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PCR-based species identification of *Agriotes* larvae

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Abstract

Click beetle larvae within the genus *Agriotes* (Coleoptera: Elateridae), commonly known as wireworms, are abundant ground-dwelling herbivores which can inflict considerable damage to field crops. In Central Europe up to 20 species, which differ in their distribution, ecology and pest status, occur in arable land. However, the identification of these larvae based on morphological characters is difficult or impossible. This hampers progress towards controlling these pests. Here, we present a polymerase chain reaction (PCR)-based approach to identify, for the first time, 17 *Agriotes* species typically found in Central Europe. Diagnostic sequence information was generated and submitted to GenBank, allowing the identification of these species via DNA barcoding. Moreover, multiplex PCR assays were developed to identify the nine most abundant species rapidly within a single-step reaction: *Agriotes brevis*, *A. litigiosus*, *A. obscurus*, *A. rufipalpis*, *A. sordidus*, *A. sputator*, *A. ustulatus*, *A. lineatus* and *A. proximus*. The latter two species remain molecularly indistinguishable, questioning their species status. The multiplex PCR assays proved to be highly specific against non-agrioted elaterid beetles and other non-target soil invertebrates. By testing the molecular identification system with over 900 field-collected larvae, our protocol proved to be a reliable, cheap and quick method to routinely identify Central European *Agriotes* species.

Keywords: barcoding, multiplex PCR, wireworms, Elateridae

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Introduction

The larvae of click beetles within the genus *Agriotes* (Coleoptera: Elateridae), also known as wireworms, are abundant ground-dwelling insects which are predominantly herbivorous (Langenbuch, 1932; Furlan, 1998; Traugott *et al*., 2008a). They feed on a wide range of plants in grasslands and arable fields, where they can cause considerable damage to crops such as potatoes and maize (Parker & Howard, 2001). Throughout Central Europe, 18 *Agriotes* species have been recorded (Cate, 2007); another two species can occur in adjacent regions in the south and east (L. Furlan, personal communication). Nine of these 20 species are widespread and of special agricultural importance: *Agriotes brevis*, *Agriotes lineatus*, *Agriotes litigiosus*, *Agriotes obscurus*, *Agriotes proximus*, *Agriotes rufipalpis*, *Agriotes sordidus*, *Agriotes sputator* and *Agriotes ustulatus* (Tóth *et al*., 2003; Furlan & Tóth, 2007). The biology and ecology of some of these species have been studied in detail (e.g. Langenbuch, 1932; Kabanov, 1975; Furlan, 1996, 1998, 2004; Traugott *et al*., 2008a), revealing differences in species-specific traits, such as feeding behaviour and phenology. Therefore, an accurate identification to species

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level is a prerequisite for any study addressing *Agriotes*' basic biology, pest status or control. Although genus-specific morphological characters (i.e. specific spiracles placed on the ninth abdominal segment) allow to clearly identify wireworms of the genus *Agriotes*, species-specific features are described for only eight of the 20 Central European species in a current larval identification key (Klausnitzer, 1994). The intraspecific variability in these morphological identification characters is unknown. Additionally, some diagnostic features (e.g. certain structures of the mandible) cannot be used reliably in field-collected larvae as they are usually worn down. Hence, in field surveys the species-specific identification of *Agriotes* larvae implies several difficulties and is usually not considered. Adults, on the contrary, can be identified morphologically (Lohse, 1979), and pheromone catches of males (Furlan & Tóth, 2007; Vernon & Tóth, 2007) can be used as a surrogate for the larval population present in the soil. *Agriotes* larvae, however, are long-lived, typically spending 2–5 years in the soil before pupating (Langenbuch, 1932; Kabanov, 1975; Furlan, 1998, 2004), which means that the adult population does not necessarily reflect the species composition in the larval population. Moreover, as pheromones are available for only some *Agriotes* species, this approach does not allow the examination of the complete *Agriotes* species spectrum.

Molecular approaches have shown a great potential to taxonomically assign 'difficult' organisms, and they constitute a valuable tool in overcoming problems entailed with conventional morphological identification methods. Sequencing of taxon-specific DNA-fragments (DNA barcoding) provides one way for molecular taxonomic assignment (e.g. Greenstone *et al*., 2005; Antonini *et al*., 2009). This approach has resulted in a steadily increasing number of diagnostic sequences available in public databases such as GenBank or BOLD (Ratnasingham & Hebert, 2007). In DNA barcoding, one DNA sequence of particular interest is the mitochondrial cytochrome *c* oxidase subunit I (COI) gene, which has been proposed as a universal 'barcode' for animals (Hebert *et al*., 2003). Aside from barcoding approaches, a number of polymerase chain reaction (PCR)-based techniques have been developed within the last decade, providing fast, yet reliable, species identification (Hinomoto *et al*., 2004; Traugott *et al*., 2006; Hosseini, 2007; Rugman-Jones *et al*., 2009a,b), also within the genus *Agriotes*. Accordingly, Ellis *et al*. (2009) recently utilized a terminal restriction fragment length polymorphism (T-RFLP) technique to identify the larval stages of three *Agriotes* species occurring in the UK.

Here, we combine DNA barcoding and multiplex PCR to molecularly identify *Agriotes* species. The objectives of our study were: (i) to generate species-specific sequence information for all Central European *Agriotes* species; (ii) to develop a multiplex PCR assay for rapid identification of the nine most widespread species in Central Europe (the 'core' *Agriotes* species); and (iii) to evaluate the molecular identification protocol by screening a large number of field-collected *Agriotes* larvae. We aimed to develop a simple protocol which is cheap, reliable and can be performed with standard molecular equipment to maximize its practical applicability.

Materials and methods

Origin of adult beetles

Adults of each *Agriotes* species occurring in Central Europe were used to establish the molecular identification system. The majority of these beetles was stored in 70–90% ethanol; rare species (*A. acuminatus*, *A. infuscatus*, *A. medvedevi*, *A. modestus*, *A. pallidulus*, *A. paludum*, *A. pilosellus*, *A. turcicus*, as well as *A. aequalis* and *A. gurgistanus*, occurring in regions bordering in the south and east, respectively) were dried and pinned and came from private beetle collections (P. Cate, L. Furlan, G. Platia), including some individuals dating back as far as 1963. Beetles were identified using a standard identification key based on morphological characters (Lohse, 1979). To account for the genetic variation within and between species, beetles collected from all over Europe were examined (table 1). Special attention was paid to the nine core *Agriotes* species, including specimens from Canada, where some of these species have been introduced from Europe more than a century ago (Vernon *et al*., 2001).

DNA extraction and PCR

Total DNA was extracted from adult beetles using tissue from legs or the abdomen. From each *Agriotes* species, several individuals were used, including, if possible, specimens collected at different localities. A CTAB-based protocol described in Juen & Traugott (2005) was employed with the following modifications to obtain amplifiable DNA from badly preserved beetles (specimens stored in ethanol <70% or dried/pinned specimens): beetles' tissue was fully immersed in 430 µl TES buffer (0.1 M TRIS, pH 8, 10 mM EDTA, 2% sodium dodecyl sulphate) and 10 µl Proteinase K (20 mg ml^{-1}, AppliChem, Darmstadt, Germany) in 1.5 ml reaction tubes, homogenized with pestles followed by an overnight incubation at 58°C. To extract DNA from pinned beetles and voucher specimens, we used a non-destructive DNA extraction method, which avoids conferring external morphological damage to the beetle. In this case, the whole beetle was placed in a 1.5 ml reaction tube containing the TES buffer–Proteinase K mix described above, incubated overnight and removed afterwards, leaving a sufficient amount of DNA for further analysis. Specimens were flushed with ethanol (99.8%) several times before replacing them to the collection. Voucher specimens for each species were either deposited in our laboratory or returned to the owner in case of rare specimens from beetle collections. All extractions were done in a separate pre-PCR laboratory; one extraction negative control was included in each batch of beetles extracted and tested with universal primers (Folmer *et al*., 1994: PCR conditions see below).

A part of the 5′-end of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene (approx. 660 bp) was amplified by PCR using the universal invertebrate primers LCO1490 and HCO2198 (Folmer *et al*., 1994). To facilitate the amplification of degraded DNA from badly preserved beetles, the following intermediate primers were employed: C1-J-1859 (Simon *et al*., 1994) and newly designed general *Agriotes* primers Agr-gen-A501 (5′-GATTYCTGTTGATCGYATATTAAT-3′), Agr-gen-A500 (5′-TGTTCCTGCDCCRTTTTC-3′) and Agr-gen-S500 (5′-GTTATYGTRACAGCACATGCWTTC-3′). The forward primer LCO1490 was used in combination with reverse primers A501 and A500, C1-J-1859 with HCO2198 and S500 with A501; amplifying approx. 350–500 bp fragments. Each 10 µl PCR contained 1.5 µl of DNA extract, 0.25 U HotStarTaq® DNA Polymerase (Qiagen, Hilden, Germany), 1 µl of 10 × PCR Buffer (Qiagen), 5 mM $MgCl_2$, 0.2 mM dNTPs (Genecraft, Lüdinghausen, Germany), 1 µM of each primer, 5 µg bovine serum albumin (BSA) and 2.55 µl of PCR-grade RNase-free

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Table 1. The 20 *Agriotes* species (except for two larvae, indicated by '∗', adults only) investigated in this study, including the nine agriculturally most important species (in boldface). For each species, the sampling localities, number of DNA extracts and COI (mtDNA) sequences obtained (not all specimens were sequenced) are provided. A '+' indicates species whose larvae are covered by the morphological identification key of Klausnitzer (1994). Sequence superscript 'ht' refers to those haplotypes used for phylogenetic analyses (see fig. 1).

Species	Sampling localities	DNA extracts	COI sequences (GenBank Accession nos)
+ ***Agriotes brevis*** Candèze 1863	Vienna / Lower and Upper Austria	3	2 (HM542015^{ht3}, HM542014^{ht3})
	Italy	3	1 (HM542017^{ht1})
	Croatia	4	2 (HM542013^{ht3}, HM542016^{ht2})
+ ***Agriotes lineatus*** (Linnaeus 1767)	Innsbruck (Tyrol)	2	
	Vienna / Lower and Upper Austria	3	2 (HM542024^{ht2})
	Germany	3	1 (HM542023^{ht2})
	France	2	2 (HM542025^{ht1}, HM542022^{ht3})
	Romania	2	
	Holland	3	
	England (UK)	3	1 (HM542021^{ht4})
	Canada	2	1 (HM542026^{ht1})
Agriotes litigiosus (Rossi 1792)	Italy (North)	5	3 (HM542028^{ht1}, HM542027^{ht2})
	Italy (South)	9	4 (^{ht3})
+ ***Agriotes obscurus*** (Linnaeus 1758)	Innsbruck (Tyrol)	5	3 (HM542033^{ht1}, HM542032^{ht1}, HM542029^{ht2})
	Vienna / Lower and Upper Austria	2	
	Holland	3	1
	England (UK)	4	1 (HM542030^{ht1})
	Canada	2	1 (HM542031^{ht1})
Agriotes proximus Schwarz 1891	Portugal	5	4 (HM542037^{ht1})
	Bulgaria	5	
Agriotes rufipalpis Brullè 1832	Greece	4	3
	Bulgaria	4	3 (HM542038^{ht1})
	Hungary	3	
Agriotes sordidus (Illinger 1807)	Italy	9	5 (HM542044^{ht1}, HM542043^{ht1}, HM542042^{ht1}, HM542041^{ht1})
	Germany	2	2 (HM542040^{ht2}, HM542039^{ht2})
	France	3	3
+ ***Agriotes sputator*** (Linnaeus 1758)	Innsbruck (Tyrol)	6	1
	Vienna / Lower and Upper Austria	8	2 (HM542051^{ht1}, HM542050^{ht1})
	Croatia	5	2 (HM542047^{ht1})
	Germany	5	4 (HM542049^{ht1}, HM542048^{ht1}, HM542045^{ht1})
	France	2	1 (HM542052^{ht1})
	Holland	2	
	England (UK)	5	1 (HM542046^{ht1})
+ ***Agriotes ustulatus*** (Schaller 1783)	Vienna / Lower and Upper Austria	3	
	Italy	7	3 (HM542057^{ht1}, HM542056^{ht1})
	Germany	7	2 (HM542054^{ht4})
	Croatia	5	1 (HM542053^{ht5})
	Slovenia	1∗	
	Hungary	7	3 (^{ht2}, HM542055^{ht3})
+ *Agriotes acuminatus* (Stephens 1830)	Vienna	1	1 (HM542011)
	Italy	2	2
Agriotes aequalis Schwarz 1891	Italy	2	1 (HM542012)
Agriotes gallicus Boisduval & Lacordaire 1835	Switzerland	6	2
	Germany	5	3 (HM542018)
Agriotes gurgistanus (Faldermann 1835)	Russia	1	1
	Bulgaria	2	1 (HM542019)
Agriotes infuscatus Desbr. des Loges 1870	Italy	4	2 (HM542020)
	Georgia	1	1
Agriotes medvedevi Dolin 1960	Slovakia	1	1
	Ukraine	1∗	1
Agriotes modestus Kiesenwetter 1858	Italy	1	
	Ukraine	1	1
+ *Agriotes pallidulus* (Illinger 1807)	Germany	2	2 (HM542034)
	France	1	1
Agriotes paludum Kiesenwetter 1859	Greece	4	1
	Turkey	1	1 (HM542035)
+ *Agriotes pilosellus* (Schönherr 1817)	Vienna / Lower and Upper Austria	1	
	France	1	1 (HM542036)
Agriotes turcicus Candèze 1863	unknown origin	1	1
	Turkey	1	

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Table 2. Non-target elaterid beetles and other soil invertebrates used to evaluate the specificity of the PCR assays.

Species	DNA extracts	COI sequences (GenBank Accession nos)
Elateridae		
Actenicerus sjaelandicus (O.F. Müller 1764)	1	1 (HM542009)
Adrastus rachifer (Geoffroy 1785)	1	1 (HM542010)
Agrypnus murinus (Linnaeus 1758)	5	1 (HM542058)
Athous bicolor (Goeze 1777)	1	1 (HM542059)
Athous vittatus (Gmelin 1790)	1	
Hemicrepidius niger (Linnaeus 1758)	5	1 (HM542060)*
Melanotus crassicollis (Erichson 1841)	1	
Melanotus villosus (Geoffroy 1785)	1	
Selatosomus aeneus (Linnaeus 1758)	1	1 (HM542061)
Cantharidae	1	
Carabidae	4	
Staphylinidae	3	
Scarabaeidae	3	
Brachycera	3	
Geophilidae	4	
Lithobiidae	1	
Acari	2	
Lumbricidae		
Aporrectodea rosea (Savigny 1826)	1	
Dendrobaena octaedra (Savigny 1826)	1	
Lumbricus terrestris Linnaeus 1758	1	
Octolasion lacteum Orley 1885	1	

*, outgroup for phylogenetic analysis (fig. 1).

water (Qiagen). The thermocycling program (executed on a Mastercycler Gradient, Eppendorf, Hamburg, Germany) consisted of an initial activation step of 15 min at 95°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C and a 10 min final extension at 72°C. PCR products were electrophoresed on 1.5% agarose gels stained with GelRed™ (Biotium, Hayward, USA) and visualized under UV light.

Sequencing and phylogenetic analysis

PCR products of specimens from several localities were purified with ExoSAP®-IT (GE Healthcare, Little Chalfont, UK) following the manufacturer's recommendation and subjected to cycle sequencing PCR (BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Foster City, USA) using the general primers described above. Following precipitation and cleanup, the resuspended PCR products were sequenced on a 3130 Genetic Analyzer (Applied Biosystems) in both forward and reverse directions. Sequences were aligned and edited manually using BioEdit Sequence Alignment Editor v7.0.9.0 (Hall, 1999) and representative sequences submitted to GenBank and BOLD (GenBank accession numbers listed in table 1). For the design of the species-specific primers a sequence alignment was generated comprising the *Agriotes* species (table 1) and the non-target elaterids (table 2).

The phylogenetic relationships among the nine core *Agriotes* species were examined using PAUP* v.4.0b10 (Swofford, 2002). As tree-building method, maximum parsimony was selected and *Hemicrepidius niger* served as an outgroup. The most parsimonious trees were inferred by a heuristic search with 100 random-addition-sequence replicates and the tree-bisection-reconnection (TBR) option. Node supports were evaluated by running 1000 replicates of bootstrap resampling (Felsenstein, 1985) and a 50%-majority-rule-consensus tree refined using MEGA v. 4.0.2 (Tamura *et al.*, 2007). Sequence divergences within and between species (same sequence alignment as used for phylogenetic tree, outgroup excluded) were calculated using the uncorrected *p*-distance option (Nei & Kumar, 2000).

Primer design and multiplex PCR

Primer Premier 5 (Premier Biosoft International, Palo Alto, USA) was used to design several primers targeting the nine core *Agriotes* species. We aimed to generate primers that amplify DNA fragments between 100–600 bp, which allows separation by simple agarose gel electrophoresis. Primers targeting conserved sites within two or more species were preferred in order to reduce the total number of primers in the reaction.

A diagnostic multiplex PCR assay was developed and optimized by gradient PCR, testing of different primer combinations and concentrations. Compared to standard singleplex PCR, multiplex PCR offers the advantage of being able to simultaneously screen for several species within one reaction, saving time and money. The optimized multiplex PCR was conducted in a total volume of 10 µl containing 1.5 µl of DNA extract, 5 µl of 2 × Multiplex PCR Master Mix (Qiagen), 1 µl of 10 × primer mix (primer concentrations provided in table 3) and 2.5 µl of PCR-grade RNase-free water (Qiagen). The thermocycling protocol included an initial activation step of 15 min at 95°C, followed by 35 cycles of 30 s at 94°C, 90 s at 61°C, 1 min at 72°C and a final step of 10 min at 72°C. Each PCR included both negative (PCR-grade RNase-free water instead of DNA) and positive controls (DNA of the *Agriotes* species under investigation). Multiplex PCR products were electrophoresed on 3% agarose gels stained with GelRed™ (Biotium) at 90 V for 35–40 min and band patterns visualized under UV light to identify the species. The peqGOLD Ultra LowRange DNA-Ladder II (25–700 bp; PeqLab, Erlangen, Germany) was used as a fragment size standard.

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Table 3. Specific primer pairs designed from COI sequences (mtDNA) to detect *Agriotes* species. Columns show the primer targets, primer names (S and A denotes forward and reverse primers, respectively), primer sequences, expected product size and concentration (Con.) of the primers in the multiplex PCRs (I, II).

	Target species	Primer names and sequences (5′-3′)	Size (bp)	Conc. (μM)
Multiplex PCR (I)	*Agriotes brevis/sputator*	Agr-gen-S212: AGATTTACAATGTTATTGTAACAGCA	168	0.3
		Agr-bre/spu-A215: AAGGTGGAAGAAATCAAAATCTC		0.45
	Agriotes rufipalpis	Agr-ruf-S214: GACTTCACTTAGCAGGGATATCT	192	0.3
		Agr-ruf-A217: GGTCTGTTAATAGTATAGTAATTGCC		0.3
	Agriotes sordidus	Agr-sor-S213: GGTATTTCTTCTATTCTTGGTGCT	225	0.25
		Agr-sor-A216: AGGGTCTCCTCCCCCC		0.25
	Agriotes lineatus/proximus	Agr-lin/pro-S211: CCCCTTCCCTCTCCCTG	293	0.45
		Agr-obs/lin/pro-A213: TGCTAAGACAGGTAAGGATAAAAGA		0.2
	Agriotes ustulatus	Agr-gen-S212: general *Agriotes* primer, listed above	323	see above
		Agr-ust-A214: TAAAATTGATGAAATTCCTGCC		0.15
	Agriotes obscurus	Agr-obs-S215: GAAATGACCAGATCTACAATGTTATC	464	0.15
		Agr-obs/lin/pro-A213: *A. obscurus/lineatus/proximus* primer, listed above		see above
	Agriotes litigiosus	Agr-gen-S212: general *Agriotes* primer, listed above	516	see above
		Agr-lit-A218: CTGCTGGGTCAAAAAATGAA		0.25
Multiplex PCR (II)	*Agriotes brevis*	Agr-gen-S212: AGATTTACAATGTTATTGTAACAGCA	168	0.4
		Agr-bre/spu-A215: AAGGTGGAAGAAATCAAAATCTC		0.2
		Agr-gen-S212: general *Agriotes* primer, listed above	462	see above
		Agr-bre-A522: TTGCCCCAGCTAATACTGGA		0.2
	Agriotes sputator	Agr-gen-S212: general *Agriotes* primer, listed above	168	see above
		Agr-bre/spu-A215: *A. brevis/sputator* primer, listed above		see above

Specificity testing of the multiplex PCR

The specificity of the multiplex PCR was evaluated using the 20 Central European *Agriotes* species (table 1). A minimum of ten individuals of each of the nine core species was tested, except for *A. rufipalpis* and *A. proximus* (seven and eight individuals only).

Non-target elaterid beetles (e.g. *Agrypnus murinus*, *H. niger*) and other soil invertebrates (mainly insect larvae), which typically can be found in the wireworms' environment, were collected in *Agriotes*-infested field sites (table 2), their DNA extracted as described above and tested.

Evaluation of the molecular identification protocol

The practical applicability of the protocol was evaluated by screening 905 *Agriotes* larvae, which were collected in several field sites in Austria. Larvae of unknown identity were stored in 96% ethanol prior to molecular analysis. A simple Chelex-based DNA extraction protocol (Traugott *et al.*, 2008b) was used to extract the DNA from these wireworms: the larval abdomen was cut to take a small piece of tissue, which was homogenized in a 1.5 ml reaction tube containing 20 μl PBS (pH 7.2, Sigma-Aldrich, St. Louis, USA) and 5 μl Proteinase K (20 mg ml^{-1}, AppliChem). Thereafter, 200 μl of 10% Chelex solution (Bio-Rad, Hercules, USA) was added, and the samples were incubated overnight at 58°C on a rocking platform followed by 15 min incubation at 94°C. Within each batch of 30 samples, two extraction negative controls were included to check for potential carry-over of DNA.

Results

Agriotes *barcoding sequences and phylogenetic analysis*

In total, 196 adult *Agriotes*, collected from 60 locations, were used to develop the molecular identification system (table 1). Fifty-three individual COI sequences were deposited in GenBank and BOLD (GenBank accession numbers see table 1), covering all *Agriotes* species investigated except *A. medvedevi*, *A. modestus* and *A. turcicus*, where sequence generation was not possible due to the low quality of the DNA. These reference sequences allow identifying DNA barcodes of 17 *Agriotes* species commonly found in Central Europe.

Basic phylogenetic analysis was conducted for the nine core *Agriotes* species, comprising 22 scorable haplotypes (table 1) with a mean interspecific sequence variability of 11.4%. Using 508 nucleotide-long-sequences, maximum parsimony tree reconstructions showed that conspecific sequences clustered together (fig. 1; 346 nucleotides were constant, 27 variable, but parsimony-uninformative, 135 parsimony-informative; tree length: 354 steps). Haplotypes of *A. brevis* and *A. sputator*, as well as *A. lineatus* and *A. proximus*, however, could not be separated clearly due to low interspecific variability between the species pairs (bootstrap-support values for nodes for *A. brevis*/*A. sputator* and *A. lineatus*/*A. proximus* were 100% and 99%, respectively). Interspecific sequences divergences between *A. brevis* and *A. sputator* ranged from 2.0% to 2.4% (intraspecific variability of *A. brevis*-haplotypes: 0.4–0.6%; *A. sputator*: one haplotype only). Likewise, interspecific sequences divergence between *A. lineatus* (intraspecific variability: 0.2–0.8%) and *A. proximus* (one individual only) ranged between 0.4–0.8% only, prohibiting the development of species-specific primers (see below).

The multiplex PCR-based identification protocol

For the nine core *Agriotes* species, primers were designed: species-specific primers for *A. litigiosus*, *A. obscurus*, *A. rufipalpis*, *A. sordidus* and *A. ustulatus*, as well as group-specific primers targeting *A. brevis/sputator* and *A. lineatus/*

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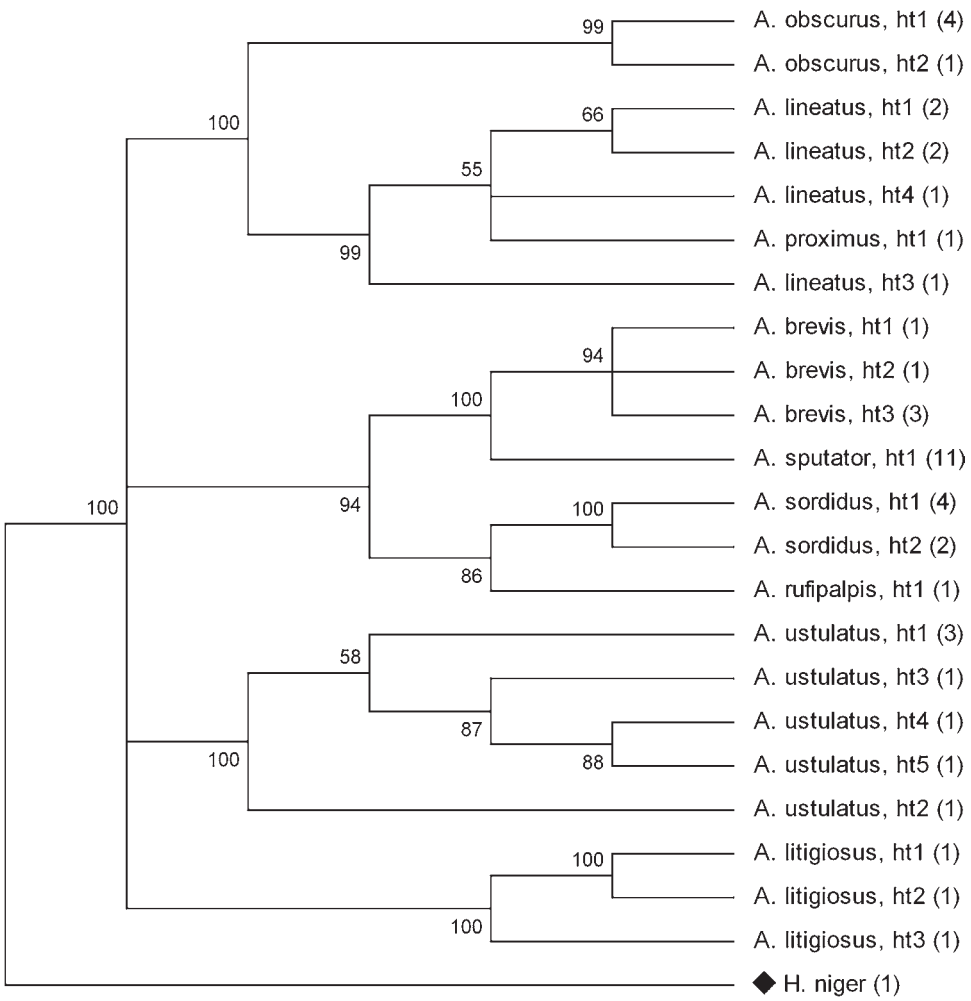

Fig. 1. Maximum parsimony tree of COI (mtDNA) sequences of *Agriotes* species. Bootstrap-support values (1000 replicates) greater than 50% are indicated above branches. Numbers beside *Agriotes* species indicate haplotypes (ht); numbers in parentheses refer to the total individual sequences. ◆, outgroup *Hemicrepidius niger*.

proximus. Eleven primers were put together in a first multiplex PCR (I), yielding DNA-fragments between 168–516 bp in size (table 3, figs 2a and A1 in Appendix) allowing to discriminate between these species/groups within a single-step reaction. Beside the desired PCR products amplified by the specific primers, additionally 530 bp and 455 bp fragments were amplified in *A. sordidus* and *A. lineatus*/*proximus*, respectively. These fragments resulted from the combination of the general *Agriotes* forward primer S212 with the reverse primers A216 and A213 (figs 2a and A1 in Appendix). Moreover, some variations in band patterns were found in several individuals: a ~180 bp fragment appeared in four out of the 11 *A. sordidus* tested, while the additional 530 bp fragment mentioned above was missing in another four larvae. In *A. ustulatus*, an additional ~500 bp fragment appeared in five out of ten individuals. These variations in band patterns were also observed when field-collected *Agriotes* larvae were assayed. Occasionally, additional non-specific faint bands could be observed (e.g. in *A. rufipalpis*; fig. 2a). The diagnostic bands, however, were always present.

To assign specimens testing positive for the *A. brevis*/*A. sputator*-primer pair to their respective species, a second multiplex PCR (II) was employed. The reaction mix and thermocycling conditions were the same as for the PCR (I), except that only three primers (S212, A215 and A522, for primer concentrations see table 3) were used and that the annealing temperature was 55°C for 3 min. Within this assay, *A. sputator* gave a single 168 bp PCR product (serving as an internal PCR control), whereas an additional 462 bp fragment was amplified with the DNA of *A. brevis* (figs 2b and A1 in Appendix).

The specificity of both multiplex PCR assays was tested with DNA extracts from all 20 *Agriotes* species (table 1), as well as from the other elaterid species and non-target invertebrates (table 2). No cross-reactivity was found within the corresponding size range (100–600 bp) of the diagnostic PCR products. Occasionally, an extra non-specific band of ~700 bp was observed for *H. niger* and two earthworm species (*Lumbricus terrestris*, *Octolasion lacteum*).

The application of the diagnostic PCR assays was evaluated using 905 field-collected *Agriotes* larvae. Eighty-three

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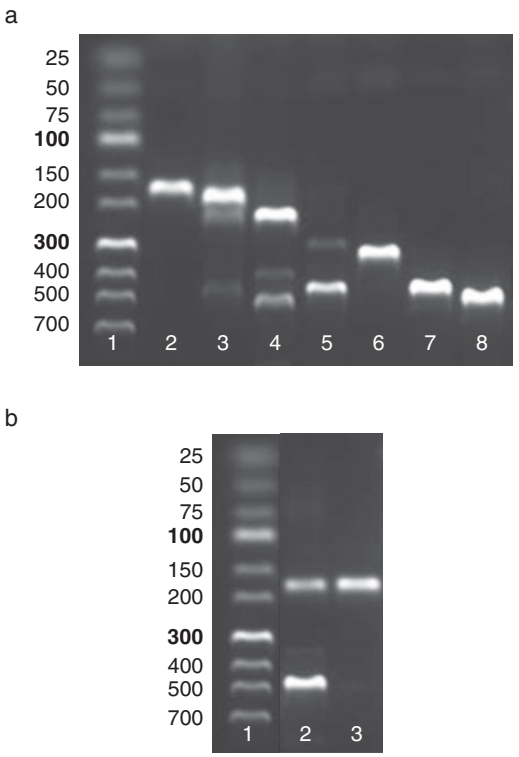

Fig. 2. (a) Multiplex PCR (I) products of the nine taxa targeted using specific primers. Lane 1: fragment size standard (25–700 bp). Samples from left to right: *Agriotes brevis/sputator*, 168 bp (2), *A. rufipalpis*, 192 bp (3), *A. sordidus*, 225 and 530 bp (4), *A. lineatus/proximus*, 293 and 455 bp (5), *A. ustulatus*, 323 bp (6), *A. obscurus*, 464 bp (7), *A. litigiosus*, 516 bp (8). (b) Multiplex PCR (II) products to identify *A. brevis* and *A. sputator*. Lane 1: fragment size standard (25–700 bp). Lane 2 and 3, *A. brevis* (168 and 462 bp) and *A. sputator* (168 bp), respectively.

percent of the larvae showed specific band patterns, allowing assignment of these specimens to one of the nine core *Agriotes* species (*A. lineatus/A. proximus* as a group). DNA extracts which failed in the multiplex PCR were retested in singleplex PCR using universal COI primers (see above). The amplified COI fragments were sequenced to assign the specimens to their species-specific DNA barcode using the blast algorithm (Altschul *et al*., 1990). This approach revealed that several '*Agriotes*' larvae were *Adrastus* sp. (Coleoptera, Elateridae), which are morphological very similar to *Agriotes* in larval stage. Less than 2% of the field-collected larvae (17 out of 905) did not provide amplifiable DNA.

Discussion

The molecular identification system developed in the present study comprises the provision of diagnostic barcoding sequences for 17 *Agriotes* species which occur in Central Europe as well as a multiplex PCR-based identification protocol for the nine most widespread and hence agriculturally most important species. These assays allow, for the first time, to reliably identify the Central European *Agriotes* species in pre-adult stage.

The present *Agriotes* barcoding is based on the 5′-end of the COI gene, which has been proposed as a universal 'barcode' (Hebert *et al*., 2003; Hajibabaei *et al*., 2007; Mitchell, 2008), resulting in a rapidly growing COI sequence database (e.g. Lindroth & Clark, 2009 for *Melanotus* wireworms occurring in the midwestern United States). Sequencing PCR products amplified with the universal COI primers (Folmer *et al*., 1994) offers reliable identification of *Agriotes* species by comparing the generated sequences with those we have deposited in GenBank. No barcodes could be generated for *A. medvedevi*, *A. modestus* and *A. turcicus* due to bad DNA quality. However, these three species have been taken into consideration for the establishment of the specificity of the multiplex PCR assays.

For the multiplex approach, we initially aimed at identifying all nine core *Agriotes* species within a single PCR. This was hampered by the limits to the number of primers which can be used within one PCR, especially with short fragments. In the present case, a subsequent, second multiplex PCR was required to separate between *A. brevis* and *A. sputator*. Likewise, Rugman-Jones *et al*. (2009a) identified six hemipteran species by multiplex PCR and were able to diagnose two additional species after digestion of PCR products with restriction enzymes. Similarly, PCR products obtained with general primers can be directly subjected to restriction enzyme digestion to provide diagnostic band patterns (e.g. Miller *et al*., 1999; Roehrdanz *et al*., 2009; Rugman-Jones *et al*., 2009b; Sumer *et al*., 2009). Ellis *et al*. (2009) were the first to molecularly discriminate between three *Agriotes* species found in the UK (*A. obscurus*, *A. lineatus* and *A. sputator*) by applying a T-RFLP technique. However, methods in addition to PCR require further investment of time and money. Moreover, Noel *et al*. (2004) found that the identification success rates were significantly lower in PCR-RFLP compared to multiplex PCR, especially when degraded DNA was examined. Our protocol, on the contrary, should also be suitable to identify badly preserved specimens and even semi-digested DNA (Lindahl, 1991; King *et al*., 2008), as we have chosen primer pairs amplifying rather short, yet easily distinguishable, fragments ranging from 168 to 530 bp. Another advantage inherent to PCR-based identification techniques is that only small amounts of tissue are necessary for successful analysis. The *Agriotes* larvae can still be used for other types of analysis (e.g. morphological examination) stored as voucher specimens or even re-analyzed after molecular analysis.

Multiple individuals from each *Agriotes* species collected from different regions in Europe and Canada were used to establish the molecular identification. This assures that both the barcoding and the multiplex PCR-based identification are not corrupted by intraspecific sequence variability. The multiplex PCR assay proved to be highly specific as the diagnostic band patterns were obtained only with the target species. Also, no cross-amplification was found with non-agrioted elaterid beetles, as well as with other soil-dwelling invertebrates. Other insect larvae, for instance those mistakenly identified as *Agriotes*, will not lead to false positive results in the case of being assayed.

The multiplex PCR-based identification protocol (see Appendix) allows rapid identification of large numbers of samples at low costs. Using this protocol, one person can process approximately 100 larvae within two days. Besides, it

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is simple enough to be implemented in most molecular labs, requiring only standard equipment, and is ideally suited for dealing with large numbers of samples, such as typically encountered in field surveys and/or routine diagnostic work. By replacing conventional agarose gel electrophoresis with automatic electrophoretic techniques such as QIAxcel (Qiagen) (Macfadyen *et al*., 2009), the efficacy of our approach can be enhanced even further. Similarly, Saccaggi *et al*. (2008) developed a molecular identification protocol which allowed differentiating between three mealybug species within four hours, demonstrating the feasibility of multiplex PCR for species identification.

The phylogenetic analysis of the nine core *Agriotes* species conducted within this study showed that sequence data is generally consistent with the current morphological species assignments (Lohse, 1979). The analysis showed a high similarity between *A. lineatus* and *A. proximus*. These species were almost identical when the complete COI gene and additional parts of the COII were examined (data not presented). Accordingly, there is also hardly any evidence for species separation when comparing morphological characters, as differences used in adult taxonomy are minor and restricted to pronotum hair coat and prothorax characteristics only (Lohse, 1979). These two species were also found to be attracted by the same pheromone baits in Portugal and Bulgaria (Subchev *et al*., 2005; Tóth & Furlan, 2005; Tóth *et al*., 2008). Unfortunately, the larvae of *A. proximus* have not yet been described (Klausnitzer, 1994), making a comparison of these two species in their larval stage impossible. Further research is required to provide deeper phylogenetic resolution, as these results raise the question of whether *A. lineatus* and *A. proximus* should still be seen as two separate species.

Our study has shown that both DNA barcoding and multiplex PCR are versatile tools for species-specific assignment of *Agriotes* larvae. This approach now facilitates studying wireworm ecology at a species-specific level, hence contributing to the development of novel control strategies against these soil pests.

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Appendix: The multiplex PCR-based identification protocol for Central European *Agriotes*

1 Storage of field-collected wireworms
- Store larvae individually in 70–90% ethanol or frozen.

2. Chelex-based DNA extraction
- Cut larval abdomen and take a small piece of tissue;
- Homogenize sample in a 1.5 ml reaction tube containing 20 µl PBS and 5 µl Proteinase K (20 mg ml^{-1}) and cool at 4°C;

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- Add 200 µl of 10% Chelex solution (slurry continuously mixed on magnetic stirrer) and incubate sample overnight at 58°C on a rocking platform;
- Incubate sample at 94°C for 15 min;
- Store DNA extract at –28°C.

3. Multiplex PCR (I) and (II)
   - Spin samples at 13,000 rpm for 5 min before using them in PCR;
   - PCR master mix:

	1× (µl)
PCR-grade water (Qiagen)	2.5
2× Multiplex PCR Master Mix (Qiagen)	5
10× primer mix MP I or II (table 3)	1
DNA extract	1.5
Total reaction volume	10

   - Thermocycling conditions:

Cycle number		°C	Time
1	Initial denaturation	95	15 min
35	Denaturation	94	30 sec
	Annealing*	61/*55*	1.5 min/*3 min*
	Extension	72	1 min
1	Final extension	72	10 min

*, Second multiplex PCR (II) with differing annealing temperature and duration in italic face.

4. Agarose gel electrophoresis
   - Use 3% agarose gel and LowRange DNA-Ladder to separate PCR products.

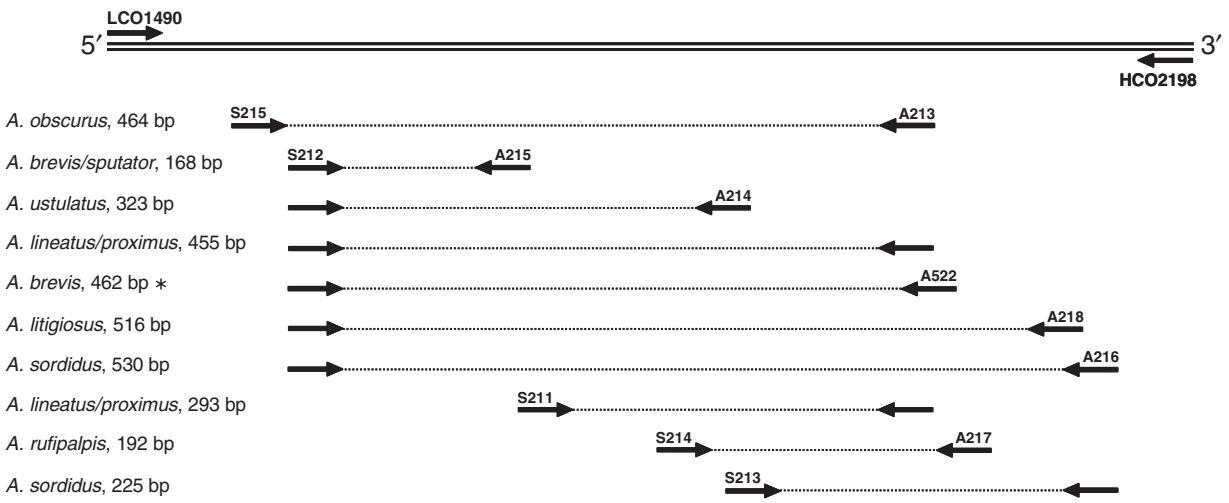

Fig. A1. Schematic overview of *Agriotes*-specific PCR products and corresponding primers (full primer names and sequences listed in table 3) targeting the COI gene (mtDNA). LCO1490 and HCO2198 are universal primers (Folmer *et al.*, 1994) comprising a 658 bp fragment. S and A denotes forward and reverse primers, respectively. A '∗' indicates the PCR product which appears in the second multiplex PCR (II) only.

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