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New internal primers targeting short fragments of the mitochondrial COI region for archival specimens from the subfamily Aphidiinae (Hymenoptera, Braconidae)

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Abstract

Archival specimens are a great resource for molecular research in population biology, taxonomy and conservation. A primary goal for researchers is to preserve specimens from collections by improving non-invasive methods for DNA extraction and to achieve successful amplification of the short fragments of a target gene in the event of DNA fragmentation. We tested the suitability of a noninvasive method of DNA extraction and amplification of the barcoding region of the mitochondrial gene cytochrome c oxidase subunit I from archival specimens of aphid parasitoids belonging to the genera *Aphidius*, *Lysiphlebus* and *Praon* (Aphidiinae, Braconidae, Hymenoptera). Using a commercial kit as a noninvasive method, we successfully extracted DNA from dry 7 to 41 year old samples of 26 different parasitoid species. However, amplification of the barcoding region failed using the standard primer pair LCO1490/HCO2198. In order to reconstruct DNA barcodes we designed internal genus-specific degenerative primers and a new amplification protocol to target the short fragments within the mitochondrial region. Novel primers were designed using as a template the reference sequences from congeners retrieved from the public database. The combination of standard primers with internal primers, in direct and nested amplification reactions, produced short overlapping subsequences, concatenated to recover long barcoding sequences. Additional analyses also confirmed that primers initially designed for *Aphidius*, *Lysiphlebus* and *Praon* can be combined in a mixture, and successfully used to obtain short fragments of disintegrated DNA from archival specimens of several other braconid species from the genera *Ephedrus* and *Monoctonus*.

Keywords
COI, archival specimens, *Aphidius*, *Ephedrus*, *Lysiphlebus*, *Monoctonus*, *Praon*, short fragments

Introduction

The DNA from an archival species is an important source of data in the areas of population genetics, conservation, taxonomy and phylogeny. In the past researchers were in conflict between the maintenance of specimens undamaged and their use in molecular analyses, which created a strong limitation for studies on museum specimens, in particular studies with rare or extinct species, or those restricted to one or a few individuals collected many years ago (Gilbert et al. 2007; Mandrioli 2008). However, archival DNA study is now a rapidly developing area of research due to the continual improvements of molecular tools with which it is possible to recover DNA information from museum specimens and dry remains, without damaging the material.

Insects are a group where these tools have received increasing attention and noninvasive techniques have been developed and used for a variety of orders (Gilbert et al. 2007; Andersen and Mills 2012). Noninvasive methods of DNA extraction from dried specimens are important in order to preserve the quality of museum specimens. Unfortunately, not all specimens contain DNA of suitable quality and in the right amount for conclusive genetic studies. Successful amplification depends on post-mortem processes of DNA degradation, which can cause miscoding lesions or physical destruction of the DNA molecule (Rizzi et al. 2012). Degradation of DNA consequently produces methodological difficulties in amplification and sequencing of the target region, processes that are limited by the small quantity of template DNA and recovery of short fragments. Besides natural processes of disintegration, another factor that makes archival specimens difficult to work with is the preservation methodology, which can over time result in DNA damage (Dillon et al. 1996; Burrell et al. 2015). In the case of parasitic Hymenoptera, Andersen and Mills (2012) determined that age was a significant factor for successful sequencing, while size and DNA concentration did not influence the amplification of the targeted nuclear and mitochondrial genes.

Parasitoid Hymenoptera are a taxonomically challenging group under frequent revision, making them a group of great interest for retrieval of genetic information from museum specimens (Andersen and Mills 2012). Among parasitoids that have been intensively surveyed by taxonomists and ecologists are aphid endoparasitoids from the subfamily Aphidiinae (Braconidae, Hymenoptera). They are distributed worldwide, closely following the distribution of their aphid hosts (Starý 1988). As solitary endoparasitoids, Aphidiinae are one of the most important natural enemies of aphids and can effectively regulate their populations (Hågvar and Hofsvang 1991). They have been commercially produced and released as classical biological control agents of aphids in many regions and have achieved significant results in diverse agroecosystems. The most important genera of aphid parasitoids used in biological control are *Aphidius* Nees, 1818; *Diaeretiella* Starý, 1960; *Ephedrus* Haliday, 1833 and *Praon* Haliday, 1833 (Boivin et al. 2012).

The subfamily Aphidiinae is a diverse group with many cryptic species complexes, and reliable identification is therefore of key importance for their use as biological control agents.

This study included aphid parasitoids belonging to the common aphidiine genera *Aphidius*, *Lysiphlebus* Förster, 1862 and *Praon*. Identification based on morphology has often been shown to be inadequate in distinguishing the species of these genera due to the limited number of valid discriminatory morphological characters, as well as their high variation on the intraspecific level (Pungerl 1983; Kavallieratos et al. 2005, 2010; Tomanović et al. 2003, 2004). Furthermore, several species have confusing taxonomic histories and are in need of revision. In fact, over the last two decades these genera have been constantly rearranged on the basis of new morphological characters and more recently obtained molecular data as well.

Mitochondrial barcoding region of the cytochrome oxidase c subunit I (COI) had been used to reconstruct phylogenetic relationships within the genera (Jafari-Ahmadabadi et al. 2011), and examine the phylogenetic affinity and diversity of Aphidiinae from different geographical regions (Lenin 2015). In addition, it has successfully detected immature stages of parasitoids inside their aphid hosts, e.g., *Lysiphlebus testaceipes* Cresson, 1880 inside its host *Aphis fabae* Scopoli, 1763 (Traugott and Symondson 2008). Either solely or in combination with morphometric methods, the barcoding method was routinely applied in revisiting and resolving the taxonomic status of many species complexes. For example, three species - *Aphidius colemani* Viereck, 1912; *A. platensis* Brèthes, 1913, and *A. transcaspicus* Telenga, 1958- were distinguished within the *A. colemani* group (Tomanović et al. 2014); three species - *A. rubi* Starý, 1962; *A. silvaticus* Starý, 1962, and *A. urticae* Haliday, 1834 were re-described within the *A. urticae* group (Jamhour et al. 2016); two new species - *Praon longicaudus* Tomanović & Starý, 2014 and *P. sambuci* Tomanović & Starý, 2014 - were described within the species complex *Praon abjectum* Haliday, 1833 (Mitrovski et al. 2013); the species status of *P. dorsale* Haliday, 1833; *P. longicorne* Marshall, 1896; *P. volucre* Haliday, 1833, and *P. yomenae* Takada, 1968 was confirmed and a new species, viz., *Praon staticobii* Tomanović & Petrović, 2014 was described within the *Praon dorsale-yomenae* s. str. group (Mitrovski et al. 2014). Apart from taxonomic revisions, the barcoding marker was successfully used to discover new allochthonous species accidentally introduced into new habitats, such as the invasive species *Lysiphlebus orientalis* Starý & Rakhshani, 2010 (Petrović et al. 2013) and *Aphidius ericaphidis* Pike & Starý, 2011 (Petrović et al. 2017).

Considering that these parasitoids are important for fundamental taxonomic and conservation research, as well as being potential biological control agents in aphid management programs, it would be of great value to investigate the possibility of recovering barcoding fragments of COI from museum specimens. Thus, the main objectives of this study were as follows: i) DNA extraction from dry archival specimens belonging to the genera *Aphidius*, *Lysiphlebus* and *Praon* using a noninvasive method; ii) PCR amplification of several short and overlapping fragments within the barcoding region of cytochrome c oxidase subunit I, iii) traditional Sanger sequencing and alignment of

different short overlapping fragments and concatenation to recover longer target barcoding region of mitochondrial DNA and iv) testing the suitability of novel primers for targeting barcodes in archival specimens of other braconid species.

Material and methods

Analyses included species from three different genera of aphid parasitoids, viz., *Aphidius*, *Praon* and *Lysiphlebus*. In total 45 specimens were submitted to molecular analyses, including 11 species of *Aphidius*, nine of *Lysiphlebus* and six of *Praon*, killed and preserved in dry condition from 7 to 41 years prior to DNA extraction (Table 1). Additionally, in order to test the suitability of these primers in amplification of other parasitiods we chose four species from the genus *Monoctonus* Haliday, 1833 and four of *Ephedrus* Haliday, 1833, all dry material up to 31 year old (Table 1).

Table 1. The list of analyzed species from the genera *Aphidius*, *Lysiphlebus*, *Praon*, *Ephedrus*, *Monoctonus* with designated aphid host/plant associations and geographic origin.

Sample code	Parasitoid species	Country of origin	Sampling year/ age of samples*	Host plant	Aphid host	Specimen condition **
AF1	*Aphidius tanacetarius*	Serbia	2011/7	*Tanacetum vulgare*	*Metopeurum fuscoviridae*	F
AF2	*Aphidius sussi*	Montenegro	2005/13	*Aconitum toxicum*	*Delphiniobium junackianum*	F
AF 3	*Aphidius sonchi*	Serbia	2010/8	*Sonchus arvensis*	*Hyperomyzus lactucae*	F
AF4	*Aphidius linosiphonis*	Montenegro	2011/7	*Galium* sp.	*Linosiphon* sp.	F
AF5	*Aphidius ribis*	Montenegro	2011/7	*Ribes petreum*	*Cryptomyzus* sp.	F
AD1	*Aphidius funebris*	Serbia	1998/20	*Crepis* sp.	*Uroleucon* sp.	D
AD2	*Aphidius absinthii*	Serbia	2001/17	*Artemisia vulgaris*	*Macrosiphoniella* sp.	D
AD3	*Aphidius sussi*	Serbia	1998/20	*Aconitum toxicum*	*Delphiniobium junackianum*	D
AD4	*Aphidius ervi*	Slovenia	2009/9	*Triticum aestivum*	*Sitobion avenae*	D
AD5	*Aphidius eadyi*	Russia	2007/11	*Pisum sativum*		D
AD6	*Aphidius eglanteriae*	Serbia	1996/22	*Rosa* sp.	*Chaetosiphon* sp.	D
AD7, AD8	*Aphidius avenae*	Montenegro	2000/18	*Salix retusa*		D
AD9	*Aphidius sussi*	Serbia	2000/18	*Aconitum pentheri*	*Delphiniobium junackianum*	D
AD10	*Aphidius arvensis*	Iran	2010/8	*Inula* sp.	*Aphis sargasi*	D
AD11	*Aphidius erysimi*	Czech Republic	1999/19	*Erisymum* sp.	*Pseudobrevicoryne erysimi*	D
AD12	*Aphidius eglanteriae*	Serbia	1998/20	*Thalictrum elatum*	*Longicaudus trirhodus*	D
AD13	*Aphidius smithi*	United States	1977/41	*Medicago sativa*	*Acyrthosiphon pisum*	D

Sample code	Parasitoid species	Country of origin	Sampling year/ age of samples*	Host plant	Aphid host	Specimen condition **
AD14	*Aphidius eadyi*	Iran	1977/41	*Medicago sava*	*Acyrthosiphon pisum*	D
AD15	*Aphidius banksae*	Israel	1979/39	*Medicago sativa*	*Acyrthosiphon pisum*	D
PF1	*Praon volucre*	Iran	2009/9	*Sonchus oleraceus*	*Uroleucon sonchi*	F
PF2	*Praon dorsale*	Serbia	2010/8	*Corylus avelana*		F
PF3	*Praon abjectum*	Serbia	2011/7	*Thallium aquile*	*L. trialeurodes*	F
PD1	*Praon longicorne*	Montenegro	2009/9	*Geranium robertianum*	*Aphis malvae*	D
PD2	*Praon dorsale*	Montenegro	2006/12	*Filipendula ulmaria*	*Macrosiphum cholodkovskyi*	D
PD3	*Praon longicorne*	Serbia	2006/12	*Rubus* sp.	*Macrosiphum funestum*	D
PD4	*Praon yomenae*	Montenegro	2009/9	*Rubus* sp.		D
PD5	*Praon yomenae*	Iran	2009/9	*Acroptilon repens*	*Uroleucon* sp.	D
PD6	*Praon longicorne*	Czech Republic	2008/10	*Rubus* sp.	*Macrosiphum funestum*	D
PD7	*Praon spinosum*	Croatia	2005/13	*Carex nigra*	*Thripsaphis verrucosa*	D
PD8	*Praon spinosum*	Croatia	2009/9	*Carex* sp.	*Thripsaphis verrucosa*	D
PD9, PD10, PD11	*Praon longicorne*	Czech Republic	1998/20	*Urtica dioica*	*Microlophium carnosum*	D
PD12	*Praon barbatum*	Serbia	2011/7	*Medicago sativa*	*Acyrthosiphon pisum*	D
PD13	*Praon necans*	Serbia	2005/12	*Typha* sp.	*Rhopalosiphum nymphaeae*	D
PD14, PD15	*Praon yomenae*	Japan	2002/16	*Hemerocallis fulva*	*Indomegoura indica*	D
LF1	*Lysiphlebus hirticornis*	Serbia	2011/7	*Tanacetum vulgare*	*Metopeurum fuscoviridae*	F
LF2	*Lysiphlebus cardui*	Serbia	2010/8	*Cirsium arvense*	*Aphis fabae cirsicanthoides*	F
LF3	*Lysiphlebus fabarum*	Serbia	2009/9	*Cirsium arvense*	*Aphis fabae cirsicanthoides*	F
LD1	*Lysiphlebus hirticornis*	Serbia	2011/7	*Tanacetum vulgare*	*Metopeurum fuscoviridae*	D
LD2	*Lysiphlebus cardui*	Serbia	2010/8	*Cirsium arvense*	*Aphis fabae cirsicanthoides*	D
LD3	*Lysiphlebus fabarum*	Serbia	2009/9	*Cirsium arvense*	*Aphis fabae cirsicanthoides*	D
LD4	*Lysiphlebus testaceipes*	Italy	2006/12	*Hedera helix*	*Aphis hederae*	D
LD5	*Lysiphlebus testaceipes*	France	2006/12	*Rubus fruticosus*	*Aphis ruborum*	D
LD6	*Lysiphlebus testaceipes*	Costa Rica	2000/18	*Eugenia wilsonii*	*Toxoptera aurantii*	D
LD7	*Lysiphlebus fritzmuelleri*	Serbia	2006/12	*Vicia cracca*	*Aphis craccae*	D
LD8	*Lysiphlebus confusus*	Iran	2005/13	*Verbascum* sp.	*Aphis verbasci*	D
LD9, LD10	*Lysiphlebus desertorum*	Iran	2005/13	*Achillea millefolium*	*Protaphis* sp.	D

Sample code	Parasitoid species	Country of origin	Sampling year/ age of samples*	Host plant	Aphid host	Specimen condition **
LD11, LD12	*Lysiphlebus fabarum*	Iran	2005/13	*Tragopogon pratensis*	*Brachycaudus tragopogonis*	D
LD13	*Lysiphlebus alpinus*	Serbia	1996/22	*Daucus carota*	*Semiaphis dauci*	D
LD14	*Lysiphlebus melandriicola*	Chech Republic	1998/20	*Carduus* sp.	*Brachycaudus cardui*	D
LD15	*Lysiphlebus fabarum*	Iran	2005/13	*Tragopogon pratensis*	*Brachycaudus tragopogonis*	D
ED1	*Ephedrus laevicollis*	Serbia	2000/18	*Rosa* sp.	*Chaetosiphon* sp.	D
ED2	*Ephedrus plagiator*	Montenegro	2004/14	*Lonicera xylosteum*	*Hyadaphis* sp.	D
ED3	*Ephedrus validus*	Finland	1987/31			D
ED4	*Ephedrus koponeni*	Finland	1987/31			D
MD1	*Monoctonus paulensis*	Canada	2005/13	*Capsicum annuum*	*Myzus persicae*	D
MD2	*Monoctonus allisoni*	USA	2001/17	*Delphinium galucum*	*Nasonovia* (*Eokakimia*) *wahinkae*	D
MD3	*Monoctonus washingtonensis*	USA	1992/26	*Triticum* sp.	*Rhopalosiphum padi*	D
MD4	*Monoctonus leclanthi*	Montenegro	2002/16	*Aconitum toxicum*	*Delphiniobium junackianum*	D

* number of years the specimens were kept dry in collections prior to DNA extraction

**Specimen condition: (F) fresh refers to specimens kept after collection in 96% ethanol; (D) dry are specimens which were kept dry in collections, pinned or glued to cardboard

DNA extraction

Dry specimens were carefully removed from the card points so that they could be re-mounted afterwards if the specimens are holotypes. The whole specimens were used for DNA extraction using the QIAGEN Dneasy Blood and Tissue Kit. In the case of parasitoid specimens used as a control, they were preserved in 96% ethanol prior to extraction. Whole specimens were placed in 2 ml Eppendorf tubes with proteinase K and ATL buffer. After incubation overnight at 56 °C insect specimens were removed from the buffer, rinsed with 96% ethanol several times, air-dried and put back in the collection. The remaining solution was treated according to the manufacturer's instructions.

PCR amplification

The first step was an attempt to amplify a barcoding region of mitochondrial gene cytochrome c oxidase subunit I from dry material using the standard primer pair LCO1490/HCO2198 (Folmer et al. 1994). Each PCR reaction was carried out in a volume of 20µl, including: 1µl of extracted DNA, 11.8 µl H_20, 2 µl High Yield Reac-

tion Buffer A with 1xMg, 1.8 µl of $MgCl_2$ 2.25 mM, 1.2 µl of dNTP 0.6 mM, 1µl LCO1490 0.5 µM, 1µl HCO2198 0.5 µM, 0.2 µl DNA polymerase 0.05U/µl. The amplification protocol included : i) initial denaturation at 95 °C for 5 min; ii) 35 cycles of 1 min at 94 °C, 1 min at 54 °C and 30 sec at 72 °C and iii) final extension at 72 °C for 7 min. Products were visualized on agarose gel.

Due to DNA fragmentation in dry specimens, internal degenerative primers were designed to amplify overlaping short fragments of COI through direct and nested PCR, which could thereafter be aligned to a longer barcoding sequence (Fig. 1). Reference COI sequences of parasitoids retrieved from the GenBank (www.ncbi.nlm.nih.gov/Genbank) were used as a template to design primers for dry material of the genera *Aphidius*, *Praon* and *Lysiphlebus* (Table 2). They were aligned and manually searched for shared conservative regions on which to place the newly designed primers.

The initial idea was to divide the barcoding fragment of COI obtained with LCO1490/HCO2198 into three overlapping subsequences, around 260 bp, 270 bp and 280 bp long respectively, and the primers designed for this were marked as for direct PCR. Furthermore, additional internal primers were designed within these three subsequences to amplify even shorter fragments through nested PCR (Fig. 1).

The genus-specific degenerative primers were used in combination with standard primers LCO1490 and HCO2198 (Fig. 1). Finally, the position of internal primers allowed diverse combinations and targeting of overlapping fragments of different length and position. Due to the shared conservative sites in COI sequences, it was possible for primers initially designed for *Aphidius* species to be also used in amplification of short fragments in combination with primers specifically designed for *Lysiphlebus* species (Aph1Rn, Aph2Fd, Aph3Rn) and for dry *Praon* specimens as well (Aph2Fn) (Fig. 1).

Prior to testing their suitability for amplification of short fragments from dry samples, the designed primers were initially tested on control specimens preserved in

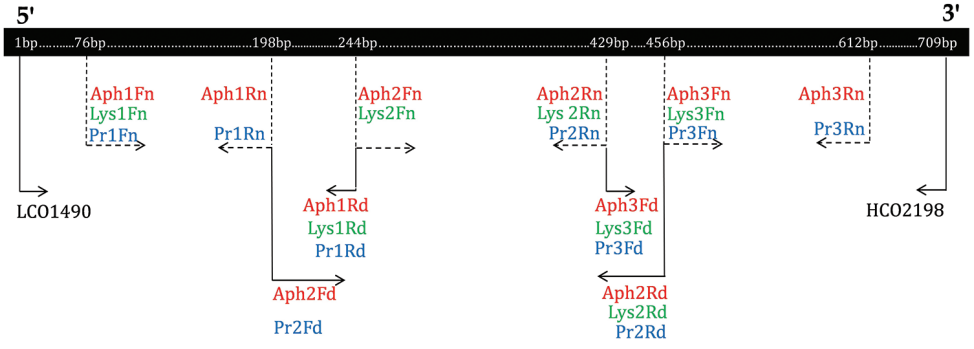

Figure 1. Position of internal degenerative primers within the barcoding region of COI. *Aphidius* - specific primers: Aph1Fn, Aph1Rn, Aph1Rd, Aph2Fd, Aph2Fn, Aph2Rn, Aph2Rd, Aph3Fd, Aph3Fn and Aph3Rn; *Lysiphlebus* - specific primers: Lys1Fn, Lys1Rd, Lys2Fn, Lys2Rn, Lys2Rd, Lys3Fd and Lys3Fn; *Praon* - specific primers: Pr1Fn, Pr1Rn, Pr1Rd, Pr2Fd, Pr2Rn, Pr2Rd, Pr3Fd, Pr3Fn and Pr3Rn. Arrows refer to the direction of the primers, forward or reverse. The exact position of internal primers is designated in comparison to the first nucleotide of the forward LCO1490 primer sequence (5' GGTCAACAAATCATAAAGATATTGG 3').

Table 2. The list of reference *Aphidiinae* species obtained from GenBank and used in designing the genus-specific primers.

Parasitoid species	Accession number
Aphidius matricariae	JN620563
Aphidius urticae	JN620590
Aphidius sonchi	JN620589
Aphidius rhopalosiphi	JN164779
Aphidius ervi	JQ723411
Aphidius microlophii	JN620566
Aphidius uzbekistanicus	JN164751
Aphidius funebris	JN620561
Aphidius rosae	JN620582
Aphidius eadyi	JN620551
Aphidius salicis	JN620585
Aphidius ribis	JN620579
Aphidius colemani	KJ615362
Aphidius transcaspicus	KJ615375
Lysiphlebus testaceipes	HQ599569
Lysiphlebus orientalis	KC237736
Lysiphlebus hirticornis	HQ724540
Lysiphlebus fabarum	JQ723416
Lysiphlebus cardui	JN620640
Lysiphlebus confusus	KM408535
Praon barbatum	JN620671
Praon yomenae	JN620693
Praon gallicum	JN620680
Praon abjectum	KC128671
Praon dorsale	KC128677
Praon exsoletum	KJ848478

96% ethanol. In total, five *Aphidius* species were submitted to initial testing (samples AF1-AF5; Table 1). Three following primer combinations were confirmed successful in direct PCR reactions: i) LCO1490/Aph1Rd, ii) Aph2Fd/Aph2Rd and iii) Aph-3Fd/HCO2198 (Fig. 2). Three species from the genus *Praon* were used for test trials (samples PF1- *P. volucre*, PF2- *P. dorsale* and PF3- *P. abjectum*; Table 1). Three individual analyses were conducted: 1. LCO1490/Pr1Rd; 2. Pr2Fd/Pr2Rd; and 3. Pr3Fd/HCO2198. All of the products with fresh samples were visualized (Fig. 3). *Lysiphlebus hirticornis* Mackauer, 1960 (LF1), *L. cardui* Marshall, 1896 (LF2) and *L. fabarum* Marshall, 1896 (LF3) were included in the initial trials (Table 1). The four following primer combinations were confirmed suitable: 1) LCO1490/Lys1Rd; 2) Aph2Fd/Lys2Rd; 3) Pr2Fd/Lys2Rd; and 4) Lys3Fd/ HCO2198 (Fig. 5).

After confirmation of their suitability, the new primers were then used in trials with dry specimens. Products of PCR were obtained in 40 μl volumes. In the direct PCR reac-

Table 3. The list of primers designed for the genera *Aphidius*, *Lysiphlebus* and *Praon* to amplify short fragments of COI barcoding region from dry specimens through direct and nested PCR analyses.

Parasitoid group	Primer name*	5 ' 3' primer sequence**	Primer direction
Aphidius	Aph1Rd	GRGGRAAAGCYATATCAGGAG	reverse
Aphidius	Aph1Fn	TAAGWTTATTAATTCGWATRGA	forward
Aphidius	Aph1Rn	CAATTWCCAAATCCWCCAATTAT	reverse
Aphidius	Aph2Fd	ATAATTGGWGGATTTGGWAATTG	forward
Aphidius	Aph2Rd	GTWCTAATAAAATTAATWGCWCC	reverse
Aphidius	Aph2Fn	CTCCTGATATRGCTTTYCCYC	forward
Aphidius	Aph2Rn	GADGAAATHCCTGCTAAATG	reverse
Aphidius	Aph3Fd	CATTTAGCWGGDATTTCYTC	forward
Aphidius	Aph3Fn	GGAGCWATTAATTTTATTAGWAC	forward
Aphidius	Aph3Rn	GTAGTATTTAARTTWCGATC	reverse
Lysiphlebus	Lys1Rd	GAGGAAAAGCYATATCWGGAG	reverse
Lysiphlebus	Lys1Fn	TAAGWTTAATTATTCGWATRGA	forward
Lysiphlebus	Lys2Rd	GTWCTAATAAAATTAATTGCHCC	reverse
Lysiphlebus	Lys 2Fn	CTCCWGATATRGCTTTTCCTC	forward
Lysiphlebus	Lys 2Rn	GAWGAAATACCWGCTAAATG	reverse
Lysiphlebus	Lys3Fd	CATTTAGCWGGDATTTCWTC	forward
Lysiphlebus	Lys3Fn	GGDGCAATTAATTTTATTAGWAC	forward
Praon	Pr1Rd	GAGGRAAAGCTATATCAGGAG	reverse
Praon	Pr1Fn	AAGWGATCAAATTTAYAATAG	forward
Praon	Pr1Rn	CAATTWCCAAAYCCWCCAATTAT	reverse
Praon	Pr2Fd	ATAATTGGAGGRTTTGGWAATTG	forward
Praon	Pr2Rd	GTTGWAATAAAATTAATWGCYCC	reverse
Praon	Pr2Rn	CATTTAGCWGGTATTTCWTC	reverse
Praon	Pr3Fd	CATTTRGCTGGWATTTCYTC	forward
Praon	Pr3Fn	GGAGCWATTAATTTTATTWC	forward
Praon	Pr3Rn	GTWGTATTTAWATTTCGATC	reverse

*the last letter in the primer's name refers to PCR reaction: *d*-direct and *n*-nested

**degenerative base designation/actual base coded: R or - A, or - G; Y or -C or - T; W or -A, or - T.

tion, 4 µl of extracted DNA was added into 36 µl of mix, following the recipe described for the LCO1490/HCO2198 primer pair. In nested PCR, 0.25 µl of a product from direct PCR was added into 39.75 µl of mix. The following protocol was developed for direct and nested PCR: i) initial denaturation at 95 °C for 5 min; ii) 37 cycles of 1 min at 95 °C, 1 min at 54 °C, and 30 sec at 72 °C; and iii) final extension at 72 °C for 7 min.

Amplified COI fragments were sequenced in both directions using an automated equipment (Macrogen Inc, Seoul, South Korea). Overlapping short fragments of the barcoding region were manually edited in FINCHTV ver.1.4.0 (www.geospiza.com), concatenated to obtain longer sequences and aligned using the CLUSTAL *W* program integrated in MEGA5 (Tamura et al. 2011). A Maximum likelihood tree was constructed using the MEGA5 software, with 500 bootstrap replicates performed to assess the branch support. The evolutionary distances were computed using the Tamura-Nei

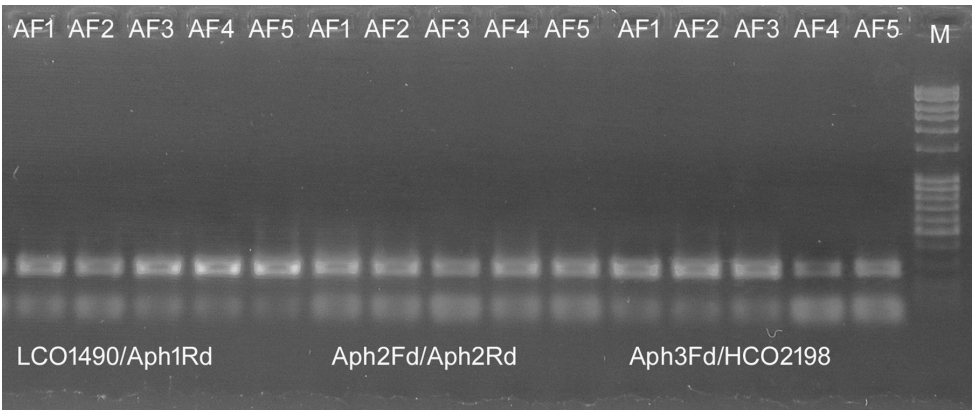

Figure 2. Agarose gel visualizing the products of direct PCR in initial trials testing the novel primers with fresh *Aphidius* samples. Three direct PCR reactions were conducted with the following primer pairs: **1** LCO1490/Aph1Rd **2** Aph2Fd/Aph2Rd; and **3** Aph3Fd/HCO2198. The species included in trials were: AF1- *A. tanacetarius*, AF2- *A. sussi*, AF3- *A. sonchi*, AF4- *A. linosiphonis* and AF5- *A. ribis*. M – marker.

method (Tamura and Nei, 1993). Phylogenetic analyses included the sequenced barcodes recovered from archival parasitoid specimens combined with the reference COI sequences of Aphidiinae from GenBank.

Results

Initial trials with dry specimens using standard primer pair for the COI barcoding region LCO1490/HCO2198 failed to give products. Thereafter, 15 dry specimens of 11 different *Aphidius* species (*A. absinthii* Marshall, 1896; *A. arvensis* Starý, 1960; *A. avenae* Haliday, 1834; *A. banksae* Kittel, 2016; *A. eadyi* Subba Rao and Sharma, 1959; *A. eglanteriae* Haliday, 1834; *A. erysimi* Starý, 1960; *A. funebris* Mackauer, 1961; *A. ervi* Haliday, 1834; *A. smithi* Subba Rao and Sharma, 1959; *A. sussi*) were submitted to molecular analyses (Table 1). Insects had been killed and stored dry in collections for 8 to 41 years prior to DNA extraction. The same three combinations of standard and degenerative primers previously confirmed as suitable in the test trials with fresh material were used with dry samples AD1-AD15 as well. Direct PCR produced amplicons in all three combinations for samples AD1 to AD6, while in the cases of samples AD7 to AD15 no product was visualized. The products from direct PCR with primer pair LCO1490/Aph1Rd were submitted to two independent nested reactions with primers LCO1490/Aph1Rn and Aph1Fn/Aph1Rd; from direct PCR with primers Aph2Fd/Aph2Rd to nested reactions with Aph2Fd/Aph2Rn and Aph2Fn/Aph2Rd; and products obtained with Aph3Fd/HCO2198 were included in nested trials with the primers Aph3Fd/Aph3Rn and Aph3Fn/HCO2198. In all six individual nested reactions short fragments of the barcoding region were amplified successfully and visualized for all of the tested samples.

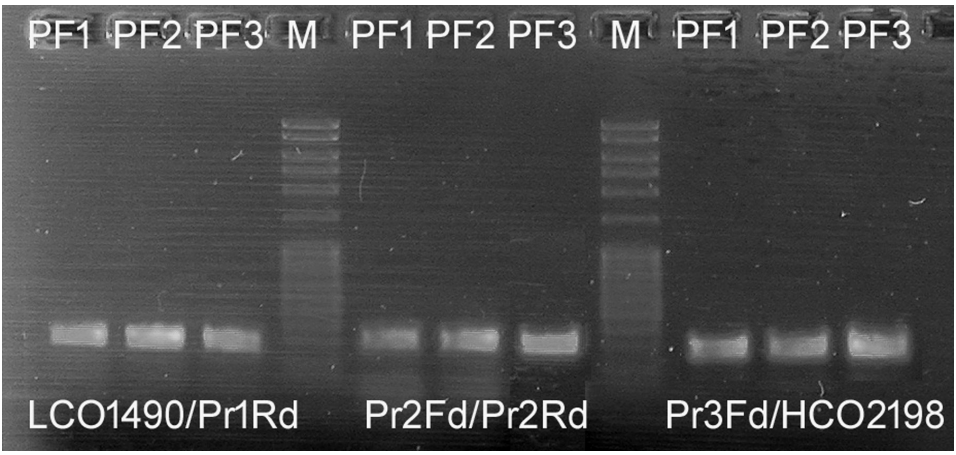

Figure 3. Agarose gel visualizing the products of direct PCR in initial trials testing the novel primers with fresh *Praon* samples. Three direct PCR reactions were conducted with primer pairs: 1. LCO1490/Pr1Rd, 2. Pr2Fd/Pr2Rd, 3. Pr3Fd/HCO2198. The species included in trials are PF1- *P. volucre*, PF2- *P. dorsale*, PF3- *P. abjectum*; M – marker.

In total 15 specimens of eight *Praon* species preserved dry for 7 to 20 years prior to DNA extraction were analysed (Table 1). We attempted to retrieve short overlapping fragments of COI barcodes from dry samples PD1-PD15 through the same three direct amplifications as with the fresh material. In analyses with primers targeting the first fragment of the barcoding sequence, all products were obtained. In the second and third reactions short fragments of barcode were amplified in samples PD1-PD11 and PD13, while no product was visualized for samples PD12, PD14 and PD15. The same methodological approach was applied here, namely using the products from direct PCR as a template for secondary nested trials. The amplicons of samples PD12, PD14 and PD15 from the trial with primer pair Pr2Fd/Pr2Rd were processed further in two nested reactions with combinations Pr2Fd/Pr2Rn and Aph2Fn/Pr2Rd, while the products of direct PCR with Pr3Fd/HCO2198 were processed in secondary analyses using the combinations Pr3Fd/Pr3Rn and Pr3Fn/HCO2198. Subsequent analyses successfully targeted short fragments within the subsequences of the barcoding region in all four nested test trials (Fig. 4).

The novel primers were tested on *Lysiphlebus alpinus* Starý, 1971; *L. confusus* Tremblay & Eady, 1978; *L. desertorum* Starý, 1965; *L. fabarum*; *L. fritzmuelleri* Mackauer, 1960; *L. hirticornis*; *L. melandriicola* Starý, 1961; *L. testaceipes*), stored dry in collections for 7 to 22 years. Three separate analyses were conducted using the primer combinations confirmed as suitable with fresh material. Amplicons were visualized in the first direct analysis with the LCO1490/Lys1Rd combination for samples LD1-LD7 and LD10-LD15. No products were visible for samples LD8 and LD9 which were further processed in nested trials with LCO1490/Lys1Rn and Lys1Fn/Lys1Rd. Products of the direct PCR conducted with the primer combination Aph2Fd/Lys2Rd were obtained in all samples except LD8, LD9 and LD12 which were thereafter processed in nested analyses with 1. Aph2Fd/Lys2Rn; and 2. Lys2Fn/Lys2Rd. In the third direct

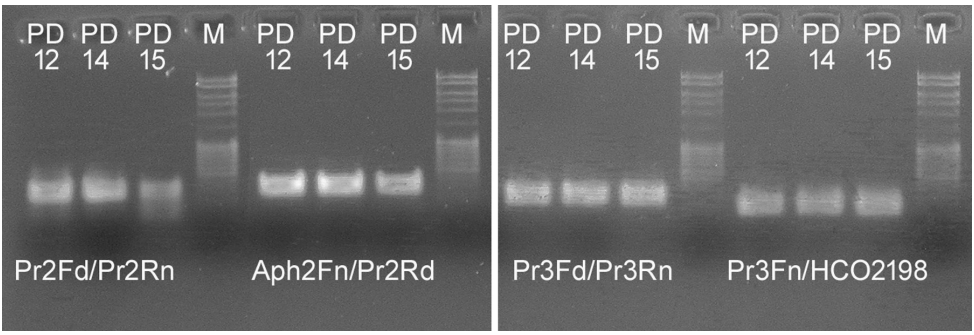

Figure 4. Agarose gel visualizing the products of nested trials with products of direct PCR for samples PD12 - *P. barbatum*, PD14 - *P. yomenae*, and PD15 - *P. yomenae*. The products from PCR with Pr2Fd/Pr2Rd were submitted to secondary nested trials with primer pairs Pr2Fd/Pr2Rn and Aph2Fn/Pr2Rd. Amplicons obtained with Pr3Fd/HCO2198 were used as the template for nested reactions with Pr3Fd/Pr3Rn and Pr3Fn/HCO2198.

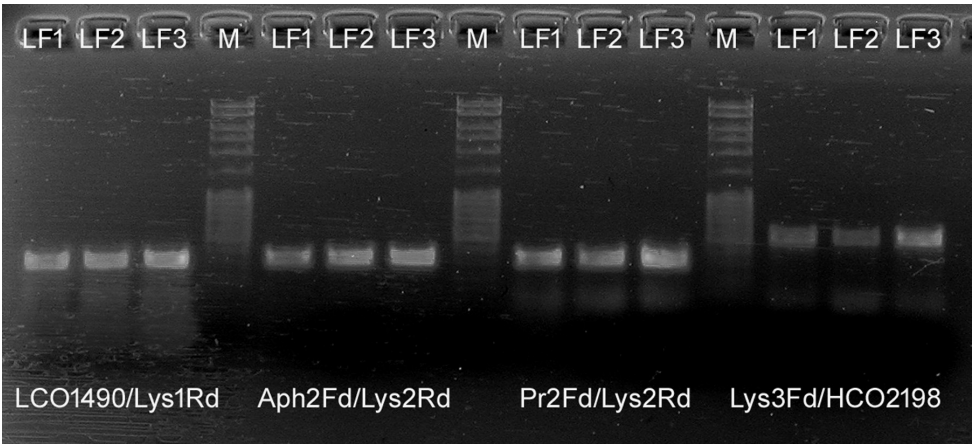

Figure 5. Agarose gel visualizing the products of direct PCR in initial trials testing the novel primers with fresh *Lysiphlebus* samples. Tested combinations of primers were: 1) LCO1490/Lys1Rd; 2) Aph2Fd/Lys2Rd; 3) Pr2Fd/Lys2Rd; and 4) Lys3Fd/HCO2198. The species included in trials were: LF1 - *L. hirticornis*; LF2 - *L. cardui*; and LF3 - *L. fabarum*; M – marker.

PCR trial, amplicons were visualized in all analyzed specimens besides LD8, LD9 and LD13 which were further submitted to analyses with primers 1. Lys3Fd/Aph3Rn; and 2. Lys3Fn/HCO2198. We obtained products in all nested trials (Fig. 6).

Our research covers different taxonomically challenging Aphidiinae, for which reason we tested suitability of the newly designed primers on several other archival specimens from the genera *Monoctonus* and *Ephedrus*. In order to preserve the limited amount of DNA obtained from dry specimens, we avoided blind PCR trials as well as testing of all possible combinations by doing initial alignment of barcode sequences of fresh material (unpublished data) and degenerative primers (Table 4). According to the alignment we chose the primers best suited to target the species of interest.

Table 4. Comparison of barcode fragments of COI for *Monoctonus* and *Ephedrus* with degenerative primers sequences.

Degenerative primer	Difference in base pair substitutions (bp)	
	***Monoctonus* sp.**	***Ephedrus* sp.**
Aph1Rd	0–2 bp	4–6 bp
AphF1n	2–5 bp	0–3 bp
Aph1Rn	0–4 bp	0–4 bp
Aph2Fd	0–4 bp	2–3 bp
Aph2Rd	0–2 bp	2–5 bp
Aph2Fn	0–2 bp	4–7 bp
Aph2Rn	1–3 bp	2–5 bp
Aph3Fd	0–3 bp	1–4 bp
Aph3Fn	0–2 bp	4–7 bp
Aph3Rn	0–1 bp	1–4 bp
Lys1Rd	0–2 bp	4–5 bp
Lys1Fn	0–4 bp	0–4 bp
Lys2Rd	0–1 bp	1–2 bp
Lys2Fn	0–2 bp	5–7 bp
Lys2Rn	1–4 bp	0–5 bp
Lys3Fd	0–3 bp	1–3 bp
Lys3Fn	0–1 bp	5–7 bp
Pr1Rd	0–3 bp	3–5 bp
Pr1Fn	1–4 bp	4–6 bp
Pr1Rn	0–4 bp	1–3 bp
Pr2Fd	0–4 bp	0–2 bp
Pr2Rd	1–2 bp	0–1 bp
Pr2Rn	1–4 bp	0–4 bp
Pr3Fd	0–4 bp	0–4 bp
Pr3Fn	1–3 bp	4–7 bp
Pr3Rn	0–2 bp	0–3 bp

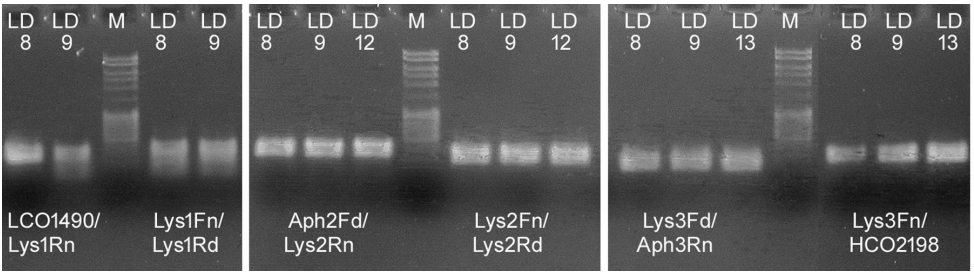

Figure 6. Agarose gel visualizing the products of nested trials with products of direct PCR for samples LD8 – *L. confusus*, LD9 – *L. desertorum*; LD12 – *L. fabarum*; and LD13 – *L. alpinus*. The products of LD8 and LD9 from PCR with LCO1490/Lys1Rd were submitted to secondary reactions combining two primer pairs, viz., 1. LCO1490/Lys1Rn; and 2. Lys1Fn/Lys1Rd. Amplicons of LD8, LD9 and LD12 obtained with Aph2Fd/Lys2Rd were submitted to secondary nested trials with primer pairs Aph2Fd/Lys2Rn and Lys2Fn/Lys2Rd. Products from direct PCR with Lys3Fd/HCO2198 were used as the template for nested reactions with Lys3Fd/Aph3Rn and Lys3Fn/HCO2198.

In the case of *Ephedrus* species, we chose two combinations for direct PCR, i.e., 1. LCO1490/Pr2Rd, and 2. Aph3Fd/HCO2198. Four species preserved in dry condition for 14 to 31 years in collections were included in the test trials, viz., *E. plagiator* Nees, 1811 (ED1); *E. laevicollis* Thomson, 1895 (ED2); *E. validus* Haliday, 1833 (ED3); and *E. koponeni* Halme, 1992 (ED4) (Table 1). Amplicons of both targeted fragments were visualized on gel for specimens ED1, ED3, and ED4, while in the case of the ED2 sample a PCR product was visible only with primer pair Aph3Fd/HCO2198. Products of the ED2 were subjected to separate nested reactions with primer pair LCO1490/Pr1Rd and Pr2Fd/Pr2Rd. Both short fragments of the barcode were successfully amplified and concatenated with the third subsequence obtained in direct PCR to retrieve a longer barcode fragment of COI.

Dry specimens of the following four *Monoctonus* species preserved for 13 to 26 years were subjected to PCR analyses: *M. paulensis* (Ashmead) (MD1); *M. allisoni* Pike and Starý, 2003 (MD2); *M. washingtonensis* Pike and Starý, 1995 (MD3); and *M. leclanthi* Tomanović and Starý, 2002 (MD4). The same approach was repeated as with *Ephedrus*, i.e., barcoding sequences of fresh material were aligned and analysed for primers suitability prior to molecular analyses (Table 4). The final choice fell on three combinations in direct PCR to retrieve three overlapping short fragments within the barcoding COI fragment: 1. LCO1490/Aph1Rd; 2. Pr2Fd/Lys2Rd; and 3. Pr3Fd/HCO2198. The final results show that the tested combinations of standard and degenerative primers successfully amplified all three short subsequences in all tested *Monoctonus* species.

The overall results of combining different primers in direct and secondary nested reactions are summarized in Fig. 7.

Short fragments of the COI barcodes obtained from direct and nested PCR analyses of the following samples were deposited in the GenBank: AD4 - *A. ervi* (MG991997), AD7 - *A. avenae* (MG991998), AD10 - *A. arvensis* (MG991999), LD1 - *L. hirticornis* (MG992000), LD4 - *L. testaceipes* (MG992001), LD7 - *L. fritzmuelleri* (MG992002), PD2 - *P. dorsale* (MG992003), PD5 - *P. yomenae* (MG992004), ED2 - *E. plagiator* (MG991993), ED4 - *E. koponeni* (MG991992), MD1 - *M. paulensis* (MG991996), MD2 - *M. allisoni* (MG991995), MD3 - *M. washingtonensis* (MG991994). Several reference COI sequences from different Aphidiinae species were obtained from the public database and used with the archival material for tree construction. A total of 31 barcoding sequences were aligned, trimmed to the same length and submitted to phylogenetic analysis. A Maximum likelihood tree shows evident clustering of congeneric species in separate lineages with substantial bootstrap support (Fig. 8), confirming the quality of COI barcoding sequences retrieved from archival parasitoids specimens by targeting the short overlapping fragments with newly designed primers.

Discussion and conclusion

The barcoding method has shown to be a useful tool in discriminating parasitoid species from the five Aphidiinae genera studied, enabling further research on their biodiversity

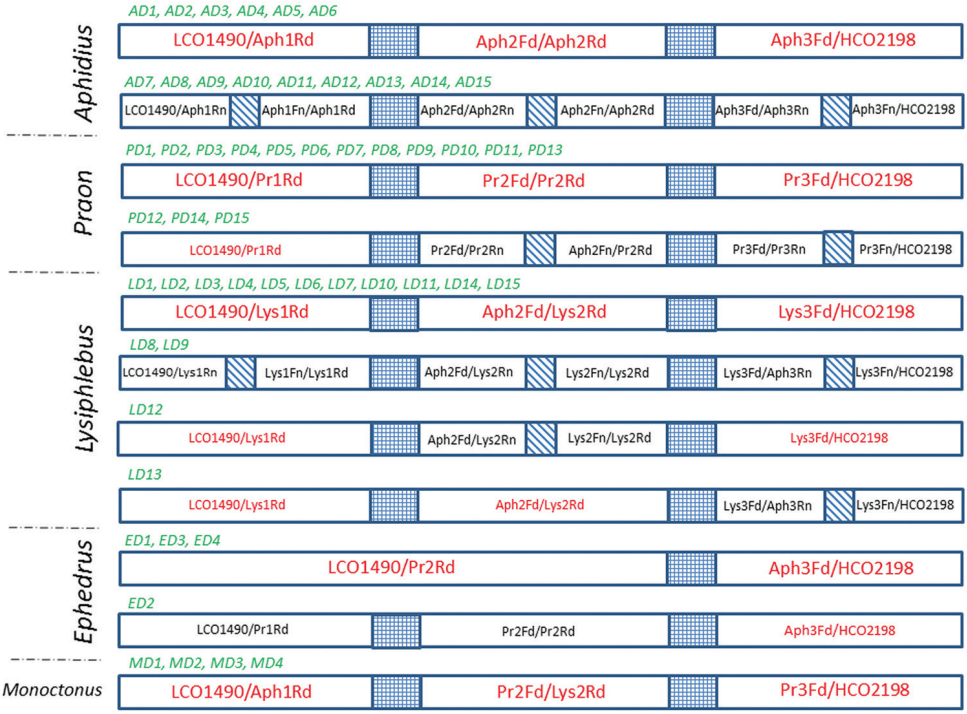

Figure 7. Scheme with overview of PCR attempts to recover the barcoding region of cytochrome c oxidase subunit I with novel primers from archival specimens from the genera *Aphidius*, *Praon*, *Lysiphlebus*, *Ephedrus* and *Monoctonus*. Primer pairs coloured red were used in direct PCR; black coloured primers were used in secondary nested reactions. Positions where short fragments within the subsequences overlap are marked with a pattern.

and phylogeny. The results presented here indicate the possibility of testing many other different combinations of primers in future research on archival specimens with the expectation of achieving success in retrieving the targeted subsequences. The position of the newly designed primers was evidently well chosen, targeting sites conservative enough to permit their multiple uses on a much wider spectrum of museum material than initially planned.

Similar to the results obtained by Andersen and Mills (2012), in our study age was apparently a limiting factor for successful amplification with the newly designed internal primers. On the other hand, the starting point in this study was awareness that museum specimens are not always available, or that the type material is sometimes restricted to a single specimen, etc., and thus cannot be manipulated in numerous trials. For this reason, blank PCR products were always further processed through secondary analyses with additional internal primers. This assumption was confirmed to be the basis of a good methodological approach with substantial success.

The results presented above refer only to combination of primers randomly selected to test their suitability in retrieving the barcoding region from *Ephedrus* and *Monoctonus* species. Without the need for further expenditure of limited DNA sources, the

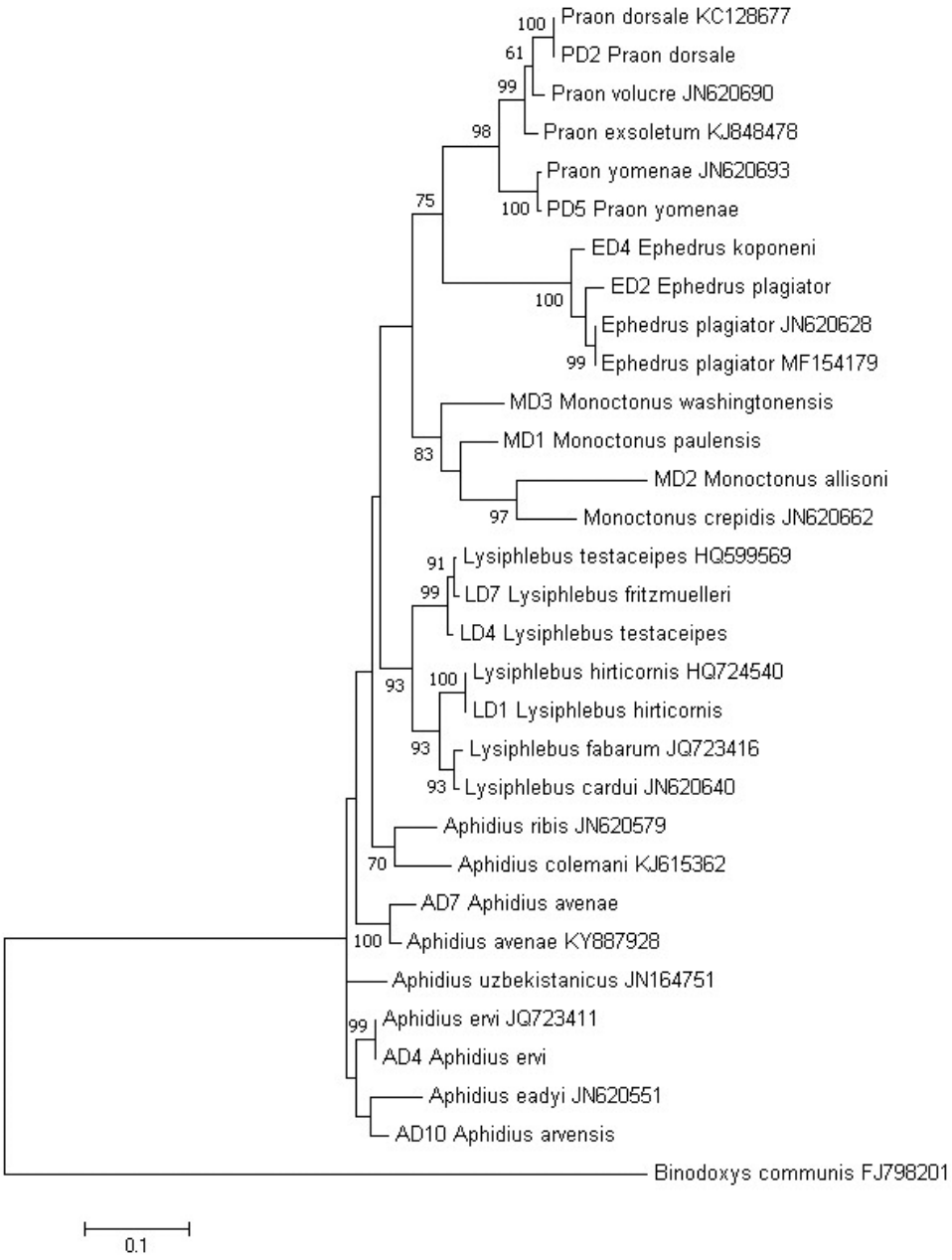

Figure 8. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood is shown. There were a total of 568 positions in the final dataset. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of replicate trees >50% in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches.

here presented overview of nucleotide differences between the barcodes of parasitoids and information about primers clearly indicate that quite a few other combinations can be tested with the expectation of successfully retrieving short fragments.

Many benefits of using novel primers in conservation genetics and phylogeny studies are recognized, above all, the possibility of analyzing archival material of Aphidiinae parasitoids with unresolved taxonomic status. To date there have been many phylogenetic studies with different hypotheses about the origin and classification of certain taxa. Many examples in the literature show the importance of an integrative approach combining molecular and morphological data in taxonomic, phylogenetic and conservation studies, but even when using such an approach, researchers are quite often left with open questions. In view of the many confronting opinions held by different groups of authors, we can assume that the involvement of archival remains of Aphidiinae in molecular analyses will prove to be of great usefulness by yielding results enabling us to resolve the problems of phylogenetic relationships and the taxonomic recognition of different parasitoid groups.

It can be predicted that the herein described method of retrieving the barcoding region in parasitoids will take on increasing importance by making it possible to include not only extinct species preserved in museums, but also endemic or rare species under threat of extinction as well. Good examples of parasitoid species with potential risk of extinction are various associations of aphid hosts/parasitoids whose distribution are restricted to habitats under constant anthropogenic pressure of degradation such as the wetlands (Tomanović et al. 2012).

Modern genomic research opened complex questions exceeding the capacity of traditional DNA sequencing technologies. The Next-generation sequencing has revolutionized the biological sciences allowing us to study biological systems at higher level. In the light of an ongoing rapid progress in the field of modern sequencing technologies, newly designed primers could meet the demands in terms of depth of information in studying genomics of different Aphidiinae by delivering an insight into DNA variation of the target mitochondrial region.

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