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With 2 figures and 4 tables

Abstract: DNA-based diet analysis of natural enemies is a valuable tool for unravelling the food choice of predators in agroecosystems. It enables the rapid identification of potential biocontrol agents of invertebrate pests. Here, we present a new multiplex PCR system for the identification of pest slug species in the diet of their natural enemies such as carabid beetles. It comprises three species-specific primers targeting the mitochondrial cytochrome c oxidase subunit I (COI) gene to detect DNA of the common garden slug, *Arion distinctus* (Stylommatophora: Arionidae), the Iberian slug, *Arion lusitanicus* (Stylommatophora: Arionidae) and the grey field slug *Deroceras reticulatum* (Stylommatophora: Agriolimacidae). We also include (super)family-specific primers for Arionidae and Limacoidea, which amplify parts of the 28S gene for ribosomal RNA (rRNA) in order to identify a wider range of slugs. The amplicons for Arionidae can be assigned to a total of seven Central European slug species of this family and the amplicons for Limacoidea to ten species. The multiplex assay showed high specificity against DNA extracts of field-collected slugs and co-occurring invertebrates. The assay also exhibited high sensitivity, which was confirmed by testing it with 223 dietary samples from field-collected carabids as potential natural enemies of slugs in agroecosystems. This methodology represents a new, cost-effective, highly sensitive and specific approach for the identification of common Central European slug species as well as for analysing trophic interactions to identify natural enemies for further biological control development. It can also be applied in any study where a rapid and reliable identification of slugs is needed.

Keywords: 28S rRNA, *Arion distinctus*, *Arion lusitanicus*, Arionidae, trophic interactions, COI, *Deroceras reticulatum*, diagnostic PCR, Limacoidea, slug primers

1 Introduction

Slug damage in arable agricultural crops and horticultural plants causes serious economic losses. The molluscicides that are frequently applied against these pests can have harmful effects on humans and other animals as well. The resulting reduction in biological diversity (Gonthier et al. 2014) also leads to decreased pest control by naturally occurring predators (Hill et al. 2017, Power 2010). Larval and adult carabid beetles (Coleoptera: Carabidae) are generalists and several species consume slugs (Pianezzola et al. 2013, Symondson et al. 2002, Thomas et al. 2009). Depending on morphological features, like mandible size, carabid species feed on eggs as well as newly hatched up to adult slugs (Hatteland et al. 2010, McKemey et al. 2001, Paill 2004). The role of carabid beetles in the regulation of slugs has long been studied in different ecosystems employing correlative analysis, enzyme-linked immunosorbent assays (ELISA) (Bohan et al. 2000) and polymerase chain reaction (PCR) based approaches (Eskelson et al. 2011, Jelaska et al. 2014). Classical ecological approaches include direct observation of feeding behaviour or, for example, manual examination of faecal samples. Molecular analyses, in particular, have been proven to be very suitable for unravelling predator-prey interactions (Harper et al. 2005, King et al. 2008) because a higher number of samples can be investigated within comparatively short time and at high taxonomic prey resolution. This is especially important when it comes to identifying potential biocontrol agents for pest species, such as slugs. The potential of PCR approaches is

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that they allow prey to be unambiguously identified – often at a resolution down to the species level. Diagnostic PCR has proven to be a robust tool that can be used in the form of multiplex assays (Harper et al. 2005, King et al. 2011, Sint et al. 2012). Species-specific primers are available for six species of slugs that are abundant in Central Europe: *Arion ater* (Linnaeus, 1758) (Stylommatophora: Arionidae), *Arion lusitanicus* (Mabille, 1868) (syn. *Arion vulgaris*; Moquin-Tandon, 1855) (Stylommatophora: Arionidae), *Arion rufus* (Linnaeus, 1758) (Stylommatophora: Arionidae), *Limax cinereoniger* (Wolf, 1803) (Stylommatophora: Limacidae), *Deroceras laeve* (Müller, 1774) (Stylommatophora: Agriolimacidae) and *Deroceras reticulatum* (Müller, 1774) (Stylommatophora: Agriolimacidae), as well as one family-specific primer pair for *Arion* species (Dodd 2004, Eskelson et al. 2011; Hatteland et al. 2011, Jelaska et al. 2014). To the best of our knowledge, however, no species-specific primer pair is available for *Arion distinctus* (Mabille, 1868) (Stylommatophora: Arionidae), a species that is known to be highly abundant in arable land. Moreover, family-specific primers for Arionidae (Gray, 1840) and superfamily-specific primers for Limacoidea (Lamarck, 1801) are also currently not available. Such (super)family-specific primers would be ideally suited to identify other relevant slug species as well and those predators that have the potential to regulate them. For example, pestiferous slugs like *Limax maximus* (Linnaeus, 1758) (Stylommatophora: Limacidae) or *Lehmannia valentiana* (Férussac, 1823; now: *Ambigolimax valentianus*) (Stylommatophora: Limacidae) are problematic, native species of Central European agroecosystems (Scaccini et al. 2020). *Deroceras invadens* (Reise et al. 2011) (Stylommatophora: Agriolimacidae) and *Deroceras panormitanum* (Lessona & Pollonera 1882) (Stylommatophora: Agriolimacidae) are actively invading agricultural land across Europe (Reise et al. 2011, Scaccini et al. 2020).

Different DNA marker genes, 12S / 16S / 28S ribosomal RNA (rRNA) and cytochrome c oxidase subunit I (COI), are typically used to distinguish between snail species or to construct molecular phylogenies. In contrast to other animal groups the COI region is not necessarily the preferred marker gene (Beese, Armbruster, Beier & Baur 2009, Cadahía et al. 2014, Koene & Schulenburg 2005). The species-specific slug primers available to date have tended to target the COI and 12S rRNA regions (Dodd 2004, Eskelson et al. 2011, Harper et al. 2005, Hatteland et al. 2011), but also 16S and 28S rRNA marker genes have been used for phylogenetic studies (Barr et al. 2009, Dayrat et al. 2001). The 12S rRNA region does not show sufficient divergence between slug species to allow for species-specific differentiation and, at the same time, is not conserved enough for broader, (super) family-specific primers. With its broad coverage of various slug species sequences in databases such as NCBI, the COI gene would be a more suitable gene to develop species-specific primers for slugs than the 12S rRNA region. The primer design for family-specific primers, however, requires an evaluation of the available sequences from different rRNA regions.

The main objective of this study is the development of a multiplex PCR assay for the identification of slug DNA, including the three dominant species, *A. distinctus, A. lusitanicus*, and *D. reticulatum*, as well as two (super) family-specific primer pairs for Arionidae and Limacoidea to account for other slug species that may be present in arable fields. As this PCR assay was developed to identify slug species in arable land that could be consumed by carabid beetles, the assays were tested for specificity in carabid gut contents, so as to exclude non-target DNA amplifications of potential predators and other co-occurring arthropods and lumbricids.

2 Methods

2.1 Sample collection and DNA extraction

In 2017, 15 slugs of the families Arionidae and Agriolimacidae as well as 231 carabids (184 individuals of the species *Poecilus cupreus* (Linnaeus, 1758) (Coleoptera: Carabidae: Harpalinae) and 47 individuals of *Pseudoophonus rufipes* (De Geer, 1774) (Coleoptera: Carabidae: Harpalinae)), were sampled in a study to examine slug predation in an organically cultivated winter wheat field in Rotholz (Tyrol, Austria). The slugs were stored individually at –28 °C prior to DNA extraction. A sample of tissue (2 mm³) was cut from each slug, dissolved in 400 µl TES buffer (0.1 M TRIS, 10 mM EDTA, 2% (w/v) SDS, pH 8), supplemented with 10 µl Proteinase K (20 mg/ml) and glass beads (10× Ø 3 mm and 5× Ø 5 mm), and homogenized in a Precellys® 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) at 5000 rpm for 120 s. All samples were then incubated overnight at 56 °C for lysis. Following the manufacturer's instructions, the DNeasy® Blood & Tissue Kit was used for DNA extraction on a Biosprint 96 extraction robotic platform (both from Qiagen, Hilden, Germany). Finally, the DNA was eluted in a volume of 200 µl 1× TE buffer (10 mM TRIS, 1 mM EDTA, pH 8) and stored at –28 °C. To check for possible cross-sample contamination, one extraction negative control (PCR-grade water instead of DNA extract) was included during this workflow. Carabids can be induced to regurgitate their mid-gut contents (Trevor 1982). We could therefore avoid whole-body DNA extracts by preserving such regurgitated gastric contents. The collection of the carabid regurgitates and subsequent DNA extraction followed the protocols described by Frei et al. (2019) and Wallinger et al. (2015). For both the slug tissue samples and carabid regurgitates, the entire process of DNA extraction was conducted in a separate pre-PCR laboratory using an UVC-equipped laminar flow hood.

2.2 PCR and Sanger sequencing

Using the universal invertebrate primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994), a ~723 bp fragment of the COI gene was amplified for each slug sample. The 10 µl PCR reactions contained 1.5 µl DNA extract, 0.5 µl bovine serum albumin (BSA) (10 mg/ml), 0.5 µl 5× Q-Solution (Qiagen), 5 µl 2× QIAGEN® Multiplex PCR Master Mix (Qiagen), 0.5 µl molecular grade water, 1 µl forward primer (10 µM) and 1 µl reverse primer (10 µM). The thermal cycling scheme consisted of 15 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 90 s at 50 °C, 60 s at 72 °C and a final extension for 10 min at 72 °C. The automated capillary electrophoresis system QIAxcel with the QIAxcel DNA Screening Kit (both from Qiagen) was used to separate and visualize the amplicons. The method AM320 with an injection time of 30 seconds was applied and results scored with the software QIAxcel ScreenGel v1.6.0 (Qiagen). Samples containing amplicons of the expected fragment size and with signal intensities above 0.1 relative fluorescence units (RFUs) were deemed to be positive. Each PCR product (diluted to ~50 ng/µl) was enzymatically cleaned with Exonuclease I (*Escherichia coli*) (ExoI) (NEB, Frankfurt am Main, Germany) and Thermosensitive Alkaline Phosphatase (TSAP) (Promega, Mannheim, Germany) by mixing each of the 7 µl PCR products with 0.1 µl ExoI (10 U/µl), 0.1 µl TSAP (1 U/µl) and 1.8 µl molecular grade water. Reactions were carried out by incubating samples for 15 min at 35 °C followed by an inactivation step for 15 min at 80 °C, in both reaction cycles while shaking. Samples were sent to Eurofins Genomics (Ebersberg, Germany) for bidirectional sequencing.

2.3 Data analysis

For visualization and processing of the sequencing data, as well as for primer design, the software tools Unipro UGENE v1.28.1 (Okonechnikov et al. 2012) and AutoDimer (Vallone & Butler 2004) were used. A NCBI BLAST search was previously done (web BLAST; status of nucleotide BLAST database: 2017-11-21) applying the nucleotide query algorithm (blastn). On the basis of the Sanger sequencing results and BLAST hits, the 15 slug individuals were identified as three different species, *A. distinctus, A. lusitanicus* and *D. reticulatum.* The multiplex assay was therefore designed to include specific primers for these three slug species. Two (super)family-specific primer pairs for Arionidae (Gray, 1840) and Limacoidea (Lamarck, 1801) were included to screen for the consumption of other slug species. Gene regions including COI, 12S rRNA, 16S rRNA, 18S rRNA and 28S rRNA were examined *in silico* to assess the genetic variability between species and families within the NCBI nucleotide database. This was done by collecting the sequencing information of 44 Central European slug species of the families Boettgerillidae (Wiktor & Likharev 1979) (one species), Agriolimacidae (Wagner, 1935) (nine species) and Limacidae (Rafinesque-Schmaltz, 1815) (20 species), in the superfamily Limacoidea, and the 14 species of the family Arionidae in the superfamily Arionoidea (Table S1, see Table S2 for NCBI accession numbers).

2.4 Multiplex PCR development and evaluation

In order to find the optimal annealing temperature and primer concentrations for the multiplex PCR assay, preliminary PCRs were carried out both in singleplex and multiplex reactions. Each 10 µl PCR reaction contained 1.5 µl DNA extract, 5 µl 2× Multiplex PCR Master Mix (Qiagen), 0.5 µl BSA (10 mg/ml), 1 µl molecular grade water, and 2 µl of each primer pair (10 µM) for singleplex PCRs or alternatively 2 µl primer mix (at equal ratios; each primer 10 µM) for multiplex PCRs. Gradient PCRs for both singleplex and multiplex assays were performed under the following thermal cycling conditions: 15 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 90 s annealing, 60 s at 72 °C, and a final extension for 10 min at 72 °C. Annealing temperatures below 60.5 °C led to additional non-specific bands for the (super)family-specific primer pairs. Consequently, annealing temperatures at 60.5 °C, 60.6 °C, 62.2 °C, 63.1 °C, 63.9 °C, 65.7 °C and 66.8 °C were examined first in singleplex and then in multiplex PCRs. To determine the optimal primer concentrations of group- and species-specific primer pairs for the final multiplex assay, 12 different primer compositions were tested (Table 1). A mixture of slug DNA from the three target slug species served as test DNA extract and also as a positive control. Using the singleplex PCRs, the sensitivity of each primer pair was assessed individually. For this purpose, dilutions in the ratios 1:50 and 1:100 of the 15 slug DNA extracts were prepared. Assay specificity was evaluated by testing the established multiplex PCR protocol with different DNA extracts of invertebrate species, families or groups (Table 2) typically found on Tyrolean arable land. Finally, the newly established multiplex assay was evaluated for its suitability for field samples by testing DNA extracts from the regurgitates of 184 *P. cupreus* and 39 *P. rufipes* (eight *P. rufipes* individuals did not regurgitate). All PCRs were checked for DNA carryover contamination by including at least one negative control (molecular grade water) and for amplification success by a positive control (mix of each slug species; equal ratio). For the evaluation of PCR products, the automated capillary electrophoresis system QIAxcel and its software was used under the parameter settings described in 2.2.

3 Results

Most of the available DNA sequences of the 44 Central European slug species (Table S1), presented in the NCBI nucleotide database, were those of COI (64%) and 16S rRNA

Table 1. Compositions of primer mixes that were initially tested in multiplex PCRs at an annealing temperature of 60.5 °C; volumes of individual primers that were applied to yield the primer mixes 1 to 12 (primer stock solution was 10 µM); to fill up to a volume of at least 10 µl, TE buffer was added where needed.

		Primer mixes											
		1	2	3	4	5	6	7	8	9	10	11	12
Targets	**Primer name**	**Volumes for each primer (µl)**											
Limacoidea	28S-Ari-Der-S580 (fw)	1.0	0.5	1.0	1.5	1.0	0.5	1.0	1.5	1.5	1.5	1.5	1.5
	Limacoidea-A594 (rv)	1.0	0.5	0.5	0.5	1.0	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Arion	Ari-dis-S578 (fw)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
distinctus	Ari-dis-A591 (rv)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Deroceras	Der-ret-S579 (fw)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
reticulatum	Der-ret-A592 (rv)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Arion	A.l.-Co1-F1 (fw)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
lusitanicus	A.l.-Co1-R2 (rv)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Arionidae	28S-Ari-Der-S580 (fw)	1.0	1.0	1.0	1.0	2.0	2.0	2.0	2.0	3.0	4.0	3.0	4.0
	Arionidae-A593 (rv)	1.0	1.0	1.0	1.0	2.0	2.0	2.0	2.0	2.0	2.0	3.0	4.0
Final volume	TE buffer		1.0	0.5									
	Primer mix	10.0	9.0	9.5	10.0	12.0	11.0	11.5	12.0	13.0	14.0	14.0	16.0

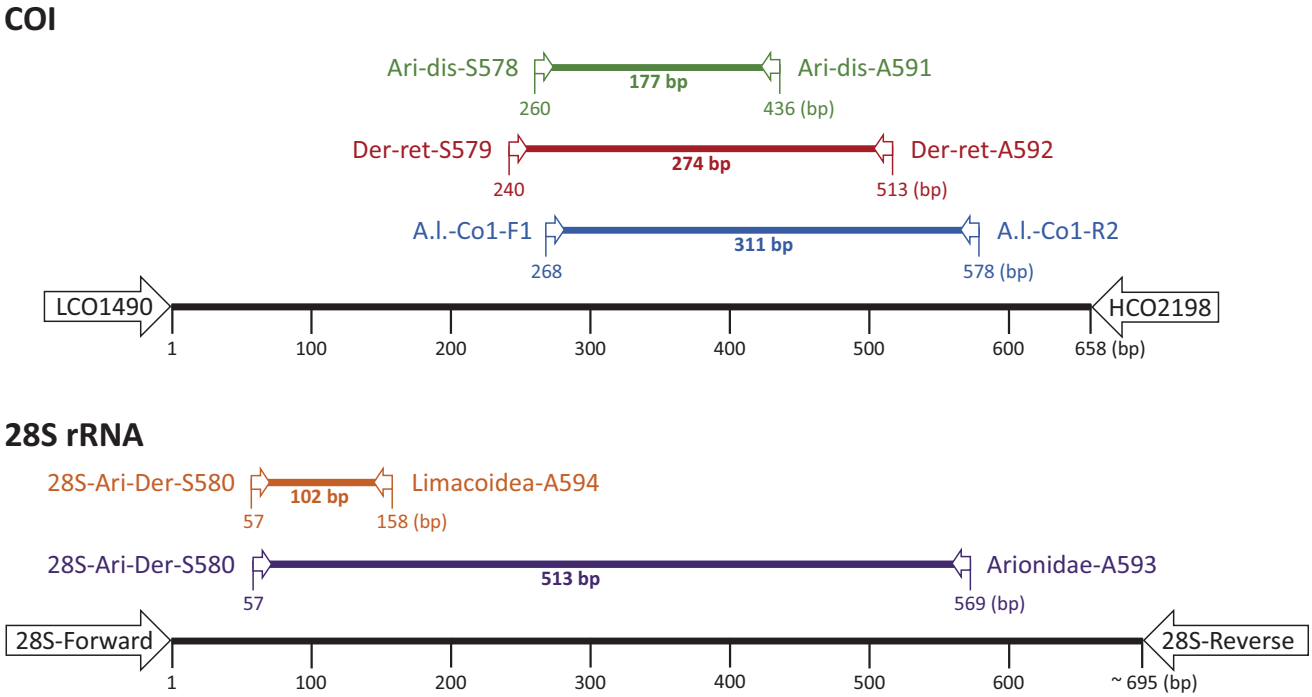

Fig. 1. Positions of the three species-specific (upper panel) and two (super)family-specific (lower panel) slug primer pairs targeting the cytochrome c oxidase subunit I (COI) and 28S ribosomal RNA (rRNA) gene, respectively.

(45%), followed by 28S rRNA (41%), 12S (18%) and 18S rRNA (11%). Given the gene variability within and between species, the COI was chosen as the preferable gene region for the design of species-specific slug primers and the 28S rRNA for the (super)family-specific primers (Fig. 1), with the latter (super)family-specific primers covering at least 17 slug species (Fig. 2). For *A. lusitanicus*, 28S rRNA sequences are currently absent in the NCBI database. Nevertheless, the family-specific primer pair for Arionidae amplified 28S rRNA fragments in DNA extracts of *A. lusitanicus*.

Except for the COI primer pair 'A.l.-Co1-F1' and 'A.l.-Co1-R2' for *A. lusitanicus* by Hatteland et al. (2011), all primer pairs were newly designed and combined in the multiplex PCR assay. The multiplex assay with the three

Table 2. List of non-target invertebrates (species, families or other groups), co-occurring in the field and used for the specificity tests of the multiplex PCR assay.

Scientific name	Rank	Family
Anchomenus dorsalis (Pontoppidan, 1763)	species	Carabidae
Agonum muelleri (Herbst, 1784)	species	Carabidae
Alopecosa trabalis (Clerck, 1757)	species	Lycosidae
Aphidius rhopalosiphi (De Stefani-Perez, 1902)	species	Braconidae
Aporrectodea caliginosa (Savigny, 1826)	species	Lumbricidae
Bembidion properans (Stephens, 1828)	species	Carabidae
Bembidion quadrimaculatum (Linnaeus, 1761)	species	Carabidae
Bembidion tetracolum (Say, 1823)	species	Carabidae
Coccinella septempunctata (Linnaeus, 1758)	species	Coccinellidae
Collembola (Lubbock, 1870)	class	Collembola (class)
Curculionidae (Latreille, 1802)	family	Curculionidae
Formicidae (Latreille, 1802)	family	Formicidae
Harpalus affinis (Schrank, 1781)	species	Carabidae
Julidae (Leach, 1814)	family	Julidae
Lithobius sp.	species	Lithobiidae
Lumbricus terrestris (Linnaeus, 1758)	species	Lumbricidae
Melolontha sp.	species	Scarabaeidae
Microplitis mediator (Haliday, 1834)	species	Braconidae
Octolasion sp.	species	Lumbricidae
Pachygnatha clercki (Sundevall, 1823)	species	Tetragnathidae
Pardosa palustris (Linnaeus, 1758)	species	Lycosidae
Pirata hygrophilus (Thorell, 1872)	species	Lycosidae
Plutella xylostella (Linnaeus, 1758)	species	Plutellidae
Poecilus cupreus (Linnaeus, 1758)	species	Carabidae
Poecilus versicolor (Sturm, 1824)	species	Carabidae
Silpha sp.	species	Silphidae
Trechus quadristriatus (Schrank, 1781)	species	Carabidae
Trochosa terricola (Thorell, 1856)	species	Lycosidae

species-specific and two (super)family-specific primer pairs was generated for amplifying DNA fragments in a size range between 102 and 513 bp (Table 3). The initial tests revealed an optimal annealing temperature of 60.5 °C for this multiplex PCR. Accordingly, the final PCR conditions were: 15 min at 95 °C, followed by 35 cycles of 94 °C for 30 s, 60.5 °C for 90 s, 72 °C for 60 s, and a final extension at 72 °C for 10 min. The evaluation of the signal intensities (highest score of RFU values per fragment) showed the highest efficiency for the primer concentrations of setup 10 (Table 1), in generating a PCR product for each target when applying these optimal cycling conditions. The final volume of a 10 µl PCR reaction mix contained 1.5 µl DNA extract, 5 µl 2× Multiplex PCR Master Mix (Qiagen), 2 µl primer mix (Table 3), 0.5 µl BSA (10 mg/ml) and 1 µl molecular grade water.

All DNA extracts of the 15 slugs produced species- and (super)family-specific DNA fragments. In the sensitivity tests, the amount of amplified DNA in the dilutions of slug DNA extracts (1:50 and 1:100) were measured via the RFU values obtained in the capillary electrophoresis (Table 4). Even for the 1:100 dilutions most of the values were larger than 1.0 RFU and only two of the 1:100 dilutions tested with the family-specific primers did not produce an amplicon. The specificity of the newly established multiplex PCR was confirmed by testing against nine ground beetle species and 19 other field relevant invertebrate taxa, which showed that no amplification occurred with non-target taxa (Table 2).

In the screening of the multiplex PCR assay with regurgitates of the field-collected carabids, target amplicons were detected in 15 samples of *P. cupreus* and two of *P. rufipes*. In these 17 carabid regurgitates, detections of multiple slug

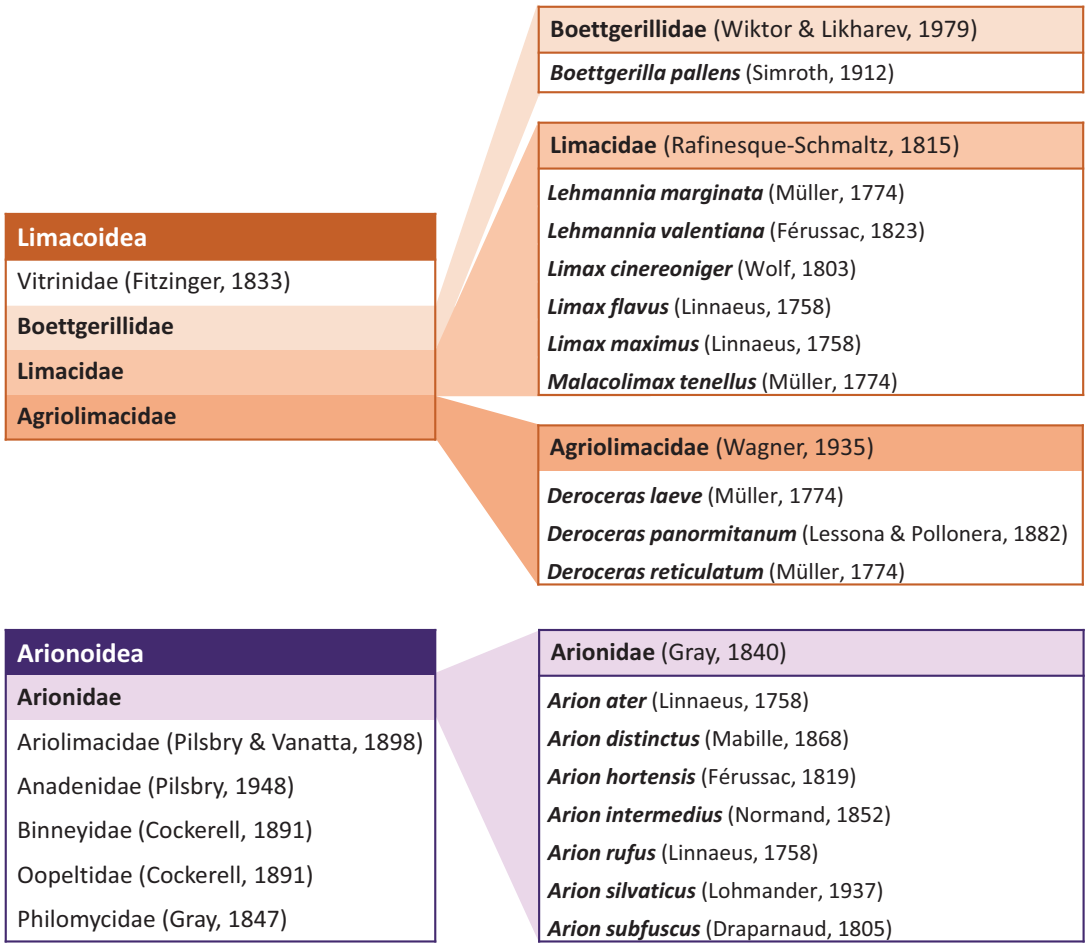

Fig. 2. List of Central European slug species with available 28S rRNA sequences to develop the (super)family-specific primers for Limacoidea (Lamarck, 1801) and Arionoidea (Gray, 1840); based on Bouchet et al. (2005) and the NCBI nucleotide BLAST database (status of 2017-11-21).

species per sample were not present. Either one of the three target slug species was detected or solely one of the (super) family-specific primers for Limacoidea or Arionidae generated an amplicon. The detections included the following: *D. reticulatum* amplicons in eight *P. cupreus* samples and *A. lusitanicus* in one *P. cupreus* and one *P. rufipes* regurgitate. Amplicons for *A. distinctus* were not detected in any of the regurgitate samples. DNA of Limacoidea were detected in three regurgitates of *P. cupreus* and one of *P. rufipes*, as well as DNA of Arionidae in three regurgitates of *P. cupreus*.

4 Discussion

The focus of this study was the development of an optimized multiplex PCR assay using specific primers for the identification of common Central European slug species. This included a bioinformatic analysis, and (super)family-specific primers were designed for the detection of at least 17 slug species (Table S1). Based on a discrimination of similar *vs.* variable sequence regions, within and among species, and a testing of cross-reactivity of primers, we were able to generate three species-specific primer pairs targeting the COI and two (super)family-specific primer pairs for the 28S region. The multiplex PCR assay was tested against target species and a variety of non-target animals that can co-occur with slugs in cereal fields and was found to be highly specific. Moreover, the testing with field samples of carabid regurgitates indicates that this multiplex approach is also capable of generating amplicons from templates with degraded DNA.

There has been an increasing use of molecular methods to assess trophic interactions as they allow the examination of feeding interactions that are difficult to track with other means of diet analysis: for example, assessing the diet of predatory arthropods which often show extraoral-digestion or masticate their prey before intake (Gómez-Martínez

Table 3. Species- and (super)family-specific primers included in the multiplex PCR setup amplifying the cytochrome c oxidase subunit I (COI) and 28S ribosomal RNA (rRNA) gene regions. Provided are the targeted slug taxon, the primer names, sequences, the targeted gene, the fragment size of the respective amplicon, the concentration of the primers in the PCR and the reference for each primer pair.

Target	Primer name and sequence (5' → 3')		Gene	Size (bp)	Concentration in MP PCR (µM)	Reference
Limacoidea	28S-Ari-Der-S580	AGTAACGGCGAGTGAAGCG	28S	102	0.214	this study
	Limacoidea-A594	CGCCCTCTGATGCGA			0.071	
Arion distinctus	Ari-dis-S578	TGACTACTACCGCCTTCTCTCTTAC	COI	177	0.143	this study
	Ari-dis-A591	TGCCCCTAAAATTGAAGACATT			0.143	
Deroceras reticulatum	Der-ret-S579	GAATAAATAATATAAGGTTTTGATTACTTCCC	COI	274	0.143	this study
	Der-ret-A592	GATCAAACAAATAATCTTAAACGTTCTATTC			0.143	
Arion lusitanicus	A.l.-Co1-F1	GCCCCCATCTTTACTTTTACTTATTTGCTCC	COI	311	0.143	Hatteland et al. (2011)
	A.l.-Co1-R2	GTATGGTAATAGCCCCCGCCAATACG			0.143	
Arionidae	28S-Ari-Der-S580	AGTAACGGCGAGTGAAGCG	28S	513	0.571	this study
	Arionidae-A593	GGCATGTCACCGCTCG			0.286	

Table 4. Amplification success for diluted DNA extracts (1:50 and 1:100) of the 15 tested slug specimens out of the three species *Arion distinctus, Arion lusitanicus* and *Deroceras reticulatum* using singleplex PCR coupled with capillary electrophoresis system where amplification strength is measured in relative fluorescence units (RFU). Species refers to used species-specific primer pair of the multiplex assay and group to the (super)family-specific (see Table 3). PCRs without amplification success are indicated by a "X" character.

Targets	PCR (RFUs)			
	species		group	
	1:50	1:100	1:50	1:100
Arion distinctus				
Slug 1	2.47	2.30	3.38	2.33
Slug 2	2.03	1.53	1.27	0.45
Arion lusitanicus				
Slug 1	5.69	5.18	2.48	1.69
Slug 2	7.03	3.84	2.91	0.45
Slug 3	0.94	1.33	0.33	X
Slug 4	5.44	2.55	0.80	X
Slug 5	9.00	7.83	3.27	3.30
Slug 6	5.62	5.67	2.51	2.53
Slug 7	7.44	6.43	4.82	1.17
Slug 8	5.26	5.04	6.67	5.41
Deroceras reticulatum				
Slug 1	4.78	2.60	4.00	2.38
Slug 2	5.18	2.78	2.65	1.69
Slug 3	3.87	3.77	2.93	2.34
Slug 4	5.28	3.08	2.37	1.75
Slug 5	3.30	3.26	1.96	1.65

et al. 2020, Gomez-Polo et al. 2016, Read et al. 2006) and trophic interactions in more difficult to access habitats such as aquatic ecosystems (Jensen et al. 2018, Lutz et al. 2020). Here, the advantage of analysing prey DNA compared to visually examining prey remains or conducting direct observations becomes apparent (Birkhofer et al. 2017, Traugott et al. 2020). Prior studies (Dodd 2004, Eskelson et al. 2011, Hatteland et al. 2011, Jelaska et al. 2014), that have examined the predation by carabids of slugs, developed sensitive PCR assays to amplify degenerated DNA but were primarily focused on the detection of European slug species, *A. ater, A. lusitanicus, A. rufus, L. cinereoniger, D. laeve* and *D. reticulatum*. The 28S family-specific primers developed here were designed to cover a broader taxonomic range of slug species than this earlier work. This is particularly useful where little is known in advance about the slug species that occur. The field samples obtained from the regurgitates of carabid beetles should be seen as a proof-of-concept test that the multiplex primers can be used for the detection of any slug DNA of European species (Table S1). However, the primers are also suitable for PCR approaches in which other DNA extracts are examined for the analysis of trophic interactions, such as DNA extracts from faeces. In addition to carabid beetles, the natural predators of slugs include amphibians, reptiles, birds and mammals, other beetles, parasitic flies, and arachnids (El Titi 2002). As an economically important pest, DNA detection of slugs is important for identifying potential predators that could improve biological control.

This multiplex PCR assay is applicable to the screening of large numbers of individual dietary samples, rapidly and at comparatively low cost. The PCR protocol can be implemented in any molecular diagnostic laboratory and data evaluation is rapid and scalable, in comparison to alternative approaches e.g. next-generation sequencing (NGS) or metabarcoding methods where considerable bioinformatic expertise is needed (Rubbmark et al. 2019). The method is particularly beneficial if a defined set of prey species are known to be present at a study site, such as is often the case in arable and horticultural systems. It is especially useful for work requiring individual-based analysis, given that it allows for a mass screening of individuals using a combination of multiplexing and fragment analysis that renders the task highly efficient.

With the present multiplex assay, it is possible to identify the presence of the three common slug species, *A. distinctus, A. lusitanicus* and *D. reticulatum* as prey. Moreover, the identification of other slugs, abundant in arable land (Scaccini et al. 2020), is possible via the two (super)family-specific primer pairs for Arionidae and Limacoidea, comprising 15 additional slug species (Fig. 2). Only those samples testing positive for these (super)family-specific primer pairs would then be sequenced and identified to species level using DNA barcoding, greatly reducing the sequencing overhead. Currently, the known number of species that can be recorded with the (super)family-specific primers is limited as no more sequences for 28S rRNA are available. However, should the 28S rRNA region be sequenced for these missing slug species, there is the possibility that these primers will detect more slug species. This, of course, has to be tested in the future and also if the (super)family-specific primers work on slug species that have not yet been tested because of missing 28S rRNA sequences. With regard to an increasing number of 28S rRNA sequences within the NCBI nucleotide database, future studies will show whether the (super) family-specific primers are capable of identifying even more slug species.

This multiplex PCR assay is highly specific, clearly amplifying the DNA of the target species, with no detections of the non-target invertebrates tested. Should the multiplex PCR be applied in different ecosystems or field sites of other regions than presented here, we recommend further testing of specificity for species that could co-occur in those samples. Due to the optimization of the annealing temperature, the comprehensive testing of balanced primer concentrations as well as the use of highly sensitive and specific primers, optimal amplification efficacies for each primer pair could be achieved. Sint et al. (2012) have shown that a comprehensive adjustment of factors such as the primer concentration and the annealing temperature are of crucial importance in order to achieve the maximum sensitivity and specificity within a multiplex PCR. In the current study, a high sensitivity for the newly established multiplex assay could be demonstrated by the high RFU values which allow for a relative quantification of the PCR product obtained. The high RFU values for the 1:100 diluted DNA extracts further indicate that even lower concentrations of the target DNA should be amplifiable with the new assays, which is essential for the identification of prey DNA in dietary samples. Previous studies have indicated that fragment length is also an important factor as with increasing digestion time longer prey DNA fragments are typically more degraded and this is associated with a decrease in fragment counts (Deagle et al. 2006, King et al. 2008, Symondson 2002). This problem can be countered, by keeping amplicon sizes as small as possible and using highly sensitive primer pairs, as implemented in this multiplex PCR assay.

In conclusion, the approach outlined here presents a new, cost-efficient multiplex PCR assay for the identification of common slug species in European agroecosystems. This highly sensitive and specific PCR assay was designed for dietary analysis but can also be applied in any study where a quick and reliable identification of slugs is required. It is therefore a valuable tool to understand the natural enemy-pest trophic relationships of arable and horticultural land, which contributes to improved knowledge of biocontrol and sustainable agriculture.

Conflict of interest: None declared.

Author contributions: DAB, MT and CW conceived and designed the study. YGG and CW performed the field sampling. YGG was responsible for the molecular work (DNA extractions, PCR screening, and primer design). YGG performed the data analysis and wrote the manuscript, which all authors revised and finally approved to be published.

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The pdf version (Adobe JavaScript must be enabled) of this paper includes an electronic supplement: **Supplement Table S1, S2**

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