

## Research Article

**Feeding habits of the exotic invasive slug *Limax maximus*: a basis for risk assessment of herbivory on forest and agricultural ecosystems**Wataru Komatsu<sup>1</sup> and Ikuyo Saeki<sup>2,3,\*</sup><sup>1</sup>Graduate School of Science and Technology, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8571, Japan<sup>2</sup>Faculty of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8571, Japan<sup>3</sup>Makino Herbarium, Tokyo Metropolitan University, 1-1 Minami-osawa, Hachioji, Tokyo 192-0397, Japan

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**Received:** 3 March 2022**Accepted:** 16 August 2022**Published:** 14 October 2022**Handling editor:** Frank H Koch**Thematic editor:** Catherine Jarnevich**Copyright:** © Komatsu and SaekiThis is an open access article distributed under terms of the Creative Commons Attribution License ([Attribution 4.0 International - CC BY 4.0](https://creativecommons.org/licenses/by/4.0/)).**OPEN ACCESS****Abstract**

Invasions of exotic herbivores often result in considerable damage to vegetation. Slugs are one of the herbivores that pose a high risk of altering vegetation when introduced to new sites. The large invasive slug, *Limax maximus*, was recently introduced into Japan. We examined its feeding behavior to assess potential effects on vegetation at introduced sites. We conducted two complementary experiments: feeding trials and DNA meta-barcoding analyses. In the feeding trials, we provided *L. maximus* with 23 test plants and calculated Acceptability Index (AI) values to evaluate palatability. *Limax maximus* clearly favored specific plants; average AI was highest for *Brassica oleracea*, followed by *Lactuca indica*, and *Sambucus racemosa*. DNA meta-barcoding analyses identified plants from six families in the feces of wild individuals. According to the generalized linear mixed model analyses, the plants that *L. maximus* preferred to eat were crops, herbaceous plants, and plants with glabrous leaves. Because native slugs and land snails do not show these preferences, *L. maximus* may exert herbivory pressure on native and agricultural plants that is different from that exerted by native molluscan communities. Our work demonstrated that combining multiple methods helps to estimate the potential risk of exotic herbivory in natural ecosystems. Additional research should examine how *L. maximus* influences forest vegetation structure and crop productivity in areas where it is introduced.

**Key words:** DNA meta-barcoding, exotic herbivore, feeding behaviour, introduced species, mollusc, palatability, vegetation

**Introduction**

Invasions by exotic herbivores can cause considerable damage to vegetation (Ehrenfeld 2010; Nuñez et al. 2010; Simberloff et al. 2013). For example, herbivory by introduced mammals, such as goats and rabbits, has altered plant species composition and diversity in many areas of the globe (Campbell and Donlan 2005; Cubas et al. 2019). Invasive vertebrates that cause changes in plant communities are often targets of eradication efforts. (Campbell and Donlan 2005; Jones et al. 2016). However, herbivory by exotic invertebrates should not be overlooked because it can also give a

serious impact on native ecosystems (Roques et al. 2009; Oduor et al. 2010). Exotic invertebrates are small in size, which hinders prevention of introduction and eradication. In addition, the basic taxonomy and ecology of many invertebrates remain largely unknown (Roques et al. 2009; Rowson et al. 2014). These factors make it difficult to assess the potential risks of herbivory by exotic invertebrates and to establish effective countermeasures.

Slugs are important herbivores in temperate grasslands and forests (Cottam 1985; Rathcke 1985; Cook and Radford 1988; Del-Val and Crawley 2004; Hahn et al. 2011; Orians et al. 2013; Shiels et al. 2014). They often affect plant diversity by selectively feeding on seedlings (Buschmann et al. 2005; Strauss et al. 2009) and can alter native vegetation once they become established. In field experiments conducted in Hawaii, exotic slugs decreased seedling survival of some endangered plant species; thus slugs can be a direct cause of plant endangerment (Joe and Daehler 2008). Hawaii has no native slugs, and so these introduced species may inflict damage of a type not previously experienced by native plants. In New Zealand, the native fern *Botrychium australe* R.Br. suffered extensive defoliation by herbivory of the introduced slug *Deroceras reticulatum* (O. F. Müller, 1774) after fire disturbance (Sessions and Kelly 2002). These cases indicate that exotic slugs have a high risk of altering native vegetation once they become established.

The Japanese archipelago has a rich gastropod fauna, with more than 800 species of terrestrial molluscs (Azuma 1995). Japan has repeatedly been exposed to invasions by introduced slugs, some of which have become numerous or even dominant. *Ambigolimax valentianus* (Férussac, 1822), which was introduced after World War II, is now common in Japan (Kurozumi 2002), causing serious agricultural damage by herbivory on vegetables. In addition, the large exotic slug *Limax maximus* (Linnaeus, 1758) was discovered in Ibaraki Prefecture, Japan in 2006 (Hasegawa et al. 2009). *Limax maximus* is native to Europe, North Africa, and Asia, and has been introduced to many countries in North and South America, Africa, the Pacific Islands, and Australia (Gaitán-Espitia et al. 2012), as well as to the islands of Hawaii (Joe and Daehler 2008) and New Zealand (Barker and McGhie 1984; Roth and Sadeghian 2006). *Limax maximus* is perceived as an invasive slug (Gaitán-Espitia et al. 2012) and can occur in a wide range of habitats, including forests, hedgerows, and gardens (Kerney and Cameron 1979; Cook and Radford 1988; Meyer et al. 2013). In some countries, the species is considered an agricultural pest (Stange et al. 2009; Kozłowski 2012). In Japan, its distribution is rapidly expanding, and occurrences have been reported from the islands of Hokkaido (Morii et al. 2018) and Honshu (Hasegawa et al. 2009). When mature, *L. maximus* (ca. 15 cm) is markedly larger than *Meghimatium bilineatum* (Benson, 1842) (ca. 5 cm), which is one of the most common native slugs in Japan. The size, population density, and rapid range expansion of *L. maximus* raise concerns about its effects on natural and agricultural ecosystems (Morii et al. 2015).

To minimize the ecological and economic damage caused by biological invasions, it is essential to understand the ecology of potentially harmful non-native species. Information on food preferences is especially important in assessing the potential effect of herbivory on natural vegetation. If exotic species select a specific food resource, they may alter plant species composition and diversity (Buschmann et al. 2005; Carlsson and Lacoursière 2005). Although *L. maximus* is among the most successful invasive slugs, its feeding ecology in introduced areas has rarely been investigated except for a limited number of studies in Hawaii (Joe and Daehler 2008; Meyer and Yeung 2011). *Limax maximus* is known to be omnivorous and eats a variety of foods including fungi, carrion, and fresh and rotting plants (Barker 1999; Kozłowski 2012). However, its diet and food preferences may change depending on the food resources available in introduced areas. Moreover, competition is possible if native and introduced species prefer similar food resources (Byers 2000; Paustian and Barbosa 2012).

To assess the potential risk to vegetation, we examined *L. maximus* herbivory in forest and agricultural ecosystems in Japan, where the introduced slug is rapidly expanding its distribution. Our initial efforts addressed: (1) Which common plant species are preferred by *L. maximus*? (2) Does *L. maximus* prefer plants with specific traits? and (3) How do plant preferences of *L. maximus* and another invasive slug compare with those of native snails and slugs? To answer these questions, we conducted two complementary experiments: feeding trials and DNA meta-barcoding analyses. In the feeding trials, the palatability to *L. maximus* of 23 plant species was evaluated and compared with other terrestrial molluscs. In the DNA meta-barcoding analyses (hereinafter referred to as “meta-barcoding analyses”), we collected feces from wild individuals of *L. maximus* at the site of a high-density invasive population, then used meta-barcoding to examine which plants are consumed by the slugs. Integrating the results of these experiments allowed us to assess the potential risk of herbivory by *L. maximus* and possible effects on vegetation.

## Materials and methods

### *Feeding trials*

Feeding trials were conducted from July 9, 2020 to November 10, 2020 at the University of Tsukuba, Ibaraki Prefecture, Japan. We selected five species of terrestrial molluscs (Table 1): two exotic slugs (*L. maximus* ( $n = 21$ ) and *A. valentianus* ( $n = 21$ )), one native slug (*M. bilineatum* ( $n = 11$ )), and two native land snails (*Acusta sieboldtiana* [Pfeiffer, 1850] ( $n = 8$ ) and *Euhadra brandtii brandtii* [Kobelt, 1875] ( $n = 10$ )); the latter three species are common in Japan and sympatric to introduced populations of *L. maximus*. All samples were collected in mixed forests with broadleaf and coniferous trees, or on roadside trees planted on the University of Tsukuba campus.

**Table 1.** List of slug and land snail species used for feeding trials.

Scientific name	Slug / Snail	Native / Exotic	Number of individuals <sup>1)</sup>	Average weight (range) (g)
<i>Limax maximus</i>	Slug	Exotic	21 (2)	3.24 (0.77–6.45)
<i>Ambigolimax valentianus</i>	Slug	Exotic	21 (2)	0.60 (0.34–1.01)
<i>Meghimatium bilineatum</i>	Slug	Native	11	1.64 (0.68–2.32)
<i>Acusta sieboldiana</i>	Snail	Native	8 (3)	1.14 (0.19–2.00)
<i>Euhadra brandtii brandtii</i>	Snail	Native	10	3.65 (1.73–6.13)

<sup>1)</sup> The total number of individuals used for feeding trials is shown in the first set of numbers. The number of individuals whose data were partially available due to death and escape were noted within parentheses.

**Table 2.** List of plant species used in feeding trials.

No.	Scientific name (Abbreviation)	Native/Exotic/Crop	Herbaceous/Woody
1	<i>Houttuynia cordata</i> (HC)	Native	Herbaceous
2	<i>Disporum smilacinum</i> (DS)	Native	Herbaceous
3	<i>Commelina communis</i> (CC)	Native	Herbaceous
4	<i>Boehmeria nivea</i> (BN)	Native	Herbaceous
5	<i>Solanum nigrum</i> (SN)	Native	Herbaceous
6	<i>Aster rugulosus</i> (AR)	Native	Herbaceous
7	<i>Lactuca indica</i> (LI)	Native	Herbaceous
8	<i>Youngia japonica</i> (YJ)	Native	Herbaceous
9	<i>Cayratia japonica</i> (CJ)	Native	Herbaceous (vine)
10	<i>Pueraria montana</i> (PM)	Native	Herbaceous (vine)
11	<i>Paederia scandens</i> (PS)	Native	Herbaceous (vine)
12	<i>Mallotus japonicus</i> (MJ)	Native	Woody
13	<i>Hydrangea paniculate</i> (HP)	Native	Woody
14	<i>Sambucus racemosa</i> (SR)	Native	Woody
15	<i>Trifolium repens</i> (TR)	Exotic	Herbaceous
16	<i>Solidago canadensis</i> (SC)	Exotic	Herbaceous
17	<i>Sonchus asper</i> (SA)	Exotic	Herbaceous
18	<i>Taraxacum officinale</i> (TO)	Exotic	Herbaceous
19	<i>Robinia pseudoacacia</i> (RP)	Exotic	Woody
20	<i>Ligustrum lucidum</i> (LL)	Exotic	Woody
21	<i>Glycine max</i> (GM)	Crop	Herbaceous
22	<i>Brassica oleracea</i> (BO)	Crop	Herbaceous
23	<i>Morus alba</i> (MA)	Crop	Woody

The collected individuals included both adult and juvenile stages. After collection, the gastropods were immediately taken to the laboratory and kept until the experiment started. The time between collection and the start of the experiment ranged approximately from 8 to 15 days. At the beginning of the experiment, we had a total of 71 individuals (Table 1). Unfortunately, some individuals died during the experiment. In that case, additional individuals were collected if available and used to continue the experiment.

We selected 23 plant species common to Japan for the feeding trials (Table 2). They include native and exotic species, herbaceous and woody plants, and wild and cultivated plants. Cultivated plants were included because *L. maximus* is known to damage agricultural plants (Stange et al. 2009; Kozłowski 2012). We tested palatability of both native and exotic plant species because in a study conducted in Hawaii, some rare native plants were more susceptible to feeding damage than common exotic plants (Joe and Daehler 2008), although a study of two slugs (*Ariolimax columbianus* (Gould, 1851) and *Arion ater* (Linnaeus, 1758)) in North America did not

show such differences (Cates and Orians 1975). Both herbaceous and woody plants were selected because Rathcke (1985) suggested that slugs prefer herbs to woody plants.

The 23 plants were offered one by one to each mollusc. For each feeding trial (i.e., one individual mollusc with one plant species), we measured the amount of plant material consumed in 24 hours by area. In each trial, we offered lettuce along with the test plant, following Dirzo's method (1980), to obtain an acceptability index (AI; Equation 1):

$$\text{AI} = \text{leaf area of test material consumed} / \text{leaf area of lettuce consumed} \quad (1)$$

Before each of the feeding trials, molluscs were kept in individual plastic cups (8 cm diameter × 5 cm height) in the laboratory, and given a constant supply of lettuce to prevent them from becoming hungry. At the beginning of a trial, the lettuce was removed and an equal amount of test plant and fresh lettuce by leaf area was put in the cup. To determine the amount of lettuce and test plant to be offered to each mollusc species, we measured the average amount of lettuce consumed by an individual of that species in 24 hours. For each trial, 40% of the average daily amount of lettuce and the same leaf area of tested plant was provided to each individual, following Hahn et al. (2011). The amount of lettuce given during the experiment ranged from 100 cm<sup>2</sup> to 600 cm<sup>2</sup>. With this method, if an individual snail or slug found the test plant as palatable as lettuce, an equal amount would be consumed (AI = 1). After we began conducting trials, we found that the native slug, *M. bilineatum*, did not eat lettuce. We provided them with sliced cucumber (*Cucumis sativus* L.), instead, and *M. bilineatum* ate it as the other molluscs ate lettuce. Because of this difference in the plants offered for feeding trials, the results for *M. bilineatum* should be interpreted with caution. The plants were given to the molluscs in the same order. We assumed that the order of plants did not affect the amount of consumption of each trial. All trials were performed in a laboratory where the temperature was maintained at 24 °C.

Before and after each trial, the lettuce or cucumber and test plants were placed on graph paper and photographed, and the area of each leaf sample was measured using an image processing application (ImageJ ver. 1.8.0; Schneider et al. 2012). We compared the two images to calculate the leaf area consumed during the trial. A total of 1416 AIs were obtained for the 23 plant species; 217 missing values (13.3% of all possible AIs) from individuals that didn't eat lettuce or died during the experiment were excluded (i.e., 71 mollusc individuals × 23 plant species – 217 missing values = 1416 AIs; Tables 1 and 2). One hundred forty nine out of 217 missing values resulted from not eating lettuce. The average of AIs was calculated for each of the plant species, and we tested whether it differed from zero by performing a one sample T-test.

We performed non-metric multidimensional scaling (NMDS) analysis of the tabulated AI values to determine differences in the palatability of plants

**Table 3.** List of individuals of *Limax maximus* and related sample information used for DNA meta-barcoding analyses.

Individual ID	Category <sup>1)</sup>	No. of feces collected for analyses	Sample no. for DNA meta-barcoding <sup>2)</sup>
A	Wild	6	1
B	Wild	4	2
C	Reared	2	3
D	Reared	2	3

<sup>1)</sup> Wild: Wild individual collected in a forest on the University of Tsukuba campus. Reared: Reared individual fed on lettuce and *Sambucus racemosa* used in feeding trials.

<sup>2)</sup> PCR products amplified from feces of individuals C and D were pooled and analyzed as Sample 3.

to the molluscs and ran permutation multivariate analyses of variance (PERMANOVA) using the Bray-Curtis distance matrices. We used data from the 28 mollusc individuals of four species (*L. maximus*, *A. valentianus*, *A. sieboldtiana*, and *E. b. brandtii*) that had no missing values. In other words, we excluded data for individuals for which AI values could not be calculated because they did not eat any lettuce in one or more feeding trials. We also excluded the data for *M. bilineatum* since it consumed only small amounts of plant material (see Results for details). In addition, we performed generalized linear mixed model (GLMM) analysis to determine if any plant traits were significantly related to AI values of *L. maximus*, *A. valentianus*, *A. sieboldtiana*, and *E. b. brandtii*. The traits included in the model were (1) plant origin (exotic, native, or crop), (2) plant type (woody or herbaceous), (3) leaf thickness, and (4) leaf pubescence (pubescent or glabrous). Leaf thickness was measured by using a QUICKmini PK-1012CPX digital thickness gauge (Mitutoyo, Kawasaki, Japan) for a total of nine mature leaves taken from three individuals of each plant species and averaged. In the GLMM analysis, a Gaussian distribution was selected, and the identification number of each mollusc was treated as a random effect. The software R 4.0.3 (R Core Team 2020) was used for these statistical analyses, along with the metaMDS and adonis functions in the package *vegan* (Oksanen et al. 2020), the *lmer* function in the packages *lme4* (Bates et al. 2015) and *lmerTest* (Kuznetsova et al. 2017). Model selection of the GLMM analysis was performed with the package *MuMIn* (Barton 2020).

### DNA meta-barcoding analyses

To determine which plants *L. maximus* consumes in natural environments, feces were collected from wild individuals, and analyzed using meta-barcoding to identify plant DNA present in the feces. Two wild individuals of *L. maximus* were collected in a forest on the University of Tsukuba campus, where the individuals for feeding trials were collected, from September 25, 2020 to November 18, 2020 (Table 3). Two individuals were taken to the laboratory and put in individual plastic bags. Feces (2–6 pieces) were collected from each individual during the next 3 days. In addition,

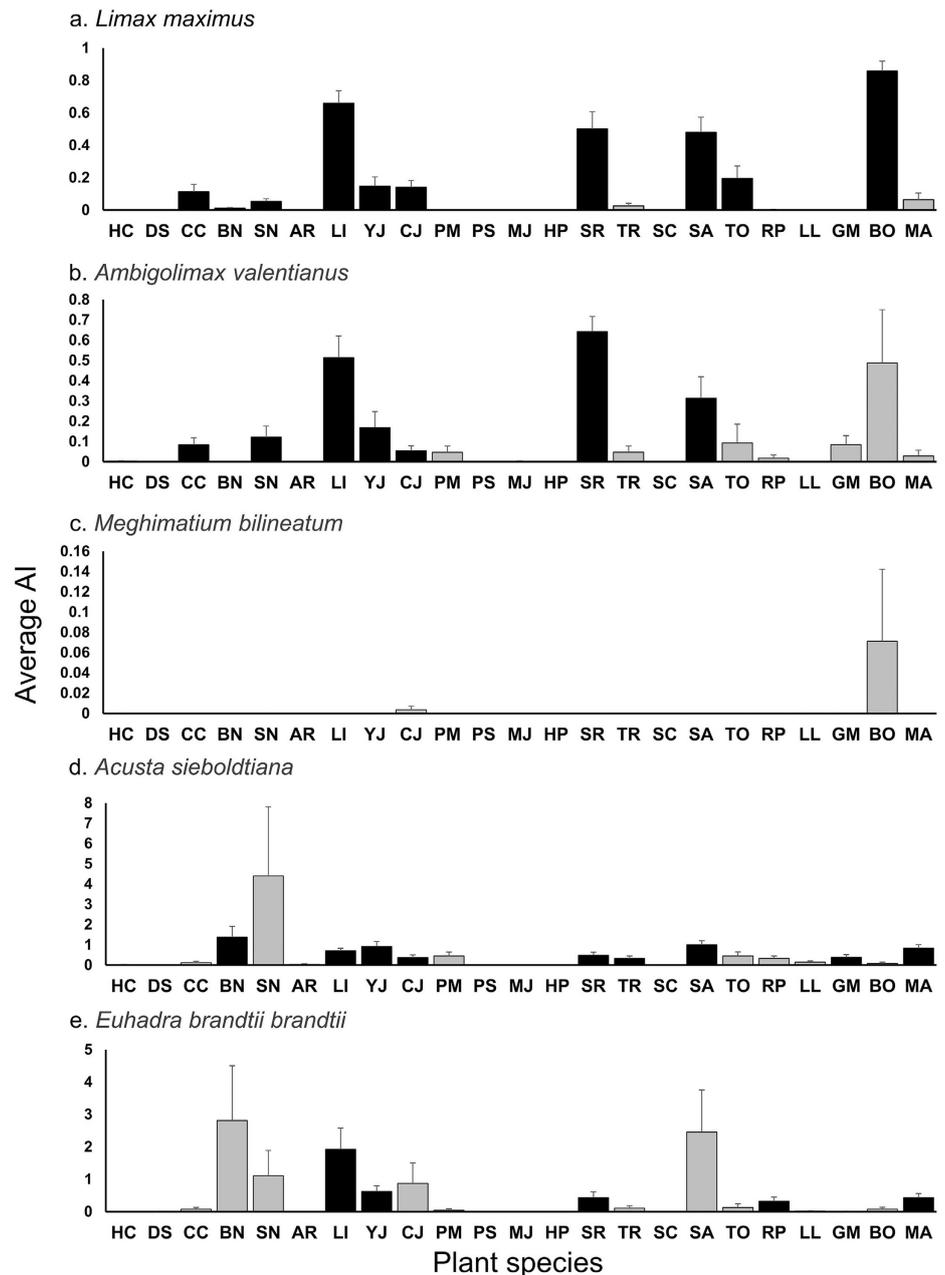
two individuals of *L. maximus* fed with lettuce and *Sambucus racemosa* L. were prepared to obtain a control sample. We tested a relatively small number of individuals (i.e., four individuals) to maximize time and cost efficiency, so the results should be interpreted with caution.

Each of the collected feces was placed in a separate 1.5-ml tube and stored in a freezer at  $-20^{\circ}\text{C}$ . Total DNA was extracted by using QIAGEN's DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) or QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocols. The two types of DNA extraction kit were used for the experiment because we liked to check if they had any differences in the performance of PCR amplification. Since there was little difference between them, we used the samples extracted by both kits. PCR was performed using a primer set designed to amplify a partial region of the *rbcL* gene (Supplementary material Table S1; Seibutsugiken Co., Ltd, Sagamihara, Japan.). The PCR mixture contained ultrapure water (3.3  $\mu\text{l}$ ), forward and reverse primers (10  $\mu\text{M}$ , 0.6  $\mu\text{l}$  for each), and PrimeSTAR Max DNA Polymerase (5.0  $\mu\text{l}$ ) (Takara Bio Co., Ltd., Kusatsu, Japan). The thermal cycling program was 35 cycles of  $98^{\circ}\text{C}$  for 10 sec,  $50^{\circ}\text{C}$  for 30 sec and  $72^{\circ}\text{C}$  for 5 sec. The PCR products were electrophoresed on an agarose gel for confirmation of amplification. Samples from the two lab-fed individuals were pooled. These were purified and used for a second round of PCR amplification (Table S2). The library obtained from the second PCR was sequenced by using a MiSeq sequencer (Illumina, San Diego, USA). Experimental procedures after the second PCR were performed by Seibutsugiken Co. Ltd (Kanagawa, Japan). The paired sequences with 300 bps length were used for the analyses. To compare the results with vegetation at the site where the wild *L. maximus* were collected, a list of vascular plants was made during a field survey on September 25, 2020. The floristic survey was conducted in the area within 200 m of the collection points. The list of plant species was compared with taxonomic groups detected by DNA meta-barcoding analyses.

## Results

### *Feeding trials*

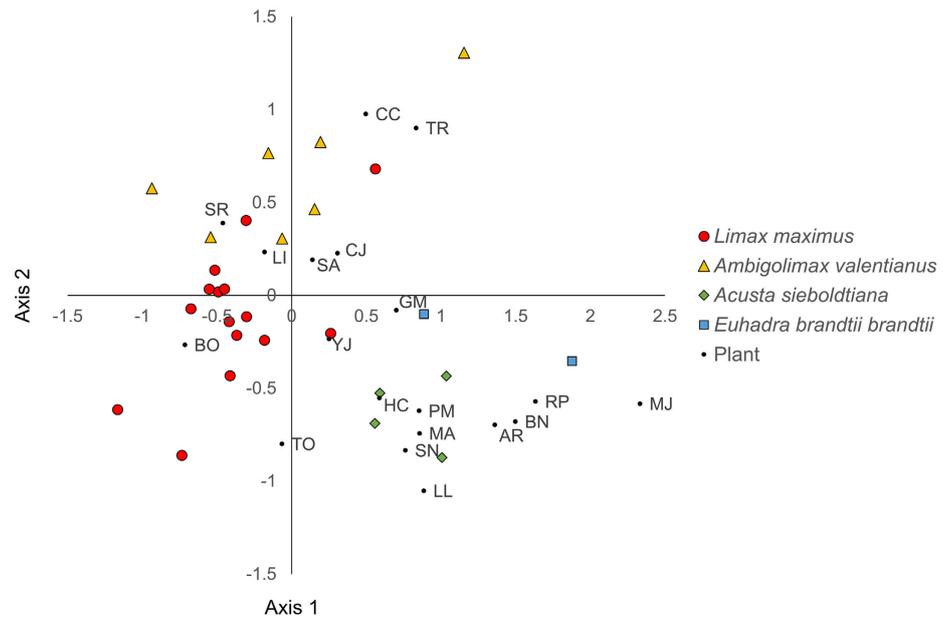
Feeding patterns differed markedly among the five molluscan species, but some plants were commonly preferred or least-preferred (Figure 1). For *L. maximus*, the highest average AI (0.86) was for *B. oleracea* (BO), followed by *L. indica* (LI; 0.66), *S. racemosa* (SR; 0.50), *Sonchus asper* (SA; 0.48), and *T. officinale* (TO; 0.20). These species, except *T. officinale*, were also preferred by *A. valentianus*. For *A. sieboldtiana*, *Solanum nigrum* L. (SN) was strongly preferred (4.40). *Euhadra brandtii brandtii* preferred *B. nivea* (2.81) and *Sonchus asper* (2.45). In contrast, the native slug *M. bilineatum* consumed only *B. oleracea* and *Cayratia japonica*. Plants with low AI values,



**Figure 1.** Results of feeding trials of seven molluscan species. The vertical axis shows the average Acceptability Index (AI) derived by using Dirzo's method (1980), and the horizontal axis shows plant species. HC, *Houttuynia cordata*; DS, *Disporum smilacinum*; CC, *Commelina communis*; BN, *Boehmeria nivea*; SN, *Solanum nigrum*; AR, *Aster rugulosus*; LI, *Lactuca indica*; YJ, *Youngia japonica*; CJ, *Cayratia japonica*; PM, *Pueraria montana*; PS, *Paderia scandens*; MJ, *Mallotus japonicus*; HP, *Hydrangea paniculate*; SR, *Sambucus racemosa*; TR, *Trifolium repens*; SC, *Solidago canadensis*; SA, *Sonchus asper*; TO, *Taraxacum officinale*; RP, *Robinia pseudoacacia*; LL, *Ligustrum lucidum*; GM, *Glycine max*; BO, *Brassica oleracea*; MA, *Morus alba*. See Table 2 for detailed information on plants. Error bars represent standard errors (SE). Black bars indicate that the mean is significantly different from zero. Note that the scale of the y-axis differs among the graphs.

i.e., unfavored plants, differed among the molluscan species, but *Hydrangea paniculata* Siebold (HP), *Solidago canadensis* L. (SC), and *Mallotus japonicus* (L.f.) Müll.Arg (MJ) were avoided by all the molluscs.

The NMDS shows patterns of plant choices among mollusc species (Figure 2). In the ordination diagram, *L. maximus* was assigned a low score



**Figure 2.** Ordination analyses (nonmetric multidimensional scaling; NMDS) of palatability of plants to four molluscan species based on the results of feeding trials. Palatability was scored by using the Acceptability Index (AI) following Dirzo’s method (1980). See Table 2 for the abbreviations of plant names.

**Table 4.** Results of the (PERMANOVA) to examine the differences of food choices among *Limax maximus* (exotic), *Ambigolimax valentianus* (exotic), *Acusta sieboldiana* (native), and *Euhadra brandtii brandtii* (native). Abbreviations are: df, degrees of freedom; SS, sum of squares; MS, mean squares; F, F-statistic;  $R^2$ , explained variance.

Parameter	df	SS	MS	F	$R^2$	P-value
1) <i>L. maximus</i> vs. other species						
Factor	1	1.40	1.40	7.79	0.23	< 0.001
Residuals	26	4.66	0.18		0.77	
Total	27	6.06				
2) Exotic vs. native species						
Factor	1	1.33	1.33	7.31	0.22	< 0.01
Residuals	26	4.73	0.18		0.78	
Total	27	6.06				

on NMDS axis 1, and the other exotic slug, *A. valentianus*, was plotted close to it with a slightly higher score on NMDS axis 2. In contrast, the two native land snails were assigned high scores on NMDS axis 1, and placed relatively close to each other. The analyses of PERMANOVA indicated plant choice differed between *L. maximus* and the other molluscs (Table 4). In addition, the exotic species were significantly differentiated from the native species.

According to the GLMM analyses, one or more plant traits significantly affected the AI of the exotic slugs (Table 5). For *L. maximus*, the model with plant origin, plant type, and leaf pubescence was selected as the best-fit with the lowest AIC value. The coefficients indicated that *L. maximus* favored plants with glabrous leaves over those with pubescence, herbaceous over woody plants, and crop species over native and exotic plants. Leaf pubescence was also included in the best-fit model for *A. valentianus*, which favored glabrous leaves over pubescent ones. However, no other traits

**Table 5.** List of best-fit models for predicting the Acceptability Index (AI) of plants for two exotic slugs (*Limax maximus* and *Ambigolimax valentianus*) and two native land snails (*Acusta sieboldiana* and *Euhadra brandtii brandtii*) in Japan, with four plant traits as explanatory variables. The analyses were performed with a generalized linear mixed model. The table shows the models with the lowest Akaike information criterion (AIC) values (difference < 2). A null model is also shown. The AI was calculated following Dirzo's method (1980). The models were constructed based on the results of feeding trials with the 23 plant species shown in Table 2.

Species	N	Model ID	Coefficient <sup>1)</sup>				AIC	delta AIC	Weight <sup>5)</sup>	
			Plant origin (native/exotic/crop) <sup>2)</sup>		Plant type (herbaceous/woody) <sup>3)</sup>	Leaf thickness				Leaf pubescence (glabrous/pubescent) <sup>4)</sup>
			Exotic	Crop						
<i>Limax maximus</i>	21	Model 1	–	–	–0.122*	–	–0.227*	149.0	–	0.51
		Model 2	0.002	0.153*	–0.127*	–	–0.212*	150.3	1.3	0.26
		Null	–	–	–	–	–	206.2	57.2	0
<i>Ambigolimax valentianus</i>	21	Model 1	–	–	–	–0.497	–0.132*	202.6	–	0.61
		Model 2	–	–	–	–	–0.138*	203.8	1.2	0.33
		Null	–	–	–	–	–	217.4	14.8	0
<i>Acusta sieboldiana</i>	8	Model 1	–	–	–	–1.424	–	543.3	–	0.30
		Model 2	–	–	–0.335	–1.972	–	544.7	1.4	0.15
		Model 3	–	–	–	–1.603	0.298	544.9	1.6	0.14
		Null	–	–	–	–	–	545.1	1.8	0.12
<i>Euhadra brandtii brandtii</i>	10	Model 1	–	–	–	1.453	–	716.1	–	0.26
		Model 2	–	–	–0.404	0.651	–	716.5	0.5	0.21
		Model 3	–	–	–0.430	–	–	717.7	1.6	0.12
		Null	–	–	–	–	–	717.7	1.7	0.11

<sup>1)</sup> An asterisk (\*) indicates a  $p$ -value < 0.05 based on  $t$ -statistics using Satterthwaite's method.

<sup>2)</sup> Plant origin was categorized as native, exotic, or crop; the coefficients shown compare exotic and crop plants against native plants.

<sup>3)</sup> Woody plants in comparison with herbaceous plants.

<sup>4)</sup> Plants with pubescent leaves in comparison with plants with glabrous leaves.

<sup>5)</sup> Akaike weight.

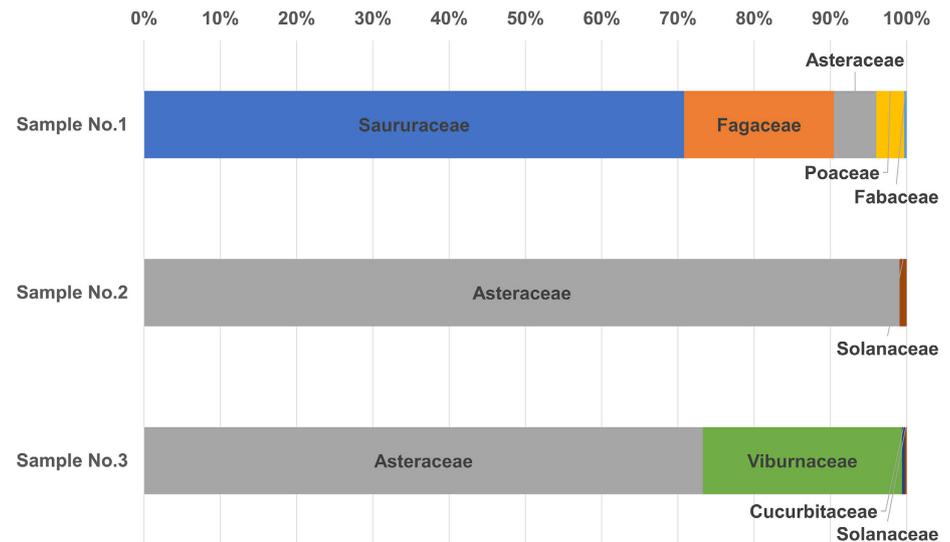
were selected as important explanatory variables. In contrast to the exotic slugs, the AIs of the two native land snails were not well predicted by these plant traits, and the AIC of the best-fit model for each species was similar to that of the null model.

### DNA meta-barcoding analyses

Asteraceae was detected in both samples 1 and 2, but otherwise the “wild” samples featured different families (Sauraceae, Fagaceae, Poaceae and Fabaceae in sample 1; Solanaceae in sample 2) (Figure 3). Plants in these families except for Solanaceae were observed at the slug-collection site (Table S3). In the control sample from slugs fed in the laboratory, plants in the families Asteraceae and Viburnaceae were predominant (Figure 3). Three days before collecting the fecal samples, the slugs were fed lettuce (Asteraceae) and *S. sieboldiana* (Viburnaceae). These results indicate that plants detected from wild individuals of *L. maximus* were likely consumed within a few days before the collection date.

### Discussion

In the present study, we combined two complementary methods to assess the herbivory behavior of *L. maximus* and compared it with that of the other molluscs. Feeding trials indicated that *L. maximus* feeds on different



**Figure 3.** Results of DNA meta-barcoding analyses for feces of wild (Samples 1–2) and reared individuals (Sample 3) of *Limax maximus* showing the proportion of sequence reads by plant family. See Table 3 for details. The total number of reads for samples 1, 2, and 3 was 46,714, 47,384, and 43,888, respectively.

plants than native snails. Its feeding behavior also differs from that of the native common slug *M. bilineatum*, which rarely ate test plants in feeding trials (Figure 1). It was surprising that *M. bilineatum* did not feed even on lettuce, which was preferred by many slug species (Dirzo 1980; Joe 2006). This slug appears not to be primarily herbivorous and is considered to be mycophagous. Thus, its main food resources seem less likely to overlap with those of *L. maximus* than the other species. On the other hand, *L. maximus* and the other exotic slug examined, *A. valentianus*, had high AI values for a wide range of plant species, including crops. Thus, these exotic slugs have a different trophic character from the common native slug in Japan and poses a potential risk of herbivory in forest and agricultural ecosystems.

According to the meta-barcoding analyses, feces from wild individuals contained a variety of plants (Figure 3), most of which were recorded at the site where *L. maximus* was collected (Table S3). Interestingly, Saururaceae was detected in the DNA meta-barcoding analysis although the plant in this family, *Houttuynia cordata*, was rarely eaten in the feeding trials (Figure 1). In the DNA meta-barcoding analyses, we used a relatively small number of samples from *L. maximus* because we focused on examining whether any individuals expressed positive signs of herbivory to complement the results of feeding trials. Additional studies with increased sample sizes are encouraged to obtain further information on this behaviour.

*Limax maximus* demonstrated marked preferences for specific plant species over lettuce in the feeding trials (Figure 1), including *B. oleracea*, *L. indica*, *S. racemosa*, *S. asper*, and *T. officinale*. Plants in the Asteraceae, such as *L. indica*, *Sonchus asper*, *Y. japonica*, and *T. officinale*, as well as *B. nivea* and *S. racemosa*, were favored by both exotic slugs and native land snails. In previous studies, plants in the Asteraceae (Rathcke 1985), Urticaceae

(Rathcke 1985; Joe 2006; Shiels et al. 2014), and the genus *Sambucus* (Rathcke 1985) were commonly eaten by slugs, which is generally consistent with our results. The NMDS and PERMANOVA analyses demonstrated that *L. maximus* and the other molluscs had different plant preferences although the other exotic slug, *A. valentianus*, had relatively similar plant preferences to that of *L. maximus* (Figure 2, Table 3). Thus, exotic slugs may cause herbivory damage to plants that are not preferred by native land snails, such as *B. oleracea* and *S. racemosa*, which were preferred by exotic slugs but not by native land snails.

The categories of plants likely to be eaten by *L. maximus* were crops, herbaceous plants, and plants with glabrous leaves (Table 5). The preference for crop species is concordant with reports that *L. maximus* damages some vegetables and horticultural plants (Stange et al. 2009; Kozłowski 2012). The stronger preference for herbaceous plants than woody plants is concordant with Rathcke (1985). Regarding leaf thickness, Dirzo (1980) and Hahn et al. (2011) showed that thin leaves were more easily eaten by *Agriolimax caruana* Polloner and *D. reticulatum*, respectively, but this was not supported for *L. maximus*. Indeed, *B. oleracea*, with thick leaves, was consumed by *L. maximus* (Figure 1). Body size may strongly affect the ability of slugs and snails to eat thick leaves. Leaf pubescence is considered to serve as a plant defense and to reduce feeding by insects (Levin 1973). Here, exotic slugs preferred plants with glabrous leaves to those with pubescent leaves, suggesting that leaf hairs reduce feeding in slugs. Herbivorous invaders often damage native plants more than exotic ones because native plants lack defense mechanisms that are effective against the invaders (Bowen and van Vuren 1997; Cubas et al. 2019). We did not observe evidence of this trend for *L. maximus* or for *A. valentianus* (Table 5). Shiels et al. (2014) reported similar results for exotic molluscs in Hawaii and mentioned that plant morphological traits are more important for predicting herbivory patterns than plant origin. Our data also support this tendency.

## Conclusions

Here, we observed that *L. maximus* shows clear herbivory behaviour, and its preferences differ from those of native terrestrial molluscs. Because of this difference, vegetation at introduction sites may experience herbivory pressure unlike that imposed by native molluscan communities. Our results imply that crops, plants with glabrous leaves, non-woody plants, and some plants in the Asteraceae (e.g., *Sonchus*, *Lactuca*, and *Youngia*) are preferred by *L. maximus*. Nevertheless, the magnitude of the impact on native vegetation remains unclear, because the extent of the damage is highly dependent on slug density and available food resources. *Limax maximus* is omnivorous and known to consume fungi and animals (Taylor 1894; Barker 1999; Meyer and Yeung 2011; Kozłowski 2012; Norden and

Williams 2015). Follow-up studies are needed to examine what food resources other than plants *L. maximus* consumes at introduced sites and how *L. maximus* influences forest vegetation structure and crop productivity in agricultural ecosystems. Our work demonstrated that combining multiple study methods helps to estimate the potential risk of exotic herbivory in natural ecosystems.

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## Author contribution

W.K. and I.S. designed the study. W.K. performed laboratory experiments and data analyses. W.K. and I.S. wrote a draft of the manuscript. W.K. and I.S. contributed to data interpretation and approved the final version of manuscript.

## Ethics and permits

All the experiments and collections were performed in accordance with the Institutional Policy on Animal Experimentation of the University of Tsukuba.

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### Supplementary material

The following supplementary material is available for this article:

**Table S1.** Primer sets used for the first round of PCR of DNA meta-barcoding analyses to examine the feeding habits of *Limax maximus*.

**Table S2.** Primer sets used for the second round of PCR of DNA meta-barcoding analyses to examine the feeding habits of *Limax maximus*.

**Table S3.** List of vascular plants at the site where the wild individuals of *Limax maximus* were collected for DNA meta-barcoding analyses.

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