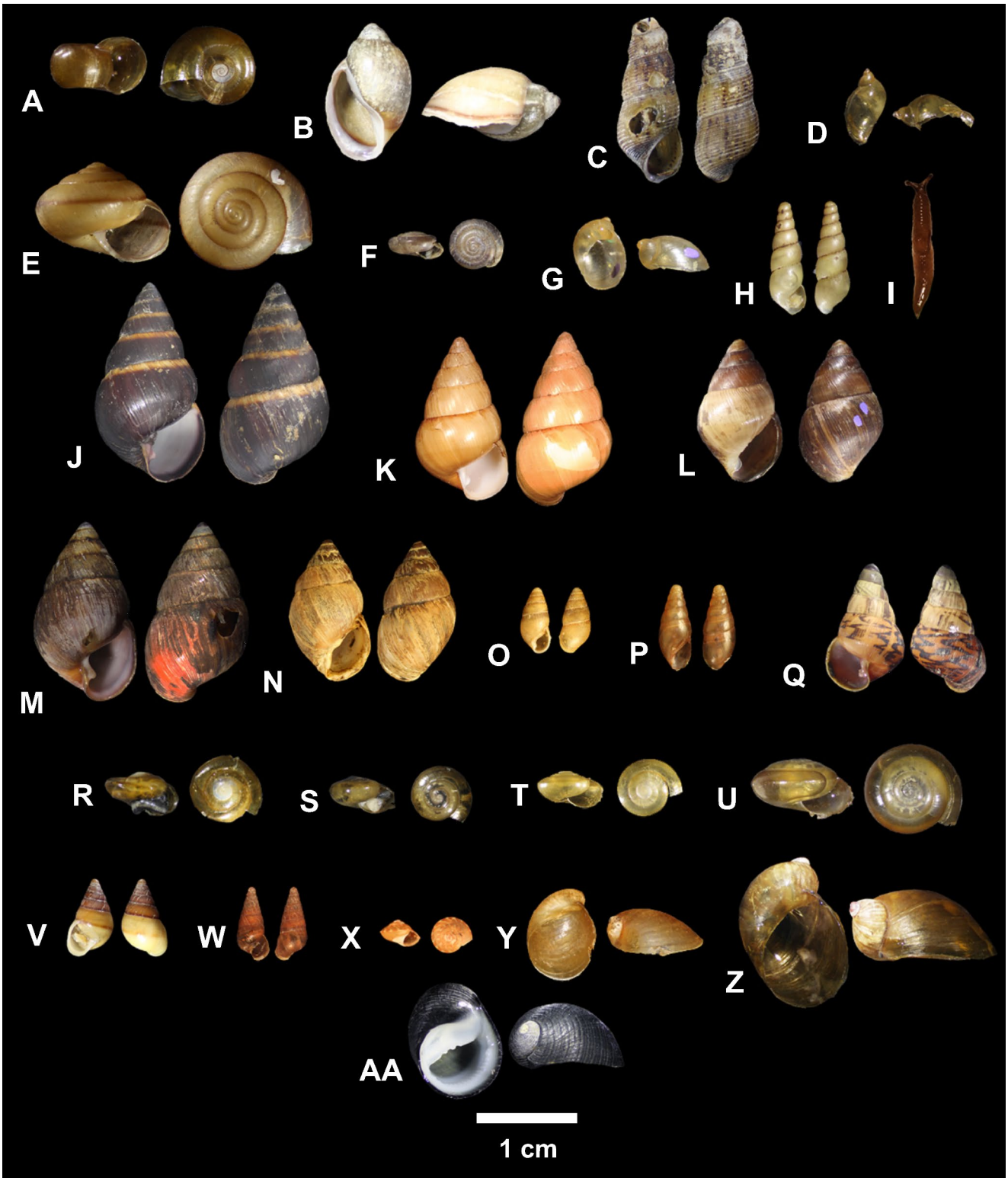

Fig. 3 Representatives of species used in FTA and tissue sampling. Invasive freshwater species **(A)***Planorbella duryi*, **(B)***Physella acuta*, **(C)***Tarebia granifera*, **(D)** and *Pseudosuccinea columella*. Invasive terrestrial species **(E)***Bradybaena similaris*, **(F)***Lacteoluna selenina*, **(G)***Succinea tenella*, **(H)***Allopeas clavulinum*, and **(I)***Deroceras laeve*. Native Amastridae species **(J)***Amastra spirizona*, **K)***Amastra micans*, **L)***Amastra rubens*, **M)***Amastra intermedia*, **N)***Amastra cylindrica*, **O)***Leptachatina crystallina*, **P)***Leptachatina cerealis*, and **Q)***Laminella sanguinea*. Native terrestrial species **R)***Philonesia* sp., **S)***Philonesia waiheensis*, **T)***Philonesia hartmanni*, **U)***Kaala subrutila*, **V)***Auriculella uniplicata*, **W)***Auriculella turritella*, **X)***Pleuropoma laciniosa*, **Y)***Catinella rotundata*, **Z)***Catinella baldwini*. Native marine species **AA)***Nerita picea*

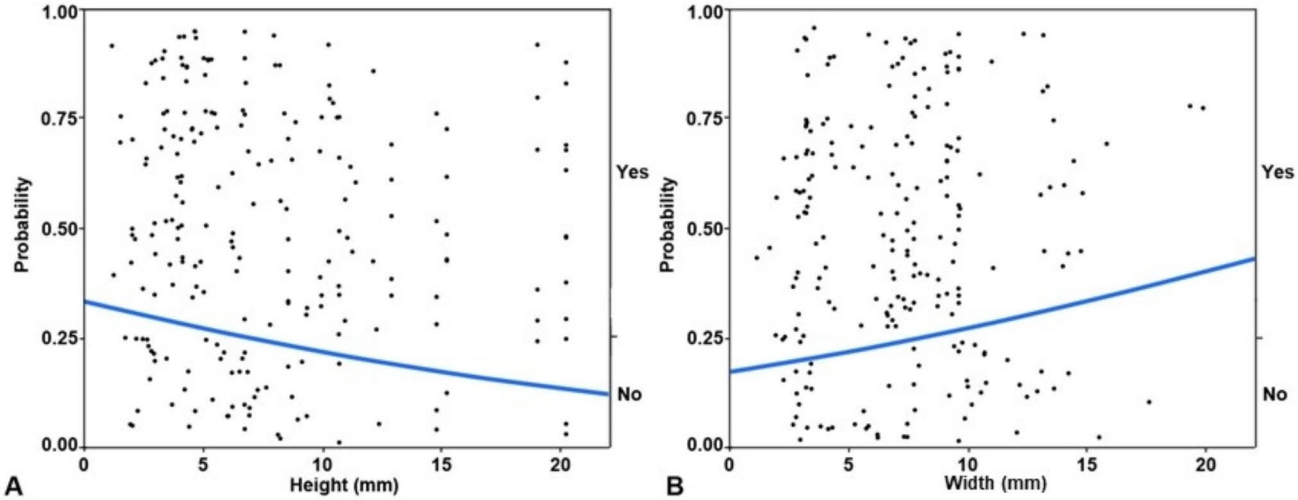

Fig. 4 Logistic regression models for shell dimensions and FTA sample COI sequencing success. The y-axis indicates probability of success (yes) or failure (no) for obtaining a COI sequence relative to the shell dimension measurement indicated by the x-axis. Lines indicate best-fit models for the relationship between shell dimension size and total success. Shell dimensions are **(A)** Height (mm), **(B)** Width (mm)

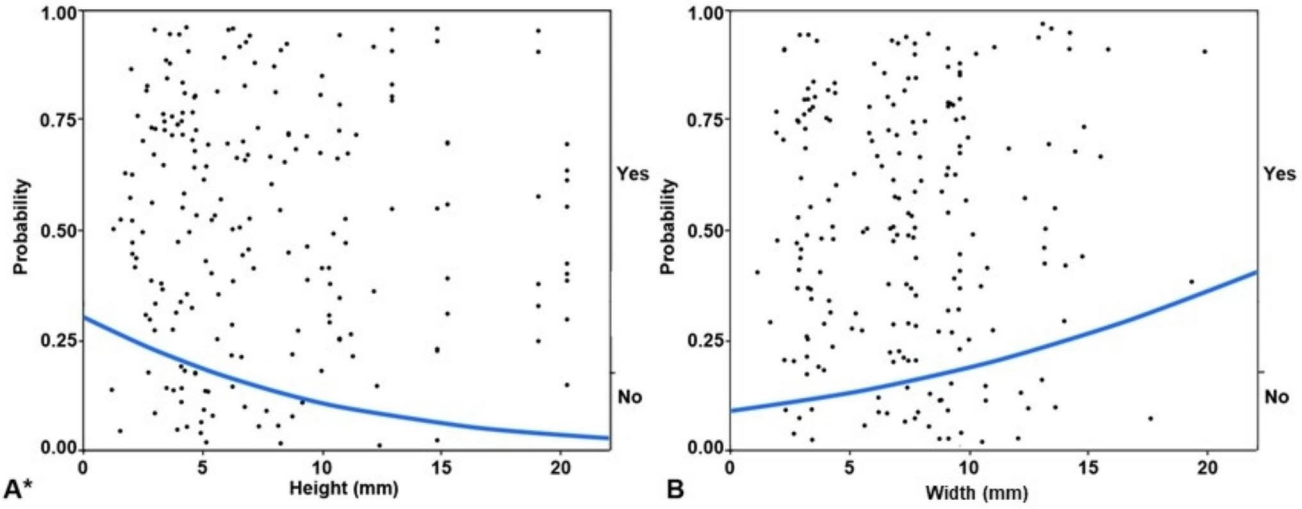

Fig. 5 Logistic regression models for shell dimensions and tissue sample COI sequencing success. The y-axis indicates probability of success (yes) or failure (no) for obtaining a COI sequence relative to the shell dimension measurement indicated by the x-axis. Lines indicate best-fit models for the relationship between shell dimension size and total success Shell dimensions are **(A)** Height (mm) and **(B)** Width (mm). * Indicates that the model had a significant Chi-square probability ($p < 0.05$)

was not assessed for either parents or offspring, definitive conclusions cannot be made about how FTA influences reproduction.

It is notable that the species for which the FTA method had significantly lower mitochondrial amplification and sequencing success than the tissue sampling method (*Nerita picea*) was operculate. This species (and *Pleuropoma laciniosa* and *Tarebia granifera*, which were also operculate and for which fewer COI sequences were generated for FTA than for tissue samples) were the most difficult to sample because of prolonged sealing of the aperture. This behavior resulted in reduced contact of the foot with the FTA card. This may have caused fewer cells to slough off and lyse, and consequently a lower initial amount of DNA in extractions. A corresponding lower concentration of DNA template in an amplification reaction could explain why these samples ultimately failed to produce a sequence.

It cannot be ruled out that some of the 27 deaths of FTA sampled individuals were due to old age, but as the age of individuals at time of collection could not be determined, it was not possible to account for this. It is also possible that there was some deleterious effect if the snails ingested part of the FTA card during sampling, but no apparent feeding was observed for any species. However, it is highly likely

that some deaths were due to handling or housing problems, and not FTA sampling (Table 3). One *Philonesia* sp. individual and two *Pseudosuccinea columella* individuals had cracked shells at time of sampling and probably died from physical damage. Also, nine deaths of FTA-sampled *Physella acuta* were probably due to inappropriate housing, as evidenced by the housing control experiment in which no deaths occurred for FTA sampled individuals that were supplied more water and vegetation (Tables 1 and 3). If these 12 deaths with ambiguous causes are removed from the analyses, the FTA sampling death rate decreases to 6.6%. However, even without correcting for deaths caused by handling or housing errors, the death rate for FTA sampled individuals (12.1%) was not quite significantly different from a control death rate (3.7%) ($p = 0.0719$). Therefore, there is no statistically supported evidence that FTA death rate is higher than the control, or that lethality should be a concern when considering FTA use for a study.

Although the tissue method resulted in significantly more COI amplifications than the FTA method, there was only a difference of 11.6%. Following the amplification step, success rate and quality of COI sequencing was similar for the two methods, and both methods worked well for obtaining 16S sequences. Because FTA cards do permanently remove a physical portion of the snail's body, unlike tissue clipping, they are clearly the preferable option for studies on rare species. For example, if a researcher requires DNA sequences from a large number of individuals, FTA cards can be used to avoid injuring every individual through tissue clipping, or removing many whole animals from their habitat to process in the lab. Although based on this study, an expected ~ 12% of these samples would fail to produce a sequence, simply sampling more individuals with FTA cards would offset the sequencing failure rate without causing negative impacts (death or physical damage) to the individual or population. However, there are several caveats for FTA use for DNA sampling of gastropods.

Even though overall FTA sampling resulted in few deaths and a high percentage of successful sequences, it failed to work well as a non-lethal approach or successfully produce sequences for certain species. The significantly higher number of deaths of the *Auriculella uniplicata* and *Auriculella turritella* sampled with FTA, some of which were sampled in the field and transported back to the lab for further observation, (Table 1) versus the *Auriculella* sp. (a close relative) controls touched with the untreated portion of the card (Table 3) is concerning. The genus *Auriculella* is endemic to Hawaii (Cooke and Kondo 1960; Yeung and Hayes 2018) and its remaining range is restricted to refugial mountain habitat with native vegetation (Solem 1990). It can be reasonably assumed that it is more sensitive to environmental stressors than the generalists that experienced no deaths post FTA sampling in this study, *Bradybaena similaris* and *Planorbella duryi*, which are widespread invasives (Cowie et al. 2008). Régnier et al. (2011) indicated that a major advantage of FTA cards is that they do not impact the chances of an individual's survival in the field, and therefore are a convenient option for non-lethal field sampling, but this study demonstrated otherwise for two endemic Hawaiian species. Therefore, it is recommended that in-lab trials for potentially sensitive taxa be conducted before FTA cards are used extensively. For example, while endemic *Philonesia* spp. and *Catinella* spp. co-occur with A*uriculella* spp. in specialized montane habitat, they did not have significant death rates in this study (Table 1). Based on the laboratory results of this study, we would recommend FTA cards for use in field studies of these rare snails. For highly sensitive taxa, fecal sampling can be explored as a non-lethal sampling option for gastropods that is less invasive than FTA cards. It requires no contact with the animal, and genetic data has been successfully obtained from sloughed-off cells in fecal samples of mammals of various sizes (Kurose et al. 2005; Rodgers and Janečka 2013; de Flamingh et al. 2023). For field studies without reliable access to cold storage, FTA cards may still be the better option, as fecal samples can be prone to degradation. In a mollusc-specific example, clam feces yielded high quality sequences, but those stored at warmer (28 °C) temperatures failed to produce a PCR band (Zhang et al. 2019).

The results of this study indicated that shell size has no bearing on whether FTA DNA sampling can be used for the amplification of a COI sequence. Lines of fit for logistic regression analyses seemed to indicate that for both FTA and tissue samples, as shell height increases, the likelihood of success of amplifying COI increases, but as greatest width increases, the likelihood of success for obtaining a COI sequence decreases; however, these trends were not significant (Figs. 4 and 5). Therefore, height and greatest width are not related to FTA sampling success. It is interesting that despite the wide scatter, the model for shell height was significant for tissue samples (Fig. 5). While this cannot be interpreted with relevance for FTA sampling success, it may suggest that height is good predictor of the size of an individual's foot, and the amount of tissue that can be used for extraction. Although the same volume was targeted for tissue sampling of each individual, larger pieces may have been taken from larger individuals, resulting in higher extraction concentrations and greater amplification success. It is notable that some of the species had large standard deviation values for their shell measurements (Table 4), suggesting that in future study, other metrics such as mass and total volume would be more accurate for analyzing the relationship between specimen size and sampling success. Also, habitat type seems to have no relationship with FTA

sampling success. The three species with the lowest success for amplifications and sequences using FTA samples were from different habitats (freshwater *Tarebia granifera*, terrestrial *Pleuropoma laciniosa* and marine *Nerita picea*).

It is notable that the species that had significantly lower COI amplification successes relative to tissue control matches were operculate (Table 5). Also, although the differences were not statistically significant, *Tarebia granifera*, had half as many mitochondrial sequences from FTA (6/15) samples than tissue samples (12/15), and *Pleuropoma laciniosa* had no mitochondrial sequences from FTA cards compared to the high success of tissue samples (5/6). These two species are also operculate. A pooled analysis indicated that that compared to the than non-operculate species, operculate species indeed had a collectively had lower COI amplification rate for FTA card samples. It has been hypothesized that the operculum evolved as a defense against predators (Checa and Jiménez-Jiménez 1998), which could correspond to the quick withdrawal and prolonged sealing behaviors observed here, which made sample collection difficult. The results demonstrated that the method is successful for obtaining sequences from non-operculate aquatic snails (*Physella acuta*, *Planorbella duryi*, and *Pseudosuccinea columella*), but more work is needed to test or optimize its utility for operculate species. It is also possible that taxonomic identity may play a role, since three species are all in the Neritimorpha or Caenogastropoda, which are evolutionarily distant and basal to the Heterobranchia, to which all other sampled species belong (Fig. 1). To distinguish whether operculum possession or taxonomic identity is the causative factor in amplification and sequencing failures, non-operculate representatives of these basal clades should be tested in the future, such as the shell-less neritimorphs in the genus *Titiscania* (family Neritopsidae).

Hawaiian land snails were used in this study as a specific example of how a well-developed non-lethal sampling method may be applied to studies that have important conservation ramifications. Amastridae is the only extant endemic Hawaiian family of either plants or animals (Zimmerman 1948), and of 325 recognized species, only 21 or 22 (< 7%) still remain, all in small populations (Yeung et al. 2018; Yeung and Hayes 2018). Here, none of 54 variably sized individuals of the eight native Hawaiian species sampled with the FTA method died (Table 4; Fig. 3). As trials with *Leptachatina* spp. demonstrated, it can also be difficult to obtain tissue from smaller live amastrids. Because of their rarity and the need to maximize genetic variation in captive breeding programs to ensure population health (reviewed by Frankham et al. 2014), it is especially important that individuals are not killed for DNA sampling. This study not only confirmed FTA sampling as a viable non-lethal DNA sampling method for this family, its successful use for amplifying and sequencing markers makes conservation studies requiring wide scale genetic sampling feasible. For example, FTA cards can be used to monitor the genetic variability of the breeding colonies of all species to the individual level, and to more carefully manage inbreeding so that fitness can be maximized in offspring (Leberg and Firmin 2008, Frankham et al. 2014).

The case of the single mountain peak extreme endemic *Kaala subrutila* is an example of FTA cards being safe to use for even the rarest of species. This species is the only one of its genus, occurs at low abundance, and has only ever been recorded from an area of less than 1 km^2 on Oahu's tallest mountain, Mt. Kaala (Baker 1940; Curry and Yeung 2013). It is under threat from invasive species predation (Curry and Yeung 2013). However, our DNA studies of this species using the FTA method are not likely to contribute to its decline, as the health of the single individual sampled was not affected. The same idea may be applied to other gastropods of extreme rarity or conservation status. The success of the FTA method for sampling three species of Succineidae, invasive *Succinea tenella* and native Hawaiian *Catinella rotundata* and *C. baldwini*, implies conservation significance on a global level. The family is distributed worldwide and the FTA method could be used to study threatened species occurring in multiple regions. Cumulatively, these findings for Hawaiian land snails may be projected to mean that FTA cards can be used on critically threatened molluscs throughout the Pacific and elsewhere, and are especially advantageous for studies or conservation applications requiring wide-scale genetic sampling.

Non-lethal and non-invasive DNA sampling methods are essential for avoiding negative impacts on rare species in studies requiring large-scale collection of genetic data. The purpose of this study was to address the insufficient development of these methods for gastropods, which are in decline almost everywhere in the world and are especially imperiled in the islands of the Pacific. The results corroborate previous findings (Régnier et al. 2011) that FTA card sampling is a viable non-lethal alternative to tissue clipping for sampling snails. By sampling taxonomic groups representing a variety of habitat types, a wide range of sizes, and distantly related evolutionary lineages, the study expanded this approach for obtaining DNA to a broad range of gastropods. However, based on higher death rates for a few species and lower success rates in obtaining sequence data from difficult-to-sample species, it is recommended that lab trials be conducted before wide scale use.

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Author contributions KL produced the data, conduced analyses, wrote the manuscript, and prepared the figures.

Data Availability The data of this study are available upon request from the author KL.

Declarations

Competing interests The authors declare no competing interests.

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