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## Identification of *Agriotes* wireworms – Are they always what they appear to be?

Identifikation von *Agriotes*-Drahtwürmern –  
Sind sie immer, was sie zu sein scheinen?

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### Abstract

Wireworms are internationally recognized as economically important pests with the most damaging wireworms in middle European arable land belonging to the genus *Agriotes*. From the five species of greater importance in Germany, three (*A. lineatus*, *A. obscurus*, *A. sputator*) are very difficult to identify in their larval stage. Precise identification is crucial when trying to determine whether there are ecological differences between these three widespread species, e.g. in the reactions to different soil types, to soil moisture or in food choice. We report on the differentiation of *A. lineatus*, *A. obscurus* and *A. sputator* wireworms by comparing the identification by morphological traits of the larvae with the identification by PCR using a part of the mitochondrial DNA. Morphological traits given in commonly used identification keys for wireworms were not always reliable. Likewise the PCR did not always produce results, in part due to cross reactions. Therefore we developed new primers for reducing cross-reactions previously encountered. In the following, samples from different populations from the three species were compared to investigate if the proportion of aberrant specimens differed at different locations.

**Key words:** Wireworm, *Agriotes*, species identification, morphology, PCR

### Zusammenfassung

Die Schnellkäfergattung *Agriotes* beinhaltet einige der in Ackerbaukulturen schädlichsten Drahtwürmer. Von den

fünf in Deutschland als wirtschaftlich bedeutend eingestuften *Agriotes*-Arten sind insbesondere die drei Arten *A. lineatus*, *A. obscurus*, *A. sputator* im Larvalstadium schwierig zu unterscheiden. Eine genaue Identifikation ist jedoch entscheidend, um mögliche Unterschiede, zum Beispiel in der Präferenz für Bodenarten, für Bodenfeuchte oder in der Nahrungspräferenz, überhaupt zu erkennen. Aber wichtige morphologische Unterschiede, die in allgemein genutzten Bestimmungsschlüsseln für Drahtwürmer angegeben werden, ermöglichten nicht immer eine korrekte Bestimmung. Ebenso war es nicht in allen Fällen möglich, die Tiere über eine PCR zu identifizieren, da teils Kreuzreaktionen auftraten. Daher entwickelten wir neue Primer für die Arten. Nachfolgend wurden Populationen von verschiedenen Standorten untersucht, um zu überprüfen, ob die Anteile aberranter Exemplare sich zwischen den Standorten unterschieden.

**Stichwörter:** Drahtwurm, *Agriotes*, Artbestimmung, Morphologie, PCR

### Introduction

Wireworms are internationally recognized as economically important pests. Sometimes the occurrence of wireworms in the fields results in remarkable crop losses, while in other cases their presence does not cause serious damage. One reason for this may be species-specific ecological differences. Several observations (SCHAERFFENBERG, 1940; FRÖMMING and PLATE, 1953) but also more detailed studies on the food choice of wireworms (SCHAERFFENBERG,

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1939; TRAUGOTT et al., 2007) indicate that not all species behave the same way: there are predatory wireworms as well as phytophagous wireworms, and there are also species which may feed on both types of food. Additionally, even phytophagous wireworms differ in their preference for plant species. It has been shown that certain vegetable baits are more attractive than others (PARKER, 1996), that DNA of some plants is found more commonly in wireworms than DNA of others (WALLINGER et al., 2012), and that there are plant components that can deter feeding (VILLANI and GOULD, 1985), indicating that wireworms are not extreme generalists but that the species may actively choose preferred plants. However, as their development stretches over several years, wireworms are unlikely to be extreme specialists. Other ecological factors like moisture, soil type, humus content, differences in temperature preferences, egg laying preferences of adults or activity periods may also contribute to differences in damage between species. BLACKSHAW and VERNON (2008) have shown clear differences in the behaviour of adult click beetles, which may contribute to differences in wireworm distribution. The timing of feeding during the course of the year can be markedly different between species, and therefore different species can affect the same crop differently, as shown by FURLAN (2014) for the species *Agriotes ustulatus*, *A. sordidus* and *A. brevis* in maize. The feeding periods of *A. ustulatus* are markedly different from those of the other two, causing less damage to the crop. The three middle-European species investigated here (*A. sputator*, *A. lineatus*, *A. obscurus*) belong to the same developmental group and are not that different, but still their timing is not exactly the same and therefore some differences are likely to occur. Therefore, to better understand the damages caused by them, *Agriotes* wireworms need to be identified at species level.

Problems with the identification especially of commonly occurring *Agriotes* wireworm species are known for a long time (e.g. STAUDACHER et al., 2011; RUSEK, 1972). That is why wireworm damage is often reported as such, without being assigned to a certain species of *Agriotes* wireworm. Consequently we do not have sufficient knowledge to tell if there are species-specific preferences for certain crops, certain soils or certain environmental conditions. Recently, PCR surveillances have been demonstrated to be a suitable tool for identifying wireworms of the genus *Agriotes* up to the species level (STAUDACHER et al., 2011; BENEFER, 2011) and have also been used for wireworms of other genera in Canada (BENEFER et al., 2013). However, though this technique is a great help to identify morphologically difficult specimens it is not always available. For the plant protection services morphological identification in routine tests may be the most reliable and swiftest option provided that the morphological traits used are reliable.

In this survey we report on the reliability of the three main morphological determinants used for separating the three most widespread *Agriotes* species in Germany. It was shown that two of these traits are reliable in the majority of individuals, but that there is a certain percentage in each species that would be misidentified based on their mor-

phology. The third trait, the angle of the mandible tooth, is much less reliable as it is subject to severe abrasion.

We also report on the differentiation of *A. lineatus*, *A. obscurus* and *A. sputator* wireworms by comparing morphological determinants of the larvae with the obtained PCR results using a part of the mitochondrial DNA. In this study we used an already sequenced mitochondrial fragment (STAUDACHER et al., 2011) and developed new primers for reducing cross-reactions previously encountered. It was shown that the newly synthesized primers are suitable to distinguish all three *Agriotes* species even in a combined (multiplex) PCR, where all three primer pairs have been applied in one reaction mix.

## Material and methods

### Origin of wireworms

Wireworms of the genus *Agriotes* were collected at different field sites for several years or reared at the Julius Kühn-Institut (JKI) in Braunschweig in 2013.

The field-collected wireworms (Tab. 1 and 2) originated mainly from a meadow near the Julius Kühn-Institut (Braunschweig, Messeweg 11/12), and were collected in 2011. The specimens from Tab. 3 were collected from potato fields in the vicinity of Uelzen (Lower Saxony), from potato fields in Deggendorf, Olching and Klotzau (Bavaria) and from a wheat field in the region of Tübingen (Baden-Württemberg) and were collected in 2013.

The specimens listed in Tab. 4 and 5 were not collected in the field. They were bred from known parents (adult beetles collected from the field) at the JKI wireworm breeding, which is described in more detail below, and from the wireworm breeding at Agroscope Reckenholz, Zürich, Switzerland (parents *A. obscurus* from Bärau, Berner Oberland and *A. lineatus* from Zürich, method after KÖLLIKER et al., 2009).

The specimens from Tab. 6 were from six different sites (Neubörger, Dasselsbruch, Westenholz from Lower Saxony in 2011, Münzenberg from Hesse in 2011, Hildburghausen and Oldisleben from Thuringia in 2013) from the nationwide click beetle and wireworm monitoring (see LEHMHUS, 2012, 2014).

Further tests were performed with about 40 morphologically unidentifiable *Agriotes* wireworms from field sites in the vicinity of Braunschweig.

To establish if there might be any indications of differences in feeding preferences from different species, wireworms from eight different potato fields were identified (Fig. 6) (Deggendorf, Olching and Klotzau in Bavaria, Natendorf, Oldendorf, Uelzen-Barnstedt, Uelzen-Borg and Zargleben in Lower Saxony).

### Wireworm breeding from click beetles at the JKI

The wireworm breeding was established to obtain larvae with known parentage for species comparison in the larval stage. The male and female click beetles for the wireworm breeding originated from an extensive meadow surrounded by, named Braunschweig in Tab. 5, and in

case of *A. obscurus* in 2014 also from Suderburg close to Uelzen, Lower Saxony. The method used for catching the adult beetles of both sexes consists of plastic sheets spread on the ground and covered with cut grass under which the click beetles aggregate (KÖLLIKER et al., 2009). Click beetles used for the laboratory rearing originated from populations from a single site, an extensive grassland close to Braunschweig. After catching, male and female click beetles of the three species were kept at 15 °C for a maximum of two weeks and were fed with bee pollen. Afterwards they were kept at 20–25 °C in pots with 25 (15 in 2014) beetles in a climatic chamber following KÖLLIKER et al. (2009). Larvae were reared using wheat as food plant instead of a mixture of four grasses as published by KÖLLIKER et al. (2009). The wheat was immediately resown when it started to look unhealthy. After seven months the largest larvae in the rearing pots were suitable for morphological identification. After 12 months the majority of wireworms had reached a length of about 2 cm. The number of larvae ranged from 120–490 specimens per pot and was lowest in the *A. sputator* pots and highest in the *A. obscurus* pots.

#### Morphological identification of wireworms

The wireworms were identified up to the species level following dichotomous identification keys using morphological traits (COCQUEMPOT et al., 1999; KLAUSNITZER, 1994). This was done under a stereo microscope (Zeiss Discovery V8). The criteria used were the angle of the “substitute” tooth of the mandible to the tip of the mandible (KLAUSNITZER, 1994; RUSEK, 1972), the occurrence or absence of granulation between the legs (COCQUEMPOT et al., 1999) and at the base of each abdominal segment (KLAUSNITZER, 1994; RUSEK, 1972), and the occurrence or absence of a small additional seta above the lateral stigma of the abdominal segments 1–8 (COCQUEMPOT et al., 1999). For determining the angle of the “substitute” tooth of the mandible, a scale with angles of 50°–130° in steps of 10° was used. Live wireworms were cooled down to 5 °C prior to morphological identification to reduce movement during identification.

#### PCR-detection of *Agriotes* species

The PCR was performed according to STAUDACHER et al. (2011). However, we generated new primers using the mitochondrial DNA sequence of *Agriotes* published in the NCBI-data bank (HM542025.1 *lineatus*, HM542029.1, *obscurus*).

After the morphological determination of the wireworms, parts of the worm were extracted and the DNA was extracted using the Qiagen DNA columns (DNeasy Plant mini Kit) according to the manufacturer’s instructions (Qiagen, Hilden). The DNA was collected and stored frozen.

A standard PCR was performed in a thermo cycler. The temperature settings were: 3' at 96 °C, 35 cycles: 30'' at 96 °C, 30'' at 63 °C, 30'' at 72 °C, and finally 5' at 72 °C. Taq-polymerase (0.5 units; ..... ) was used with an appropriate nucleotide mixture. The PCR-product was then separated on a 0.7% agarose gel and photographed.

#### Sequences of the primers used

The following primer pairs for the detection of the three wireworm species were used:

<i>Agriotes sputator</i> :	Aspuf: 5' tccaccttcattgtctctctattaatg 3'
<i>Agriotes lineatus</i> :	Aspur: 5' tactgtagagataaaagtagcaataaa 3'
	Alinf: 5' cccctccctctccctg 3'
	(STAUDACHER et al., 2011)
	Alinr: 5' atttaagtttcggcagtaataagtatt 3'
<i>Agriotes obscurus</i> :	Aspuf: 5' cagtggaatcactgcactac 3'
	Aspur: 5' gaacaggtgctgataaaggatg 3'

The three primer pairs were used in a 10 µMol concentration and added to the reaction mix prior to the PCR-amplification step.

## Results

#### Differentiation of *Agriotes sputator* from *Agriotes lineatus* and *Agriotes obscurus*

Wireworms were identified morphologically, dead and alive. According to relevant keys *A. sputator* can fairly easily be separated from the other two widespread *Agriotes* wireworms causing damage in Germany. The identification traits used are described as follows: *A. sputator* has a region of small granules at the base of each abdominal segment, as described by KLAUSNITZER (1994), and additionally small granules on the coxae between the legs, as described by COCQUEMPOT et al. (1999). Both these traits are found to be well visible if present and are shown in Fig. 1, while Fig. 2 depicts the absence of these traits.

When determining live wireworms compared to wireworms killed prior to their identification, a higher error rate occurred in the live, and therefore moving wireworms (Tab. 1). In contrast, morphological identification of dead larvae appeared straightforward following the two keys (KLAUSNITZER, 1994; COCQUEMPOT et al., 1999). Using PCR it was difficult to assign wireworms to a single species for unknown reasons. Here, 65.6% (40 out of 61 wireworms) were identified as *A. sputator* by PCR and morphological identification. 14.8% (nine wireworms) were morphologically identified as *A. sputator* but no clear results were obtained by PCR. Seven specimens were morphologically identified as *A. sputator* when alive but as other species when dead.

A subsample of 12 of these morphologically identified larvae was analyzed by a further laboratory (LTZ Augustenberg) using the same PCR-method. For this subsample the results obtained for *A. sputator* were similar for all institutions and methods while the results for other species differed. Although the PCR-protocol used followed STAUDACHER et al. (2011) in both laboratories, cross-reactions with primers for two species (in most cases *A. lineatus/A. obscurus*) were again observed in the JKI laboratory but not in the LTZ Augustenberg laboratory though some wireworms here also showed a very weak reaction with the primers for a 2<sup>nd</sup> species.

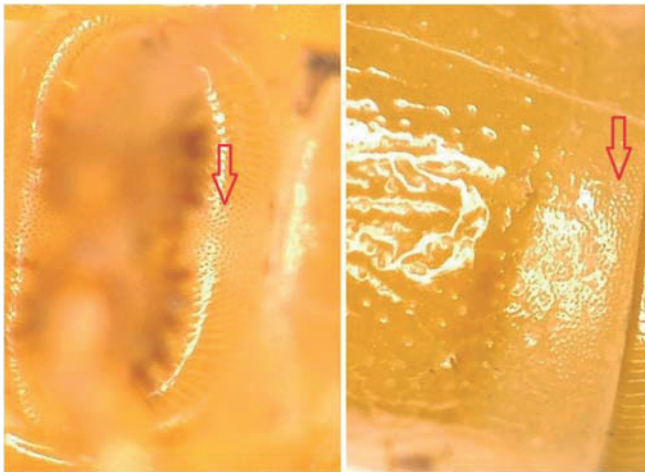


Fig. 1. Granules between the legs (left) and at the base of abdominal segments (right) identify *Agriotes sputator*. Photographed specimen from the wireworm rearing at JKI.

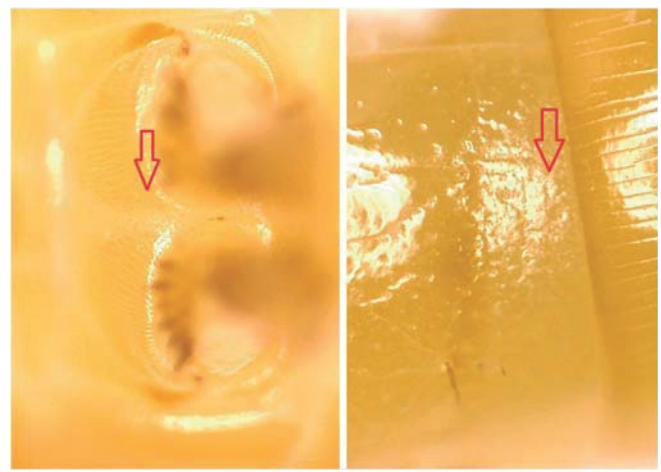


Fig. 2. No granules between the legs (left) and no granules at the base of abdominal segments (right) indicate that this is either *A. lineatus* or *A. obscurus*. Photographed specimen from the wireworm rearing at JKI, in this case *A. obscurus*.

Tab. 1. Identification results 2011 for 61 *Agriotes* wireworms from Braunschweig (Messeweg 11/12), a site where *A. sputator* is the dominant species. Identification has been performed by the presence or absence of fine granules at the base of each abdominal segment (KLAUSNITZER, 1994) and fine granules between the legs (COCQUEMPOT et al., 1999)

Samples	Morphological identification of live specimens	Morphological identification of dead specimens	PCR	Number of cases	%
31	<i>Agriotes sputator</i>	<i>Agriotes sputator</i>	<i>Agriotes sputator</i>	40	65.6
9	Not possible since too active	<i>Agriotes sputator</i>	<i>Agriotes sputator</i>		
3	<i>Agriotes sputator</i>	<i>Agriotes sputator</i>	No reaction specimen identified as several species	9	14.8
6	<i>Agriotes sputator</i>	<i>Agriotes sputator</i>			
6	<i>Agriotes sputator</i>	other species	other species specimen identified as several species	7	11.5
1	<i>Agriotes sputator</i>	other species			
5	other species	other species	other species	5	8.2

Tab. 2. Identification results 2011 for 13 *Agriotes* wireworms from Braunschweig, Messeweg 11/12 (these are included above), a site where *A. sputator* is the dominant species. PCR by two laboratories

Species	Morphological identification of living specimens/JKI-A	Morphological identification of dead specimens/JKI-A	PCR/LTZ Augustenberg	PCR/JKI-EP
<i>A. sputator</i>	8	7	7	7
<i>A. obscurus</i>	5	6	4	0
<i>A. lineatus</i>	0	0	2	0
<i>A. lin./obs.</i>	0	0	0	6*

\* reaction with primers for both species

Further PCR tests had the same result with cross reactions occurring in about 40 specimens from the vicinity of Braunschweig that were morphologically either *A. lineatus* or *A. obscurus*, but were not definitely identifiable due to abrasion. With the PCR following the method by STAUDACHER et al. (2011) each individual was assigned to both species. The reason remained unknown. Therefore, the primer development as the following step was undertaken for *Agriotes lineatus* and *Agriotes obscurus*.

#### Development of new mitochondrial primers to differentiate between *A. lineatus* and *A. obscurus*

The DNA sequences of the *Agriotes* species from the NCBI-databank revealed almost similar sequences of the mitochondrial cytochrome oxidase subunit 1 gene, differing only by several nucleotides within each single species. For the generation of suitable primers we chose those regions which differed in several point mutations from each other (results are not shown). A typical multiplex PCR is depicted in Fig. 3? when the primer combination (see 'Material and methods') of the three *Agriotes* species was used.

*Agriotes lineatus* is lacking the smaller seta of two setae close to the stigma in abdominal segments 1–8 while *A. obscurus* (and also *A. sputator*) shows this seta (see Fig. 5) according to the key provided by COCQUEMPOT et al. (1999). Additionally the angle of the small "substitute" tooth to the tip of the mandible is narrower in *A. lineatus* than in *A. obscurus* (Fig. 4), but the difference appears often less marked than suggested in the identification keys using this trait (SCHAERFFENBERG, 1940; KORSCHESKY, 1941; KLAUSNITZER, 1994).

The 48 specimens from Tab. 3 were collected from potato fields in the vicinity of Uelzen (Lower Saxony), from potato fields in Deggendorf, Olching and Klotzau (Bavaria) and from a wheat field in the region of Tübingen (Baden-Württemberg).

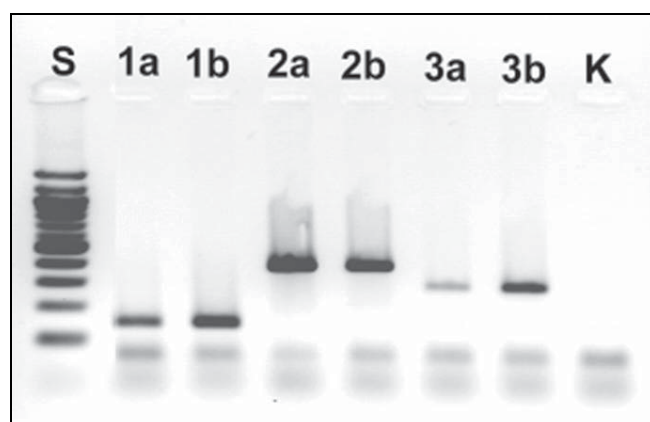


Fig. 3. Multiplex-PCR with the three *Agriotes* species *obscurus* (1, 146 bp), *lineatus* (2, 289 bp) and *sputator* (3, 289 bp) in two repeats (a, b). K represents the water control and S the size standard (100 bp ladder). There is no cross-reaction occurring between the single species, when applying the multiplex-PCR. The size of the PCR-fragments obtained from the species can easily be differentiated.

In 21 of the 48 specimens (43.8%) no morphological identification was possible following the mandible, compared to only two specimens (4.2%), where no morphological identification was possible following the setae. However, in 9 cases (18.8%) differences between morphological identification and identification by PCR were observed. 10 of these wireworms (20.8%) did not react to any of the primers, although it was ensured that DNA was in the samples.

To test the accuracy of the results obtained, 31 larvae with known parentage (11 *A. lineatus*, 10 *A. obscurus* and 10 *A. sputator*) from the wireworm breeding at the JKI, established in 2013, were subjected to morphological and molecular species determination. Their parentage was known as they were offspring from adult click beetles caught at a single field site (Schandelaher Wohld) (methods according to KÖLLIKER et al., 2009, modified). These click beetles were morphologically identified following LOHSE (1979) and LAIBNER (2000).

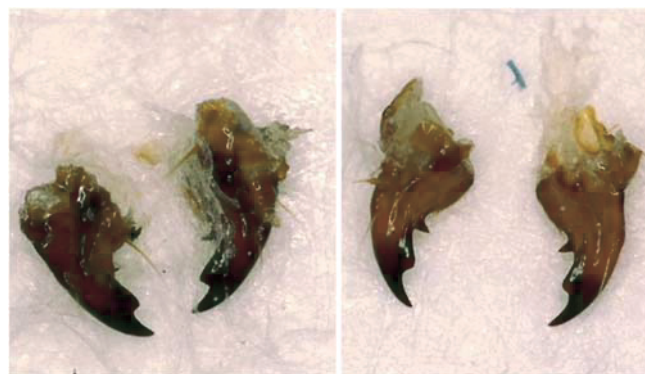


Fig. 4. "Substitute" tooth angle of *A. lineatus* (left) and *A. obscurus* (right). The angle is narrower in *A. lineatus* than in *A. obscurus*, but the difference appears often less marked than suggested in the identification keys using this trait (SCHAERFFENBERG, 1940; KORSCHESKY, 1941; KLAUSNITZER, 1994). Specimens from the wireworm rearing at JKI.

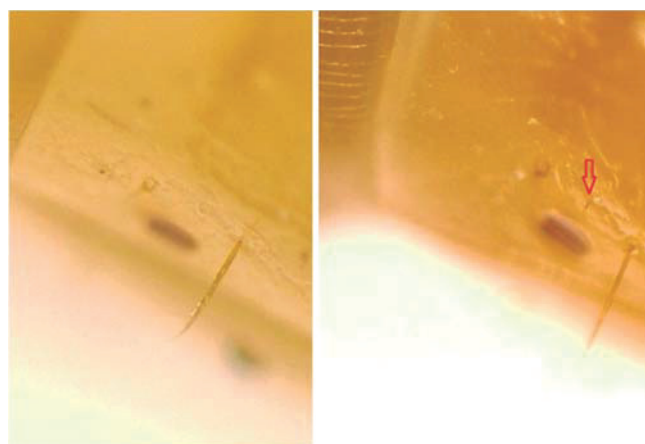


Fig. 5. Setae above the stigma of *A. lineatus* (left) and *A. obscurus* (right). Apart from the large seta to the right of the stigma a fairly small, sometimes stubby seta is found above the stigma in *A. obscurus* and *A. sputator*. This seta is supposed to be absent in *A. lineatus* according to COCQUEMPOT et al. (1999). Photographed specimens came from the wireworm breeding at JKI.

**Tab. 3. Separation of *Agriotes lineatus* and *Agriotes obscurus*, 48 wireworms tested (alcohol specimens). *lin* = *A. lineatus*, *obs* = *A. obscurus*, \* reasons unknown as DNA was in the sample. Differences between morphological and PCR results are highlighted in bold print**

No. of cases	Morphology mandible (angle of “substitute tooth” to tip of mandible)	Morphology setae (2 <sup>nd</sup> seta above lateral stigma in abdominal segments 1–8 absent or present)	PCR
7	<i>obs</i>	<i>obs</i>	<i>obs</i>
14	too abraded	<i>obs</i>	<i>obs</i>
2	<i>obs</i>	3 segments without 2nd seta	<i>obs</i>
2	<i>obs</i>	<i>obs</i>	No reaction*
6	too abraded	<i>obs</i>	No reaction*
1	<b><i>obs</i></b>	<b><i>obs</i></b>	<b><i>lin</i></b>
5	<b><i>lin</i></b>	<b><i>obs</i></b>	<b><i>obs</i></b>
1	<b><i>lin</i></b>	<b><i>obs</i></b>	<b><i>lin</i></b>
3	<i>lin</i>	<i>obs</i>	No reaction*
1	<b>too abraded</b>	<b><i>obs</i></b>	<b><i>lin</i></b>
1	<b><i>lin</i></b>	<b><i>lin</i></b>	<b><i>obs</i></b>
1	<i>lin</i>	<i>lin</i>	No reaction*
4	<i>lin</i>	<i>lin</i>	<i>lin</i>

The wireworm species identification via PCR confirmed all larvae to be the species expected from the parents used in the breeding. The morphological identification of

the wireworms was less straightforward. Concerning the setae in the abdominal segments already two specimens of *A. obscurus* occurred in this sample where morphological identification was difficult. Furthermore, in one specimen of *A. lineatus* morphological identification following the setae would have resulted in identification as *A. obscurus* (Tab. 4).

For further investigation into the frequency of aberrant individuals in populations of the three species, a number of individuals of each species *A. lineatus*, *A. obscurus* and *A. sputator* from different populations from wireworm breedings at the JKI and the Agroscope Reckenholz were examined morphologically. The wireworm specimens from JKI being alive, the angle of the substitute tooth could be examined only in those opening their mandibles. The results concerning the frequency of aberrant individuals from these populations of the three species are given below (Tab. 5). The average angle of the “substitute” tooth to the tip of the mandible differs between species, but it was not possible to safely determine the species from the mandible. The angle differed markedly even between individuals of the same species, while there was considerable overlap between species. The second distinguishing trait used was the granulation between legs and at the base of each segment, which only 2% of the *A. sputator* did not show. The third trait was the small seta that should be present in *A. obscurus* (and *A. sputator*), but absent in *A. lineatus*. Contrary to the key, this seta was absent in some *A. obscurus* and present in some *A. lineatus* in all populations.

These data originated only from populations from 3 field sites for *A. obscurus*, from 2 field sites for *A. lineatus*

**Tab. 4. Separation of *Agriotes lineatus*, *Agriotes obscurus* and *Agriotes sputator*, 31 wireworms from the JKI wireworm breeding from 2013 tested (alcohol specimens, parentage known)**

From wireworm rearing of species	Morphology mandible (angle of “substitute tooth” to tip of mandible)	Morphology setae	PCR	Comments/Morphological description
<i>A. sputator</i>	10 <i>A. sputator</i>	10 <i>A. sputator</i>	10 <i>A. sputator</i>	All specimens with a mandible angle wider than 90° and with 2nd seta. All specimens with granules at the base of abdominal segments and between legs.
<i>A. obscurus</i>	10 <i>A. obscurus</i>	10 <i>A. obscurus</i>	10 <i>A. obscurus</i>	All specimens with a mandible angle wider than 90° and with 2nd seta. No granules at the base of abdominal segments and between legs. Two specimens with extremely small 2nd seta in abdominal segments 1–8, but normally sized in other segments.
<i>A. lineatus</i>	11 <i>A. lineatus</i>	10 <i>A. lineatus</i> 1 <i>A. obscurus</i>	11 <i>A. lineatus</i>	All specimens with a mandible angle of 80° or smaller. No granules at the base of abdominal segments and between legs. One specimen with small 2 <sup>nd</sup> seta in all abdominal segments. Two specimens with small 2nd seta in two segments, 8 specimens without the 2nd seta in abdominal segments 1–8.

**Tab. 5. Frequency of aberrant individuals of *Agriotes lineatus*, *Agriotes obscurus* and *Agriotes sputator* from the wireworm breedings at JKI and Agroscope Reckenholz. Braunschweig and Suderburg specimens examined alive, Zürich and Bärau specimens in alcohol. As some specimens did not open their mandibles or mandibles were too abraded, the angle of the substitute tooth to the tip of the mandible could not be examined in all cases. n = number of specimens examined**

Species	Site	Number of specimens n	Angle of "substitute" tooth to tip of mandible (following KLAUSNITZER, 1994)	"Aberrant" specimens concerning granulation and 2 <sup>nd</sup> seta (following COCQUEMPOT et al., 1999)
<i>A. obscurus</i>	Bärau (CH) 2013	44	105° (80–140°) n = 43	7 (15.9%)
<i>A. obscurus</i>	Suderburg (DE) 2014	52	106° (80–120°) n = 33	7 (13.5%)
<i>A. obscurus</i>	Braunschweig (DE) 2013	200	101° (60–120°) n = 121	18 (9.0%)
<i>A. obscurus</i>	Braunschweig (DE) 2014	51	105° (80–140°) n = 29	4 (7.8%)
<i>A. lineatus</i>	Zürich (CH) 2013	22	90° (70–130°) n = 18	3 (13.6%)
<i>A. lineatus</i>	Braunschweig (DE) 2013	200	78° (60–120°) n = 144	13 (6.5%)
<i>A. sputator</i>	Braunschweig (DE) 2013	200	94,8° (60°–120°) n = 157	4 (2.0%)

"Aberrant" specimens: *A. sputator*: Specimens with only weak granules at the base of abdominal segments or between legs. *A. obscurus*: Specimens with extremely small, nearly invisible 2<sup>nd</sup> seta in all segments or with second seta missing in several segments. *A. lineatus*: Specimens with small 2<sup>nd</sup> seta in all abdominal segments.

and from 1 field site for *A. sputator*. In other populations of these three species the percentage of aberrant specimens may be different. Therefore, in a comparison of the PCR and morphological determination in different *Agriotes* populations 60 alcohol-conserved specimens from six different sites, collected in the German click beetle monitoring, were examined. In this assessment wireworms were identified morphologically with the key of COCQUEMPOT et al. (1999). For the PCR the newly developed primers were used. In 50 cases congruent results were found between PCR and morphological identification, but in 10 cases results differed. Three (= 5% of the 60 specimens) morphologically not reliably identifiable specimens due to extreme abrasion (mandibles reduced to stumps and most setae lacking) were identified as *A. obscurus* via PCR. One wireworm (= 1.7% of the 60 specimens) appeared to be *A. obscurus* morphologically, showing no obvious granules between legs and at the base of abdominal segments 1–8, but was assigned to *A. sputator* by PCR. Six wireworms (= 10%) were identified as *A. lineatus* via PCR, but had been assigned morphologically to the species *A. obscurus* due to the occurrence of the small second seta above the stigma of the abdominal segments 1–8. Three of these aberrant *A. lineatus* specimens were from a single site (Westenholz, Lower Saxony) while the others came from different sites. Out of six different sites, four had morphologically aberrant wireworms even in this comparatively small number of samples.

Some further *A. lineatus* populations were already tested from three sites in Lower Saxony: Uelzen (PCR 41

*A. lineatus*, 14.6% with 2<sup>nd</sup> seta on more than two segments, 31.7% with 2<sup>nd</sup> seta on at least one segment), Dasselsbruch (PCR 14 *A. lineatus*, 21.4% with 2<sup>nd</sup> seta on all segments, 50% with 2<sup>nd</sup> seta on at least one segment) and Vahlde (PCR 13 *A. lineatus*, one individual, 7.7% with 2<sup>nd</sup> seta on all segments).

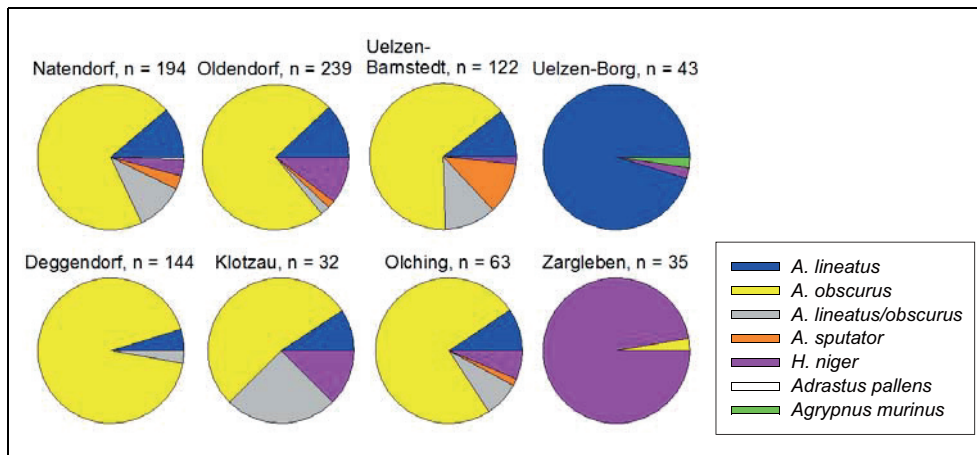
The importance of correct species identification is illustrated by the species spectrum of wireworms from eight different sites in potato. Substantial numbers of wireworms were collected in potato from eight sites (four sites close to Uelzen/Lower Saxony, three sites in Bavaria, one site in eastern Lower Saxony) to determine whether a species preference for a certain crop might occur. The identification was done mainly morphologically, but problematic specimens were additionally tested by PCR. These data suggest that there may indeed be preferences of certain species to certain crop situations, although some wireworms were too severely damaged during the potato harvest or during postal transport to be assigned to species. They were only identified as *A. lineatus*/*A. obscurus*. The data showed some similarities for six sites with the dominance of *A. obscurus* and occurrence of two to three further species, while the two other sites differed markedly (Fig. 6).

## Discussion

The three wireworm and click beetle species investigated (*A. sputator*, *A. lineatus*, *A. obscurus*) are the most widespread species in agricultural areas in Germany (LEHMUS,

**Tab. 6.** Separation of wireworms (mainly *Agriotes lineatus* and *Agriotes obscurus*) from six different sites, 60 wireworms tested, 10 from each site (alcohol specimens). Few specimens of *Agriotes sputator* also occurred in these samples. *lin* = *A. lineatus*, *obs* = *A. obscurus*, *sput* = *A. sputator*, ? = identification traits too abraded for morphological identification. Differences between morphological and PCR results are highlighted in bold print

No.	Site											
	Neubörger Lower Saxony 2011		Dasselsbruch Lower Saxony 2011		Westenholz Lower Saxony 2011		Münzenberg Hesse 2011		Hildburghausen Thuringia 2013		Oldisleben Thuringia 2013	
	morph	PCR	morph	PCR	morph	PCR	morph	PCR	morph	PCR	morph	PCR
1	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<b>obs</b>	<b>lin</b>	<i>lin</i>	<i>lin</i>	<i>obs</i>	<i>obs</i>	<i>obs</i>	<i>obs</i>
2	<i>lin</i>	<i>lin</i>	<b>obs</b>	<b>lin</b>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>obs</i>	<i>obs</i>	<i>obs</i>	<i>obs</i>
3	<i>lin</i>	<i>lin</i>	<i>obs</i>	<i>obs</i>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>obs</i>	<i>obs</i>	<i>obs</i>	<i>obs</i>
4	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>spu</i>	<i>spu</i>	<i>obs</i>	<i>obs</i>	<b>obs</b>	<b>spu</b>
5	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>obs</i>	<i>obs</i>	?	<b>obs</b>
6	<i>lin</i>	<i>lin</i>	<i>obs</i>	<i>obs</i>	<b>obs</b>	<b>lin</b>	<i>lin</i>	<i>lin</i>	<i>obs</i>	<i>obs</i>	?	<b>obs</b>
7	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>obs</i>	<i>obs</i>	<i>obs</i>	<i>obs</i>
8	<i>lin</i>	<i>lin</i>	?	<b>obs</b>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>obs</i>	<i>obs</i>	<i>obs</i>	<i>obs</i>
9	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>spu</i>	<i>spu</i>	<i>obs</i>	<i>obs</i>	<i>lin</i>	<i>lin</i>
10	<i>lin</i>	<i>lin</i>	<i>lin</i>	<i>lin</i>	<b>obs</b>	<b>lin</b>	<i>lin</i>	<i>lin</i>	<b>obs</b>	<b>lin</b>	<b>obs</b>	<b>lin</b>
<b>Aberrant specimens [%]</b>	<b>0</b>		<b>20</b>		<b>30</b>		<b>0</b>		<b>10</b>		<b>40</b>	



**Fig. 6.** Results of wireworm identification from samples from potato. Identification by morphology, of problematic specimens additionally by PCR. Some damaged specimens could not be identified by PCR. Four sites close to Uelzen/Lower Saxony (upper row), three sites in Bavaria (lower row), one site eastern Lower Saxony (Zargleben, lower row, right). n = number of wireworms.

2012; LEHMUS, 2014) and in neighbouring countries (JOSSI et al., 2008; ELLIS et al., 2009; LOLE, 2010). In contrast to the adult *Agriotes* click beetles, wireworms of the genus *Agriotes* show only a few traits suitable for morphological identification. For example, a major trait helping to separate *A. lineatus* from *A. obscurus*/*A. sputator* is the absence or presence of an additional seta (bristle) above the stigma on the first to the eighth abdominal segment (COCQUEMPOT et al., 1999). Additionally, the shape of the small additional tooth of the *Agriotes* wireworm mandible and its angle to the tip of the mandible were used for identification (KLAUSNITZER, 1994; KORSCHESKY, 1941; RUSEK, 1972; DOLIN, 1978). *A. sputator* shows an additional trait, an area covered by fine regular granules at the base of each abdominal segment (KLAUSNITZER, 1994) and between the

legs (COCQUEMPOT et al., 1999). These three traits seem to enable an easy identification of *Agriotes* wireworms from the three different species. We also tried to find further species-distinguishing morphological traits but none were consistent throughout the samples so far, though there is a tendency for *A. lineatus* larvae to be lighter in colour and to appear slightly shorter in comparison to width than the other two species.

Since different *Agriotes* species can show differences in their biology and therefore can differ in their impact on a crop (FURLAN, 2014), correct identification of species is important in practice. But if the commonly used keys do not result in correct identification of *Agriotes* species, the clarification of which species cause damage in which crop becomes problematic. In addition, in the majority of



studies on *Agriotes* ecology and behaviour field-collected larvae are used. The outcome of experiments may be seriously affected by the difficulties in morphological identification of these cryptic pests. Therefore, morphological and molecular determination of *Agriotes* wireworms was compared here directly.

The morphological identification traits in *Agriotes* are vulnerable and may in part be lost. We consider the likely explanation an abrasion caused by the wireworm movement in the soil. With a range of 60°–120° for the mandible tooth angle for each of the three species, it seems that abrasion of the mandible can alter the angle in both directions, making it either narrower or broader. Abrasion makes this trait so variable that differences between species emerge only in the average angle (e.g. for Braunschweig 2013: *A. sputator* 94,8°, *A. obscurus* 100,6°, *A. lineatus* 78,3°). The differences between the angles were much less marked than the angles given in KLAUSNITZER (1994), i.e., *A. sputator* 90°, *A. obscurus* 120°, *A. lineatus* 60°. This trait is therefore only usable as additional information but not as a key feature for identification.

Though the setae are less vulnerable to abrasion, they also may break due to movements through the soil. If this happens it may be possible to see the socket where the seta is inserted, but only under good magnification. Abrasion is therefore one reason for specimens being not reliably identifiable.

Yet not all difficulties are explained by abrasion or loss of setae. The regular occurrence of individuals showing mixed traits, e.g. a mandible like *A. lineatus* and the setae pattern of *A. obscurus* (with the additional small seta) or vice versa, complicates the morphological differentiation. The occurrence of *A. lineatus* specimens showing the additional small seta clearly demonstrates that not all identification errors occur due to abrasion of important key features (Tab. 3–6). The PCR to support the morphological identification results was used to differentiate between the *Agriotes* specimens via genetic differences. The PCR results (following STAUDACHER et al., 2011, and using self-developed primers) clearly show that morphological identification of wireworms of the genus *Agriotes* is not always possible.

In general, the morphological key provided by COCQUEMPOT et al. (1999) for *Agriotes* wireworms from France is also correct for most individuals of the same species in Germany. But the results showed that there are aberrant individuals that have morphological characters contradicting the results produced by PCR. When used on wireworms from the JKI rearing, the PCR corresponded with the morphological species identification of the parents (= adult click beetles). Thus it is assumed that the PCR with the newly developed primers produces the correct species identification. The populations of the three most common wireworm species (*A. sputator*, *A. obscurus* and *A. lineatus* from the wireworm breeding) originating from sites in Northern Germany and in Switzerland all held aberrant specimens. In these populations a range from 2.0% (*A. sputator*) up to 15.9% (*A. obscurus*) of the individuals, depending on species, would have been

misidentified using the key by COCQUEMPOT et al. (1999). In smaller samples from other sites this percentage varied further and was sometimes either higher or lower. Morphological identification of *A. sputator* may be easier than of *A. lineatus* and *A. obscurus*, because the granules at the base of the segments appear to be less vulnerable to abrasion than setae or mandible teeth. The latter trait appears to be the most sensitive and therefore the least reliable trait.

The data from further wireworm monitoring sites indicate that the percentage of aberrant specimens may vary between populations from different sites, but also that the majority of sites holds aberrant specimens. The frequency of misidentifications may therefore strongly vary between different sites. Others, e.g. JOSSI et al. (2008) and STAUDACHER et al. (2011) mention similar difficulties with morphological determination of these three important species in their larval stage. Therefore, at the moment there is no morphological key to reliably differentiate between all specimens of these *Agriotes* larvae. A variable portion of the larvae, depending on the location, will be misidentified just relying on morphological keys. Nevertheless the majority of larvae are correctly identified with morphological keys.

However, when exact identification of each *Agriotes* specimen is needed, it is necessary to combine the morphological differentiation with the now developed PCR-detection. Our newly synthesized primers are suitable to distinguish all three *Agriotes* species in a combined (multiplex) PCR, where all three primer pairs have been applied in one reaction mix. With the new primers, the difficulties in differentiating between *A. lineatus* and *A. obscurus* encountered before with the primers given by STAUDACHER et al. (2011) did not occur.

Precise identification is crucial when trying to determine whether there are ecological differences, e.g. in food preferences of these three widespread species. ELLIS et al. (2009) and LOLE (2010) conclude similarly that stating ecological differences between these three species is hampered by the lack of a method to distinguish between the wireworms merely by morphological (physical) features, and they emphasize the necessity of PCR-methods for precise identification.

Frequent determination errors, together with species differences in their effects on a given crop, would make a prognosis of damage difficult. For example, PARKER and HOWARD (2001) state that the effort to predict the likely level of damage to potatoes caused by a given wireworm population has not been consistently successful. This may in part also be due to incorrect wireworm species identification. The wireworm species spectrum from eight different potato field sites indicates a possible preference of *A. obscurus* for the potato crop even though only part of the wireworms was identified via PCR. Similarly, KEMPKENS et al. (2004) also found a dominance of *A. obscurus* (above 90%) and 9% *Hemicrepidus niger* in potato. But in their work only morphological identification after KLAUSNITZER (1994) was performed, indicating that part of the *Agriotes obscurus* might have belonged to other *Agriotes* species.

In view of the results presented here, this work will be continued with further wireworm populations from the German click beetle and wireworm monitoring, including further wireworm samples from additional crops.

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