Otiorhynchus spp. (Coleoptera: Curculionidae) as pests in horticulture - genetics and management options with entomopathogenic fungi

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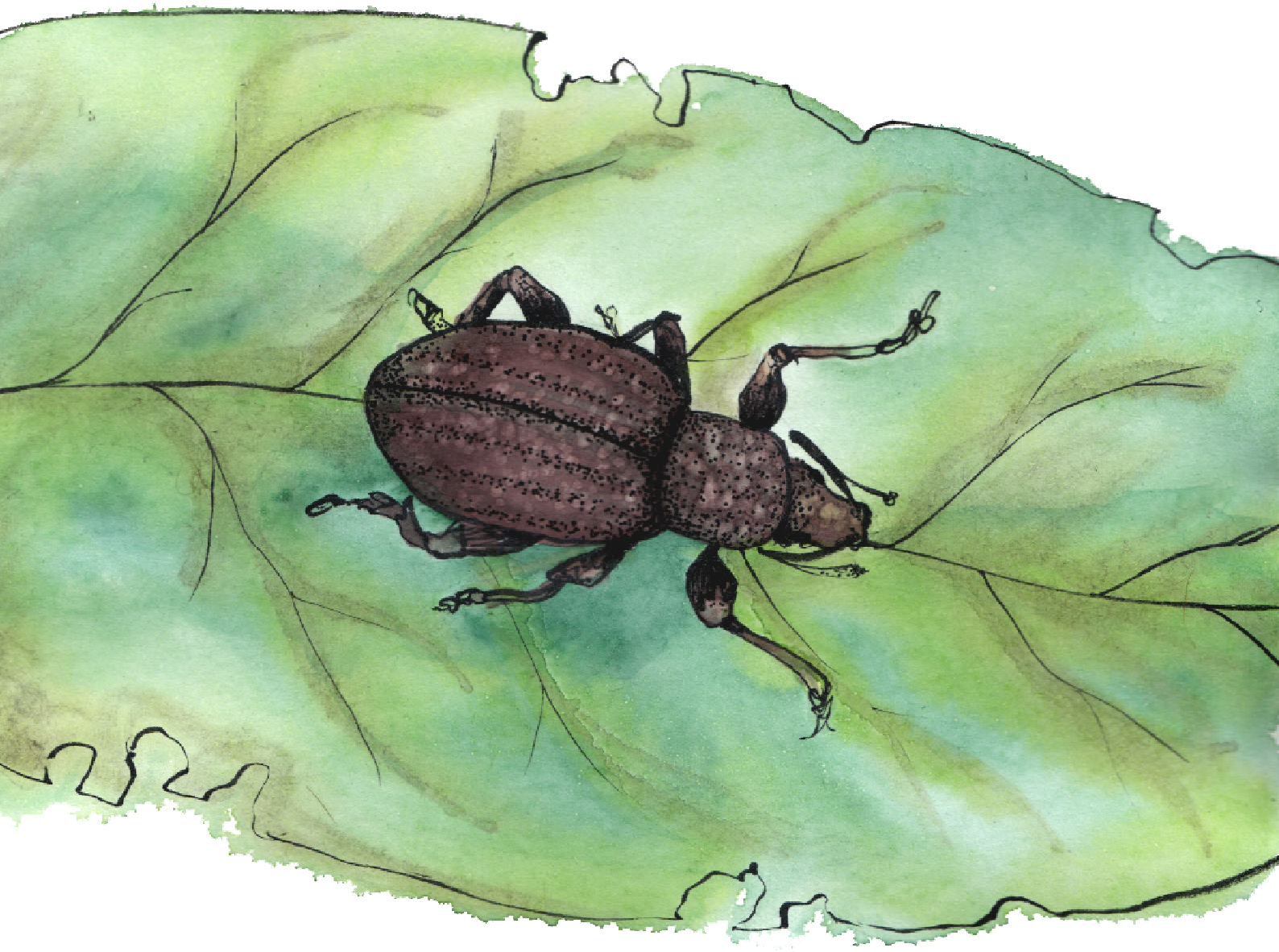


Content

List of abbreviations

ATCC	American Type Culture Collection
BCA	biological control agents
bp	base pair(s)
BVL	Bundesamt für Verbraucherschutz und Lebensmittelsicherheit
CFU	colony forming units
COI / coxA	cytochrome oxidase subunit I
COII	cytochrome oxidase subunit II
°C	degree Celsius
d	day
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
EEC	European Economic Community
e.g.	for example
EPF	entomopathogenic fungi
EPN	entomopathogenic nematodes
etc.	et cetera
EU	European Union
fwd	forward
g	gramme
h	hour
ITCC	Indian Type Culture Collection
ITS	internal transcribed spacer
kb	kilobase
l	litre
L:D	light-dark cycle
µ	micro
mg	milligramme
MID	multiplex identifier
min	minute
ml	millilitre
mM	millimolar
mtDNA	mitochondrial deoxyribonucleic acid
n	nano

NGS	next generation sequencing
p	pico
PCR	polymerase chain reaction
%	percentage
PPP	plant protection products
®	registered trademark
rDNA	ribosomal deoxyribonucleic acid
rev	reverse
RFLP	restriction fragment length polymorphism
rRNA	ribosomal ribonucleic acid
s	second
s.d. / SD	standard deviation
SE	standard error
sp.	species (singular)
spp.	species (plural)
SSCP	single-strand conformation polymorphism
SSR	simple sequence repeats
TGGE	temperature gradient gel electrophoresis
™	trademark
U	unit
US	United States
UV	ultraviolet
V	volt
var.	variety
WG	wettable granule
z. B.	zum Beispiel

List of tables

List of figures

1 Zusammenfassung

Rüsselkäfer der Gattung *Otiorhynchus* (Coleoptera: Curculionidae) verursachen durch Wurzelfraß als Larven sowie durch Blattfraß als adulte Tiere weltweit einen wirtschaftlichen Schaden an zahlreichen gartenbaulichen Kulturen. Neben dem bekanntesten Schädling dieser Gattung, dem Gefurchten Dickmaulrüssler *Otiorhynchus sulcatus*, traten in den vergangenen Jahren diverse andere *Otiorhynchus* Arten zunehmend als Schaderreger auf. Die nachtaktiven Käfer sowie die bodenbewohnenden Larven sind grundsätzlich schwer mit biologischen oder chemischen Pflanzenschutzmitteln zu bekämpfen. Da jede *Otiorhynchus* Art unterschiedlich empfindlich gegenüber Pflanzenschutzmitteln reagieren kann sowie ihre eigene Phänologie aufweist, ist die exakte Artbestimmung dieser Schädlinge eine Grundvoraussetzung für die Entwicklung von effektiven Bekämpfungsstrategien. Adulte Rüsselkäfer lassen sich anhand ihrer Morphologie gut bestimmen. Die morphologische Artunterscheidung von *Otiorhynchus* Eiern, Larven und Puppen ist jedoch nahezu unmöglich. Deshalb wurde im Rahmen der vorliegenden Doktorarbeit ein molekulares Diagnoseverfahren entwickelt, das es ermöglicht 16 *Otiorhynchus* Arten und acht weitere Rüsselkäferarten, unabhängig von ihrem Entwicklungsstadium, zu bestimmen. Dieses Verfahren kann zukünftig als schnelle und kostengünstige Methode zur sicheren Identifikation von Rüsselkäfern im Pflanzenschutz eingesetzt werden.

Zur biologischen Bekämpfung von *O. sulcatus* Larven werden in der Praxis häufig entomopathogene Nematoden verwendet. Eine weitere biologische Bekämpfungsmöglichkeit stellen entomopathogene Pilze dar. Bislang wurden sowohl entomopathogene Nematoden als auch Pilze hauptsächlich gegen den Gefurchten Dickmaulrüssler eingesetzt. Über die Wirksamkeit gegenüber anderen *Otiorhynchus* Arten ist jedoch nur wenig bekannt. Deshalb wurden die entomopathogenen Pilze *Beauveria bassiana*, *Isaria fumosorosea* und *Metarhizium anisopliae,* welche in den kommerziell erhältlichen Präparaten Naturalis®, PreFeRal® WG bzw. GranMet-P® enthalten sind, im Labor gegen unterschiedliche Entwicklungsstadien verschiedener Rüsselkäferarten der Gattung *Otiorhynchus* getestet. Es konnte gezeigt werden, dass ver-

schiedene *Otiorhynchus* Arten als adulte Käfer unterschiedlich empfindlich gegenüber den getesteten entomopathogenen Pilzen sind. Außerdem wurde im Freiland eine Methode zur Quantifizierung der Effizienz von entomopathogenen Pilzen gegenüber adulten Käfern der Gattung *Otiorhynchus* etabliert.

Entomopathogene Pilze, insbesondere die Art *B. bassiana*, werden weltweit zur biologischen Schädlingsbekämpfung von Insekten eingesetzt. Dabei kann das Kultursubstrat beim Topfen von Pflanzen mit entomopathogenen Pilzen gemischt werden oder die entomopathogenen Pilze werden direkt auf die Pflanze oder auf den Boden ausgebracht. Während die Wirkung von entomopathogenen Pilzen auf Insekten gut untersucht ist, ist bisher nur wenig über deren Persistenz, Verbreitung und Einfluss auf die im Boden natürlich vorkommenden Pilze bekannt. Pilze spielen z. B. als Zersetzer von totem organischem Material oder als Mykorrhiza-Symbionten von Pflanzen eine wichtige Rolle im terrestrischen Ökosystem. Neue Erkenntnisse über die Persistenz, Verbreitung sowie Interaktion von entomopathogenen Pilzen mit den im Boden lebenden Mikroorganismen könnten deshalb wichtige Informationen zur Risikobewertung von biologischen Pflanzenschutzmitteln basierend auf entomopathogenen Pilzen liefern. Aus diesem Grund wurde der Einfluss des entomopathogenen Pilzes *B. bassiana* Stamm ITCC 4688 auf die indigene Pilzdiversität einer landwirtschaftlich genutzten Ackerfläche untersucht. Es konnte gezeigt werden, dass sich *B. bassiana* während des Untersuchungszeitraumes von sieben Wochen im Feld etablierte und verbreitete, sowie dass *B. bassiana* keinen Effekt auf die Diversität der natürlich vorkommenden Pilz-Gemeinschaft hatte.

Wie bereits erwähnt, haben in den letzten Jahren vermehrt verschiedene Rüsselkäferarten der Gattung *Otiorhynchus* weltweit Schäden an zahlreichen gartenbaulichen Kulturen verursacht. Die zunehmende Ausbreitung der Arten ist wahrscheinlich auf den Klimawandel und/oder auf den verstärkten globalen Handel von befallenen Pflanzen zurückzuführen. Viele *Otiorhynchus* Arten sind extrem polyphag, besitzen das Potential sich an neue Wirtspflanzen anzupassen und vermehren sich durch Parthenogenese. Diese Fähigkeiten könnten die Etablierung einer *Otiorhynchus* Art in einem vorher unbesiedelten Gebiet begünstigen. Das Potential, sich an neue Wirtspflanzen anzupassen sowie die parthenogenetische Fortpflanzung könnten so-

wohl in der genetischen Ausstattung der Käfer als auch in einer Vergesellschaftung mit endosymbiontischen Bakterien begründet sein. Deshalb wurde im Rahmen der vorliegenden Dissertation das Endosymbiontenspektrum von vier verschiedenen *Otiorhynchus* Arten untersucht. Dabei konnte gezeigt werden, dass unter anderem Bakterien der Gattungen *Rickettsia* sowie „*Candidatus* Nardonella“ in den untersuchten Käfern vorkamen. Die biologische Funktion dieser Endosymbionten ist bisher spekulativ. Erkenntnisse darüber könnten jedoch zukünftig zur Entwicklung von neuen Bekämpfungsstrategien gegenüber Rüsselkäfern der Gattung *Otiorhynchus* genutzt werden.

2 Summary

Worldwide, weevils of the genus *Otiorhynchus* (Coleoptera: Curculionidae) cause damage with detrimental economic effects to many horticultural crops due to the root feeding of their larvae as well as foliage feeding of their adults. Aside from the black vine weevil *Otiorhynchus sulcatus*, which is the best-known pest within this genus, numerous other *Otiorhynchus* species have been increasingly recognized as pests in recent years. Nocturnal adult weevils and soil-inhibiting larvae are in principle difficult to control with biological or chemical plant protection products. In addition, each *Otiorhynchus* species shows a different phenology or may have a varying susceptibility towards plant protection products. Therefore, the exact species identification of the respective weevil pest is a prerequisite for the development of efficient control strategies. While adult weevils can be distinguished by phenotypical characteristics, the determination of *Otiorhynchus* eggs, larvae and pupae, only on the basis of morphological features, is nearly impossible. For that reason, a molecular diagnostic method, which allows the species determination of 16 *Otiorhynchus* and eight other weevil species, independent of their developmental stage, was developed. This diagnostic method might be used in future for fast and cost-efficient species identification of weevils in plant protection.

The application of entomopathogenic nematodes is a well established method for biological control of *O. sulcatus* larvae. Another biocontrol strategy is the application of entomopathogenic fungi. So far, both entomopathogenic fungi and nematodes have been used mainly against the black vine weevil. As less is known about the effectiveness of entomopathogenic fungi against different *Otiorhynchus* species, the present thesis analysed the efficacy of the entomopathogenic fungi *Beauveria bassiana*, *Isaria fumosorosea* and *Metarhizium anisopliae* (formulated in the commercially available products Naturalis®, PreFeRal® WG and GranMet-P®, respectively) against different developmental stages of several *Otiorhynchus* species under laboratory conditions. Infection experiments revealed that different species of adult *Otiorhynchus* weevils show a different susceptibility to the analysed entomopatho-

genic fungi. In addition, a method to quantify the efficacy of entomopathogenic fungi against adult *Otiorhynchus* spp. under field conditions was developed.

Worldwide, entomopathogenic fungi, in particular the species *B. bassiana,* are used for biological insect pest control. They are either incorporated into the potting media at the time of planting or applied directly onto the plant or onto the surface of the soil. While the effect of entomopathogenic fungi against insects has been well studied, little is known so far about their fate, spread and influence on the naturally occurring soil fungi. As decomposers of dead organic material or as mycorrhizal symbionts of plants, fungi play an important role in the terrestrial ecosystem. New insights into the fate, spread and interactions of entomopathogenic fungi and soil-inhabiting microorganisms could therefore provide important information for proper risk assessment of plant protection products which are based on entomopathogenic fungi. For this reason, the influence of the entomopathogenic fungus *B. bassiana* strain ITCC 4688 on the indigenous fungal diversity of an agricultural field was analysed. During the seven weeks of study *B. bassiana* became established within the field. In addition, a natural spread, as well as no effect on the diversity of the indigenous soil fungi was detected.

As previously mentioned, in recent years additional different *Otiorhynchus* species have caused damage to numerous crops which are grown for horticultural purposes worldwide. The increasing extension of their geographic range is probably caused by climate change and/or an intensified international trade of infested plants. Several *Otiorhynchus* species are extremely polyphagous, have the potential to adapt to new host plants and reproduce by parthenogenesis. Those abilities may favour the establishment of an *Otiorhynchus* species in a newly colonised habitat. The genetic equipment of the weevils or the association with endosymbiotic bacteria may be responsible for the potential to adapt to new host plants or the parthenogenetic mode of reproduction. Therefore the endosymbiotical spectrum of four *Otiorhynchus* species was investigated. As one of the results, bacteria of the genera *Rickettsia* and "*Candidatus* Nardonella" were detected. So far, the biological function of bacterial endosymbionts in *Otiorhynchus* spp. is speculative. However, new insights into the association of bacteria and weevils may be used in future to develop novel strategies for the control of *Otiorhynchus* pests.

3 General introduction

3.1 *Otiorhynchus* spp. as pests in horticulture

The weevil genus *Otiorhynchus* Germar belongs to the Coleopteran family Curculionidae. Within this genus there are many extremely polyphagous species which are known as pests on numerous horticultural crops (Backhaus 1996). Among them, the black vine weevil *Otiorhynchus sulcatus* Fabricius (Figure 1, A) is considered as the most important *Otiorhynchus* pest worldwide (Moorhouse et al. 1992). Furthermore it is listed by the Royal Horticultural Society in 2010 and 2011 within the top ten garden pests in the United Kingdom (Royal Horticultural Society 2012). However, aside from the black vine weevil, many other *Otiorhynchus* species apparently extended their geographic range, and concurrently their significance as pests in horticulture increased over the past years. This may either be a consequence of climatic change and/or increased spreading of these weevils due to an intensified international movement of horticultural plants and products (Wheeler 1999, Majka and MacIvor 2009, Dehnen-Schmutz et al. 2010, Staverløkk 2010).

All species of the genus *Otiorhynchus* cause largely similar symptoms and damage on their respective host plants. Adult weevils produce typical crescent shaped notches on leaves (Figure 2, A-C) and flowers. This is usually an aesthetic damage and does not profoundly influence the vitality of the plants. However, it may reduce their market value in particular when ornamentals are affected. In contrast, soil-borne weevil larvae (Figure 2, D) feed on roots of their host plant which may lead to nutrient and water deficiencies and subsequently results in wilting, stunting or the plant's death (Smith 1932, Moorhouse et al. 1992). Therefore, larvae are regarded as the most harmful developmental stage of *Otiorhynchus* weevils.

Otiorhynchus weevils are nocturnal and flightless. They are hardly seen in the daytime, as they hide in the soil, in the leaf litter near their host plants or under the pots of infested plants. Therefore the above described characteristic crescent notches on leaves (Figure 2, A-C) and flowers are normally the initial recognizable symptoms for

the presence of adult *Otiorhynchus* spp. At first sight, growers usually link those feeding traces with the occurence of the black vine weevil. However, this is not necessarily the case, as a recent investigation in the Pacific Northwest and in Germany showed that there are frequently plenty of other *Otiorhynchus* species present in nurseries and that *O. sulcatus* is not always the dominant species (Collman 2001, Sprick 2009). In Germany, 16 *Otiorhynchus* species have been found aside from the back vine weevil, in tree nurseries as well as horticultural and hop cultivation farms, inflicting damage on their respective host plants (Sprick 2009) (Figure 3).

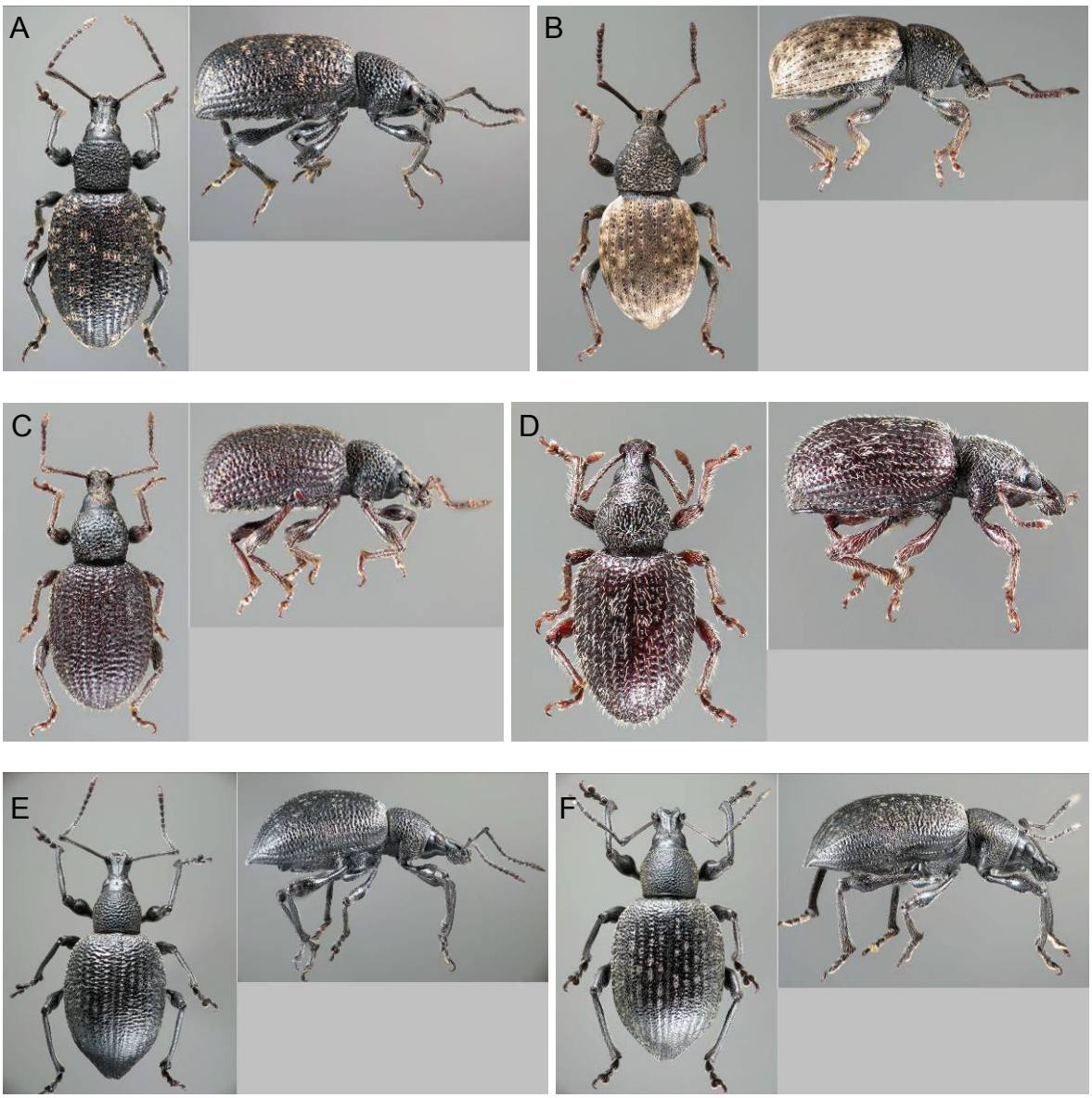


Figure 1: Adult weevils of the species (A) *O. sulcatus*, (B) *O. raucus*, (C) *O. rugosostriatus*, (D) *O. dieckmanni*, (E) *O. salicicola* and (F) *O. armadillo* (photos: P. Stüben). Please note, the illustrations are not to scale.

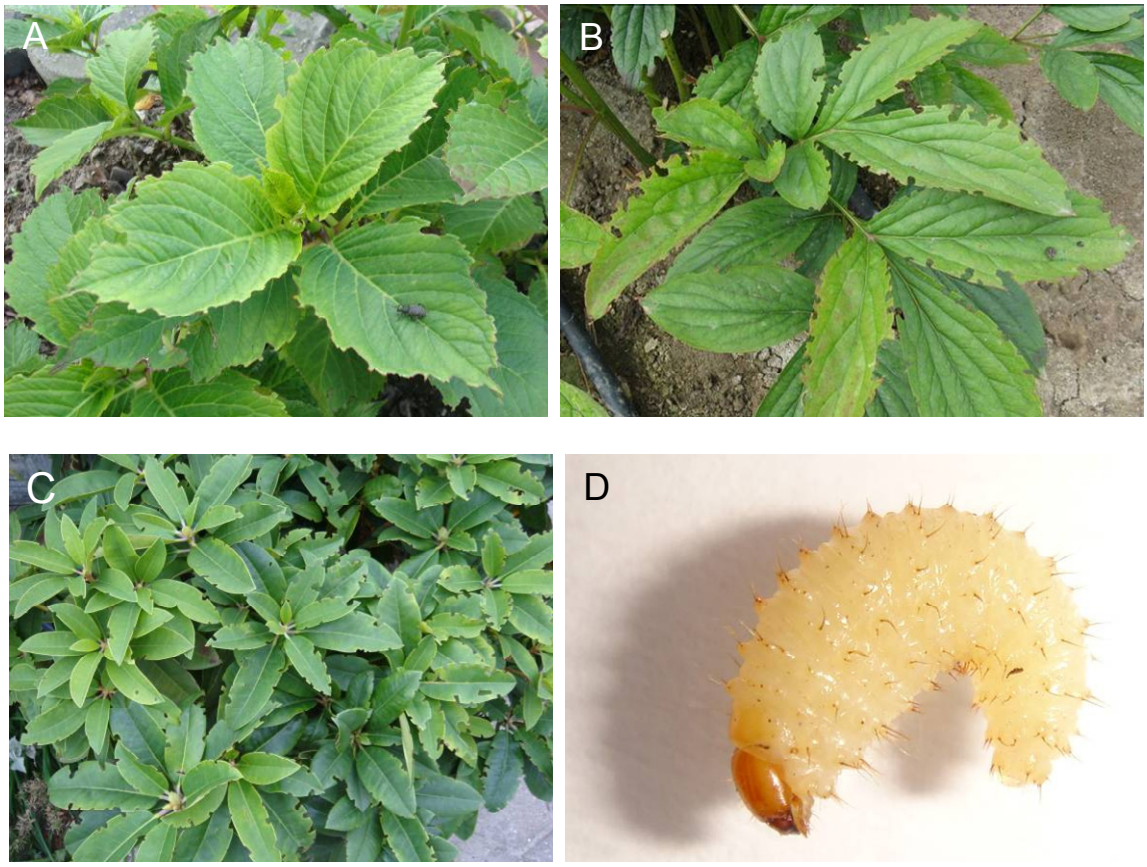


Figure 2: Characteristic crescent shaped notches on (A) *Hydrangea*, (B) *Paeonia* and (C) *Rhododendron* plants inflicted by *Otiorhynchus* spp. (D) *Otiorhynchus* sp. larva. Photos (A), (C) and (D): P. Sprick. Photo (B): G. Kubach.

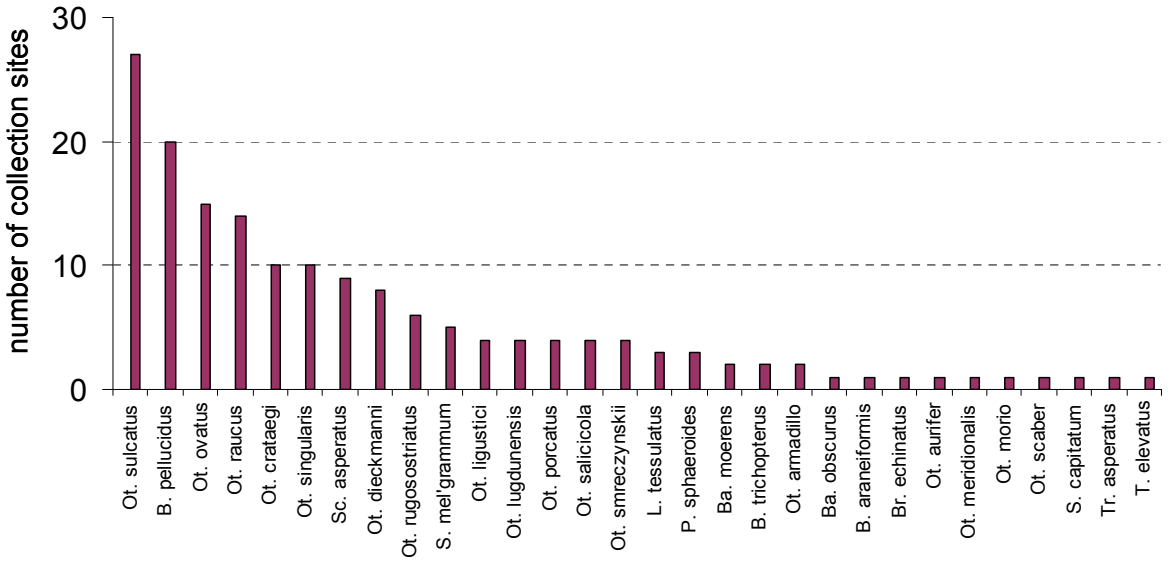


Figure 3: Occurence of weevil species in selected collection sites (Sprick 2009). Abbreviations: B.: *Barypeithes*, Ba.: *Barynotus*, Br.: *Brachysomus*, L.: *Liophloeus*, Ot.: *Otiorhynchus*, P.: *Peritelus*, Sc.: *Sciaphilus*, S.: *Strophosoma*, T.: *Tropiphorus*, Tr.: *Trachyphloeus*.

Figure 4: Commercial plantation of perennial ornamentals: (A) peonies and (B) switchgrass.

Damage to cultivated plants produced by *O. sulcatus* adults and larvae were already documented in 1834 in Germany (Smith 1932). At this time, the black vine weevil was mainly distributed to central Europe (Lundmark 2010). Today however, *O. sulcatus* may be found as a cosmopolitan pest of agricultural and horticultural crops in many parts of Europe, North and South America, Central Asia, New Zealand etc. (Lundmark 2010). For this reason, up to date numerous studies on the biology of the black vine weevil have been conducted and diverse control strategies against this pest have been developed. In contrast so far, less attention was paid to other *Otiorhynchus* species. For instance, Sprick (2009) and Collmann (2001) commonly found the species *Otiorhynchus raucus* Fabricius (Figure 1, B and Figure 3) and *Otiorhynchus rugosostriatus* Goeze (Figure 1, C and Figure 3) in their surveys. These weevils were also the most dominant species inflicting damage in a commercial plantation of perennial ornamentals (peonies and switchgrass) near Wiesbaden (Germany) (Figure 4) in recent years (Reineke et al. 2011). Furthermore, besides the two mentioned weevils, the species *Otiorhynchus dieckmanni* Magnano (Figure 1, D) occured within this plantation (personal observation). *Otiorhynchus dieckmanni* may be found in diverse European countries (Heijerman and Raemakers 2001) and is also listed by Sprick (2009) as *Otiorhynchus* species which may become harmful as a horticultural pest in the near future. In addition, the species *Otiorhynchus salicicola*

Heyden (Figure 1, E) and *Otiorhynchus armadillo* Rossi (Figure 1, F) became an increasing problem in several European countries such as Germany, the Netherlands and Switzerland (Heijerman and Hellingman 2003, van Tol et al. 2004a, Sprick 2009). For these reasons, the present thesis deals with the biology, genetics and biological control of several economically important *Otiorhynchus* species to gain crucial information, which will help to manage different *Otiorhynchus* species as pests in horticulture.

3.2 Life cycle and host plants of *Otiorhynchus* spp.

Weevils of the genus *Otiorhynchus* are phytophagous insects and major pests in many horticultural crops, partly also in viticulture (Englert 1996, Lykouressis et al. 2004) and forestry (Wulf and Berendes 1996). During the life cycle of *Otiorhynchus* weevils, damage to plants is caused primarily by root feeding larvae but also the notching of adults on the plant's foliage is of economic importance.

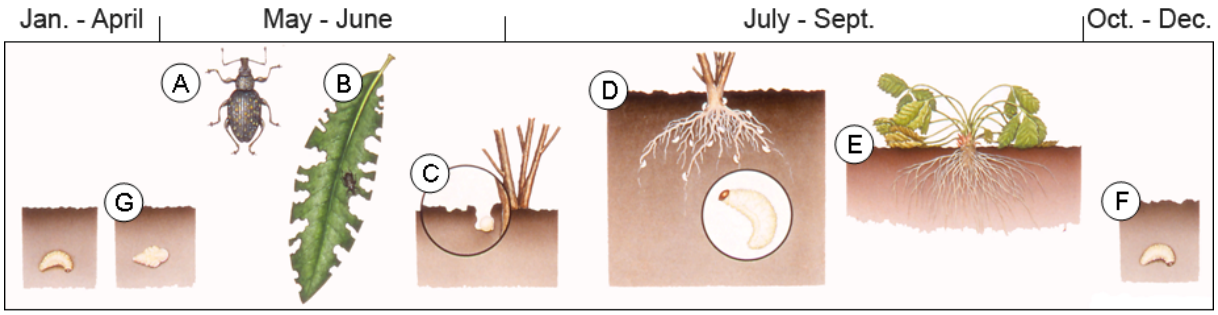


Figure 5: Schematic life cycle of the black vine weevil *O. sulcatus* (Griegel 2009, modified by Hirsch, 2012). (A) Adult weevil. (B) Damage inflicted by adult weevils on leaves. (C) Eggs in soil. (D) Root feeding larvae of the black vine weevil. (E) Root feeding behaviour of larvae may cause wilting of the plant. (F) Hibernating larva. (G) Pupa of the black vine weevil.

The life cycle described in Figure 5 and the different developmental stages of the black vine weevil are most widely representative for all *Otiorhynchus* species. However, the timing of the occurence of adult weevils, as well as the duration of the different immature life stages of *Otiorhynchus* spp. depend on the respective *Otiorhynchus* species (Vainio and Hokkanen 1993, Sprick 2009) and may vary for an "outdoor" or "greenhouse" population (LaLone and Clarke 1981) as temperature plays a key role in their development (Moorhouse et al. 1992, Umble and Fisher

2000, Son and Lewis 2005). For instance, Sprick (2009) documented that the species *O. dieckmanni* and *O. raucus* emerge early in the year (April - May), whereas *O. rugosostriatus* shows a different phenology with the occurence in June or July. In addition, personal observations in the switchgrass field in the nursery near Wiesbaden (Figure 4) in January and October 2011 revealed, that *O. dieckmanni* and *O. raucus* may also hibernate as immature adults in soil. Within the genus *Otiorhynchus* there are parthenogenetic and bisexual species (Suomalainen 1954). For example, the species *O. dieckmanni*, *O. rugosostriatus* and *O. raucus* reproduce mainly by parthenogenesis and *O. armadillo* and *O. salicicola* are bisexual. The life cycle of the parthenogenetic species *O. sulcatus* is described in Figure 5. This life cycle has to be complemented by mating of weevils for bisexual species.

The adult black vine weevil emerges from the pupa stage usually in spring or early summer (Cowles 1995) (Figure 5, A). After maturation feeding, the time period when foliar feeding which is required for egg development becomes most noticeable (Figure 5, B), eggs are laid during the night in soil crevices or on the soil surface (Smith 1932, Cowles 1995) (Figure 5, C). Weevil larvae hatch from the eggs, dig down into the ground and start feeding on the root system of their host plants (Figure 5, D). *Otiorhynchus* spp. larvae are apodous, usually white grubs with light brownish heads and a ventrally curved C-shape, which is characteristic of older larval stages (Moorhouse et al. 1992) (Figure 5, D and Figure 2, D). Larval feeding may be lethal for the host plants, especially when younger plants or freshly transplanted cuttings are affected (Moorhouse et al. 1992) (Figure 5, E). *Otiorhynchus* grubs moult several times during larval development (Smith 1932). For the black vine weevil six to seven larval stages are described (Smith 1932, LaLone and Clarke 1981). *Otiorhynchus sulcatus* mainly hibernates as larvae (Moorhouse et al. 1992) (Figure 5, F) before starting pupating in early spring (Figure 5, G). The black vine weevil has usually one generation per year. However, there may be some overlap between the generations, as hibernating adult weevils may lay eggs before the freshly emerged adults have completed maturation feeding (Cowles 1995).

Maturation feeding on leaves and flowers, as well as root feeding of larvae, can cause serious economic damage especially when ornamentals are affected.

Ornamentals such as *Hydrangea* (Figure 2, A), *Paeonia* (Figure 2, B), *Rhododendron* (Figure 2, C), *Taxus*, *Cyclamen*, *Euonymus* and many others are known as potential host plants for *Otiorhynchus* spp. (Smith 1932, Warner and Negley 1976, Fisher 2006). So far, the potential host plant range of *O. sulcatus* is the best studied one among all *Otiorhynchus* species (Smith 1932, Warner and Negley 1976). However, van Tol et al. (2004b) argue that the current list of host plants, which comprises more than 100 species (Smith 1932, Warner and Negley 1976, Masaki et al. 1984), may also contain several "bad" hosts, which are only accepted by *O. sulcatus* due to the absence of alternatives and the limited dispersal capability of flightless adult weevils (Maier 1978). In addition, van Tol et al. (2004b) suggest that the black vine weevil may have the potential to adapt to new hosts. This assumption, the ability of *O. sulcatus* to feed and reproduce in a low frequency on "bad" hosts and its parthenogenetic nature are key factors in the successful establishment of black vine weevils in new habitats (van Tol et al. 2004b, Lundmark 2010). These key factors may also play an important role in the establishment of other *Otiorhynchus* species as pests in horticulture.

3.3 Control of *Otiorhynchus* spp.

Many species of the weevil genus *Otiorhynchus* have a broad host plant range and reproduce by parthenogenesis (Smith 1932, Suomalainen 1954, Warner and Negley 1976). Therefore, a single parthenogenetic weevil, transported accidentally into a new habitat e.g. nursery, may establish a new population and thus may lead to an infestation of the nursery (Bruck 2007). Once established, *Otiorhynchus* weevils are difficult to control because of the ground-dwelling larvae and the nocturnal activity of the adult beetles.

In natural environments *Otiorhynchus* weevils have a number of enemies such as shrews, birds, lizards, entomopathogenic fungi and nematodes, carabids or parasitic dipterans and hymenopterans, which may help to maintain the population at a low level (Zimmermann 1981, Moorhouse et al. 1992). In horticultural systems however, those enemies are mostly not present or not sufficient to keep the weevil population under the economic threshold level.

In the past, several chemical and biological management strategies have been developed and are applied to control black vine weevil infestations:

Worldwide, for chemical control of weevils, insecticides based on active ingredients such as chlorpyrifos, bifenthrin, imidacloprid or thiacloprid are used. To limit an infestation with larvae of the black vine weevil in container-grown plants, growers typically incorporate those chemical substances into the potting media of the plants (Cowles 2001, Beltz et al. 2010). Chemical insecticides can be used as larvicides as described above or as foliage spray treatments against adult weevils (Cowles 1996). In addition, systemic insecticides applied to containerized ornamentals also have the potential to reduce larval infestation as well as survival and feeding of adult *O. sulcatus* (Reding and Persad 2009, Reding and Ranger 2011). For *Otiorhynchus* pest control in Germany, there are plant protection products (subsequently abbreviated as PPP) based on e.g. acetamiprid, imidacloprid or thiacloprid available for usage in allotments and private gardens, as well as for commercial purposes (for further information on the current list of available PPP, please visit the following website: https://portal.bvl.bund.de/psm/jsp/). For instance, the thiacloprid based insecticide Exemptor® (Bayer CropScience Deutschland GmbH), which is incorporated into the potting media, shows good effectiveness against *Otiorhynchus* larvae, but not against adult weevils (Beltz et al. 2010). There is currently a lack of insecticides with high efficacy against adult *Otiorhynchus* weevils (Beltz et al. 2010).

Regarding biological control, the application of entomopathogenic nematodes (subsequently abbreviated as EPN) of the genera *Heterorhabditis* and *Steinernema* is the leading method for biocontrol of *Otiorhynchus* larvae worldwide. Parasitic nematodes usually penetrate insects through natural body openings such as the mouth, the anus or stigmata. In the haemocoel of the host insect, nematode-associated symbiotic bacteria of the genus *Xenorhabdus* or *Photorhabdus* are released, causing blood poisoning and finally the death of the insect (Forst and Nealson 1996). In general, EPN are dissolved in water and subsequently applied to the soil e.g. of infested container-grown ornamentals. Entomopathogenic nematodes work as contact insecticides. That means, for successful pest control, EPN must “hit” the target pest, usually at larval insect stages, at the time of application. In addition, environmental

conditions such as soil temperature (usually temperatures above 12°C are recommended for application of EPN) are critical for nematode efficacy (Georgis et al. 2006). Due to the life cycle of *Otiorhynchus* spp. (Figure 5) and the required soil temperature, there are two seasonal windows for successful control of weevil larvae: either applying EPN in late spring targeting overwintering larvae, and/or an application in late summer/early autumn reducing the newly developed larval population (Sampson 1994, Georgis et al. 2006). Although adult weevils are usually regarded as less susceptible towards EPN, recently an investigation showed that adult *O. sulcatus* can be successfully controlled using a “lure and kill”-method. This method consists of an artificial wooden shelter, which acts as a hiding-place for the weevil (“lure”), filled on the bottom side with a gel-like formulation containing EPN (“kill”) (Ufer et al. 2010). Only recently, this trap has become commercially available (Sautter & Stepper, Ammerbuch, Germany). However, further studies are needed to evaluate the suitability of these traps for horticultural purposes (Ufer et al. 2010). Although nematode-based products are an environmentally compatible alternative to chemical pesticides, they have a limited market share attributable to relatively high costs (Georgis et al. 2006), a short shelf-life (Koppenhöfer 2007) and a potentially compromised efficacy, due to environmental conditions such as soil temperature, which is considered to be the most limiting factor for successful control of *Otiorhynchus* spp. larvae (Georgis et al. 2006). In addition, recent investigations revealed that the potting media of plants may also significantly influence the efficacy of different entomopathogenic nematode species in controlling infestations of *O. sulcatus* larvae (Ansari and Butt 2011).

Biological management strategies show the best effect when used under glass or to control an infestation of *Otiorhynchus* spp. in container-grown plants (Pickett et al. 1996). This also applies to chemical PPP. In field-grown crops, especially with deep-rooted perennial plants, such as the commercial plantation of peonies and switchgrass near Wiesbaden (Figure 4), the major problem is to ensure contact between the target insect and the exerted control agent. As *Otiorhynchus* larvae have been found in varying soil depths up to more than 40 cm (Smith 1932), neither EPN nor chemical insecticides can easily be applied through the soil profile of deep-rooted field-grown crops (Sampson 1994, Cowles 2004). This consequently may lead

to large unprotected parts of the root system, which thus may be attacked by *Otiorhynchus* larvae. In order to solve this problem, chemical insecticides are often sprayed on the foliage of the plants to reduce the population of adults and consequently the number of root feeding larvae (Cowles 1996). However, this approach is linked with some prerequisites to be effective. To identify the optimum time of application, it is necessary to determine the activity of adult weevils by a continuous monitoring (Cowles 1995). Multiple applications, preferably after sunset or at least in the evening, are needed to eliminate 1.) hibernating adult weevils, and in the following weeks 2.) freshly emerged adults, to prevent egg laying in order to stop the development of weevil offspring (Cowles 1996, Umble and Fisher 2002). Furthermore, for the effective control of adult weevils, an adequate coverage of the plants' foliage with insecticides is required. As a result of this, insecticides are often over-applied or over-diluted (Cowles 1996).

Currently, there is no chemical control of *Otiorhynchus* larvae with satisfactory results, and large-scale biological control of larvae with EPN is expensive (van Tol et al. 2004a). Therefore, interest in alternative biocontrol opportunities has increased considerably in recent years.

A number of entomopathogenic fungi are naturally occurring pathogens of larvae and adults of *O. sulcatus,* including *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* (Marchal 1977), which recently has been phylogenetically reclassified to *Isaria fumosorosea* (Humber 2007). In several studies the efficacy of different entomopathogenic fungi as biological control agents (subsequently referred to as BCA) have been tested in the field and under greenhouse conditions against the black vine weevil (Zimmermann 1981, Beck 1996, Bruck 2006, Bruck and Donahue 2007, Oddsdottir et al. 2010). In addition, recent laboratory and glasshouse studies showed that a combined use of entomopathogenic nematodes and fungi works synergistically and is highly efficacious against larvae of *O. sulcatus* (Ansari et al. 2008, 2010). Although in the past, many control strategies have been developed against *O. sulcatus,* and different entomopathogenic fungal species have been isolated as well from other *Otiorhynchus* species (Kleespies et al. 2008), so far only few reports are available, which investigate the susceptibility of other *Otiorhynchus*

species towards entomopathogenic fungi (Vainio and Hokkanen 1993, Sabbahi et al. 2008). Further information regarding the interaction of entomopathogenic fungi and *Otiorhynchus* weevils are given in chapter 3.4.1.

3.4 Entomopathogenic fungi (EPF)

The term “entomopathogenic fungi” (subsequently referred to as EPF) describes a non-monophyletic group of approximately 750 fungal taxa, which are natural pathogens of a wide variety of insects and other arthropods (Hegedus and Khachatourians 1995). The present thesis concentrates on the following three EPF: *Metarhizium anisopliae* (Metschnikoff) Sorokin, *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin, and *Isaria fumosorosea* Wize, which are widely used as biological control agents. The latter two species are currently placed in the family Cordycipitaceae, whereas *M. anisopliae* is placed within the Clavicipitaceae (both families belong to the order Hypocreales within the phylum Ascomycota).

The EPF mentioned above have a worldwide distribution and are widespread in natural and agricultural ecosystems (Domsch et al. 1980, Meyling and Eilenberg 2006, Quesada-Moraga et al. 2007). Within the terrestrial ecosystem, EPF have diverse functions. Whereas all three species have been isolated as entomopathogens from insects or as saprophytes from the soil, mainly species of the genus *Beauveria* were isolated from plants as endophytes (Vega 2008, Vega et al. 2008). Although EPF are used worldwide as BCA for insect pest control, so far their different roles in the terrestrial ecosystem have rarely been studied (Vega et al. 2009). However, studying the ecology of EPF is a prerequisite to use them effectively for designing plant protection strategies (Vega et al. 2009).

There are plenty of scientific publications explaining the different steps of the infection process of EPF in detail. For this reason, only the basic mechanisms are summarized subsequently (for further information see Hegedus and Khachatourians 1995). The mode of infection of EPF consists in general of the following four steps: 1.) attachment of the fungal spores on the insect’s cuticle (Figure 6, A-B) and subsequent germination (Figure 6, C), 2.) mechanical and enzymatical penetration of the cuticle layers (Figure 6, D), 3.) proliferation of the fungus within the body of the host

(Figure 6, E) which finally causes the host's death (Figure 6, F) due to several factors, including release of fungal toxins, invasion of organs, water and nutrients depletion and physical obstruction, 4.) re-emergence of the fungus from the host with characteristic outgrowth of fungal mycelia on the cadaver followed by sporulation of the fungus (Figure 6, F and Figure 7, A and B).

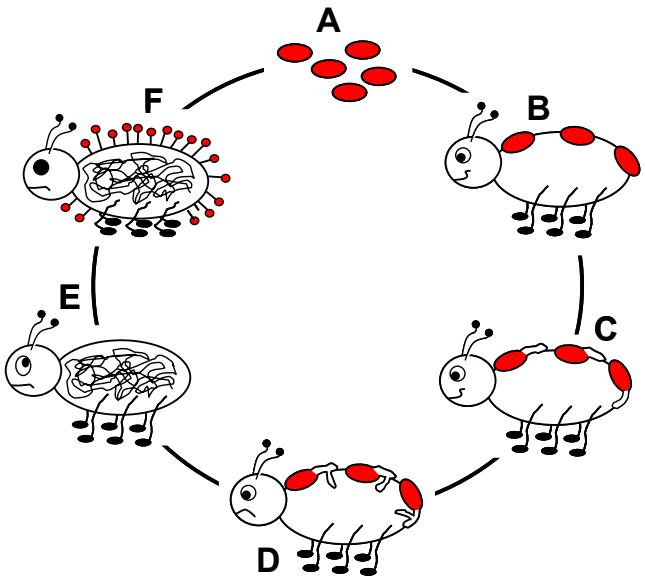


Figure 6: Schematic presentation of the infection cycle of EPF (extracted from the technical sheet of PreFeRal® WG (Biobest N.V., Westerlo, Belgium), modified by Hirsch, 2012). (A) Fungal spores. (B) Spore attachment on insects' cuticle. (C) Spore germination. (D) Cuticle penetration. (E) Internal proliferation of the fungus causing finally the host's death. (F) Dead insect and external sporulation of the fungus.

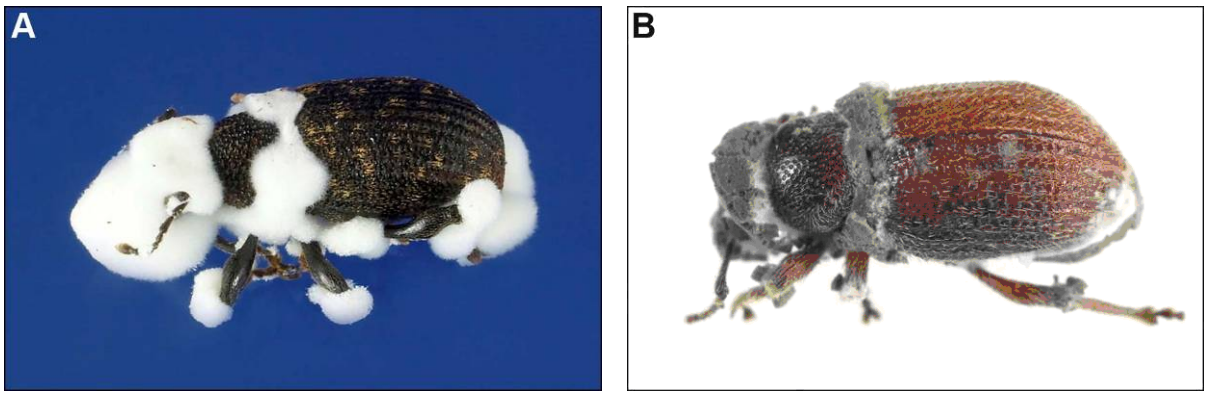


Figure 7: External sporulation of EPF on adult weevils. (A) Infected *O. sulcatus* showing "white muscardine" caused by *Beauveria* sp. (B) Infected *O. dieckmanni* showing "green muscardine" caused by *Metarhizium* sp. Please note, the illustrations are not to scale (photos: W. Schönbach).

For *Beauveria* spp. and *Metarhizium* spp., the colour of the sporulating fungus gives the cadaver a characteristic white (Figure 7, A) and green (Figure 7, B) appearance, which is known as white and green muscardine, respectively. Spore dispersal of EPF is passive and relies mainly on wind, water and insect dispersal (Meyling and Eilenberg 2007).

Beauveria bassiana, *M. anisopliae* and *I. fumosorosea* have a wide host range, which spans numerous groups of organisms within the Arthropoda, such as members of the Coleoptera, Lepidoptera or Arachnida. However, those fungal species also comprise many isolates which may have a considerably reduced host range (for review see Zimmermann 2007a, 2007b and 2008). All three species are known to be infective for *O. sulcatus* (Leatherdale 1970, Marchal 1977, Zimmermann 1981, Kleespies et al. 2008). However, the pathogenicity (the ability of a pathogen to cause disease on a given host) and virulence (which describes the quantity of pathogenicity e.g. less or highly virulent) of those EPF depends on the respective isolate and is influenced by a number of abiotic and biotic factors, such as solar radiation, moisture or microorganisms present in the soil or inside or outside of the host insect (Schabel 1976, McCoy et al. 1992, Inglis et al. 2001, Panteleev et al. 2007, Wraight et al. 2007).

Microorganisms such as fungi or bacteria may interact with each other. If this interaction is detrimental for at least one of the involved organisms, then it is called antibiosis. Antibiosis has been documented e.g. for the interaction of microorganisms associated with the cuticle of the pales weevil *Hylobius pales* Herbst and *M. anisopliae* (Schabel 1976). Therefore antibiosis may be regarded as one biotic factor which may influence the efficacy of EPF.

Regarding the environment, soil microorganisms may play a major role in fungistasis (= mycostasis). Fungistasis describes the phenomenon when viable fungal propagules do not germinate in non-sterile soil and growth of fungal hyphae is terminated, even if moisture and temperature are suitable for germination or growth of fungi (Watson and Ford 1972). Fungistasis may be of biological origin, due to microbial activity, which is referred as “microbial mycostasis” (Dobbs and Gash 1965), as it does not occur in sterilized soils (Lockwood 1964). Zimmermann (2007a) mentioned

this phenomenon for *B. bassiana* in his review. Moreover, Müller-Kögler and Zimmermann (1986) and Shimazu et al. (2002) documented a decrease of *B. bassiana* over time, when conidia were mixed in field soil samples, indicating that *B. bassiana* conidia did not germinate in field soils. Although the mechanisms inducing soil fungistasis are still unknown, it is likely that inhibitory substances released by microorganisms present in the soil are involved in this phenomenon (Zimmermann 2007a). Therefore, studying microbial diversity in soil and subsequently the interaction of *B. bassiana* with soil microorganisms is one of the prerequisites for the effective use of *B. bassiana* as a biocontrol agent against insect pests. On the other hand, as less is known about the interaction of EPF with soil microorganisms, there may be as well a detrimental influence of an applied entomopathogenic fungus to the present soil microbiota, which might have subsequent implications for soil ecosystem functioning. Therefore, the effect of EPF to non-targets, in this case soil microorganisms, should also be taken into consideration for proper risk assessment of PPP based on EPF.

3.4.1 EPF as biological control agents

Entomopathogenic fungi have demonstrated considerable potential as environmentally friendly BCA for the control of diverse arthropod pests (Hajek and Delalibera 2010). One of the prerequisites is the restricted host range of the respective fungal isolate, allowing insect pest control with limited harm to non-target organisms such as mammals, birds, earthworms, honeybees or beneficial insects (Sterk et al. 2001, Schmutterer and Huber 2005, Thungrabeab and Tongma 2007). Moreover, the development of resistance to EPF, as known for several chemical pesticides (Whalon et al. 2008), is unlikely because of the number of factors which are involved in the infection process (Schmutterer and Huber 2005), but cannot be excluded with absolute certainty (Shelton et al. 2007). In addition, EPF are also compatible with several types of chemical insecticides, fungicides or EPN, which even may increase their efficacy (Santos et al. 2007, Bruck 2009, Ansari et al. 2010, Paula et al. 2011).

Worldwide, over the past circa 50 years, approximately 130 commercially available products based on EPF have been developed, of which two-thirds are products containing *B. bassiana* and *M. anisopliae* isolates (de Faria and Wraight 2007). The

present thesis focuses on the use of the commercially available products Naturalis® (based on *Beauveria bassiana* strain ATCC 74040) and PreFeRal® WG (*Paecilomyces fumosoroseus* Apopka strain 97, which recently has been phylogenetically reclassified to *Isaria fumosorosea* (Humber 2007)), as well as on the entomopathogenic fungus *Metarhizium anisopliae* var. *anisopliae* strain Ma43 which is a synonym for the *Metarhizium anisopliae* var. *anisopliae* strain BIPESCO 5/F52 (Eilenberg et al. 2008).

In the EU, the active substance *Beauveria bassiana* strain ATCC 74040 (Naturalis®), *Paecilomyces fumosoroseus* Apopka strain 97 (PreFeRal® WG) and *Metarhizium anisopliae* var. *anisopliae* strain BIPESCO 5/F52 are listed in Annex I of Directive 91/414/EEC, which is a prerequisite for registration of these EPF as PPP in all EU member states (Hauschild 2010). Currently, *B. bassiana* strain ATCC 74040 is registered in eleven European countries with the intended use against whiteflies, thrips and aphids in tomatoes under glass and in the field (European Commission 2012, final review report). In addition, *B. bassiana* strain ATCC 74040 (Naturalis®) has been recently submitted to the BVL (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit) for registration as a plant protection product in Germany against spider mites and whiteflies in vegetables and strawberries in greenhouses (personal communication U. Quentin, Intrachem Bio Deutschland GmbH & Co. KG). *Paecilomyces fumosoroseus* Apopka strain 97 has a registration in six countries of the EU e.g. against whiteflies under glass (European Commission 2012, review report). *Metarhizium anisopliae* var. *anisopliae* strain BIPESCO 5/F52 is approved in five European countries with the intended use in open fields, particularly in nurseries, viticulture or Christmas greenery plantations against e.g. *O. sulcatus* (European Commission 2012, final review report). Currently however, none of these fungal strains has a permanent registration as a plant protection product against *Otiorhynchus* spp. and other insects in Germany.

Despite the commercial availability of approximately 130 products based on EPF, these products represent just a minor fraction of the biopesticide market in the US and EU (Jaronski 2010). The main reasons for this limited use are costs, product quality, shelf-life, as well as aspects of regulatory restrictions on the ecological

repercussions such as the fate of the EPF in the environment or effects on non-target organisms (Jackson et al. 2010, Jaronski 2010). The efficacy of EPF as BCA is mainly influenced by the interaction of the fungus and the host insect (Hajek and St. Leger 1994). For instance, physiological features of the fungus, e.g. successful enzymatical penetration of the insect's cuticle and production of toxins within the host, may determine the efficacy. In addition, environmental factors such as temperature and moisture are crucial. Moreover, the physiology of the host insect e.g. defence mechanisms or the respective developmental stage of the pest, may play an important role for the efficacy of an entomopathogenic fungus as a biocontrol agent.

Regarding *Otiorhynchus* weevils, Beck (1996) documented in laboratory trials, that different developmental stages of *O. sulcatus* have shown a varying susceptibility towards various *M. anisopliae* isolates. Whereas all tested fungal isolates showed high mortality rates for larvae, differences in efficacy of the tested isolates were documented for eggs and adults of the black vine weevil (Beck 1996). Moreover, investigations performed by Zimmermann (1982) showed, that *M. anisopliae* could effectively infect freshly laid eggs (one day old), whereas older eggs (six and eight days old) were less susceptible. Both authors assume that the chorion/cuticle composition of the respective developmental stage of *O. sulcatus* is initially determining the successful penetration of the respective entomopathogenic fungus (Zimmermann 1982, Beck 1996). Furthermore the selection of the appropriate fungal species and even the isolate may be crucial for successful control of *Otiorhynchus* weevils. Vainio and Hokkanen (1993) tested in laboratory trials the efficacy of *B. bassiana* and *M. anisopliae* against *Otiorhynchus ovatus* Linnaeus and *Otiorhynchus nodosus* (= *O. dubius*) Müller larvae. As a result of this study, *B. bassiana* was found to be less effective than *M. anisopliae*, indicating that different EPF may have a varying efficacy against *Otiorhynchus* larvae. Moreover, Sabbahi et al. (2008) detected a variation in virulence of different *B. bassiana* isolates against adult weevils of the species *O. ovatus*.

Entomopathogenic fungi are particularly interesting for the control of beetle pests, because viral and bacterial diseases are rare among Coleoptera (Hajek and St. Leger 1994). The identification of an appropriate fungal pathogen for development as bio-

insecticide may be complex and expensive till market launch (Jackson et al. 2010). Therefore the present thesis investigates whether the EPF already listed in Annex I and formulated in the commercial products Naturalis® and PreFeRal® WG, as well as the *M. anisopliae* strain Ma43, provide satisfactory *Otiorhynchus* spp. control, in order to speed up the national undergoing registration process and consequently the market launch, to provide growers and nurserymen with a potential alternative in *Otiorhynchus* pest control.

3.5 Endosymbiotic bacteria in insects

The expression symbiosis originates from the Greek syn- “together” + bios “life” and was first used in 1879 by Anton de Bary to describe the “living together” of differently named organisms (Gil et al. 2004). Symbiosis may include both exo- and endo-symbiosis. In contrast to exosymbiosis, in which one of the organisms lives on the exterior of the other, endosymbiosis describes the association, when one of the involved organisms lives inside the other organism (Gil et al. 2004). Endosymbiosis may be of mutualistic (beneficial to both organisms), commensal (beneficial to one without effect on the other) or parasitic (beneficial to one and harmful to the other) nature and occurs frequently in insects, which may serve as hosts for micro-organisms such as bacteria and fungi (Buchner 1953). Although fungi play an equally important role in insect symbiosis (for review see Gibson and Hunter 2010), so far, most research has been performed on bacteria-insect-associations. Insects may be associated with single or multiple bacteria species - sometimes forming complex communities - with various effects on their hosts (Ferrari and Vavre 2011). These effects may include reproductive manipulations (Engelstädter and Hurst 2009), nutritional relationships (Gosalbes et al. 2010), host plant specialization (Leonardo and Muiru 2003, Tsuchida et al. 2004, Janson et al. 2008) or protection against natural enemies (Kaltenpoth et al. 2005, Scarborough et al. 2005, Panteleev et al. 2007, Oliver et al. 2010, Vorburger et al. 2010), to name but a few.

Endosymbionts are divided into primary endosymbionts (P-endosymbionts) and secondary endosymbionts (S-endosymbionts). The latter ones usually have a beneficial role and are not essential for the insect host reproduction (Moran et al. 2008). There-

fore they are also called facultative endosymbionts. Although facultative endosymbionts are not essential, they may have a strong influence on the ecology and evolution of the infected insects (Oliver et al. 2010). In contrast, P-endosymbionts are obligate to support normal insect development (Moran et al. 2008) and therefore are known as obligate endosymbionts. P-endosymbionts are in general restricted to special cells, termed mycetocytes or bacteriocytes, which may form an organ, called mycetome or bacteriome, respectively (Buchner 1953). S-endosymbionts may be found as well in bacteriocytes but may also occur e.g. in cells of the reproduction organs or extracellularly in the haemolymph of the insect (Moran et al. 2008). Obligate endosymbionts are passed from one generation to the other by vertical transmission from mother to offspring, whereas facultative endosymbionts may be transmitted vertically as well as horizontally (Fukatsu and Shimada 1999).

The best studied endosymbionts in insects are bacteria of the genus *Wolbachia* (Werren et al. 2008). It is estimated that more then 60% of all insects are infected with those bacteria (Hilgenboecker et al. 2008). *Wolbachia* plays an important role in the manipulation of host reproduction such as cytoplasmic incompatibility (Bourtzis et al. 2003), feminization (Kageyama et al. 2002, Negri et al. 2006), male killing (Hurst et al. 1999, Fialho and Stevens 2000) and parthenogenesis (Huigens and Stouthamer 2003) in numerous insect species. However, male killing and parthenogenetic reproduction are phenomena which also may be linked in insects to an association with bacteria of the genus *Rickettsia* (Lawson et al. 2001, Schulenburg et al. 2001, Hagimori et al. 2006, Giorgini et al. 2010), which are closely related to *Wolbachia* bacteria (Williams et al. 2007). Occurrence of *Wolbachia* is also known for several *Otiorhynchus* species (Stenberg et al. 2003, Son et al. 2008, Lachowska et al. 2010) and may be necessary for normal development of *O. sulcatus* eggs (Son et al. 2008). Although bacterial associations in the genus *Otiorhynchus* have been historically mentioned by Buchner (1953), few attempts have been made so far, to extend knowledge about bacterial endosymbionts in *Otiorhynchus* spp. In addition, Buchner (1953) mentioned that bacterial endosymbionts of *Otiorhynchus* spp. are located within a bacteriome. Although nowadays, bacterial endosymbionts may be accurately localized via in situ hybridization, as it is described for bacteria present in the bacteriome of larvae and the apex of an ovary from an adult female of the weevil

genus *Curculio* (Toju et al. 2010), such studies are still lacking for *Otiorhynchus* weevils.

Arthropod symbiosis may be efficiently used for designing strategies in insect pest control. For instance, the pest status of an insect may be determined by its bacterial associates, which has been proven for bacteria associated with pest stink bugs on crop legumes (Hosokawa et al. 2007). Moreover, some insects are vectors of bacterial pathogens which may cause plant diseases. In the case of vector transmitted diseases, such as the Pierce's disease of grape, paratransgenesis might be a promising "symbiotic control" strategy (Bextine et al. 2004). Paratransgenesis is a technique which uses genetically modified insect symbionts, which are able to express molecules that may disrupt pathogen development or pathogen transmission by the vector (Coutinho-Abreu et al. 2010). Furthermore, the incompatible insect method, which is based on *Wolbachia* introduced cytoplasmic incompatibility, may be used for instance as an environmentally friendly technique for the control of cherry fruit flies which are a major problem in cherry production (Zabalou et al. 2004). Further examples for arthropod-symbiont interactions with impact on biological control are reviewed in Zindel et al. (2011). In conclusion, the identification of symbionts in arthropods and subsequently the understanding of their interactions harbour enormous potential for the development of novel pest- and disease control strategies in crop protection (Douglas 2007).

3.6 Molecular markers and methods used for taxonomic affiliation

Identification of organisms to species level is an essential element in many biological research approaches, either in basic or applied sciences. Traditionally, the identification of species is mainly based on morphological characteristics. Sometimes however, morphological features are not available or not sufficient for an accurate species determination. For instance, immature life stages of weevils such as larvae are often cryptic and therefore cannot be identified based on their morphology (Antonini et al. 2009). In addition, accurate species identification often requires the appropriate literature and taxonomic expertise (Hebert et al. 2003). For these reasons nowadays, DNA-based identification methods, so called molecular markers, are frequently

applied. These methods usually focus on particular DNA sequences in the genome of the respective organism, which may be referred to as genetic “barcodes” (Hebert et al. 2003). DNA barcoding mainly concentrates on the mitochondrial cytochrome oxidase subunit I (COI, frequently also abbreviated as coxA) gene, which serves as the appropriate gene for species identification in insects and other animals (Hebert et al. 2003). Although the COI gene is widely used as genetic barcode for species determination (Hebert et al. 2003, Kerr et al. 2007, Hubert et al. 2008) it is not per se suitable for all groups of organisms. For instance, bacterial species are mainly characterised by their ribosomal 16S rDNA and species determination of fungi is often based on the internal transcribed spacer (ITS) gene region (Begerow et al. 2010). In addition, the cytochrome oxidase subunit II (COII) gene seems to be a good molecular marker for identification of *Otiorhynchus* weevils (Hirsch et al. 2010). For DNA based species identification, the above mentioned genes may be amplified via polymerase chain reaction (PCR) and subsequently sequenced. Afterwards, obtained sequence reads are blasted against a database e.g. GenBank to assign the reads to the respective organism. However, sequencing of DNA fragments requires the appropriate technical equipment, and for sequence analysis some understanding of bioinformatics is necessary. Therefore, several molecular tools have been applied to facilitate DNA based species determination. For instance, PCR products may be digested with restriction enzymes, generating, after electrophoretical separation, a species-specific fragment pattern, which allows an accurate species identification on the basis of the number and size of generated fragments (Brown et al. 1999, Muraji and Nakahara 2002, Hirsch et al. 2010). This molecular method is called polymerase chain reaction (PCR)- restriction fragment length polymorphism (RFLP).

As many bacterial and fungal species, e.g. endosymbionts of insects or bacteria and fungi present in the soil, are difficult to culture or cannot be cultured outside of their natural environment, many culture-independent molecular methods have been applied to depict microbial diversity (Kirk et al. 2004, Kikuchi 2009, van Elsas and Boersma 2011). For instance, techniques such as denaturing gradient gel electrophoresis (DGGE) or single-strand conformation polymorphism (SSCP) are widely applied to describe microbial diversity in soil and insects (Mohr and Tebbe 2006, Adams et al. 2010, Hussain et al. 2011). These two methods separate electrophore-

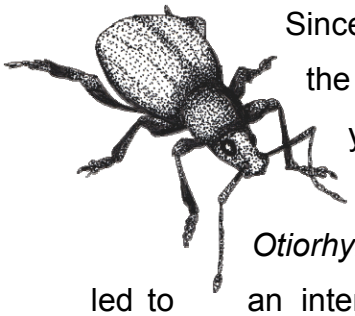
tically DNA fragments, which have been amplified previously via PCR, based on their sequence composition, and subsequently result in community specific band patterns of the present microbiota. Although these methods are suitable to detect community shifts, reflected by the presence or absence of taxa (indicated by the presence or absence of bands), they do not give any information on the taxonomic affiliation of the community members (O'Brien et al. 2005). Taxonomic affiliation may be achieved by subsequent sequencing of the respective "bands" which is however time-consuming and costly. Therefore, for the description of microbial communities, e.g. in soil, often metagenomic approaches are applied (Daniel 2005). The term "metagenomics" describes the usage of genomic methods for the analysis of microbial communities directly in their natural habitat, without the need for isolation and cultivation of the respective species (Chen and Pachter 2005). Traditional metagenomic approaches usually construct gene clone libraries with subsequent sequencing of clones (Daniel 2005, Kimura 2006). However, the construction of clone libraries or the application of DGGE or SSCP are laborious and costly methods to describe whole microbial communities in soil or insects. Recently, next generation sequencing (NGS) technologies like 454 pyrosequencing have become available. 454 pyrosequencing is a fast and cost-efficient PCR based method, as thousands of sequence reads may be analysed in parallel in one sequencing run (Harkins and Jarvie 2007). This technique may be regarded as a modern metagenomics approach, as whole microbial communities such as those present in the soil or as endosymbionts in insects, may be described without the need for isolation and cultivation of the respective species, as well as construction of time-consuming clone libraries (Buée et al. 2009, Lim et al. 2010, Hail et al. 2011, Hirsch et al. 2012).

Some entomopathogenic fungal species, such as *B. bassiana* embody a cryptic phylogenetic species complex (Rehner et al. 2011), which does not allow a strain-specific identification of the respective isolate only based on morphological characteristics. However, studies analysing the fate or the interaction of an artificially applied entomopathogenic fungus with other microorganisms present in the soil, require a strain-specific identification of the applied fungus (Enkerli et al. 2001, Rehner and Buckley 2003). For this reason, a molecular method based on microsatellite markers, also referred to as simple sequence repeats (SSR), has been

established for the determination of different *Beauveria brongniartii* (Saccardo) Petch (Enkerli et al. 2001) and *B. bassiana* (Rehner and Buckley 2003, Bischoff-Schaefer et al. in preparation) isolates. Microsatellite markers are short (one to six bases), tandemly repeated, non-coding DNA sequences which are polymorphic between individuals, as the number of DNA sequence repeats, and thus the size of the amplified PCR product may vary between different isolates (Enkerli et al. 2001). In addition, microsatellite markers are a fast and culture-independent molecular method, to detect fungal strains in bulk soil DNA, isolated from samples collected in the field or from different potting media (Schwarzenbach et al. 2007, Bischoff-Schaefer 2010, Sandhya et al. 2011).

Nowadays, many molecular markers and techniques are available to describe diversity on different taxonomic levels. Each marker and each method has its advantages and disadvantages. Therefore, the combination of several markers and/or techniques, as described in chapter 4, 7 and 8, is an appropriate strategy to overcome limitations of taxonomic affiliation of organisms with the respective molecular tools.

3.7 Aims of this thesis



Since the first documentation of damage to cultivated plants caused by the black vine weevil in Germany in 1834 (Smith 1932), almost 200 years have passed with intensive studies on its biology and control. In recent years however, the damage produced by other *Otiorhynchus* species has increased (Collman 2001, Sprick 2009). This led to an intensified interest in the biology of these species and raised the question whether implemented control strategies against the black vine weevil may be adopted as well for other *Otiorhynchus* species.

In the present thesis, I have been investigating intensively the biology and genetic diversity of different *Otiorhynchus* species as well as the prospects of biological control of weevils with EPF. In addition, the influence of an artificially applied entomopathogenic fungus on the naturally occurring soil microbiota in an agricultural field in India was analysed. The experimental setup and the results of the described research are illustrated in the following chapters.

Larvae are the most harmful developmental stage of *Otiorhynchus* weevils. However, *Otiorhynchus* larvae cannot be distinguished at species level due to a lack of morphological characteristics. As species determination is a prerequisite for developing pest management strategies, in **chapter 4** a cost-effective, robust and fast molecular method, based on a PCR-RFLP approach, is described to identify cryptic larvae of economically important weevil species in horticulture.

Chapter 5 focuses on prospects of biological control of *Otiorhynchus* spp. with different EPF. Several bioassays were performed under laboratory conditions to analyse the susceptibility of different developmental stages of *Otiorhynchus* spp. to the commercially available products Naturalis® and PreFeRal® WG as well as the entomopathogenic fungus *M. anisopliae* strain Ma43.

The effectiveness of an entomopathogenic fungus as biological control agent against *Otiorhynchus* weevils may differ in laboratory trials and under field conditions. Therefore, a method to quantify the efficacy of *B. bassiana* strain ATCC 74040 (Naturalis®), under field conditions for the control of *Otiorhynchus* spp. in peony plantations, was established, which is described in **chapter 6**.

The entomopathogenic fungus *B. bassiana* is used as biocontrol agent against many insect pests worldwide. Although the interaction of *B. bassiana* and its host insect is quite well studied, little is known about the influence of artificially applied *B. bassiana* on the indigenous soil fungal communities. In addition, less information on the fate and spread of *B. bassiana* is available, when applied onto the soil. These aspects have been analysed in the present thesis by using microsatellite markers and multitag 454 pyrosequencing and are described in **chapter 7**.

The rapid spreading of several *Otiorhynchus* species is supported by the global trade of infested plant material, whereas their establishment as pests in different horticultural crops may be influenced by their asexual mode of reproduction and their potential to adapt to new host plants. Asexual reproduction and adaptation to new host plants are mechanisms, which may be linked to the presence of endosymbiotic bacteria. Therefore, in **chapter 8** the bacterial endosymbiotic community of four eco-

nomically important *Otiorhynchus* species was investigated via multitag 454 pyro-sequencing.

The final **chapter 9** intends to summarize the most important results of this thesis and to discuss them against the background of current knowledge. Moreover, this chapter outlines potential prospective research approaches, which may assist to expand the range of current knowledge for developing new pest management strategies for the control of *Otiorhynchus* spp.

4 Molecular identification of larval stages of different *Otiorhynchus* species based on PCR-RFLP analysis

Parts of this chapter have been published as:

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Gosik, R., **Hirsch, J.** & Sprick, P. (2010): Biology and molecular determination of *Pachyrhinus lethierryi* (Desbrochers, 1875) with description of the mature larva and pupa (Coleoptera, Curculionidae, Entiminae: Polydrusini). SNUDEBILLER 11, Studies on taxonomy, biology and ecology of Curculionoidea, 80-95, Mönchen-gladbach, CURCULIO-Institute.**

* **Author contributions:** J. Hirsch and A. Reineke conceived the study design. P. Sprick performed weevil collection and morphological determination of weevils. J. Hirsch performed molecular analysis and evaluation of results.

** **Author contributions:** R. Gosik and P. Sprick performed description of biology and morphology of *P. lethierryi* and J. Hirsch did molecular analysis.

4.1 Abstract

A couple of different members of the Coleopteran genus *Otiorhynchus* (Coleoptera: Curculionidae) are becoming increasingly important as pests of nursery and ornamental plants in global horticulture. Although adult weevils are morphologically distinguishable by skilled personnel, high potential for misidentification is given for cryptic larval stages. For developing and applying efficient pest management strategies, the determination of the respective species is however a prerequisite, because each species may have a different phenology or a varying susceptibility to pesticides. The present study reports on the development of a diagnostic polymerase chain reaction (PCR)- restriction fragment length polymorphism (RFLP) method for differentiation among 16 *Otiorhynchus* and eight other weevil species independent of their developmental stage. A ~780 bp fragment of the mitochondrial cytochrome oxidase subunit II was amplified and subsequently digested with at most four restriction enzymes generating species-specific fragment patterns. The assay was validated on a total of 143 individuals and the obtained fragment patterns correctly identified 24 different weevil species. The PCR-RFLP method reported here is cost-effective, robust and fast and could be used in the future by plant protection services for diagnostic purposes.

4.2 Introduction

Several members of the weevil genus *Otiorhynchus* Germar are extremely polyphagous and are known as serious pests in a wide variety of horticultural crops. Among them, the black vine weevil, *Otiorhynchus sulcatus* Fabricius (Coleoptera: Curculionidae), is the most important worldwide (Moorhouse et al. 1992). Soil-borne larvae of *Otiorhynchus* species feed on the roots of their host plants, which could be lethal especially for younger plants or recently transplanted cuttings (Smith 1932, Moorhouse et al. 1992). Nocturnal adults cause damage by feeding on the foliage of the plant. This may not greatly affect the vitality of the plant but may alter the decorative appearance of ornamentals and consequently reduces their market values. Besides *O. sulcatus*, a recent survey in Germany and other European countries has revealed that a couple of other *Otiorhynchus* species are present on various horticultural crops (Sprick 2009), causing more or less similar visible symptoms on the leaves of their host plant as does *O. sulcatus*. Among them, *Otiorhynchus salicicola* Heyden, *Otiorhynchus crataegi* Germar or *Otiorhynchus singularis* Linnaeus were found in numbers high enough to assume the infliction of a significant damage on their respective host plant. Some of these species have apparently extended their geographic range maybe either as a result of climate change and/or intensified global trade of plant material. As a result, characteristic visible notches on leaves resulting from adult feeding are at first sight associated with the presence of *O. sulcatus*, which is in fact not necessarily the case. A recent survey in the Pacific Northwest showed as well that the black vine weevil is not exclusively responsible for weevil damage in nurseries (Collman 2001).

In the past, different pest management strategies against the black vine weevil have been developed and are implemented by the majority of ornamental and nursery plant growers. Beside treatments of plants with chemical insecticides (Cross et al. 1995, Cross and Burgess 1997, Rosetta et al. 1999), there is considerable and increasing interest in the application of alternative control strategies, mainly due to toxicological and environmental requirements (Collman 2001, Shah et al. 2007, Ansari et al. 2008, Kowalska 2008). Biological control agents such as entomopathogenic fungi (Bruck 2006, Bruck and Donahue 2007), nematodes (Kakouli-Duarte et

al. 1997, Lola-Luz et al. 2005, Lola-Luz and Downes 2007), and microsporidia (Bruck et al. 2008) have been successfully applied to control larvae of the black vine weevil. However, besides studies on the effectiveness of entomopathogenic fungi and nematodes against *O. sulcatus*, only a small number of reports are published assessing the susceptibility of a few other *Otiorhynchus* species, such as *Otiorhynchus ovatus* Linnaeus, *Otiorhynchus ligustici* Linnaeus and *Otiorhynchus nodosus* (= *O. dubius*) Müller towards these biological control agents (Vainio and Hokkanen 1993, Simser and Roberts 1994, Shields et al. 1999, Neumann and Shields 2008, Sabbahi et al. 2008).

Generally, both chemical as well as biological control measures are targeting the larval stage of *Otiorhynchus* species. For this reason, a correct identification of the respective weevil species in its larval stage is a prerequisite for applying the appropriate control strategy and for evaluating its efficiency. However, although the taxonomic identification of adult weevils is yet difficult but feasible by trained personnel, the morphological determination of weevil larvae is almost impossible because of a lack of sufficient phenotypical characteristics (Scherf 1964). Larvae of the family Curculionidae are usually legless, white grubs with light brownish heads and a ventrally curved, crescent form, showing no typical species-specific characters. Thus, larvae found in the soil or in potting media may be misidentified by growers or plant protection advisors. Such a risk of a false determination of weevil species might have resulted in the failure of costly control measures in the past. Therefore, a rapid, inexpensive and reliable diagnostic method is needed to positively identify different *Otiorhynchus* species both in the larval as well as the adult stage.

The use of molecular markers based on the amplification of regions of genomic DNA by polymerase chain reaction (PCR) provides a valuable addition or alternative to traditional phenotypic methods of species recognition (Wagener et al. 2004, Carletto et al. 2009, Kim et al. 2009). In insects, molecular DNA markers have become the most common yardstick for measuring intra- and interspecific genetic differences (Behura 2006). They also allow the determination of cryptic species or morphologically cryptic immature life stages such as larvae (Brown et al. 1999, Barcenas et al. 2005, Antonini et al. 2009, Ellis et al. 2009). These advantages have surely

propelled the barcoding of life project, which aims to establish a global bioidentification system for animals by using mitochondrial DNA (Hebert et al. 2003). Commonly used genetic regions are nuclear ribosomal DNA (e.g. internal transcribed spacers) or mitochondrial DNA (Caterino et al. 2000). Mitochondrial DNA is often used as a tool for species identification because it can be readily amplified by using universal primers for the highly conserved cytochrome oxidase subunit I (COI) and subunit II (COII) genes (Zhang and Hewitt 1997). Moreover, parts of the mtDNA can be used for genotyping and thus enabling species identification by a combination with the restriction fragment length polymorphism (RFLP) technique (Brown et al. 1999, Muraji and Nakahara 2002).

The present study reports on the development of a genetic fingerprint system based on PCR-RFLP, to positively and quickly identify larval stages of *Otiorhynchus* species. This molecular diagnostic tool is based on the amplification and subsequent digestion of a COII fragment and generates species-specific banding patterns in 24 different weevil species, with most of them having a potential pest status in global horticulture.

4.3 Materials and methods

4.3.1 Biological material and DNA extraction

In total, 16 *Otiorhynchus* and eight other weevil species were collected, conserved in 70-96% ethanol and subsequently genetically analysed. The number of examined individuals per species, geographic origin and contextual data are summarized in Table 1. Total genomic DNA was extracted from three to six legs per adult weevil by using the method described by Reineke et al. (1998). An exception was the small species *Barypeithes pellucidus* Boheman and *Pachyrhinus lethierryi* Desbrochers, where the whole body was processed. The remaining weevils (except for *B. pellucidus*, *P. lethierryi* and larvae) were conserved in 96% ethanol and are deposited as vouchers (see catalogue numbers in Table 1; no numbers were allocated for *P. lethierryi* individuals) at the Museum of Natural History (Mainz, Germany). DNA was also extracted from weevil larvae (E-2009/1090-E-2009/1092). In this case, the surface of soil-dwelling larvae was sterilized with 70% ethanol before

using the whole body for DNA extraction. Extracted DNA (from adult weevils or larvae) was resuspended in 50 µl sterile water and stored at -20°C until needed.

4.3.2 PCR amplification

A fragment (~780 bp) of the mitochondrial genetic marker COII was amplified using the primers TL-J-3037 (5′-TAATATGGCAGATTAGTGCATTGGA-3′) and TK-N-3785 (5′-GAGACCATTACTTGCTTTCAGTCATCT-3′) described by Machado et al. (2008). The PCR amplification was set up in a total volume of 20 µl consisting of 10x reaction buffer, 10 pmol of each primer, 2.75 mM $MgCl_2$, 0.25 mM dNTPs, 0.01 mg of bovine serum albumin (BSA), 0.5 U of Taq polymerase (Metabion, Martinsried, Germany) and 1-2 µl of DNA (at least 20 ng/µl) for all species except *P. lethierryi*. For the amplification of the COII fragment of *P. lethierryi* individuals Phire™ Hot-Start DNA Polymerase and 5x reaction buffer were used as described by the manufacturer (Finnzymes, Espoo, Finland). The PCR reaction was performed using 2 min at 94°C initial denaturation, followed by 35 cycles of 10 s at 94°C, 20 s at 54.4°C and 30 s at 72°C. A final extension step at 72°C for 5 min was added. An aliquot of 4 µl of each PCR product was checked for correct size (~780 bp) on an 1% agarose gel. As size marker, a 1-kb ladder (Metabion) was used. Electrophoresis was carried out at 80 V for 30 min. Gels were visualized under UV light and subsequently photographed. In each PCR-run, a negative control, using sterile water instead of template DNA, was included. The remaining PCR products were stored at -20°C.

4.3.3 Sequence analysis and preliminary identification of restriction sites

Initially, four COII PCR products obtained from four different species (E-2009/1000, E-2009/1057, E-2009/1087 and E-2009/1093) were purified (Hi Yield® PCR Clean-up/Gel Extraction Kit, Süd-Laborbedarf GmbH, Gauting, Germany), cloned into a plasmid vector, and subsequently sequenced (GU182418, GU182451, GU182474 and GU182477-GU182481). Furthermore, 83 COII amplicons (at least two per species) were directly sequenced in both directions using the same primers as in the PCR reactions. Cloning and sequencing of clones and PCR products was carried out commercially (AGOWA, Berlin, Germany). All sequences were deposited in GenBank

with the accession numbers GU182417-GU182502 and GU810915-GU810920 (Table 1).

For two individuals of *Otiorhynchus raucus* Fabricius (E-2009/1030 and E-2009/1031), a second faint ~650 bp PCR product was sometimes simultaneously amplified with the ~780 bp mitochondrial target sequence (Figure 8, B). This fragment was purified from the agarose gel, reamplified with the COII primers, and cloned and subsequently sequenced in both directions as described above. For restriction fragment length polymorphism analysis only PCR amplicons of *O. raucus* individuals showing a single ~780 bp mitochondrial target fragment were used.

For evaluation of inter- and intraspecific variation within the respective COII fragment and to indicate that the analysed sequences have originated from mtDNA, all sequences, gained from direct sequencing of PCR products as well as those obtained from GenBank (EF583371, EF583362, EF583315, EF583334, DQ009615, DQ836075 and DQ836074), were aligned manually in MEGA4 (Tamura et al. 2007), and a neighbour-joining consensus tree based on the number of base pair differences was calculated (Figure 10). As parameter gaps/missing data, complete deletion was chosen. Bootstrap values were calculated from 1000 replicates.

For initial in silico calculation of restriction fragment sizes, the four cloned and subsequently sequenced target COII PCR products served as templates for length standards (779-785 bp). For all other sequences, the restriction sites were estimated using Serial Cloner1.3 (http://serialbasics.free.fr/Home/Home.html) and predicted fragments were compared to the length standard to calculate the respective lengths of restriction fragments (Figure 11).

After digestion of COII PCR products with restriction enzyme *Taq*I all *O. ovatus* individuals showed several times nonconforming restriction band patterns as those estimated using Serial Cloner1.3 (compare Figure 8, A lane 14, C lane 1 and Figure 11). Therefore, a COII PCR product of *O. ovatus* (E-2009/1093) was cloned into a plasmid vector, and eight different clones were subsequently sequenced as described above.

4.3.4 RFLP analysis of COII PCR products

In a first assay, all COII PCR products, except for *P. lethierryi*, were digested using the enzyme *Taq*I (Metabion). The digestion was performed in 20 µl reaction volumes with 10 µl of unpurified PCR product and 10 U *Taq*I in the presence of BSA including appropriate buffer provided by the manufacturer. The incubation took place at 65°C for 1 h. Obtained restriction fragments were electrophoresed on a 3% agarose gel at 80 V for 60 min. All PCR products obtained from *P. lethierryi* were digested using the FastDigest® *Taq*I restriction enzyme (Fermentas GmbH, St. Leon-Rot, Germany). The digestion was performed using the appropriate buffers provided by the manufacturer. The incubation took place at 65°C for 5 min. Obtained restriction fragments were electrophoresed on a 3% agarose gel at 120 V for 30 min. As size standard, a 1-kb ladder (Metabion) was used respectively. DNA fragments were visualized and photographed under UV light.

For a number of weevil species, a secondary digest with appropriate restriction enzymes was subsequently performed: The COII PCR products of *Otiorhynchus morio* Fabricius, *Peritelus sphaeroides* Germar, *Barypeithes pellucidus* Boheman, *Barynotus moerens* Fabricius and *O. sulcatus* were as well digested with 10 U of *Taa*I, products obtained from *Otiorhynchus smreczynskii* Cmoluch and *Barypeithes trichopterus* Gautier with 10 U of *Xba*I and amplicons from *O. salicicola* and *Otiorhynchus coecus* Germar with 10 U of *Mnl*I, respectively. Conditions for restriction digest and electrophoretic separation were as described above, except that incubation temperature was set at 37°C for *Xba*I and *Mnl*I. All restriction digests were performed using the appropriate buffers provided by the manufacturer (Fermentas GmbH, St. Leon-Rot, Germany).

Table 1: Data on place, individual numbers (n), date of collection and catalogue numbers of *Otiorhynchus* and other weevil species (except for *P. lethierryi*) used in the present study including GenBank accession numbers for respective sequences of the COII gene. * All locations are in Germany except one.

Species	n	Catalogue numbers	Location*	Collection date	GenBank accession numbers COII
O. dieckmanni Magnano	4	E-2009/1000-E-2009/1003	Hannover-Nordstadt	June 2008	GU182417-GU182419
	2	E-2009/1004, E-2009/1005	Bottrop	May 2008	GU182420
	2	E-2009/1006, E-2009/1007	Heilbronn	May 2008	GU182421
	2	E-2009/1008, E-2009/1009	Wiesbaden	May 2008	GU182422
	1	E-2009/1010	Bad Zwischenahn	July 2008	-
O. rugosostriatus Goeze	4	E-2009/1011-E-2009/1014	Wiesbaden	July 2008	GU182423-GU182424
	2	E-2009/1015, E-2009/1016	Rellingen	July 2008	GU182425
	2	E-2009/1017, E-2009/1018	Hannover-Linden	May 2009	-
O. crataegi Germar	2	E-2009/1019, E-2009/1020	Braunschweig	June 2008	GU182426-GU182427
	2	E-2009/1021, E-2009/1022	Bad Zwischenahn-Rostrup	July 2008	-
	4	E-2009/1023-E-2009/1026	Bad Zwischenahn	July 2008	-
	3	E-2009/1027-E-2009/1029	Wahlsburg-Lippoldsberg	Aug. 2008	GU182428-GU182429
O. raucus Fabricius	2	E-2009/1030, E-2009/1031	Wiesbaden	July 2008	GU182430-GU182431
	1	E-2009/1032	Bottrop	June 2008	GU182432
	1	E-2009/1033	Braunschweig	June 2008	GU182433
	1	E-2009/1034	Rellingen	July 2008	GU182434
	1	E-2009/1035	Hannover-Herrenhausen	May 2008	GU182435
O. smreczynskii Cmoluch	4	E-2009/1036-E-2009/1039	Hannover-Nordstadt	June 2008	GU182436-GU182437
O. porcatus Herbst	2	E-2009/1040, E-2009/1041	Nordstemmen	May and Aug. 2008	GU182438-GU182439
	2	E-2009/1042, E-2009/1043	Hannover-Herrenhausen	Aug. 2008	GU182440-GU182441
O. armadillo Rossi	3	E-2009/1044-E-2009/1046	Neckarsulm-Amorbach	June 2008/July 2009	GU182442
	1	E-2009/1047	Oedheim-Degmarn	Sept. 2008	GU182443
	3	E-2009/1048-E-2009/1050	Niefern-Öschelbronn	July 2009	GU182444

Table 1 (continued)

Species	n	Catalogue numbers	Location*	Collection date	GenBank accession numbers COII
O. salicicola Heyden	6	E-2009/1051-E-2009/1056	Hamburg-Lohbrügge	Sept. 2008	GU182445-GU182449
	2	E-2009/1057, E-2009/1058	Heilbronn-Leingarten	June 2008	GU182450-GU182452
O. lugdunensis Boheman	5	E-2009/1059-E-2009/1063	Hannover-Linden	June 2008/ May 2009	GU182453-GU182454
O. singularis Linnaeus	1	E-2009/1064	Bottrop	May 2008	GU182455
	3	E-2009/1065-E-2009/1067	Hannover-Herrenhausen	May 2008	GU182456-GU182458
O. morio Fabricius	3	E-2009/1068-E-2009/1070	Nordstemmen	May 2008	GU182459-GU182460
O. coecus Germar	1	E-2009/1071	Lusen (mountain)	June 2008	GU182461
	1	E-2009/1072	*Austria: Neukirchen Bramberg	June 2009	GU182462
O. meridionalis Gyllenhal	2	E-2009/1073, E-2009/1074	Heilbronn	July, Sept. 2008	GU182463-GU182464
	2	E-2009/1075, E-2009/1076	Heilbronn	July 2009	GU182465-GU182466
O. ligustici Linnaeus	2	E-2009/1077, E-2009/1078	Frankfurt (Oder)	May 2008	GU182467-GU182468
	2	E-2009/1079, E-2009/1080	Oberulrain	April 2009	GU182469-GU182470
	3	E-2009/1081-E-2009/1083	Lübbenau-Kittlitz	April 2009	-
O. sulcatus Fabricius	2	E-2009/1084, E-2009/1085	Amtsberg-Dittersdorf	June 2008	GU182471-GU182472
	2	E-2009/1086, E-2009/1087	Wahlsburg-Lippoldsberg	Aug. 2008	GU182473-GU182474
	1	E-2009/1088	Hamburg-Lohbrügge	Sept. 2008	GU182475
	1	E-2009/1089	Hannover-Linden	May 2009	-
	3	E-2009/1090-E-2009/1092	Bad Zwischenahn	Mar. 2009	-
O. ovatus Linnaeus	2	E-2009/1093, E-2009/1094	Hannover-Nordstadt	June 2008	GU182476-GU182482
	2	E-2009/1095, E-2009/1096	Rellingen	July 2008	-
	3	E-2009/1097-E-2009/1099	Wahlsburg-Lippoldsberg	Aug. 2008	-
Sciaphilus asperatus Bonsdorff	1	E-2009/1100	Nordstemmen	Aug. 2008	GU182483
	4	E-2009/1101-E-2009/1104	Hannover-Herrenhausen	April 2008/ May 2009	GU182484-GU182485
Notaris scirpi Fabricius	2	E-2009/1105, E-2009/1106	Bad Zwischenahn	Aug. 2008	GU182486-GU182487
Peritelus sphaeroides Germar	2	E-2009/1107, E-2009/1108	Bottrop	May 2008	GU182488-GU182489
Barynotus moerens Fabricius	1	E-2009/1109	Hannover-Herrenhausen	Aug. 2008	GU182490
	1	E-2009/1110	Hameln	June 2009	GU182491

Table 1 (continued)

Species	n	Catalogue numbers	Location*	Collection date	GenBank accession numbers COII
Barypeithes trichopterus Gautier	6	E-2009/1111-E-2009/1116	Vaihingen (Enz)	May 2008	GU182492-GU182495
Barypeithes pellucidus Boheman	4	E-2009/1117-E-2009/1120	Vaihingen (Enz)	May 2008	GU182496-GU182499
Strophosoma melanogrammum Forster	1	E-2009/1121	Hannover-Herrenhausen	May 2008	GU182500
	5	E-2009/1122-E-2009/1126	Braunschweig	June 2009	GU182501-GU182502
Pachyrhinus lethierryi Desbrochers	5	-	Heilbronn-Horkheim	May 2008/ March 2009	GU810919-GU810920
	2	-	Berlin-Rudow	June 2009	GU810915
	3	-	Braunschweig	June 2009	GU810916
	2	-	Braunschweig-Rüningen	June 2009	-
	2	-	Bad Zwischenahn	June 2009	GU810917
	2	-	Niefern-Öschelbronn	June 2009	GU810918

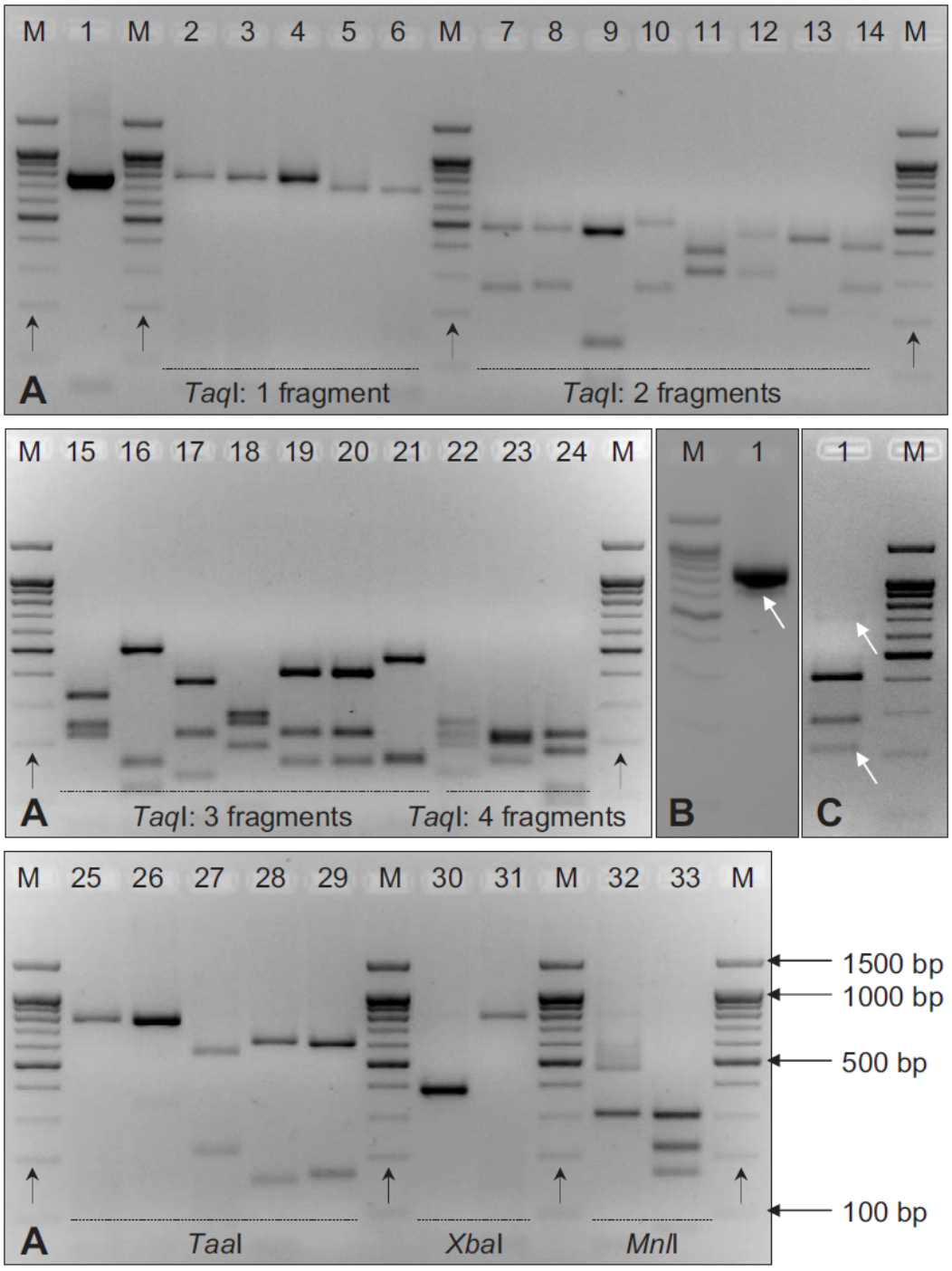


Figure 8: (A) Agarose gels (3%) illustrating in lane 1: the amplified COII fragment (undigested) of *O. sulcatus* (E-2009/1086) and lanes 2-24: restriction fragment patterns of the COII PCR product produced by *Taq*I. Species-specific banding patterns are shown in lane 2: *P. sphaeroides* (E-2009/1108), 3: *B. pellucidus* (E-2009/1117), 4: *O. morio* (E-2009/1069), 5: *O. smreczynskii* (E-2009/1037), 6: *B. trichopterus* (E-2009/1116), 7: *B. moerens* (E-2009/1109), 8: *O. sulcatus* (E-2009/1086), 9: *S. melanogrammum* (E-2009/1125), 10: *O. lugdunensis* (E-2009/1059), 11: *O. rugosostriatus* (E-2009/1014), 12: *O. dieckmanni* (E-2009/1000), 13: *O. armadillo* (E-2009/1047), 14: *O. ovatus* (E-2009/1093), 15: *N. scirpi* (E-2009/1106),

16: *O. raucus* (E-2009/1032), 17: *O. crataegi* (E-2009/1020), 18: *O. porcatus* (E-2009/1042), 19: *O. salicicola* (E-2009/1053), 20: *O. coecus* (E-2009/1071), 21: *O. meridionalis* (E-2009/1076), 22: *O. singularis* (E-2009/1067), 23: *S. asperatus* (E-2009/1102), 24: *O. ligustici* (E-2009/1080). Lanes 25-29: restriction fragment patterns of the COII PCR product produced by *Taa*I. Species-specific banding patterns are shown in lane 25: *B. pellucidus* (E-2009/1117), 26: *B. moerens* (E-2009/1109), 27: *P. sphaeroides* (E-2009/1108), 28: *O. morio* (E-2009/1069), 29: *O. sulcatus* (E-2009/1086). Lanes 30-31: restriction fragment patterns of the COII PCR product produced by *Xba*I. Species-specific banding patterns are shown in lane 30: *O. smreczynskii* (E-2009/1037), 31: *B. trichopterus* (E-2009/1116) 32-33: restriction fragment patterns of the COII PCR product produced by *Mnl*I. Species-specific banding patterns are shown for lane 32: *O. salicicola* (E-2009/1057), 33: *O. coecus* (E-2009/1071). (B) Agarose gel (1%) of the amplified COII target fragment of *O. raucus* (E-2009/1030). White arrow in lane 1: amplified fragment of a non-functional copy of the original COII gene. (C) Agarose gel (3%) of the COII amplicon of *O. ovatus* (E-2009/1099) digested with *Taq*I. White arrows in lane 1: fragments generated by an underrepresented COII haplotype of *O. ovatus*. (A-C) M, DNA size marker: 100 bp steps until 1000 bp with size of some marker bands indicated on the right. Black arrow: only fragments >100 bp are used for species determination.

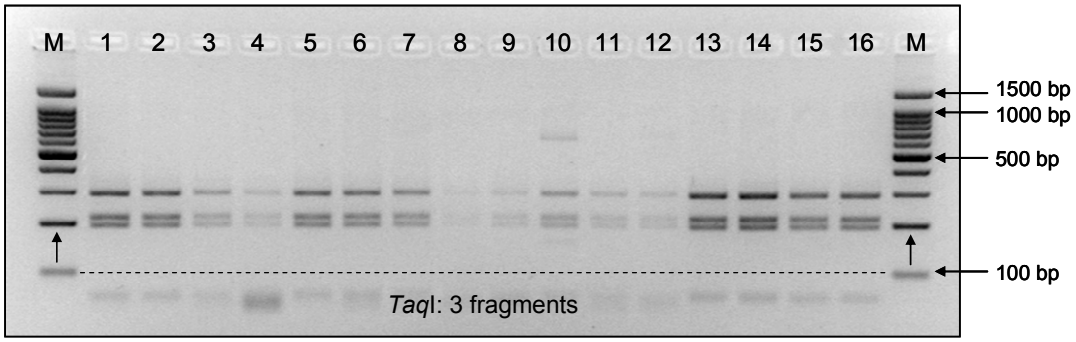
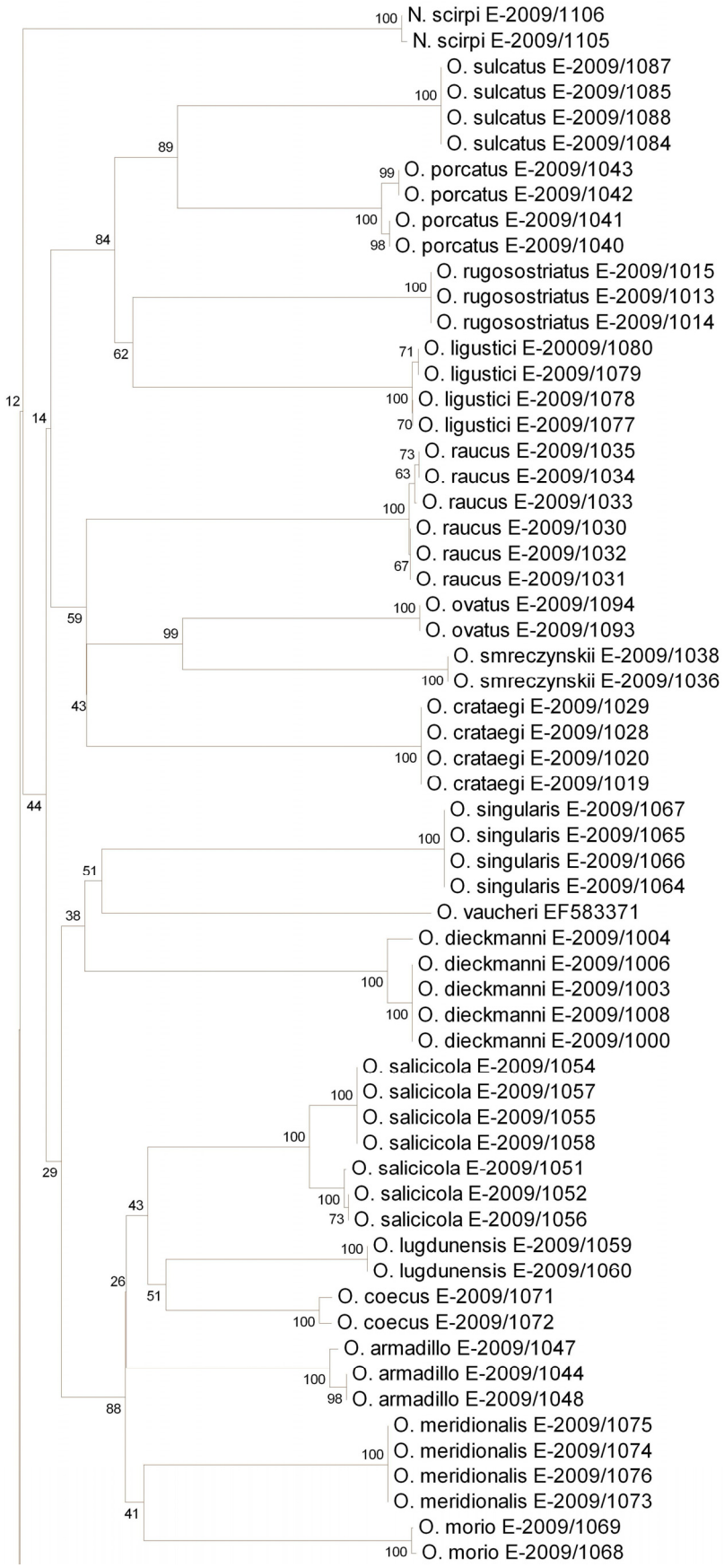


Figure 9: Agarose gel (3%) illustrating in lane 1-16 restriction fragment patterns of the COII PCR product produced by *Taq*I for *P. lethierryi* from six different German populations. M: DNA size standard: 100 bp steps until 1000 bp. Black arrow: only fragments >100 bp are used for species determination.



Please note, phylogenetic tree continues on next page.

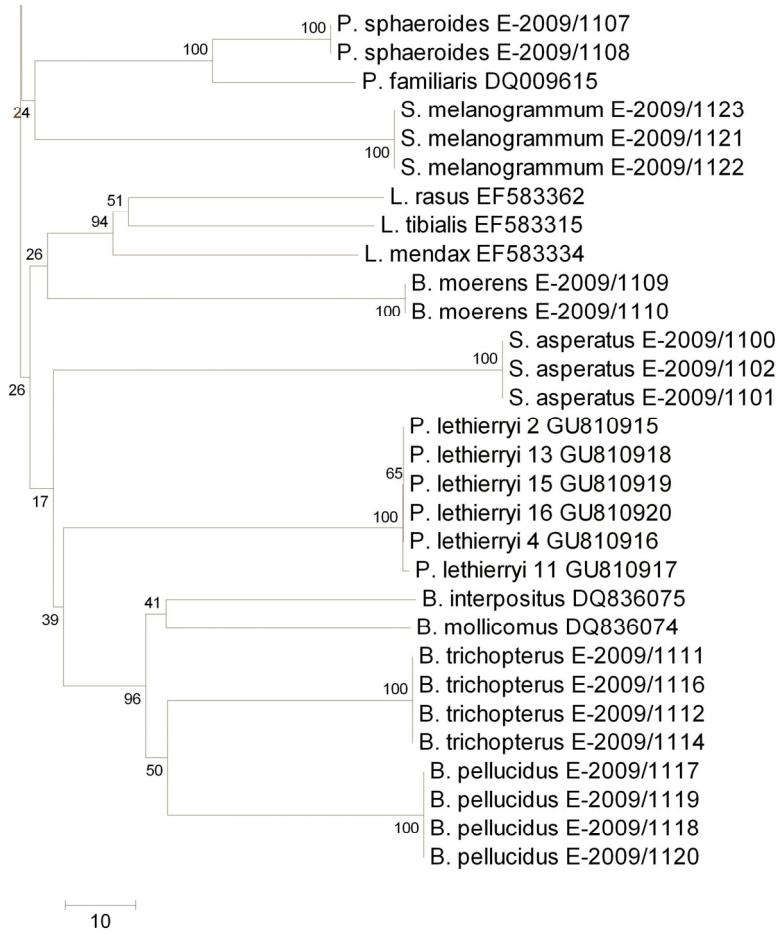


Figure 10: Neighbour-joining consensus tree showing genetic variation between COII sequences of all weevils analysed in this study and weevil sequences obtained from GenBank (accession numbers mentioned). Tree was constructed in MEGA4. As substitution model the number of nucleotide differences was chosen. Bootstrap values were calculated from 1000 replicates and are indicated at each node.

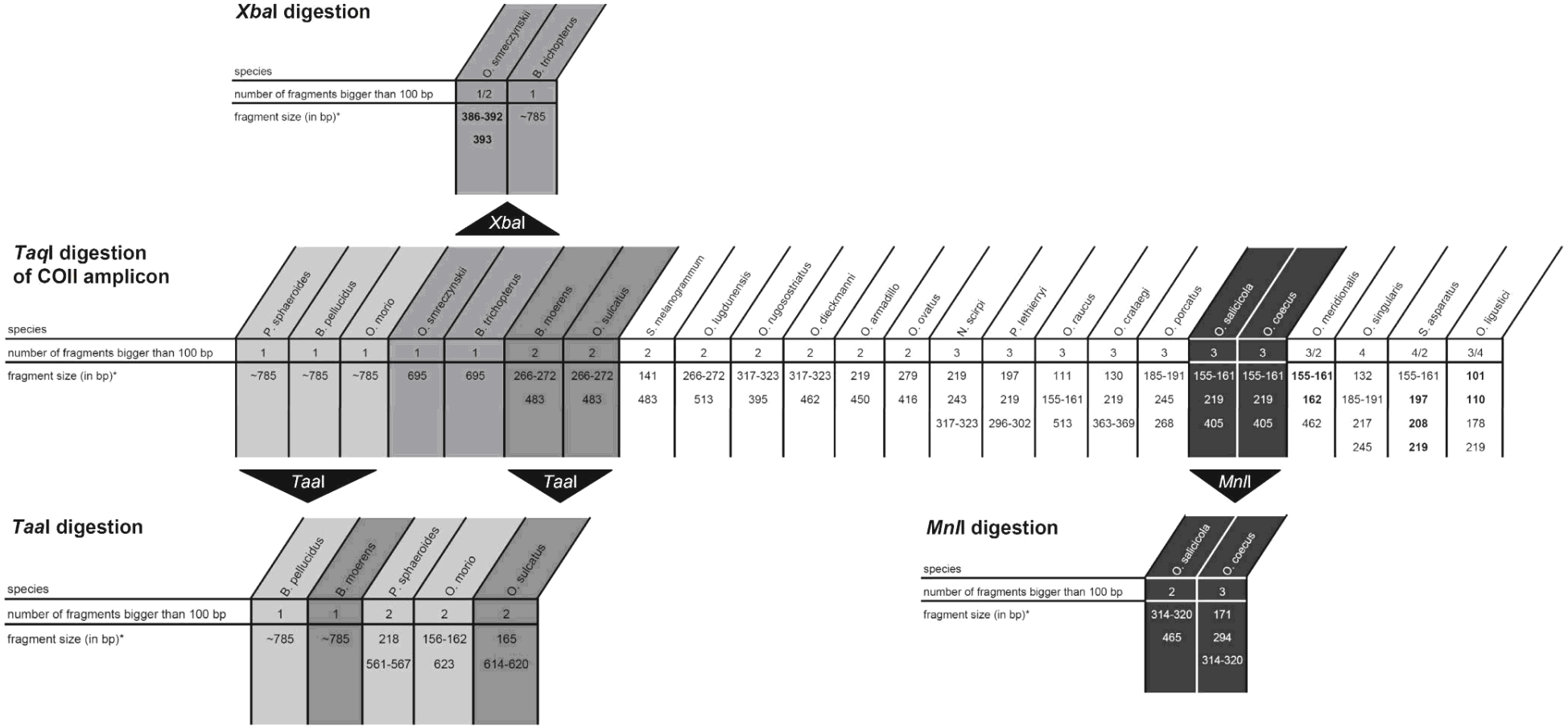


Figure 11: Schema of the PCR-RFLP assay of a COII fragment developed for identification of weevil species. Fragment sizes printed in bold letters have equal lengths and are therefore only visible as a single band on 3% agarose gels. For the diagnostic assay, only restriction fragments >100 bp are included. * Calculation of fragment size (in bp) depends on the length of four cloned and subsequently sequenced COII PCR products.

4.4 Results

4.4.1 Inter- and intraspecific variation within the COII

To test whether a fragment of the COII gene would be feasible for molecular identification of members of the Coleopteran genus *Otiorhynchus*, DNA samples from 143 individuals (E-2009/1000- E-2009/1126 and 16 *P. lethierryi* individuals) were amplified via PCR using a set of universal COII primers. This yielded in most cases a single ~780 bp COII gene fragment (Figure 8, A lane 1).

For assessment of inter- and intraspecific variation within this COII fragment and to indicate that the sequences originated from mtDNA, all sequences, gained from direct sequencing and those obtained from GenBank, were aligned manually in MEGA4 and a neighbour-joining consensus tree based on the number of base pair differences was calculated. The resulting dendrogram (Figure 10) showed that the analyzed COII gene region was derived from mtDNA and offers sufficient interspecific variability to differentiate between 17 *Otiorhynchus* spp. as well as 14 other weevil species resulting in 31 clear, well-separated branches. Intraspecific variability was in most cases low or not present which is an important prerequisite for the development of a molecular diagnostic marker. If intraspecific variability was present it was not affecting presence of restriction sites used in the PCR-RFLP assay described below.

4.4.2 Development of a PCR-RFLP assay for differentiation of several *Otiorhynchus* species

For determination of the exact length of the COII gene fragment in different *Otiorhynchus* species, PCR products obtained from single *O. sulcatus*, *O. salicicola*, *Otiorhynchus dieckmanni* Magnano and *O. ovatus* individuals were cloned into a plasmid vector and subsequently sequenced (GU182474, GU182451, GU182418 and GU182477- GU182481). The obtained sequences varied in length from 779-785 bp. This size range was set as the maximal standard sequence length.

The COII sequences were used to develop a PCR-RFLP assay for species determination of *Otiorhynchus* spp. larvae. In a first step, the sequences were screened in silico for restriction fragment sites revealing the enzyme *Taq*I as a suitable candidate

providing characteristic banding patterns for differentiation between 11 *Otiorhynchus* and four other weevil species (Figure 11). For an exact identification of nine other species, in silico sequence analysis suggested a second digestion either with *Taa*I, *Xba*I or *Mnl*I, respectively (Figure 11). For the diagnostic assay, only PCR-RFLP fragments >100 bp were included in the subsequent analysis.

To validate results of in silico digestion of the COII gene fragments, PCR products from 24 weevil species (143 individuals) were subsequently digested with *Taq*I, *Xba*I, *Taa*I or *Mnl*I resulting in clearly separated fragments achieved by electrophoresis on 3% agarose gels (Figure 8, A and Figure 9). Restriction digest of the COII PCR product with *Taq*I generated one fragment with slightly different lengths in five weevil species, two fragments in eight, and three or four fragments in eight and three species, respectively (Figure 8, A lanes 2-24 and Figure 9). A second digest with the above mentioned enzymes resulted in an exact identification of further nine species (Figure 8, A lanes 25-33). The strategy for choosing appropriate restriction enzymes and for correct identification of the respective species is illustrated in Figure 11. Difficulties in clear discrimination of restriction patterns of *Taq*I-digested COII PCR products of *Otiorhynchus meridionalis* Gyllenhal, *O. ligustici* and *Sciaphilus asperatus* Bonsdorff and *Xba*I-digested COII PCR products of *O. smreczynskii* were found due to very slight differences in restriction fragment sizes (compare Figure 8, A lanes 21, 23, 24, 30 and Figure 11).

In silico restriction digestion with *Mnl*I of *O. coecus* (E-2009/1071 and E-2009/1072) COII PCR products suggested the presence of three fragments with lengths of 171 bp, 294 bp and 314-320 bp, respectively (Figure 11) However, after *Mnl*I digestion of this product, three fragments were in fact visible on agarose gels, with the 294 bp fragment showing an atypical shifting (Figure 8, A lane 33). This banding pattern was reproducible and checked in two independent replicates of each individual.

In order to validate results of in silico digestion of *P. lethierryi* COII gene fragments, PCR products of all 16 individuals were subsequently digested with *Taq*I and electrophoretically separated in 3% agarose gels. This separation resulted mostly in clearly separated and visible fragments, with a characteristic fragment pattern for

P. lethierryi (Figure 9). Only one individual (Figure 9, lane 10) showed an atypical fragment pattern. Despite the three typical fragments (197, 219 and 296-302 bp) a fourth band with approximately 700-800 bp is visible. It is assumed that this band might be an undigested COII PCR product.

4.4.3 Amplification of two COII fragments in *O. raucus*

Although in most *Otiorhynchus* samples analysed only a single COII fragment was present, in two individuals of *O. raucus* (E-2009/1030 and E-2009/1031) a second faint ~640 bp PCR product was sometimes simultaneously amplified with the ~780 bp mitochondrial target sequence (Figure 8, B). Presence of this extra band was reproducible and was checked in two independent replicates of the PCR reaction using the respective individuals. This fragment was cloned, sequenced and subsequently aligned against the full length COII sequences. The alignment revealed the presence of several deletions of different lengths and no continuous open reading frame could be generated. Therefore, it is assumed that this amplified fragment is a nonfunctional copy of the original COII gene.

4.4.4 Determination of mitochondrial heteroplasmy in *O. ovatus*

After digestion with *Taq*I, all *O. ovatus* individuals analysed in the current study sometimes showed nonconforming restriction banding patterns as those that were predicted by in silico analysis (compare Figure 8, A lane 14, C lane 1 and Figure 11). In this case, the two predominant fragments were flanked by additional faint bands (Figure 8, C). To elucidate the reason for these extra fragments, a COII PCR product of a single *O. ovatus* specimen (E-2009/1093) was cloned into a plasmid vector and eight clones were sequenced resulting in five different haplotypes (GU182477-GU182481). One of these haplotypes (GU182479) differs extremely in its nucleotide composition, also affecting *Taq*I restriction sites and hence generating a different restriction pattern as the one predicted. For diagnostic purposes the digestion of COII PCR products of *O. ovatus* with *Taq*I generates two main and clearly visible fragments with lengths of 279 bp and 416 bp respectively. These fragments could be flanked by two slightly visible fragments of ~210 bp and 700 bp subjected to the amount of amplified underrepresented COII haplotypes present in *O. ovatus*.

4.5 Discussion

A couple of different members of the Coleopteran genus *Otiorhynchus* are becoming increasingly important as pests of nursery and ornamental plants. For applying appropriate control strategies a correct identification of the respective weevil species is indispensable; however, although adult weevils are difficult to determine, weevil larvae are morphologically cryptic hampering an accurate taxonomic identification. The present study presents a molecular approach based on PCR-RFLP analysis of a fragment of the COII gene, allowing a clear distinction between 24 weevil species even in the larval stage. Sequence analysis of the mitochondrial COII gene fragment revealed a high inter- and a low intraspecific variation between members of the Coleopteran genus *Otiorhynchus*. Thus, COII can be considered as an appropriate genetic region for species identification, as one of the required prerequisites for a good molecular marker system are met. PCR-RFLP of the COII fragment therewith also allows the determination of morphologically cryptic immature life stages like larvae, which is also one of the aims the barcoding of life project focuses on (Hebert et al. 2003). In most cases the intraspecific sequence variation of the COII fragment was negligible low, not altering any restriction sites and thus not affecting results of the PCR-RFLP fingerprint. During PCR-RFLP analysis of different *Otiorhynchus* species, three nonconforming results were generated, which needed further validation:

1. For the species *O. coecus*, in silico predicted banding patterns of the COII fragment produced by *Mnl*I are in contrast to those actually generated in the laboratory. It is known that *Mnl*I may remain associated with the digested DNA and therefore may cause atypical band shifting in agarose gels (http://www.fermentas.com, product sheet for *Mnl*I). It is assumed that this might be the reason for the atypical band shifting of the 294 bp fragment in *O. coecus*.

2. Mitochondrial DNA is increasingly being used for species determination by combination with RFLP technique in insects (Brown et al. 1999, Brunner et al. 2002, Muraji and Nakahara 2002). However, this method may cause serious problems due to mtDNA sequence variations of amplified fragments caused by mtDNA heteroplasmy (Frey and Frey 2004). Within-individual sequence variation, like it has been detected

in *O. ovatus* specimens, was also shown for the mitochondrial COI gene in *Thrips tabaci* Lindeman (Frey and Frey 2004). In *O. ovatus*, the frequency of observed haplotype variants was low but did sometimes affect the restriction banding pattern. However this nonconforming banding pattern could not be mixed up with other species specific fingerprints, therefore not affecting results of the PCR-RFLP assay. For all other species analysed, no heteroplasmy which might negatively affect the PCR-RFLP diagnostic assay was evident.

3. For two individuals of *O. raucus*, a second ~640 bp PCR product was sometimes simultaneously amplified with the ~780 bp target sequence. Sequence analysis suggested that this fragment is a nonfunctional copy of the original COII gene. During the evolution of Metazoa, duplication of genes in mitochondrial genomes have occurred quite frequently (Higgs et al. 2003). If genes are duplicated, mutations or deletions can occur in either but not in both copies of each duplicate until one copy remains (Higgs et al. 2003). This ~640 bp fragment is apparently still replicated because it takes less resources and time to copy a smaller molecule as the original longer molecule (White et al. 2008). For PCR-RFLP assay only PCR amplicons having the single mitochondrial target fragment were used, so the presence of such a nonfunctional additional copy of the COII gene was not affecting results of the PCR-RFLP assay presented here.

Besides these few exceptions of nonconforming restriction patterns, the present PCR-RFLP fingerprint allows for the first time the discrimination of 16 *Otiorhynchus* and eight other weevil species already in the larval stage and thus may be an important tool for future decisions on the choice of appropriate control strategies. The PCR-RFLP method reported here is cost-effective, robust and provides reliable results in at most five hours after DNA isolation. Including DNA extraction as described here, the determination of a single individual by means of the present molecular approach would roughly cost ~US$1.44 (~US$0.14, DNA isolation; US$0.56, PCR; US$0.16, gel analysis; and US$0.58, “conventional” digestions). By using “fast digest” enzymes, as done for *P. lethierryi* samples, the duration of digestion can be reduced by 55 min. However, “fast digest” enzymes are more

expensive than “conventional” restriction enzymes. The present PCR-RFLP assay may be used for example for diagnostic purposes by plant protection services.

Beside our first results nothing is known about the intraspecific variation of the COII gene in different *Otiorhynchus* species. Additional individuals from geographically distant regions in Europe and the United States should be analysed accordingly, to validate the worldwide potential of the present assay. Moreover, for determination of adult weevils a digital visual approach based on phenotypical characteristics and modern digital photographing technology is currently being developed (Digital Weevil Determination Project of the Curculio Institute). Therefore, in the future, two different ways can be pursued to correctly identify weevils collected in the field or present in containers or on nursery plants: Adults can easily be identified using the digital illustrated keys (e.g. http://www.curci.de/weevilnews/no/30/einleitung.html). For determination of all life stages but especially for the larval stages the PCR-RFLP fingerprint is indispensable. Both methods could be in future complementary strategies for correct identification of *Otiorhynchus* species.

In the future, both the increasing global trade of plant material as well as climate change may result in the establishment of new insect species in a given area. For advisors in plant protection, their correct identification is relevant to recommend a correct control strategy, but is often hampered by a lack of literature or taxonomic expertise for the relevant species. Strategies for species identification based on molecular methods may thus become increasingly important in the future.

5 Efficacy of commercially available entomopathogenic fungi formulations against different *Otiorhynchus* species

5.1 Introduction

Several weevils within the genus *Otiorhynchus* Germar (Coleoptera: Curculionidae) are becoming increasingly important as pests in horticulture (Collman 2001, Sprick 2009). In all species, adults cause mainly aesthetic damage by notching on the aerial parts of the plant such as leaves and flowers, whereas larvae feed on the roots below ground, which often stunt or kill the plants (Smith 1932, Moorhouse et al. 1992). Various pest management strategies from chemical insecticides (Reding and Persad 2009, Reding and Ranger 2011) to biological control agents (BCA), such as entomopathogenic nematodes (Lola-Luz and Downes 2007, Haukeland and Lola-Luz 2010), entomopathogenic fungi (EPF) (Bruck and Donahue 2007, Oddsdottir et al. 2010), and microsporidia (Bruck et al. 2008) have been successfully applied against *Otiorhynchus sulcatus* Fabricius. However, only a few reports document the susceptibility of other *Otiorhynchus* species to these BCA (Vainio and Hokkanen 1993, Neumann and Shields 2008, Sabbahi et al. 2008).

Several previous investigations have shown that EPF have a high potential for biological insect pest control (Hajek and Delalibera 2010). The commercially available products Naturalis® (*Beauveria bassiana* strain ATCC 74040) and PreFeRal® WG (*Paecilomyces fumosoroseus* strain Apopka 97, recently taxonomically transferred to *Isaria fumosorosea* (Humber 2007)) are based on EPF and are registered as insect BCA in some European countries and the United States. So far however, they have no registration as plant protection products (PPP) in Germany. In the present thesis, experiments using these two products and the entomopathogenic fungus *Metarhizium anisopliae* (strain Ma43, obtained from Julius Kühn-Institute, Darmstadt, Germany; synonym *Metarhizium anisopliae* var. *anisopliae* BIPESCO 5/F52, active ingredient e.g. in the product BIO 1020), which has currently no

registration as plant protection product in Germany, were performed against different developmental stages of several *Otiorhynchus* species.

5.2 Materials and methods

5.2.1 Fungal strains

The commercially available product Naturalis® (Intrachem Bio Italia S.p.A., Grassobbio, Italy) as well as the product PreFeRal® WG (Biobest N.V., Westerlo, Belgium) were used in the infection experiments. Naturalis® containes 69.1 g/l *Beauveria bassiana* isolate ATCC 74040 as an active ingredient with a concentration of at least 2.3×10^7 viable *B. bassiana* conidiospores per millilitre. The fungal propagules are formulated in an oil dispersion. The product PreFeRal® WG contains 2×10^9 CFU/g (CFU = Colony Forming Units; blastospores) of *Paecilomyces fumosoroseus* (synonym: *Isaria fumosorosea*) Apopka strain 97 and is formulated as a wettable granule (WG) formulation. Before use, both products were dispersed/diluted in sterile water as described by the manufacturer[1] to obtain a 3% (~ 6.9×10^5 conidia/ml) and 3.75% (~8.6×10^5 conidia/ml) suspension of Naturalis® and a 0.1% suspension of PreFeRal® WG (2×10^6 blastospores/ml). Besides those two products, *Metarhizium anisopliae* strain Ma43 (subsequently referred to as Ma43) was used, which was obtained from the Julius Kühn-Institute (Darmstadt, Germany) and grown on potato dextrose agar for conidia production. A synonym for this strain is *Metarhizium anisopliae* var. *anisopliae* BIPESCO 5/F52 (Eilenberg et al. 2008) which is the active ingredient e.g. in the products GranMet-P® or BIO 1020. For the infection experiments an aqueous solution with 2.23×10^7 conidia/ml was used. To test whether there is an influence of the pure oily formulation of Naturalis® (obtained from Intrachem Bio Italia S.p.A., Grassobbio, Italy) on the survival of infected weevils, an aqueous suspension of 3.75% Naturalis® formulation without fungal spores was also included in the infection experiments. As a negative control, sterile water was applied.

[1] Technical sheet for Naturalis® "Biologisches Insektizid zur Befallsverminderung von Drahtwürmern an Kartoffeln" available from Intrachem Bio Deutschland GmbH & Co. KG, Bad Camberg, Germany and technical sheet for PreFeRal® WG "PreFeRal® WG is a new biological insecticide that provides excellent control of the greenhouse whitefly in protected cultures of tomato and cucumber" available from Biobest N.V., Westerlo, Belgium.

5.2.2 Insects

Adults of the species *Otiorhynchus raucus* Fabricius, *Otiorhynchus dieckmanni* Magnano and *Otiorhynchus rugosostriatus* Goeze were captured with dry pitfall traps (Figure 15) in 2010 and 2011 in a peony and switchgrass plantation at a nursery near Wiesbaden, Germany (Figure 4) and kept for several weeks in the laboratory to verify their vitality prior to the experiments. Adults and larvae of the species *O. sulcatus* were mainly obtained from a rearing from the Hochschule Osnabrück or collected in the wild. Eggs of the species *Otiorhynchus salicicola* Heyden and *Otiorhynchus armadillo* Rossi were obtained from adults collected in the wild and eggs of *O. sulcatus* were gained from individuals reared at the Hochschule Osnabrück, as well as collected in the wild.

5.2.3 Infection experiments with EPF against eggs of *Otiorhynchus* spp.

The efficacy of 3% Naturalis® was tested against eggs of *O. salicicola*, *O. armadillo* and *O. sulcatus* and the effectiveness of 0.1% PreFeRal® WG was analysed against eggs of *O. salicicola* and *O. armadillo*. Eggs (<11 d old) were surface sterilized according to Hosokawa et al. (2007). Eggs were inoculated by dropping 50 µl of each treatment individually on 35-45 eggs of each species (for *O. sulcatus* and Naturalis® treatment only 12-14 eggs were used). Sterile water was used as a control. Eggs were kept individually on moistened filter paper in a 5.5 cm diameter Petri dish in the dark at 24°C for 14 d. Afterwards individuals were classified as dead or alive. Obtained data were corrected for natural mortality (Abbott 1925) and statistical analysis was performed with Statistica 7 software using a Chi²-test. The obtained percentage values for efficacy were rounded to the nearest whole number.

5.2.4 Infection experiments with EPF against larvae of *O. sulcatus*

Efficacy of EPF against *O. sulcatus* larvae was determined using potted grapevine plants (cv. Müller-Thurgau; potting media: ED 73, Patzer, Sinntal, Germany), which were artificially infected with five larvae (larval stage L4-L6) per pot (diameter 13 cm, volume 0.88 l/pot). Pots were watered once with 100 ml of 3% Naturalis® or 0.1% PreFeRal® WG, and with water as a control. Each treatment was replicated five times. Plants were kept in the greenhouse for four weeks with 23 ± 2°C, 55-75% relative humidity and watered when needed. For analysis, each pot was checked for

dead and living larvae and the number of individuals was counted. Missing larvae were counted as dead individuals, as it was assumed that these larvae were already decomposed within the time frame of the experiment. Percentage efficacy data from the infection experiment with larvae of *O. sulcatus* were corrected for mortality according to Abbott (1925) and statistical analysis was performed with Statistica 7 software using a Tukey's HSD test. Percentage values gained for efficacy of EPF were rounded to the nearest whole number.

5.2.5 Infection experiments with EPF against adult weevils of several *Otiorhynchus* species

Experiments with adult weevils were performed with *O. sulcatus*, *O. rugosostriatus, O. dieckmanni* and *O. raucus*. Weevils were inoculated either with 3.75% Naturalis®, 0.1% PreFeRal® WG, pure 3.75% Naturalis® formulation (without fungal spores), Ma43 suspension (2.23 x 10^7 conidia/ml) or sterile water as a control. Between 30-35 weevils were tested for each species and treatment. A volume of 50 µl of each suspension was individually dropped on the weevil's dorsum. Due to the limited number of individuals collected in the field, experiments with the species *O. rugosostriatus* and *O. dieckmanni* were performed once either in 2010 or 2011 (except for *O. dieckmanni* and 3.75% Naturalis®, which was repeated twice in 2011). The weevils were kept individually in 5.5 cm diameter Petri dishes with moistened filter paper and pieces of *Prunus* sp. as food source and were incubated at room temperature (2010: 23 ± 2°C and 2011: 25 ± 2°C) and 12:12 L:D for 28 d. Afterwards, dead, living and missing adults were counted. For further statistical analysis, missing individuals were treated as not available data. Mortality data were corrected according to Abbott (1925) and statistical analysis was performed with Statistica 7 software using a Chi²-test. The percentage values obtained for efficacy of applied EPF were rounded to the nearest whole number.

5.3 Results

Application of 3% Naturalis® and 0.1% PreFeRal® WG against eggs of several *Otiorhynchus* species caused 100% mortality for each weevil species compared with the control (Figure 12, A-C). Growth of fungal mycelia was clearly visible on eggs

from all species inoculated with Naturalis®, and almost no larvae hatched (Figure 12, D). In contrast, in most cases inoculation with PreFeRal® WG did not prevent eclosion of *Otiorhynchus* spp. larvae, but caused subsequent fungal infection and death of newly hatched larvae (Figure 12, E).

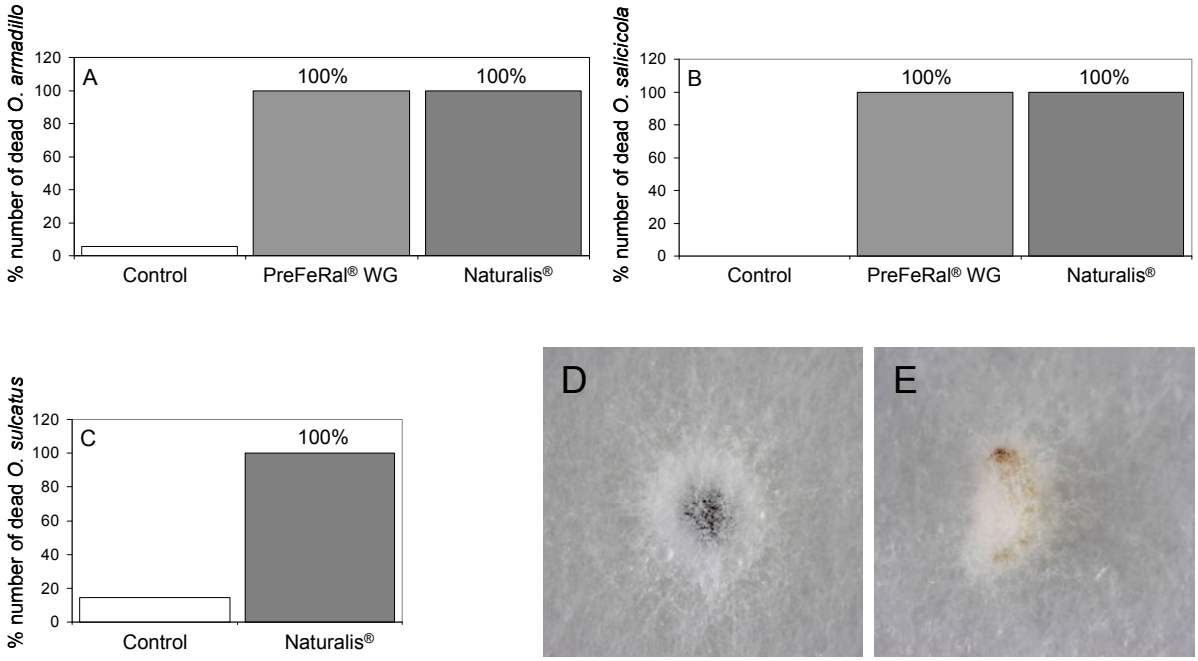


Figure 12: Percentage number of dead (A) *O. armadillo* (B) *O. salicicola* and (C) *O. sulcatus* eggs after treatment with entomopathogenic fungi. In case of significance (p-value < 0.01, Chi²-test) degree of efficacy (Abbott 1925) is indicated on top of the bars. (D) Egg of *Otiorhynchus* sp. infected with *B. bassiana* strain ATCC 74040 (Naturalis®). (E) Larva of *Otiorhynchus* sp. infected with *I. fumosorosea* (PreFeRal® WG). Photos (D) and (E): W. Schönbach.

When poured onto pots containing grapevine plants and *O. sulcatus* larvae, Naturalis® resulted in a significantly higher larval mortality (92% efficacy; p-value < 0.05, Tukey's HSD test) than PreFeRal® WG (33% efficacy; p-value > 0.05, Tukey's HSD test). Mean numbers of dead larvae for the control, Naturalis® and PreFeRal® WG were 2.6 (± 1.65 s.d.), 4.8 (± 0.45 s.d.) and 3.4 (± 0.89 s.d.), respectively.

Significant difference in mortality of adult *O. sulcatus* weevils was observed after application of Naturalis® (48% and 65% efficacy) and Ma43 (69% and 51% efficacy) compared to the control in 2010 and 2011 respectively (Figure 13, A-B). In 2010

PreFeRal® WG showed as well a degree of 32% efficacy against *O. sulcatus* (Figure 13, A). However, this result could not be confirmed in 2011 (Figure 13, B).

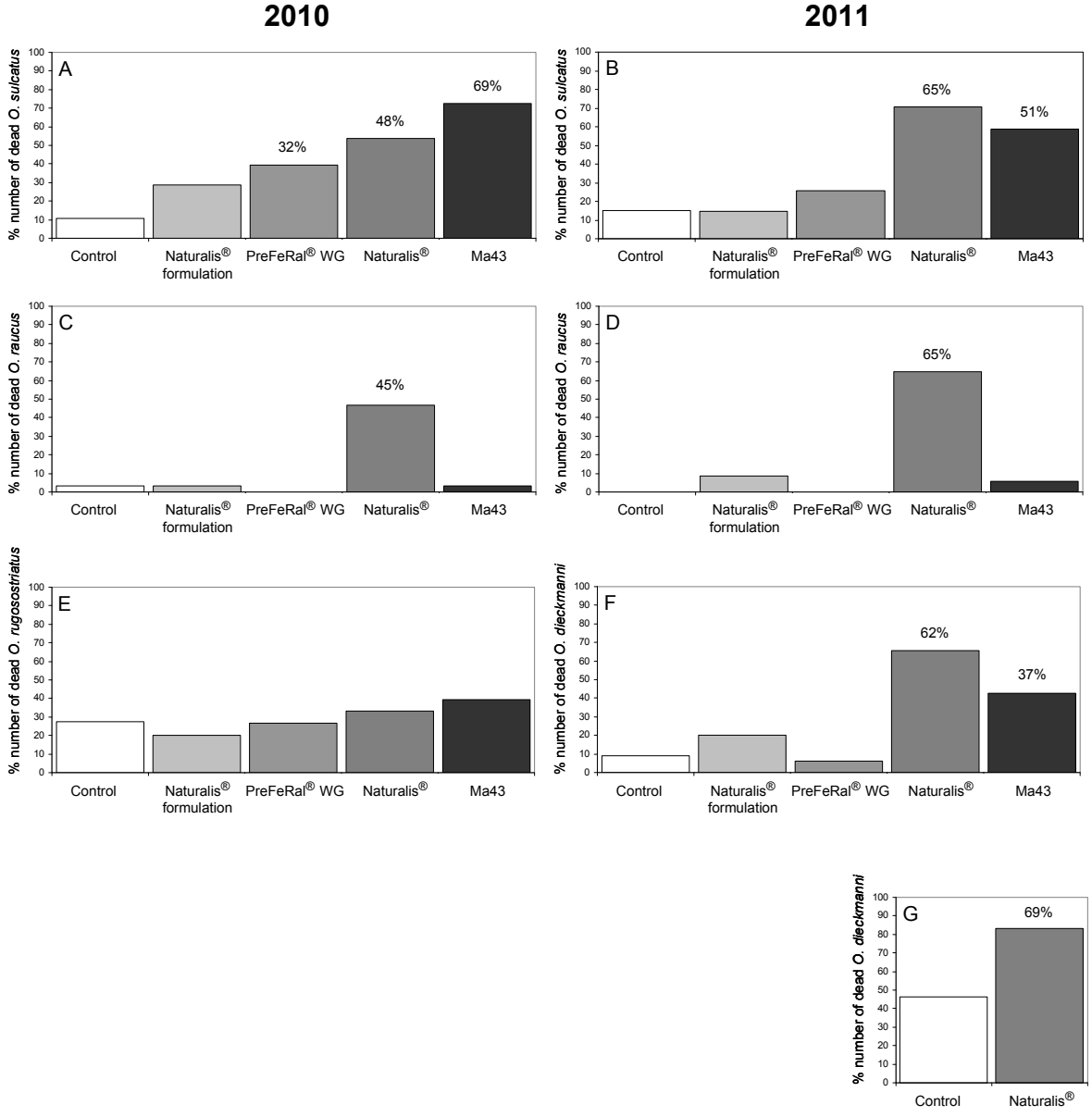


Figure 13: Percentage number of dead (A-B) *O. sulcatus*, (C-D) *O. raucus*, (E) *O. rugosostriatus* and (F-G) *O. dieckmanni* adults after treatment with entomopathogenic fungi in 2010 and 2011. In case of significant difference to the control (p-value < 0.05, Chi²-test) degree of efficacy (Abbott 1925) is indicated on top of the bars.

For *O. raucus* only Naturalis® resulted in a significant higher adult mortality compared to the control in both years (45% and 65% efficacy) (Figure 13, C-D). In 2011, significant differences in mortality of *O. dieckmanni* were detected after application of

Naturalis® (62% and 69% efficacy) and Ma43 (37% efficacy) (Figure 13, F-G). In 2010, no significant mortality attributable to any entomopathogenic fungus occurred in *O. rugosostriatus* (Figure 13, E).

5.4 Discussion

Results of this study showed that adult weevils of different *Otiorhynchus* species differ in their susceptibility to various EPF. The species *O. sulcatus* was susceptible to Naturalis® and Ma43 in both years and PreFeRal® WG also showed an efficacy of 32% in 2010. In contrast, *O. raucus* was only sensitive to Naturalis® in both years. Naturalis® and Ma43 caused significant mortality rates to the species *O. dieckmanni* in 2011. *Otiorhynchus rugosostriatus* was not susceptible to any fungal biocontrol agent in 2010. These results were either obtained in 2010 or 2011 and therefore infection experiments for *O. dieckmanni* and *O. rugosostriatus* have to be repeated in the future. Overall, the present results may be a first indication of defence mechanisms in the respective weevil species, which may be involved in protection against fungal infection. As is known for other insects, either the immune system (Lamberty et al. 2001, Imler and Bulet 2005), endosymbionts (Panteleev et al. 2007), physical barriers such as the insect's cuticle (Mazza et al. 2011) or microorganisms present on the surface of the cuticle (Schabel 1976) may be involved in defence mechanisms. Polar compounds extracted from the surface of adult red palm weevils for instance inhibited the germination of *B. bassiana* spores, whereas the growth of *M. anisopliae* was not affected (Mazza et al. 2011). Moreover, Shelton et al. (2007) mentioned that changes in the cuticle composition may result in resistance to EPF. As adult weevils were not surface sterilized before spore treatment, spore germination might have been suppressed by antibiosis due to microorganisms present on the weevil's cuticle. Antibiosis, due to fungal and bacteria contaminants, has been documented in experiments performed with adult weevils of the species *Hylobius pales* Herbst and *M. anisopliae* (Schabel 1976). In addition to exosymbionts, microbial symbionts inside the body of the host insect may play an important role in determining the efficacy of EPF. For instance, females of *Drosophila melanogaster* Meigen infected with *Wolbachia* endosymbionts, were less susceptible to *B. bassiana* (Panteleev et al. 2007) than uninfected ones. Therefore, host features, such as

specific cuticle characteristics or associated microorganisms (exo- and endosymbionts) may play a crucial role in the successful infection of adults of different *Otiorhynchus* species with various EPF.

Furthermore, the formulation of a plant protection product based on an entomopathogenic fungus may be important for the efficacy of the respective fungus to control insect pests. For application, Naturalis® (*B. bassiana*) is formulated as an oily suspension-concentrate and PreFeRal® WG (*I. fumosorosea*) is formulated as a water dispersible granule. It is assumed that the oily formulation of Naturalis® helped in keeping the fungal spores attached to the adult weevil's cuticle more effectively than the PreFeRal® WG or Ma43 (*M. anisopliae*) spore solution. Prior et al. (1988) tested oil and water formulations of *B. bassiana* against the cocoa weevil and demonstrated that the oily formulation was more efficient, propably due to the increased adhesion of conidia on the lipophilic insect's cuticle. In addition, a high relative humidity is required for germination of spores (Gillespie 1988). However, oily formulations of EPF enable fungal propagules to remain active even under low humidity conditions (Bateman et al. 2002). As EPF act as contact insecticides, a well-adherent formulation of a plant protection product based on an entomopathogenic fungus, which also ensures the required amount of moisture for germination of spores (as it is often the case for oily formulations), may be crucial for the successful control of insect pests.

Eggs (<11 d old) of the weevil species *O. sulcatus*, *O. salicicola* and *O. armadillo* were treated with a spore solution of *B. bassiana* containing ~6.9 x 10^5 conidia/ml. As a result, *B. bassiana* successfully infected weevil eggs and caused 100% mortality for each species. In contrast, studies assessing the susceptibility of *O. sulcatus* eggs (12 h old) towards several species of EPF showed that *B. bassiana* isolate Bb 142, applied in a comparable concentration, was not infectious for *O. sulcatus* eggs (Poprawski et al. 1985). In general, freshly laid eggs are regarded as more sensitive for fungal infection, which has been shown previously for *O. sulcatus* eggs and *M. anisopliae* treatment (Zimmermann 1982). However, it is known that different fungal isolates may vary in their pathogenicity and virulence against different developmental stages of *Otiorhynchus* spp. (Beck 1996, Sabbahi et al. 2008). Furthermore, the efficacy of PreFeRal® WG (*I. fumosorosea*) against eggs of *O. salicicola*

and *O. armadillo* was analysed. In contrast to *B. bassiana*, *I. fumosorosea* did not infect the eggs in most cases but caused 100% mortality to freshly emerged larvae. Similar observations were made by Zimmermann (1982) who documented that freshly emerged larvae could be infected by *M. anisopliae,* although eggs were not sensitive. To summarize, further experiments should consider more precisely the age of tested weevil eggs and experiments for *O. sulcatus* should be repeated with Naturalis®, as in the present thesis only 12-14 *O. sulcatus* eggs were analysed.

Naturalis® and PreFeRal® WG were poured onto potted grapevine plants infested with *O. sulcatus* larvae. Although PreFeRal® WG was applied in a higher concentration, it was less effective than Naturalis®. Previous investigations performed by Vainio and Hokkanen (1993) also showed in laboratory trials that different EPF may have a varying efficacy against *Otiorhynchus* spp. larvae. Vainio and Hokkanen (1993) tested larvae of *Otiorhynchus nodosus* (= *O. dubius*) Müller and *Otiorhynchus ovatus* Linnaeus against different isolates of *M. anisopliae* and *B. bassiana,* with *M. anisopliae* isolates being more effective against *Otiorhynchus* larvae.

Eggs, larvae and adults of *Otiorhynchus* spp. were susceptible to different EPF with a varying degree of efficacy for the respective weevil species, developmental stage and applied fungal strain. *Beauveria bassiana* strain ATCC 74040 (Naturalis®) was successfully applied against eggs, larvae and adults of the black vine weevil. The efficacy of an entomopathogenic fungus as biocontrol agent depends, inter alia, on the developmental stage of an insect. Dembilio et al. (2010) for instance, showed that eggs of the red palm weevil, *Rhynchophorus ferrugineus* Olivier were more sensitive to *B. bassiana* infection than adults. In addition, Beck (1996) documented that adult weevils of *O. sulcatus* were less suceptible to *M. anisopliae* infection than immature life stages. Therefore, further studies are required to analyse the efficacy of Naturalis® and other EPF against several developmental stages of *Otiorhynchus* spp. Moreover, as different *Otiorhynchus* species in the adult stage showed a differential susceptibility to Naturalis® and Ma43, the analysis of the efficacy of both fungi (*B. bassiana* and *M. anisopliae*) in a combined application is recommended. A combined application may be also a promising strategy to control *Otiorhynchus* infestation, especially if different *Otiorhynchus* species are present in a nursery.

6 Establishment of a method to quantify the efficacy of *Beauveria bassiana* for the control of adult *Otiorhynchus* spp. in the field

6.1 Introduction

Worldwide, weevils of the genus *Otiorhynchus* Germar (Coleoptera: Curculionidae) are important pests on many horticultural plants grown in the field or in nursery greenhouses. Adult weevils feed on the foliage, whereas larvae feed on the roots of their host plant, thereby causing economically significant damage (Smith 1932, Moorhouse et al. 1992, Backhaus 1996).

During a survey on the occurrence and phenology as well as a mark-release-recapture study of weevil species in a peony plantation in Germany (Figure 4), particularly high numbers of *Otiorhynchus rugosostriatus* Goeze and *Otiorhynchus raucus* Fabricius were documented (Reineke et al. 2011). In addition, within this plantation many weevils of the species *Otiorhynchus dieckmanni* Magnano were detected (data unpublished). All three species are flightless, mostly parthenogenetic and important pests on several horticultural crops, which was shown in a monitoring of *Otiorhynchus* species in Germany (Sprick 2009).

The use of entomopathogenic fungi (EPF) as biological control agents against *Otiorhynchus* species might be an alternative to chemical insecticides or entomopathogenic nematodes (Bruck 2007, Bruck and Donahue 2007). In previous laboratory experiments, the efficacy of several EPF against different life stages of *Otiorhynchus* spp. was evaluated (see chapter 5). Adults of *O. raucus* and *O. dieckmanni* were found to be highly susceptible to *Beauveria bassiana* strain ATCC 74040 (which is formulated in the commercial product Naturalis®, Intrachem Bio Italia S.p.A., Grassobbio, Italy), whereas adults of *O. rugosostriatus* showed less susceptibility. However, these results were gained under optimized conditions e.g. high relative humidity, high doses of infective conidia etc. in the laboratory and may

not necessarily be transferred to field conditions with complex abiotic and biotic interactions (Hajek and Goettel 2007).

Entomopathogenic fungi are relatively slow acting pathogens, allowing the insect host to be alive for several days after infection. However, assessing the efficacy of EPF in a field environment is rather difficult, as the cadavers of killed insects are removed rapidly by scavengers (Wraight et al. 2007). Therefore, it is necessary to collect potentially infected insects in the field and keep them in enclosures till death (Wraight et al. 2007). By doing so, the amount of death insects may be measured and subsequently the efficacy of the applied entomopathogenic fungus in the field may be quantified.

The aim of this study was to develop a field trial design to quantify the efficacy of a combined foliar and soil application of *B. bassiana* strain ATCC 74040 (Naturalis®) against adult weevils of different *Otiorhynchus* species in peony plantations.

6.2 Materials and methods

6.2.1 Study area and installation of dry pitfall traps

The field study was performed in a nursery near Wiesbaden (Germany) in which populations of *O. raucus, O. dieckmanni* and *O. rugosostriatus* coexist in a plantation of peonies and switchgrass (Figure 4). In 2010, within this plantation, four plots, each with a size of 1 m², bordered by a commercially available aluminium snail fence (R+M Gartenbedarf, Rehling-Unterach, Germany) were arranged (Figure 14, A). A single peony plant was located in the middle of each plot and two dry pitfall traps were installed on two sides of the plant (Figure 14, B). Each trap consisted of a tin (diameter 10 cm, depth 17.5 cm) with small holes in the bottom, to ensure drainage of rainwater (Figure 15). Those traps were put into a plastic pipe (25 cm length) which was previously dug into the ground at-grade (Figure 15). Some leaves were placed in each trap as shelter for the weevils, to protect them from predators, rainfall or sunshine (Figure 15). In 2011, a similar experimental setup was installed with nine plots and four dry pitfall traps per plot. In both years, the distance between the plots

in one row was 2-3.5 m and between the rows approximately 3 m in 2010 and 7-8 m in 2011 (Figure 14, A).

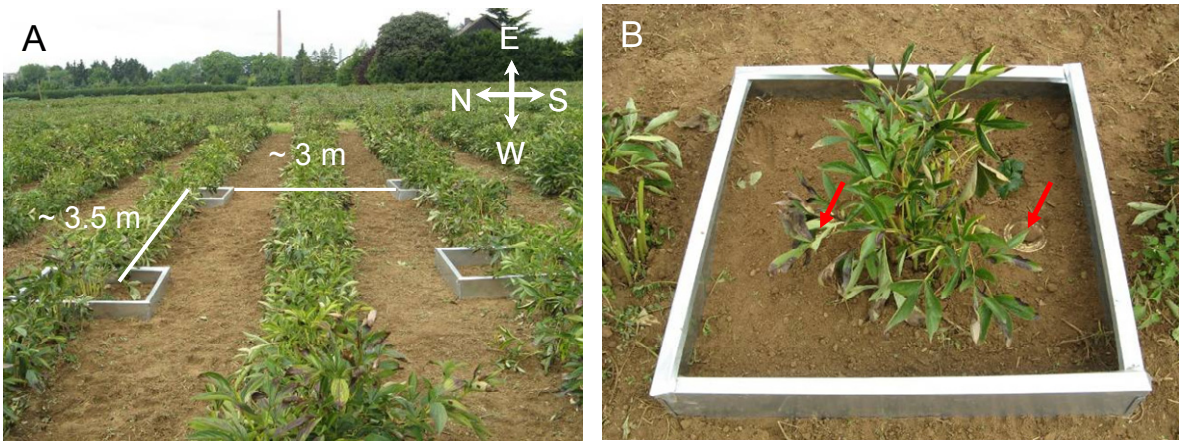


Figure 14: Field design in 2010: (A) Installation of four plots within the peony plantation. (B) Plots were bordered by a snail fence and two dry pitfall traps were installed on the right and the left side of each peony (red arrows).

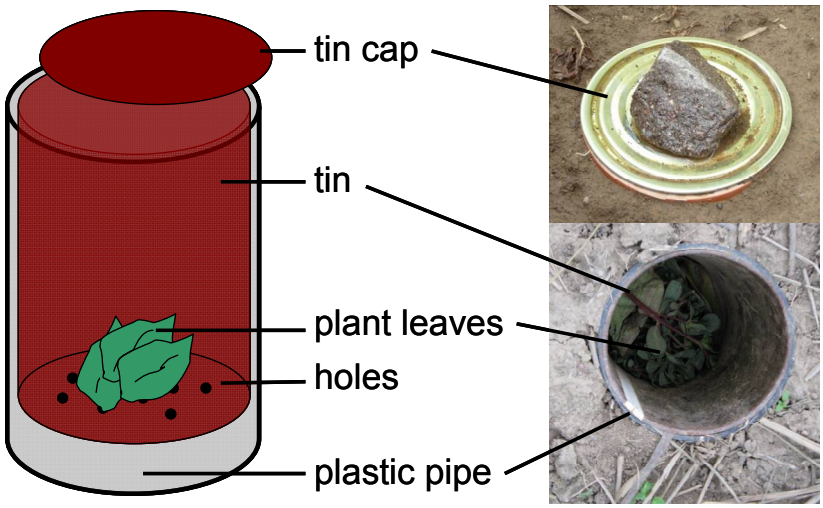


Figure 15: Design of dry pitfall trap (left) and installation of the trap in the field (right).

6.2.2 Application of *B. bassiana* and recapture of weevils

At the beginning of August in 2010 (11.08.2010), healthy adult weevils of the species *O. raucus* and *O. rugosostriatus* (respectively 100 individuals per species), mass trapped with dry pitfall traps in the same field approximately two weeks before and maintained in the laboratory to check their vitality, were released in each of the four plots with closed pitfall traps. After 24 hours, 1 l of 3% Naturalis® (~6.9 x 10^5 conidia/ml, with an application of ~6.9 x 10^8 conidia in total per plot, treatment of both

southern plots, Figure 14, A) and 1 l of water (treatment of both northern plots, Figure 14, A) as control were applied homogeneously on the soil and foliage of the plant with a commercially available application leverage (Mesto Resistent 3610, Mesto, Germany).

Due to heavy rainfalls in the following days, dry pitfall traps were opened seven days after treatment. Weevils subsequently captured in the pitfall traps (20, 22 and 24.08.2010, respectively) were counted according to species and kept in the laboratory in plastic boxes (length 14.8 cm, width 11.5 cm, height 7.3 cm) filled with moistened tissue paper and cherry and peony leaves as food source, for five weeks. The tissue paper and the leaves were replaced when needed. Weevils were kept in groups of at most 50 individuals per box. Mortality was checked weekly and dead weevils were counted and kept in a dark humid chamber at 25°C to accelerate fungal growth.

In 2011, field trials were repeated twice with nine plots installed in the peony plantation. Forty weevils of the species *O. raucus* and *O. dieckmanni* were released in each of the plots (with closed pitfall traps) at the beginning of June (08.06.2011). Those individuals were mass trapped with dry pitfall traps in the weeks before, in the switchgrass field adjacent to the peony plantation, and kept in the laboratory till release, to check their vitality. After 24 hours, 0.5 l of 3.75% (~8.6×10^5 conidia/ml, ~3.45×10^8 conidia in total per plot) and 1.875% (~4.3×10^5 conidia/ml, ~2.15×10^8 conidia in total per plot) Naturalis® and water as control were applied randomly to the respective plots. After four days (13.06.2011), pitfall traps were opened and weevils were recaptured in the following days (15, 17, 20, 22 and 25.06.2011). Already dead weevils, sometimes found in the pitfall traps, were not considered for further evaluation. Recaptured weevils were kept for five weeks in the laboratory at room temperature and 12:12 L:D. Dead individuals were transferred to Petri dishes with moistened filter paper and kept next to the boxes with weevils, as this place seemed to be appropriate to induce fungal growth which was documented in previously performed laboratory infection experiments.

The above described experiment was repeated at the end of August/beginning of September 2011 with 60 *O. raucus* released per plot (21.08.2011). Before release, all

plots were moved southwards to avoid a contamination with *B. bassiana* before application. Spray treatment was performed as described above, with the same randomly chosen application schema as in the first experiment. Traps were opened four days after treatment and subsequently, weevils were recaptured (29 and 31.08.2011 as well as 2, 5 and 8.09.2011).

Information on environmental conditions, from the day of spray treatment till last day of recapture, were obtained from Wiesbaden weather station approximately 1.5 km from the experimental site and are availalble online (http://www.wunderground.com).

6.2.3 Statistical analysis

In 2010 and 2011, recaptured weevils in the field were counted and statistical analysis was performed for recaptures with Statistica 7 software using a t-test in 2010 and a Mann-Whitney U-test in 2011. Moreover, in both years the number of dead weevils at the end of the experiment was counted and in 2010, data were statistically analysed with R 2.13.2 software using a Welch-t-test, and in 2011 using a Kruskal-Wallis test in Statistica 7. In both years, mean efficacy (average of all plots, with obtained values rounded to the nearest whole number) of *B. bassiana* per treatment was calculated using Abbott's formula (Abbott 1925).

6.3 Results

In 2010, approximately 39% of *O. raucus* and 27% of *O. rugosostriatus* were recaptured during a time period of 13 days after treatment. In June and August/September 2011, approximately 43% and 57% of *O. raucus* were recaptured respectively within 17 days after treatment. In addition, approximately 4% of *O. dieckmanni* were trapped in June 2011. There was no significant difference in re-captures of *O. raucus* and *O. rugosostriatus* in 2010 (t-test with p-value = 0.143131). In 2011, significantly more *O. raucus* than *O. dieckmanni* were trapped (Mann-Whitney U-test with p-value = 0.000313).

Although there was no evidence for a significant difference in recaptures of *O. raucus* and *O. rugosotriatus* in 2010, the efficacy of 3% Naturalis® differed for both species. Significantly more *O. raucus* (Welch t-test with p-value = 0.02752; 98% mean

efficacy) than weevils of the species *O. rugosostriatus* (Welch t-test with p-value = 0.6042; 0% mean efficacy) died while maintaining them in the laboratory after treatment with 3% Naturalis® in the field compared with the control (Figure 16, A and B).

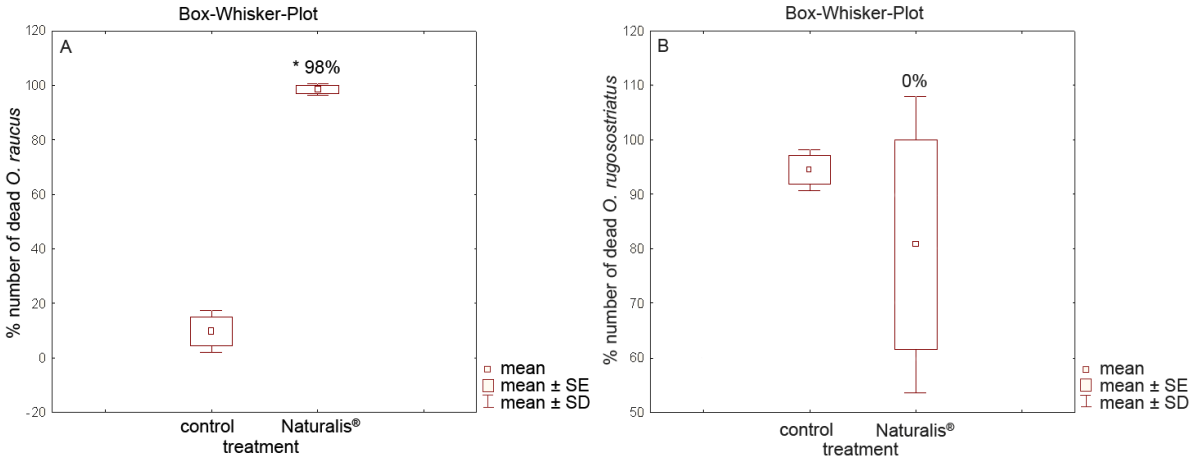


Figure 16: Percentage number of dead *O. raucus* (A) and dead *O. rugosostriatus* (B) weevils after recapture in the field and rearing in the laboratory for five weeks after treatment of field plots with water (control) and 3% Naturalis®. Out of 100 weevils released per plot, on average 46 *O. raucus* weevils were recaptured in the control and 32 in the treatment plots, respectively. For *O. rugosostriatus* on average 36 weevils were recaptured in the control and 19 in the treatment plots, respectively. * Indicates significant differences. Degree of mean efficacy (Abbott 1925) is indicated on top of the bars.

In June 2011 only 14 individuals of 360 released *O. dieckmanni* were recaptured (approximately 4% recapture rate). Due to the low number of recaptures of *O. dieckmanni,* no statistical analysis was performed. Although high numbers of *O. raucus* were recaptured in June and in August/September 2011, no significant difference between the treatments of 1.875% Naturalis® (mean efficacy of 5% and 8%) and 3.75% Naturalis® (mean efficacy of 0% and 6%) and water as control was evident (Kruskal-Wallis test with p-value = 0.4128 and p-value = 0.2881, respectively).

6.4 Discussion

In the present study, a method to quantify the efficacy of an application of EPF to control adult *Otiorhynchus* weevils in the field was developed. The field design

consists of several plots which were bordered by commercially available snail fences and within each plot, two to four pitfall traps were installed for recapture of weevils. In 2010 and 2011, 27-57% of released weevils of the species *O. raucus* and *O. rugosostriatus* were recaptured. Although not all weevils were caught - it is assumed that the uncaptured number of weevils might have been already dead and removed by scavengers - dry pitfall trapping seems to be an appropriate method to collect potentially infected *Otiorhynchus* weevils in the field. A mark-release-recapture study performed by Reineke et al. (2011) within the same plantation, also showed, that the application of dry pitfall traps is an appropriate method to capture *Otiorhynchus* weevils in the field. In the present study, one exception was the species *O. dieckmanni*, which has been recaptured only in small numbers in June 2011. In general, *O. dieckmanni* occurs early in the year (Sprick 2009). Therefore it is assumed that individuals of *O. dieckmanni* released in June were quite inactive and thus were not recaptured.

In 2010, the application of Naturalis® had a significant negative impact on the survival of *O. raucus*. Whereas *O. raucus* showed high mortality, when plots were treated with Naturalis® in the field, no effect was documented for *O. rugosostriatus*. These results are in accordance with previously performed laboratory trials, showing a significant influence of *B. bassiana* strain ATCC 74040 (Naturalis®) against *O. raucus* and less efficacy against *O. rugosostriatus* (see chapter 5). However, a high natural mortality of *O. rugosostriatus* was documented in the control plots in 2010, which cannot be explained.

In 2011, field experiments were repeated twice with nine plots and two different concentrations of Naturalis® application. In both experiments, none of the treatments had an effect on the survival of adult *Otiorhynchus* weevils. In 2010, ~6.9 x 10^8 *B. bassiana* conidia, dissolved within 1 l of water, were homogeneously applied onto the peony and the soil. In 2011, approximately half and one third of the amount of *B. bassiana* conidia (~3.45 x 10^8 and ~2.15 x 10^8 conidia/m²) used in 2010, were applied with 0.5 l of water to the plots. In addition, in 2010 recapture started seven days after treatment. However, in 2011 only four days passed between treatment and the opening of pitfall traps. Based on the present results, it is assumed that on the

one hand, the amount of applied conidia determines the success of an application of EPF and on the other hand, the period of time, which allows the insect to get into contact with the fungus, is crucial. Beck (1996) successfully used 10^{11}-10^{12} spores/m² of *Metarhizium anisopliae* applied to the soil and the root crown to control *O. sulcatus* in a stock of roses grown under glass. Therefore, it is assumed that at least ~6.9 x 10^8 conidia/m² (as used in 2010) are required to control *Otioryhnchus* weevils in the field. In addition, for further studies it is proposed to start recapture no earlier than seven days after treatment, to increase the chance of catching already infected weevils. Moreover, it must taken into account that Beck (1996) performed the experiment under controlled greenhouse conditions, while the present investigation was also exposed to environmental influences, which may affect the efficiency of EPF (Wraight et al. 2007). Although EPF were applied in the late afternoon with a cloudy sky and weather conditions (rainfall and temperature) seemed to be acceptable for the trial in 2010 and for the second experiment in 2011, it is assumed that the relatively dry months in spring/early summer 2011 might have influenced the experiment in June 2011. As suggested by Wraight et al. (2007), in future, field experiments should monitor as many environmental parameters as possible.

Despite the high mortality of weevils in the treated plots in 2010, only some individuals showed slight growth of fungal mycelia. This has also been observed in 2011. Fungal emergence and sporulation takes time and may occur only under favourable conditions (Hajek and St. Leger 1994). Therefore, it is suggested to extend the time period of incubation of weevils in a humid chamber after death. In addition, microsatellite markers applied for amplification of *B. bassiana* isolate ATCC 74040 (Naturalis®) specific bands in bulk soil DNA (Bischoff-Schaefer 2010) may be used as well, to detect infection of *B. bassiana* strain ATCC 74040 in *Otiorhynchus* spp.

Usually EPF are applied to control immature life stages of *Otiorhynchus* weevils. Therefore, plants grown in containers are artifically infested with *Otiorhynchus* eggs or larvae and after application of fungal propagules, the number of live and infected individuals is determined (Bruck 2007). In addition, in order to quantify the success of an application of EPF indirectly, the root damage caused by weevil larvae may be assessed (Oddsdottir et al. 2010). For an indirect quantification, weevil damage

caused by adult beetles on leaves (characteristic crescent shaped notches) may be measured as well. However, as adult weevils do not feed constantly throughout the year, no or less damage does not necessarily mean that there are no or only few weevils present at a site. Moreover, Beck (1996) counted the number of holes present in the soil from freshly emerged adult beetles, to quantify the efficacy of an application of EPF. However, the above mentioned opportunities are difficult to apply in perennial deep-rooted field-grown crops, as neither larvae may be released and subsequently recaptured without much effort, nor roots or holes of freshly emerged weevils may be assessed easily.

The occurence of *Otiorhynchus* spp. as pests in horticulture has increased over the last years (Collman 2001, Sprick 2009), calling for the availability of a method to quantify the efficacy of EPF against *Otiorhynchus* spp. under field conditions. The present study describes such a method, which was successfully verified for a combined foliar and soil application of *B. bassiana* strain ATCC 74040 (Naturalis®) against adult weevils of *Otiorhynchus* spp. in a peony plantation.

7 Effects on soil fungal community structures and fate of an artificially applied *Beauveria bassiana* strain assessed through 454 pyrosequencing

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* **Author contributions:** J. Hirsch, G. Sandhya, K. U. Devi, and A. Reineke conceived the study design. J. Hirsch and G. Sandhya performed sample collection and template preparation for 454 pyrosequencing analysis. G. Sandhya carried out microsatellite analysis and J. Hirsch, S. Strohmeier, and M. Pfannkuchen performed analysis of 454 sequence reads.

7.1 Abstract

The entomopathogenic fungus *Beauveria bassiana* is widely used as a biological control agent for insect pest control, with fungal propagules being either incorporated into the potting media or sprayed directly onto the foliage or soil. In order to gain a better understanding of entomopathogenic fungal ecology when applied as a bio-control agent to the soil environment, multitag 454 pyrosequencing of fungal ITS sequences was used to assess the fate and potential effects of a *B. bassiana* strain on soil fungal community structures. Results show that the overall fungal diversity was not influenced by application of *B. bassiana*. In addition, pyrosequencing and strain specific microsatellite markers indicated both an establishment of *B. bassiana* in the treated plot as well as a spread of fungal propagules to the non-treated plot during the first two weeks after its application. These results might be important for proper risk assessment of entomopathogenic fungi based biological control agents.

7.2 Introduction

Fungal entomopathogens are used worldwide as microbial biocontrol agents (BCA) against arthropod pests (Hajek and Delalibera 2010). Of the roughly 130 commercially available products based on entomopathogenic fungi, around two-thirds of them consist of conidial preparations of the two most widely studied entomopathogens, *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin (both Ascomycota: Hypocreales) (de Faria and Wraight 2007, Jackson et al. 2010, Jaronski 2010). Fungal propagules can be incorporated into the potting media at the time of planting (Bruck and Donahue 2007) or are sprayed directly onto the plant or onto the soil. *Beauveria bassiana* is known to infect a wide range of insects (Zimmermann 2007a) but may as well survive as an endophyte inside the plant or as a saprophyte in the soil (Vega et al. 2008). While the interactions between entomopathogenic fungi and their host insects are quite well studied (Hajek and St. Leger 1994, Roy et al. 2006), aspects of fungal ecology regarding putative interactions between the entomopathogen and the soil microbiota including indigenous fungal communities have been rarely assessed so far (Shimazu et al. 2002, Anderson and Cairney 2004, Meyling and Hajek 2010). However, as soil fungi are involved in many key processes in soil ecosystem functioning like decomposing organic matter or as mycorrhizal symbionts of plants (Brussaard et al. 1997), any effect exerted by the application of an entomopathogenic fungus to the structure and diversity of indigenous fungal communities in the soil, might have important implications for various ecological processes and functional soil biodiversity. Consequently, these aspects should be taken into account during the process of risk assessment required for registration of the respective entomopathogenic fungi-based commercial product.

In the past, selective media were used to study the impact of an application of entomopathogens like *B. bassiana* on soil microorganisms (Shimazu et al. 2002). As many soil microorganisms are hardly or even not culturable and their morphological determination is often difficult, cultivation-independent approaches have been applied subsequently (Bridge and Spooner 2001, Kirk et al. 2004). Different DNA fingerprinting techniques like denaturing/temperature gradient gel electrophoresis (DGGE

or TGGE), single-strand conformation polymorphism (SSCP) or traditional metagenomic approaches with clone library-based techniques have been used to define community structure of soil microbiota (for review see Anderson and Cairney 2004 and Kirk et al. 2004). However, these community profiling techniques are time-consuming and costly, especially if taxonomic affiliations of respective organisms are analysed. In addition, staining based methods like DGGE or TGGE are often not sensitive enough to detect the whole diversity within the given sample in particular if rare members of a community are present (Anderson and Cairney 2004). Lately, the accessibility of next generation sequencing technologies like 454 pyrosequencing represent new, cost-efficient and fast strategies to depict microbial diversity without the need for culturing the respective organisms, allowing an analysis of thousands of sequence reads in parallel (Harkins and Jarvie 2007). Accordingly, multitag 454 pyrosequencing techniques have been recently applied in metagenomic studies on plant- or soil-associated fungal communities (Buée et al. 2009, Lumini et al. 2010, Unterseher et al. 2010).

All the above mentioned techniques including 454 pyrosequencing are based on polymerase chain reaction (PCR) and aim at amplifying segments of microbial DNAs in bulk soil DNA samples. As targets for PCR, fragments of the 18S rDNA or the internal transcribed spacer (ITS) region have been used in studies analyzing soil fungal communities (Anderson and Cairney 2004, Buée et al. 2009, Lim et al. 2010). As the non-coding ITS region shows faster evolution rates compared with the more conserved coding 18S rDNA region, ITS sequences generally provide deeper taxonomic resolution (Lord et al. 2002). In addition, the ITS region is generally applied as a validated DNA barcode marker for the taxonomic classification of fungal species (Seifert 2009, Begerow et al. 2010).

In the present study, we report on the application of multitag 454 pyrosequencing of fungal ITS sequences for characterizing the fungal community structure in an agricultural field in India and for assessing both the fate and potential effects of an artificially applied *B. bassiana* strain on soil fungal community structures.

7.3 Materials and methods

7.3.1 Study site, fungal treatment and sample collection

Experiments were carried out from October till December 2010 on a cultivated agricultural field near Visakhapatnam (Andhra Pradesh, India) with a standing crop of chili. So far, no naturally occurring entomopathogenic fungal epizootics were documented on this field and no artificial introduction of *B. bassiana* had been made prior to this survey (R. Kongara, personal communication). Within the field, two plots each of 50 m² (control (C) and treatment (T) plot, respectively) and a distance of approximately 15 m between each other were selected. Before the application of *B. bassiana* to the (T) plot, seven soil cores (approximately 4 x 4 x 15 cm depth) were collected separately every three meters along a 22 m transect in each plot. Accordingly, *B. bassiana* strain ITCC 4688 (Indian Type Culture Collection, IARI, Delhi, India) was manually applied once at the beginning of the experiment to the (T) plot of the chili field. For this purpose, 200 g of rice containing conidiated *B. bassiana* strain ITCC 4688 was dissolved in 30 l water and 2 ml Tween80 to give a final concentration of 1×10^9 conidia/ml. Germination of conidia was analysed in the laboratory (Ekesi et al. 2001) before application and was found to be more than 90%. For assessing effects of this *B. bassiana* strain on indigenous soil fungal community structure, soil samples were collected as described above at weekly intervals for a duration of seven weeks in both the untreated (C) and the treated (T) plot. Two weeks elapsed between the last and the penultimate sample collection. For our experiment we used a cultivated agricultural field. Accidentally, cow dung slurry flowed into parts of our treatment plot. Therefore, from the fourth week after *B. bassiana* application onwards, only five soil samples were taken from the unaffected parts of the treatment plot. A total of 92 soil samples were collected over the whole duration of the survey from the (C) and (T) plot. After soil sample collection, samples were transported to the laboratory in an ice chest (8°C) and stored at 4°C (for a maximum duration of 48 h) or frozen at -20°C until further processing. Soil parameters such as pH (6.86), organic matter (0.74 mg/kg) and clay content (44%) were determined commercially (Lotus Granges India Ltd, Visakhapatnam, India). Rainfall data for the duration of the experiment were obtained from Mandal Revenue Office (Anandapuram, India).

7.3.2 DNA isolation, ITS amplification and pyrosequencing

Soil samples were independently homogenized and genomic DNA was extracted from each of the 92 samples using PowerSoil® DNA Isolation Kit (Süd-Laborbedarf GmbH, Gauting, Germany) according to the manufacturer's instructions. The variable region of the ITS-1 was amplified with fungal specific primers as described in Buée et al. (2009), which were modified for multitag 454 GS-FLX amplicon pyrosequencing by adding a four-base library "key" sequence (TCAG) and a multiplex identifier (MID) tag sequence specific to each soil sample. Primer design and selection of MIDs was performed according to the guidelines for GS FLX Titanium Series Lib-A Chemistry (Roche, Technical Bulletin No. 013-2009). PCR amplifications were set up in a total volume of 30 µl consisting of 2-6 µl of undiluted soil DNA, 15 pmol primers and 15 µl GeNei™ Red Dye PCR Master Mix (2X) (GeNei, Bangalore, India). The PCR reactions were performed at 94°C for 4 min, followed by 30 cycles of 30 s at 94°C, 55°C for 1 min and 72°C for 90 s, and a final elongation at 72°C for 10 min. An aliquot of 4 µl of each amplification product was analysed for correct size (~400 bp) on a 1% agarose gel and was afterwards purified with Hi Yield® PCR Clean-up/Gel Extraction Kit (Süd-Laborbedarf GmbH, Gauting, Germany). In total, 92 fungal PCR products, tag-encoded according to sampling date and plot, were pooled at equimolar concentrations and 454 pyrosequencing was performed commercially (LGC Genomics GmbH, Berlin, Germany).

7.3.3 Microsatellite analysis of *B. bassiana* strain ITCC 4688

As the ITS-1 gene region is not suitable for strain-specific identification of an artificially applied *B. bassiana* isolate, three microsatellite (SSR) markers (Ba01, Ba08, and Ba13; Rehner and Buckley 2003) were used in order to verify the presence of *B. bassiana* isolate ITCC 4688 in the respective soil samples. The allele sizes of the respective SSR loci of this *B. bassiana* strain were previously determined as being 121 bp, 260 bp, and 176 bp, respectively, for the three markers applied (Bischoff-Schaefer et al. in preparation). To allow fluorescent labelling and multi-plexing of the PCR products, a M13(-21) tail was placed at the 5'-end of each forward primer and a fluorescently labelled CY5 or IRD700 universal primer M13(-21) was added to PCR reactions according to the method described by Schuelke (2000). PCR

amplifications were set up in a total volume of 15 µl consisting of 90-100 ng DNA, 10x reaction buffer with 1.5 mM $MgCl_2$, 5 pmol of each primer, 0.5 µl 100x BSA, 0.2 mM dNTPs and 0.5 U of DreamTaq™ Polymerase (Fermentas, St. Leon-Rot, Germany). PCR reactions were carried out at the following conditions: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 60°C for 45 s and 72°C for 45 s and a final extension step at 72°C for 10 min. Each PCR product was checked for successful amplification on a 2% agarose gel and subsequently analyzed for size of SSR alleles via capillary electrophoresis in a multiplex analysis on a Beckman GenomeLab GeXP DNA Genetic Analysis System.

7.3.4 Sequence editing and analysis of the reads with MEGAN, EstimateS and Metastats

Clipping and sorting of 454 sequence reads by MID tags was performed by LGC Genomics GmbH (Berlin, Germany). Accordingly, individual sequences were evaluated using BLASTn 2.2.25+ with word length of 28 against the NCBI nt database. Data were imported in MEGAN version 4.61.5 (MEtaGenome ANalyzer, Center for Bioinformatics, Tübingen, Germany; Huson et al. 2007) and parameters for the Lowest Common Ancestor (LCA)-assignment algorithm were set as follows: min support 5, minscore 35.0, top percent 10.0, win score and min complexity 0.0. Calculation of diversity (Shannon index) was performed using EstimateS software package version 8.20 (http://viceroy.eeb.uconn.edu/EstimateS). Statistical comparison between control (C,1 and C,2) and treatment (T,1 and T,2) samples was made using Metastats (White et al. 2009; http://metastats.cbcb.umd.edu/) with 1000 permutations and a p-value threshold of 0.05 to determine differentially abundant taxa after application of *B. bassiana*. Samples C,3-C,7 and T,3-T,7 were excluded from the Metastats analysis due to the spread of *B. bassiana* to the control plot.

7.4 Results

7.4.1 Analysis of 454 pyrosequencing reads

In the 92 soil samples analysed in the present study, a total of ~63000 fungal ITS PCR amplicons were sequenced via 454 pyrosequencing. After clipping of se-

quences 29109 reads were analysed in MEGAN version 4.61.5 (Huson et al. 2007). These reads were compared against the GenBank database of known ITS sequences using BLASTn 2.2.25+. Most of the sequence reads were assigned (28318, ~97%) and only a small percentage (less than 3% in total) lacked a taxonomic annotation or showed no hits in MEGAN (Table 2). Approximately 71% of the assigned reads were classified as belonging to the kingdom of fungi (Figure 17). Within the subkingdom Dikarya, the most dominant phyla were Ascomycota and Basidiomycota, according to the assignment of the majority of sequence reads (Figure 17). In total, 57 fungal taxa were identified by collapsing the phylogenetic tree in MEGAN at species level (Table 3). At the beginning of our experiment (Table 3, soil samples C,0 and T,0) no indigenous *B. bassiana* strain or any other fungus belonging to the family Cordycipitaceae was present in the samples according to 454 pyrosequencing results. However, after application of *B. bassiana* strain ITCC 4688 to the treated plot, fungal sequences showing homology to Cordycipitaceae were amplified in all soil samples collected from this plot (T,1 - T,7, Table 3). In the control plot, none of the sequence reads were assigned to the family Cordicipitaceae one and two weeks (C,1 and C,2, Table 3) after *B. bassiana* application to the neighbouring treated plot, respectively. However, from the third week after application onwards (C,3 - C,7, Table 3) presence of *B. bassiana* was as well documented in the control plot, as 6.9% to 37.4% of the obtained sequence reads in fact belonged to the family Cordycipitaceae.

Table 2: Number of 454 sequence reads of fungal ITS sequences in 92 soil samples as assigned by MEGAN.

	Number of reads
Total	29109
Assigned	28318
Unassigned	483
Reads with no hits	308

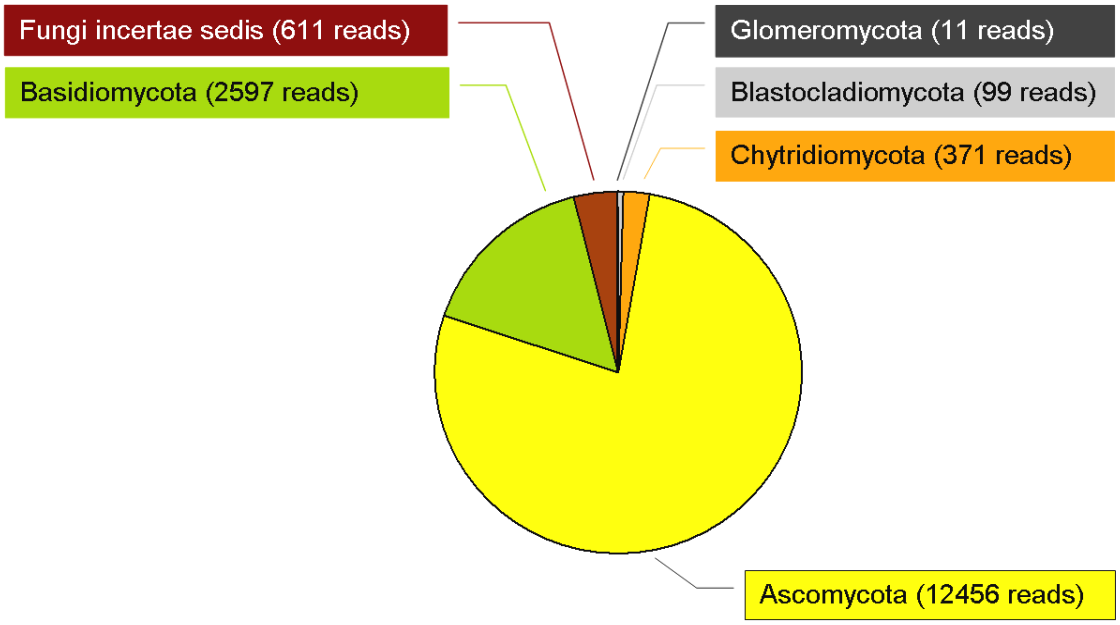

Figure 17: Distribution of fungal ITS pyrosequencing reads from 92 soil samples to phyla within the kingdom of fungi.

Table 3: Releative abundance (% pyrosequence reads) of fungal taxa present in 92 soil samples in an agricultural field in India. Taxa were assigned by MEGAN by collapsing the tree at species level according to the Lowest Common Ancestor (LCA) parameter values with *B. bassiana* assigned to the familiy Cordycipitaceae. C = control plot and T = treatment plot, numbers refer to weeks after application of *B. bassiana* isolate ITCC 4688 to the treated plot, which is indicated by a bold line in the table.

Phylum	Fungal Taxa	Percentage reads present in soil samples:													
		C,0	T,0	C,1	T,1	C,2	T,2	C,3	T,3	C,4	T,4	C,5	T,5	C,7	T,7
Blastocladiomycota	*Catenophlyctis* sp. JEL298							1.6						3.8	
	Allomyces arbuscula	5		5.3		3			2.7					2.3	
Chytridiomycota	Rhizophydiales							1.6			2.7		5.8		
	Olpidium brassicae	2.5													
	Rhizophlyctis rosea	4.5					5.4	3.5		2.4		3.7		3.5	
Ascomycota	Ascomycota sp. MA5351					2.2		2.9		1.3					
	Lecanoromycetidae	3					3.7		2.7	2.1	3.9	2.2	5.8	1.8	
	Dothideomycetidae											1.6			
	unclassified Pleosporales	3.5	4.4				2.2	1.9		1.8		2.5	5.1		
	Diaporthales				7.3	1.9	1.5			2.1	2		5.8		
	Helotiaceae sp. LM229														
	Montagnulaceae sp. LM15							1.9							
	Cordycipitaceae				19		37.4	9.6	14.4	7.6	11.3	6.9	19.9	18.7	29.2
	Sordariaceae		9.6	5.7	7.3	7.8	2.4	5.3	8	6.3	3.1	3.4	3.2	3.2	4.4
	Xylariaceae													1.5	
	mitosporic Orbiliaceae	3	3.7		5.6	7.8	1.7	1.3	3.2	4.7	3.9	3.4	5.8	1.8	5.8
	Pezizaceae	3.5													13.9
	Cercophora				2.8					1.3		1.6			
	Zopfiella	10.9		11		19.3	5.7	9.4	5.9	13.2	4.3	9.1		4.4	
	Stachybotrys	3					1.1								
	Myrothecium										2				
	Fusarium sp. BBA 70872							2.1						3.5	
	Cylindrocladiella						1.5								
	Paecilomyces	2.5			3.4	2.6	1.7	2.1	7.4	2.1	2	2.2	7.1		
	Bionectria	2.5		1.9											
	Westerdykella											2.5			
	Pyrenochaeta sp. 14009							8							
	Humicola		6.7				1.1		3.7					2.3	

Table 3 (continued)

Phylum	Fungal Taxa	Percentage reads present in soil samples: C,0	T,0	C,1	T,1	C,2	T,2	C,3	T,3	C,4	T,4	C,5	T,5	C,7	T,7
Ascomycota	*Scolecobasidium*		5.9	2.3	0	3.3	2	2.7		2.1	2.3	1.6		2	
	Cladorrhinum samala			7.6	3.9			8.8	4.3	7.6	3.1	4.4	5.1	2.9	
	Cladorrhinum bulbillosum		6.7				7.2								
	Chaetomium atrobrunneum										2.3				
	Scedosporium aurantiacum		4.4			1.9									
	Acremonium alcalophilum	5.5	4.4	3.8	2.8	3.7	4.8	9.4	7.4	7.6	2	4.7		3.2	4.4
	Spiromastix warcupii	3.5	11.1	4.9	5	3.3	3	2.1	5.9	3.2	2.7	3.4	7.1	3.5	
	Penicillium pimiteouiense					1.9									
	Aspergillus penicillioides											1.6		5	7.3
	Cladophialophora modesta	3.5										2.2			
	Cochliobolus lunatus								2.7						
	Cephaliophora tropica	9	5.9	20.2	11.7	13	3.9	17.4	13.3	19.5	4.7	22.8	9	18.4	18.2
	Ascobolus crenulatus	6	5.9	2.7	6.1	3.3	2.4			2.6		3.1			
Basidiomycota	Basidiomycota incertae sedis	3				1.9									
	Lycoperdaceae	11.4	7.4	2.7		6.3	1.7	1.3	3.2	2.1		4.1		3.2	4.4
	Conocybe			17.9											
	Pisolithus					2.2									
	Micropsalliota		4.4		2.8										
	Fibulobasidium murrhardtense	6	15.6	4.2	8.9	9.6	3.7	4.5	12.8	8.4	45.7	5	16.7	5.3	7.3
	Amanita nauseosa	3		3			2.2						3.8	3.2	5.1
	Flavodon flavus		3.7												
	Ganoderma lucidum			2.3											
	Laetisaria arvalis														
	Thanatephorus cucumeris				10.1							1.6			
Fungi insertae sedis	*Basidiobolus*													2	
	Mortierella sp. 18-M-4	5.5		2.3	3.4	5.2	2.6	2.4	2.7	1.8		4.7		2.6	
	Mortierella sp. FMR23-12			2.3											
	Endogone lactiflua						1.1								
Glomeromycota	*Glomus mosseae*										2				

7.4.2 Strain-specific identification of *B. bassiana* strain ITCC 4688 using SSR markers

As a strain specific identification of members of the family Cordycipitaceae was not possible on the basis of the obtained ITS sequences, *B. bassiana* ITCC 4688 strain specific SSR markers were amplified from the same soil DNA samples (Bischoff-Schaefer et al. in preparation). Overall, results were in accordance with 454 pyrosequencing results: While in both the control and treatment plot, respectively, no *B. bassiana* strain specific SSR alleles were amplified before the artificial application of the fungus, alleles with the respective size were evident in the treated plot starting from the first week after application until the end of the experiment seven weeks later (T,1 to T,7, Table 4). In DNA isolated from the control plot, a few samples started to show minor peaks of the respective allele size two weeks after *B. bassiana* strain ITCC 4688 application (C,2, Table 4), with a more prominent amplification being evident during the following weeks also in samples from the control plot (Table 4). SSR marker Ba08 amplified alleles of the respective size in soil samples from the treatment plot only one and two weeks after *B. bassiana* strain ITCC 4688 application, confirming previous observations on a lower sensitivity of this marker for amplification of respective sequences from bulk soil DNA samples (Bischoff-Schaefer et al. in preparation).

7.4.3 Assessment of fungal community structures

To assess whether artificial application of an entomopathogenic fungus had any effect on indigenous fungal communities present in the control and the treatment plot, a calculation of richness (Shannon index) of fungal taxa present in each plot and at each collection date was performed. The mean Shannon index ranged from 2.05 to 2.91 over all plots throughout the whole duration of the experiment (Table 5). Thus, no difference in diversity of fungal taxa was detected between the different plots and after the application of *B. bassiana* during the seven weeks of survey. In addition, an analysis using Metastats of differentially abundant taxa between the control (C,1-C,2) and treatment (T,1-T,2) plot during the first two weeks after application of *B. bassiana* revealed that only one fungal species, *Allomyces arbuscula* was significantly more abundant in the control compared to the treatment plot (p-value = 0.045).

Table 4: Amplification of strain-specific alleles of three SSR loci (Ba01, Ba08 and Ba13) of *B. bassiana* strain ITCC 4688 in soil DNA samples. Presence (+) or absence (-) of alleles of the correct size are shown. C = control plot and T = treatment plot, numbers refer to weeks after application of *B. bassiana* isolate ITCC 4688 to the treated plot, which is indicated by a bold line in the table.

	SSR marker		
Soil sample	Ba01	Ba08	Ba13
C,0	-	-	-
T,0	-	-	-
C,1	-	-	-
T,1	+	+	+
C,2	+	-	+
T,2	+	+	+
C,3	+	-	+
T,3	+	-	+
C,4	+	-	+
T,4	+	-	+
C,5	+	-	+
T,5	+	-	+
C,7	+	-	-
T,7	+	-	-

Table 5: Mean Shannon values of fungal diversity in control (C) and treatment (T) plots before and after the application of *B. bassiana* isolate ITCC 4688. Numbers refer to weeks after *B. bassiana* application to the treated plot, which is indicated by a bold line in the table.

	C,0	T,0	C,1	T,1	C,2	T,2	C,3	T,3	C,4	T,4	C,5	T,5	C,7	T,7
Shannon mean	2.91	2.61	2.51	2.53	2.65	2.50	2.73	2.59	2.66	2.11	2.85	2.40	2.79	2.05

7.5 Discussion

Here, we assessed the composition of soil fungal communities via tag-encoded 454 pyrosequencing to obtain insights on the effects of artificial application of an entomopathogenic fungus on indigenous fungal species present in the soil. The plot chosen for this experiment was an agricultural field in the tropical savannah climate zone (Aw zone according to the Köppen-Geiger climate classification, Peel et al. 2007) of India, which was cultivated according to conventional small-scale Indian farming standards and was planted with a standing crop of chili during this experiment. We selected this particular location, as we considered it to be important to perform such experiments under tropical conditions (Aw climate zone), with a monthly mean temperature above 18°C, with less than 60 mm precipitation within the driest month (Peel et al. 2007), as these conditions are favourable for spread and establishment of entomopathogenic fungi. In addition the tropical savannah climate (Aw zone) is one of the most common type of climate by land area with about 11.5% worldwide (Peel et al. 2007). Moreover we selected that field, as entomopathogenic fungal epizootics have never occurred in this area before and we condsidered it to be important to perform such a trial under managed conditions with as much practical relevance for farmers as possible. Accordingly, in this study we both obtained a first insight in fungal communities associated with this type of agricultural practice in the given geographic location and we were able to assess the fate and the dynamics of spread of a fungal biocontrol agent artificially applied to this field. Most of the discovered fungal taxa belonged to the phylum Ascomycota with only the genus *Paecilomyces* known to contain members of insect pathogenic fungi. Other fungi detected in the respective field included species known as saprobes on decaying plant material (e.g. Montagnulaceae, Sordariaceae), as plant pathogens (e.g. *Olpidium brassicae*, *Cochliobolus lunatus*, *Thanatephorus cucumeris*) or as mycorrhizal fungi (e.g. *Glomus mosseae*, *Pisolithus* spp.).

At the outset of our experiment no *B. bassiana* strain or any other fungal species of the family Cordycipitaceae was detected in the plots used for the present study via 454 pyrosequencing. This result is in agreement with the history of the selected location, as neither an artificial introduction nor a natural occurrence of *B. bassiana* or a related fungus had been made prior to this study in the respective field. However,

after an artificial application of *B. bassiana* strain ITCC 4688 in the treatment plot, SSR marker profiles and 454 pyrosequencing data proved the presence of this fungus in the treated plot for a duration of at least seven weeks. From a methodological point of view, this confirms previous studies on the suitability of SSR markers for effective and efficient monitoring of the presence of fungal biocontrol agents in the respective environment (Enkerli et al. 2004, Schwarzenbach et al. 2007). It also indicates that 454 pyrosequencing reads can both confirm the presence of certain indicator species and identify organisms not yet encountered in a given environment. With both molecular methods a natural spread of the respective *B. bassiana* isolate from the treatment to the control plot was documented during the course of our study. As water plays an important role in the movement of fungal pathogens (McCoy et al. 1992, Madden 1997, Bruck and Lewis 2002, Jaronski 2010) we assume that several rainfall events from October till December 2010 may have favoured the dispersal of *B. bassiana* conidia in this field. In addition, wind, arthropods and agricultural cultivation practices have been shown to be effective dispersal mechanisms of entomopathogenic fungal conidia (for review see Meyling and Eilenberg 2007) and may have contributed to this apparent spread of *B. bassiana*. Such a natural spread and establishment of *B. bassiana* is in agreement with the concept of classical biological control (Hajek and Delalibera 2010), where controlled introductions of a small inoculum of entomopathogens are performed, while it is anticipated at the same time that a natural increase in population density and a permanent establishment of the respective entomopathogenic organism will take place. Including fungal entomopathogens in a classical biological control approach is for sure of interest for small-scale farmers, where such a strategy represents a long-lasting and cost-efficient avenue for environmentally friendly insect pest control. However, it should be added that our molecular approach does not allow statements on the viability and virulence of the *B. bassiana* fungal propagules apparently present in the plots, as cultivation-independent methods such as SSR markers and 454 pyrosequencing will also amplify any DNA from dead fungal cells or senescent conidia. A combination of molecular methods and baiting techniques such as the *Galleria* bait method (Zimmermann 1986) would help to clarify this aspect of fungal virulence.

A second goal of our study was to address the question whether an artificial application of a microbial biocontrol agent causes a shift in the indigenous fungal community present in the respective plot e.g. indirectly due to competition for nutrients or directly due to suppression or antibiosis. During the seven weeks of our investigation no effect of artificially applied *B. bassiana* on the indigenous fungal communities was evident. The Shannon index was overall similar between the control and treatment plot over the whole duration of the experiment, indicating that there was no shift in fungal taxa richness and eveness of distribution as a consequence of artificial application of an entomopathogenic fungal strain. Similar results were obtained in a study by Shimazu et al. (2002) using selective media and analysing the density of forest soil microorganisms in the presence or absence of *B. bassiana*. Those authors speculate that the density of other fungi was not affected by *B. bassiana* attributable to non-germinated conidia with low metabolic activity when mixed in fresh (non-sterile) field soil. In addition, Schwarzenbach et al. (2009) assessed effects of an application of a *B. brongniartii* based biological control agent on soil fungal community structures in a controlled environment (soil microcosms) and detected as well only little effects. Moreover, the same authors assume that small effects caused by fungal biocontrol agents to soil fungal communities may be undiscovered in the field due to high ecosystem variation and fast compensation effects.

Although the Shannon index was overall similar, a comparison between the control (C,1-C,2) and treatment (T,1-T,2) plot by Metastats analysis revealed that the watermold species *Allomyces arbuscula* was differentially adundant in both plots during the first two weeks after application of *B. bassiana*. Watermolds may occur in aquatic and terrestrial ecosystems (Mer 1992, Prabhuji 2011), where they are involved in degradation of organic matter (Khallil and Omar 1992). In addition, watermolds undergo a seasonal fluctuation in their occurrence with October till mid-December found to be the best period of fungal growth in India (Upadhyay and Palni 2010). Therefore, the differential abundance of *A. arbuscula* in the control and treatment plot may be rather a consequence of natural fluctuation than an effect ascribed to the application of *B. bassiana*. However, as many microorganisms show seasonal dynamics, long-term studies are necessary to evaluate the effect of *B. bassiana* on the indigenous soil microbiota.

For registration purposes of fungal based biocontrol agents, any risks concerning the persistence of the applied fungal inoculum have to be evaluated in order to assess the organism's potential to spread and to become established in the environment (Scheepmaker and Butt 2010). In addition, registration authorities of the European Union require information on long-term non-target effects such as potential competitive displacement of soil microorganisms as well as information on the natural background level of a particular entomopathogenic fungus (Scheepmaker and Butt 2010). Both requirements may be achieved by using multitaq 454 pyrosequencing investigations as obtained sequence reads give a comprehensive description of the fungal diversity (Buée et al. 2009, Lim et al. 2010), and read abundance allows a quantification of the applied fungus and the present soil fungal community with some limitations as described in Amend et al. (2010).

A more detailed insight in the dynamics and interactions of entomopathogenic fungi like *B. bassiana* with other microorganisms present in the soil is crucial for a better understanding of factors influencing fungal survival and persistence and thus also for estimating success rates of applications of these organisms for biological insect pest control. A combination of new molecular methods like 454 pyrosequencing and classical approaches like bait methods represent powerful tools to acquire a more thorough knowledge on entomopathogenic fungal ecology in the near future.

8 Assessment of bacterial endosymbiont diversity in *Otiorhynchus* spp. larvae using a multitag 454 pyrosequencing approach

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* **Author contributions:** J. Hirsch and A. Reineke conceived the study design. J. Hirsch performed sample collection and template preparation for 454 pyrosequencing analysis. J. Hirsch, S. Strohmeier, and M. Pfannkuchen performed phylogenetic analysis.

8.1 Abstract

Weevils of the genus *Otiorhynchus* are regarded as devastating pests in a wide variety of horticultural crops worldwide. So far, little is known on the presence of endosymbionts in *Otiorhynchus* spp. Investigation of endosymbiosis in this genus may help to understand the evolution of different reproductive strategies in these weevils (parthenogenesis or sexual reproduction), host-symbiont interactions, and may provide a future basis for novel pest management strategy development. Here, we used a multitag 454 pyrosequencing approach to assess the bacterial endosymbiont diversity in larvae of four economically important *Otiorhynchus* species.

High-throughput tag-encoded FLX amplicon pyrosequencing of a bacterial 16S rDNA fragment was used to characterise bacterial communities associated with different *Otiorhynchus* spp. larvae. By sequencing a total of ~48000 PCR amplicons, we identified 49 different operational taxonomic units (OTUs) as bacterial endosymbionts in the four studied *Otiorhynchus* species. More than 90% of all sequence reads belonged either to the genus *Rickettsia* or showed homology to the phylogenetic group of "*Candidatus* Blochmannia" and to endosymbionts of the lice *Pedicinus obtusus* and *P. badii*. By using specific primers for the genera *Rickettsia* and "*Candidatus* Blochmannia", we identified a new phylogenetic clade of *Rickettsia* as well as "*Candidatus* Nardonella" endosymbionts in *Otiorhynchus* spp. which are closely related to "*Candidatus* Blochmannia" bacteria.

Here, we used multitag 454 pyrosequencing for assessment of insect endosymbiotic communities in weevils. As 454 pyrosequencing generates only quite short sequences, results of such studies can be regarded as a first step towards identifying respective endosymbiotic species in insects. In the second step of our study, we analysed sequences of specific gene regions for a more detailed phylogeny of selected endosymbiont genera. As a result we identified the presence of *Rickettsia* and "*Candidatus* Nardonella*"* endosymbionts in *Otiorhynchus* spp. This knowledge is an important step in exploring bacteria-insect associations for potential use in insect pest control.

8.2 Introduction

It is estimated that more than 65% of insects are associated with symbiotic bacteria, among them *Wolbachia* spp. being the most common genus (Duron et al. 2008, Hilgenboecker et al. 2008). The range of the symbiotic relationships between insect hosts and bacteria varies from being mutualistic and commensal to a pathogenic one (Gil et al. 2004, Moya et al. 2008, Kikuchi 2009). Accordingly, intracellular symbionts in insects are usually referred to as primary or secondary endosymbionts (P- and S-symbionts, respectively), with P-symbionts being obligate for the insect e.g. due to providing nutrients, while S-symbionts have a beneficial but not essential role for host insect survival (for reviews see Moya et al. 2008 and Moran et al. 2008). In many insects, endosymbionts are located in specialized organs (referred to as bacteriomes or mycetomes) and their inheritance usually follows a strict vertical transmission from mother to offspring.

Understanding relationships between insect hosts and their endosymbiotic bacteria is not only relevant from an evolutionary point of view, but can also aid in the identification of new targets for insect pest control (Douglas 2007) as well as for biotechnology and biomedicine (Moya et al. 2008). Yet, since many of the relevant microorganisms cannot be cultured, their identification and functional characterization was so far difficult or not possible at all. Lately, the accessibility of novel genomic techniques, in particular next generation sequencing (NGS) technologies represent new, cost-efficient and fast strategies to depict microbial diversity without the need for culturing the respective organisms (Harkins and Jarvie 2007). With these techniques thousands of sequence reads can be analysed in parallel allowing an extensive assessment of bacterial diversity within insects. As a target for bacterial NGS projects, ribosomal DNA genes (rDNA) like the 16S rDNA, also used for the taxonomic classification of bacterial species (Head et al. 1998), have frequently been applied for analysing the bacterial microbial community in metagenomic studies of soil (Acosta-Martínez et al. 2008, Teixeira et al. 2010), mines (Edwards et al. 2006), the deep sea (Sogin et al. 2006) or oral human microflora (Keijser et al. 2008).

In this study, we used high-throughput tag-encoded FLX amplicon pyrosequencing (Meyer et al. 2008) to characterise bacterial communities associated with four different weevil species of the genus *Otiorhynchus* Germar (Coleoptera: Curculionidae). Members of this genus are polyphagous and are regarded as pests of a variety of ornamental and nursery plants worldwide. Their soilborne larvae feed on the host plants' roots which may be lethal in particular for younger plants or recently transplanted cuttings. Further, feeding damage of adults on the plants foliage may reduce the market value of ornamentals. For these reasons weevils are often controlled by intensive insecticide applications (Moorhouse et al. 1992). Moreover, *Otiorhynchus* spp. can serve as a model genus for understanding the evolution of asexual reproduction, since it includes species both reproducing mostly parthenogenetically (like *Otiorhynchus sulcatus* Fabricius and *Otiorhynchus rugosostriatus* Goeze) as well as sexually (like *Otiorhynchus salicicola* Heyden and *Otiorhynchus armadillo* Rossi) (Suomalainen 1954, Magnano et al. 2008).

Here, by applying 454 sequencing technology, we show that weevils of the genus *Otiorhynchus* are associated with several endosymbiotic bacteria. This study is the first to report *Rickettsia* and "*Candidatus* Nardonella" endosymbionts - the ancestral endosymbiont of weevils - in *Otiorhynchus* spp. Identifying endosymbionts in the genus *Otiorhynchus* can expand to our understanding of the evolution of both endosymbiont-host insect interactions as well as of different reproductive strategies of insects and may provide a future basis for novel pest management approaches.

8.3 Materials and methods

8.3.1 Insect rearing, bacterial DNA isolation and species determination of larvae

All experiments were performed with four different *Otiorhynchus* species (*O. sulcatus, O. rugosostriatus, O. salicicola* and *O. armadillo*) collected in the field and kept in the laboratory until egg deposition. During that period of time weevils were fed with leaves of *Prunus* sp., *Potentilla* sp. or *Fragaria* sp. Freshly laid weevil eggs (at most 10 days old) were collected and surface sterilized according to the method developed by Hosokawa et al. (2007). The eggs were air dried under the clean bench and trans-

ferred individually with sterile featherweight forceps in Petri dishes filled with sterile TSA (40,0 g/l Difco™ Tryptic Soy Agar, pH 7.3 ± 0.2; Voigt Global Distribution Inc, Lawrence, Kansas). In order to enlarge the contact of egg and TSA agar and to check the success of surface sterilisation, eggs were rolled several times over the agar plate. For further analysis only eggs with no bacterial growth on TSA were included. Eggs were kept usually at 21-24°C until eclosion. Freshly emerged larvae (approximately 24-72 hours old) without egg material were individually collected from the TSA agar plates, and were stored frozen at -80°C until further processing. Total metagenomic DNA (~20-40 ng/µl DNA per larva) was extracted from the complete larvae using the MasterPure™ DNA Purification Kit (Epicentre Biotechnologies, Madison, Wisconsin). Taxonomic identity of each larva was confirmed according to a diagnostic PCR-RFLP pattern of the COII region (Hirsch et al. 2010). For metagenomic analysis seven individuals of each *Otiorhynchus* species were included.

8.3.2 Bacterial 16S rDNA PCR amplification and 454 pyrosequencing

Universal bacteria primers (fwd: 5'-MGAGTTTGATCCTGGCTCAG-3' and rev: 5'-GCTGCCTCCCGTAGGAGT-3'; Hamp et al. 2009), amplifying an approximately 450 bp fragment of the 16S rDNA, were used in the present study. These primers are covering the V1-V2 regions of the 16S rDNA gene and showed good phylogenetic resolution from phylum to family level in a recent study by Hamp et al. (2009). Primers were modified by the addition of a GS FLX Titanium Key-Primer A and B (A: CGTATCGCCTCCCTCGCGCCA and B: CTATGCGCCTTGCCAGCCCGC), a four-base library "key" sequence (TCAG) and a multiplex identifier (MID) sequence specific to each *Otiorhynchus* species. The MID sequences (forward/reverse) were as follows for the respective weevil species: *O. salicicola* (ATCGCG/CGCGAT), *O. rugosostriatus* (ATAGCC/GGCTAT), *O. sulcatus* (CCATAG/CTATGG) and *O. armadillo* (CTTGAG/CTCAAG). PCR reaction mixture consisted of 0.1 µl of Phire® Hot Start II DNA Polymerase (Finnzymes Oy**,** Espoo, Finland), 0.2 mM dNTPs (Metabion, Martinsried, Germany), 10 pmol primers and 40-80 ng of DNA template in a final volume of 20 µl. The PCR parameters (C1000™ Thermal Cycler, Bio-Rad Laboratories GmbH, München, Germany) were 95°C for 3 min followed by 35 cycles of 93°C for 60 s, 50°C for 60 s and 72°C for 70 s. A final extension step at 72°C for 5 min was added. An aliquot of 4 µl of each PCR product was checked for correct

size (~450 bp) on a 1% agarose gel and was afterwards purified with Hi Yield® PCR Clean-up/Gel Extraction Kit (Süd-Laborbedarf GmbH, Gauting, Germany). Bacterial 16S rDNA PCR products generated from all 28 *Otiorhynchus* individuals were mixed at equal molar concentrations according to species, and next generation 454 pyrosequencing was performed commercially (LGC Genomics GmbH, Berlin, Germany). The GenBank accession numbers for sequences obtained via 454 pyrosequencing are listed in Table 6.

Table 6: Endosymbiotic bacterial diversity and abundance in the four analysed *Otiorhynchus* species. Only the closest relatives and their 16S rDNA accession numbers (see appendix, additional file 1: 16S rDNA gene-based phylogeny of endosymbionts in four different *Otiorhynchus* spp. larvae) are mentioned.

Bacteria from weevil species	GenBank accession No.	Number of reads	% of total reads	Closest phylogenetic match and 16S rDNA accession number	Class
O. salicicola (in total 6073 reads)	JN563736	5516	90.83	AB478978, endosymbiont of *Pedicinus obtusus* and AJ245596 endosymbiont of *Camponotus balzanii* (referred to as "*Candidatus* Blochmannia" endosymbionts throughout the text)	γ-Proteobacteria
	JN563737	121	1.99	DQ417336, *Schlegelella aquatica*	β-Proteobacteria
	JN563738	96	1.58	FJ268988, uncultured *Acinetobacter*	γ-Proteobacteria
	JN563739	69	1.14	CU927677, uncultured bacterium	-
	JN563740	48	0.79	FJ534956, uncultured bacterium	-
	JN563741	44	0.72	EF210100, *Enterobacter hormaechei*	γ-Proteobacteria
	JN563742	34	0.56	AY923125, *Streptococcus* sp.	Bacilli
	JN563743	26	0.43	EU464962, uncultured bacterium	-
	JN563744	25	0.41	EU766013, uncultured bacterium	-
	JN563745	23	0.38	FJ393126, uncultured *Bacteroides* sp.	Bacteroidetes
	JN563746	18	0.30	EU721814, uncultured epsilon proteobacterium	ε-Proteobacteria
	JN563747	17	0.28	AY953252, *Prevotella* sp.	Bacteroidetes
	JN563748	15	0.25	FJ799146, bacterium enrichment culture clone LA29	-
	JN563749	11	0.18	EU802152, uncultured bacterium	-
	JN563750	10	0.16	AY568512, *Burkholderia fungorum*	β-Proteobacteria
O. rugosostriatus (in total 8584 reads)	JN563751	7800	90.87	AB021128, *Rickettsia* sp.	α-Proteobacteria
	JN563752	396	4.61	EF633744, *Candidatus* Neoehrlichia lotoris	α-Proteobacteria

Table 6 (continued)

Bacteria from weevil species	GenBank accession No.	Number of reads	% of total reads	Closest phylogenetic match and 16S rDNA accession number	Class
O. rugosostriatus (in total 8584 reads)	JN563753	338	3.94	AB478978, endosymbiont of *Pedicinus obtusus* and AJ245596 endosymbiont of *Camponotus balzanii* (referred to as "*Candidatus* Blochmannia" endosymbionts throughout the text)	γ-Proteobacteria
	JN563754	17	0.20	AB021128, *Rickettsia* sp.	α-Proteobacteria
	JN563755	11	0.13	EF633744, *Candidatus* Neoehrlichia lotoris	α-Proteobacteria
	JN563756	7	0.08	AB021128, *Rickettsia* sp.	α-Proteobacteria
	JN563757	6	0.07	AB021128, *Rickettsia* sp.	α-Proteobacteria
	JN563758	5	0.06	FJ868862, uncultured bacterium	-
	JN563759	4	0.05	GQ845011, *Nevskia* sp.	γ-Proteobacteria
O. sulcatus (in total 6412 reads)	JN563760	6358	99.16	AB021128, *Rickettsia* sp.	α-Proteobacteria
	JN563761	35	0.55	EF633744, *Candidatus* Neoehrlichia lotoris	α-Proteobacteria
	JN563762	19	0.30	EF633744, *Candidatus* Neoehrlichia lotoris	α-Proteobacteria
O. armadillo (in total 6311 reads)	JN563763	5900	93.49	AB478978, endosymbiont of *Pedicinus obtusus* and AJ245596 endosymbiont of *Camponotus balzanii* (referred to as "*Candidatus* Blochmannia" endosymbionts throughout the text)	γ-Proteobacteria
	JN563764	60	0.95	FJ823944, uncultured *Comamonas* sp.	β-Proteobacteria
	JN563765	54	0.86	FJ868862, uncultured bacterium	-
	JN563766	43	0.68	FJ823944, uncultured *Comamonas* sp.	β-Proteobacteria
	JN563767	35	0.55	FJ544375, *Comamonas aquatica*	β-Proteobacteria
	JN563768	31	0.49	EU560802, uncultured bacterium	-
	JN563769	23	0.36	DQ407746, primary endosymbiont of *Liposcelis decolor*	-
	JN563770	21	0.33	DQ469223, uncultured bacterium	-
	JN563771	21	0.33	GQ845011, *Nevskia* sp.	γ-Proteobacteria
	JN563772	20	0.32	DQ860049, uncultured bacterium	-

Table 6 (continued)

Bacteria from weevil species	GenBank accession No.	Number of reads	% of total reads	Closest phylogenetic match and 16S rDNA accession number	Class
O. armadillo	JN563773	11	0.17	AF006670, *Shewanella putrefaciens*	γ-Proteobacteria
(in total 6311	JN563774	11	0.17	X82133, *Shewanella putrefaciens*	γ-Proteobacteria
reads)	JN563775	11	0.17	EU801479, uncultured bacterium	-
	JN563776	10	0.16	EF019306, uncultured proteobacterium	-
	JN563777	9	0.14	AY953252, *Prevotella* sp.	Bacteroidetes
	JN563778	8	0.13	EU464962, uncultured bacterium	-
	JN563779	8	0.13	EU536078, uncultured bacterium	-
	JN563780	8	0.13	GQ068015, uncultured bacterium	-
	JN563781	8	0.13	L16490, *Porphyromonas asaccharolytica*	Bacteroidetes
	JN563782	8	0.13	AY351787, uncultured marine bacterium	-
	JN563783	6	0.10	EF648074, uncultured *Azoarcus* sp.,	β-Proteobacteria
	JN563784	5	0.08	EF648074, uncultured *Azoarcus* sp.,	β-Proteobacteria

8.3.3 Sample assignment and analysis of 454 sequencing data

Sequence reads were assembled independently by Geneious Pro Version 5.0 (Drummond et al. 2009) and WiMSeEx (Window Match Seed Extension)-Algorithm (unpublished). Results of both procedures for diversity and sequence identity were compared. Only high quality reads that did accurately match the four-base library "key" sequence (TCAG) and the multiplex identifier (MID) sequence were used for Geneious Pro assembly. Geneious Pro assembly was performed with medium sensitivity, a maximum of 120 contigs and default settings. Consensus sequences were extracted manually from all contigs. WiMSeEx assembly was performed for each tag with all raw data reads and the following parameters: minimum seed size: 200 bp, window size: 60 bp. The four-base identifier and 20 bp of the primer were chosen for seed detection. Each assembly run was stopped by reaching 500 kb sequence data. Resulting sequences of both procedures were then aligned independently using MAFFT version 5 (Katoh et al. 2005) and consensus sequences were extracted manually from clustered sequences and redundant sequence data were removed. Afterwards the sequence identifier and the primer sequence were eliminated from each consensus sequence. All consensus sequences extracted from Geneious Pro contigs were found in the WiMSeEx consensus sequences assembly data and vice versa.

8.3.4 Amplification of selected genes of most dominant endosymbionts

For accurate phylogenetic analysis of the most dominant endosymbionts in *Otiorhynchus* spp., specific 16S rDNA and cytochrome c oxidase subunit I (coxA) primers for the genus *Rickettsia* (Weinert et al. 2009) as well as 16S rDNA primers for "*Candidatus* Blochmannia*"* bacteria (Wernegreen et al. 2009) were used for amplification of the respective sequences from 2-4 *Otiorhynchus* individuals per species. PCR reactions were set up in a final volume of 20 µl consisting of 0.1 µl of Phire® Hot Start II DNA Polymerase (Finnzymes Oy, Espoo, Finland), 0.25 mM dNTPs (Fermentas GmbH, St. Leon-Rot, Germany), 10 pmol primers and 40-80 ng of DNA template. The PCR parameters (C1000™ Thermal Cycler, Bio-Rad Laboratories GmbH, München, Germany) were 95°C for 2 min followed by 40 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1 min. A final extension step at 72°C for 10 min was added. An aliquot of 4 µl of each PCR product was checked for correct size on a

1% agarose gel and was afterwards purified with Hi Yield® PCR Clean-up/Gel Extraction Kit (Süd-Laborbedarf GmbH, Gauting, Germany). Direct sequencing of the resulting PCR product was performed commercially (LGC Genomics GmbH, Berlin, Germany). As we did not detect any bacterial sequence variation within one weevil species (except for *O. sulcatus* and the 16S rDNA amplified with "*Candidatus* Blochmannia" specific primers), only one sequence per *Otiorhynchus* species and gene region was submitted to GenBank (accession numbers JN394465-JN394471, JN563785-JN563788).

8.3.5 Phylogenetic analysis

Consensus sequences gained from 454 pyrosequencing were included into an alignment of more than 260000 (SSURef_102_SILVA_NR_99_18_02_10_opt.ARF) bacterial 16S rDNA sequences (Pruesse et al. 2007) and best positions in the resulting phylogenetic tree were found including all nucleotides (positions) from the 454 assemblies using the parsimony algorithm of the ARB 5.1 software package (Ludwig et al. 2004). The here presented trees are subregions of the complete tree (see appendix, additional file 1: 16S rDNA gene-based phylogeny of endosymbionts in four different *Otiorhynchus* spp. larvae) including the sequences assembled from the 454 sequencing approach reported in this chapter and the most similar sequences available from public databases. More distantly related or unrelated sequences were included in the calculation but are not shown.

Additional 16S rDNA sequences amplified with specific primers for "*Candidatus* Blochmannia" and *Rickettsia* endosymbionts were included in the above mentioned alignment and a neighbour joining analysis was inferred using the neighbour joining algorithm included in the software package ARB 5.1 like described above. In addition, sequences of part of the coxA gene amplified in *Otiorhynchus* spp. were included in an alignment of sequences used by Weinert et al. (2009) and a neighbour joining tree was calculated accordingly.

8.4 Results and discussion

8.4.1 454 pyrosequencing and identification of endosymbionts in *Otiorhynchus* spp.

A total of ~48000 PCR amplicons were sequenced via GS FLX titanium 454 sequencing, of which ~27000 reads were assembled after having passed the additional quality controls. These sequences were summarized into 49 consensus sequences (Table 6), representing the total retrieved endosymbiotic bacterial diversity in the four different *Otiorhynchus* species. Sequence abundances of the respective OTUs were different in each weevil species analysed. We expect these differences in sequence abundance within the 16S rDNA amplicons to reflect the respective bacterial abundances in the sample.

In addition to the most abundant reads, which belonged either to the genus *Rickettsia* or were similar to "*Candidatus* Blochmannia" bacteria and endosymbionts of the lice *Pedicinus obtusus* and *P. badii*, numerous reads with low sequence frequency were detected (Table 6). Indeed, we can not fully exclude the possibility that these sequences of putative rare endosymbionts are rather artefacts e.g. due to PCR contaminations.

8.4.2 Phylogenetic analysis of *Otiorhynchus* spp. endosymbionts

Phylogenetic analysis of 454 sequence data was performed to establish the relationship of the partial 16S rDNA sequences to each other and to related sequences gained from public databases. Among all studied weevil species, *O. sulcatus* showed the lowest bacterial endosymbiotic diversity (Table 6). The vast majority of sequences in *O. sulcatus* (~99% of the total reads) and *O. rugosostriatus* (~91% of the total reads) belonged to the genus *Rickettsia* (see appendix, additional file 1: 16S rDNA gene-based phylogeny of endosymbionts in four different *Otiorhynchus* spp. larvae, Table 6). Bacteria similar to the endosymbionts of the lice *Pedicinus obtusus* and *P. badii* (Allen et al. 2009, Fukatsu et al. 2009) and the genus "*Candidatus* Blochmannia" were dominant in *O. salicicola* (~91% of the total reads) and *O. armadillo* (~93% of the total reads) (see appendix, additional file 1: 16S rDNA gene-based phylogeny of endosymbionts in four different *Otiorhynchus* spp. larvae,

Table 6). These bacteria were also found in a less dominant manner in *O. rugosostriatus* (~4% of the total reads). To determine the phylogenetic position of *Rickettsia* and putative "*Candidatus* Blochmannia" like endosymbionts detected via 454 pyrosequencing in a more precise way, genus specific primers (Weinert et al. 2009, Wernegreen et al. 2009) were used to amplify a ~750 bp fragment of the *Rickettsia* and "*Candidatus* Blochmannia" specific 16S rDNA and a ~800 bp fragment of the *Rickettsia* cytochrome c subunit I (coxA) gene, respectively. Phylogenetic analysis of these sequences placed the *Otiorhynchus* spp. specific *Rickettsia* into a new clade within the genus *Rickettsia* (Figure 18 and Figure 19). Sequences gained by using "*Candidatus* Blochmannia" specific primers were grouped within the clade of "*Candidatus* Nardonella" bacteria, which are closely related to "*Candidatus* Blochmannia" endosymbionts (Figure 20). Accordingly, the additional analysis of these endosymbionts using gene specific primers revealed for the first time the presence of *Rickettsia* and "*Candidatus* Nardonella" bacteria within the genus *Otiorhynchus*.

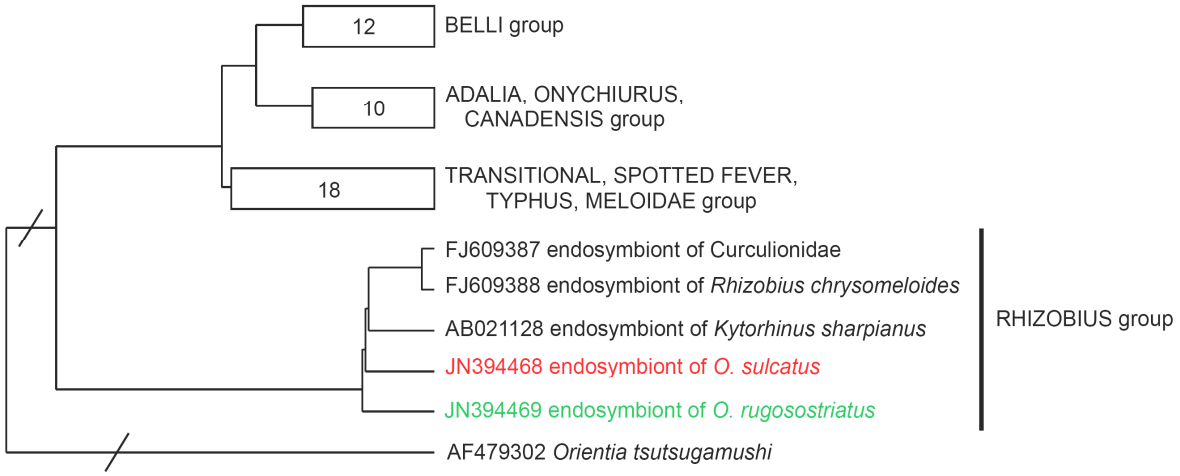

Figure 18: Neighbour joining tree of *Rickettsia* endosymbionts using sequences of 16S rDNA. Sequences obtained in the present study are coloured and phylogenetic groups were constructed according to Weinert et al. (2009). The amount of sequences included in the groups are indicated by numbers. Branch lengths were reduced in two positions (marked with diagonal slashes).

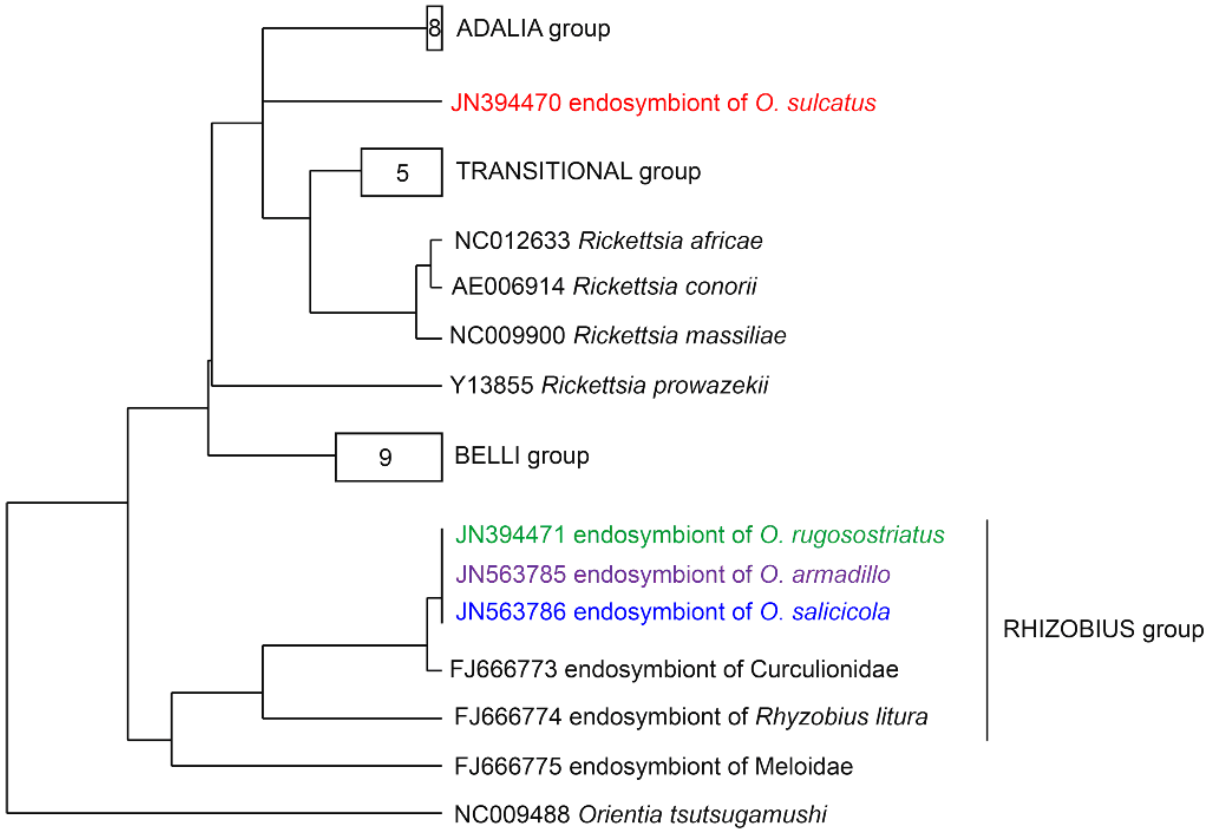

Figure 19: Neighbour joining tree of *Rickettsia* endosymbionts using sequences of coxA gene. Sequences obtained in the present study are coloured. Sequences were combined in groups according to Weinert et al. (2009). The amount of sequences included in the groups are indicated by numbers.

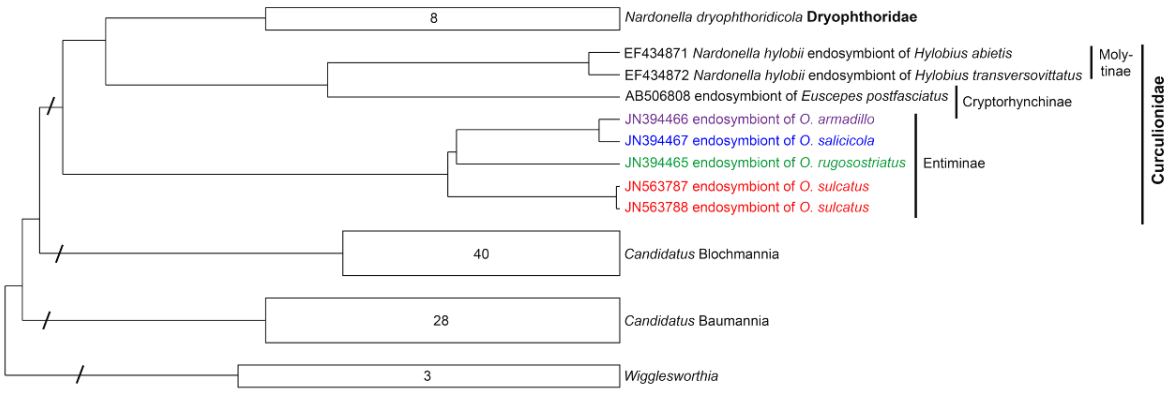

Figure 20: Neighbour joining tree of "*Candidatus* Nardonella" endosymbionts using sequences of 16S rDNA. Sequences obtained in the present study are coloured. Branch lengths were reduced in four positions (marked with diagonal slashes). The amount of sequences included in the groups are indicated by numbers.

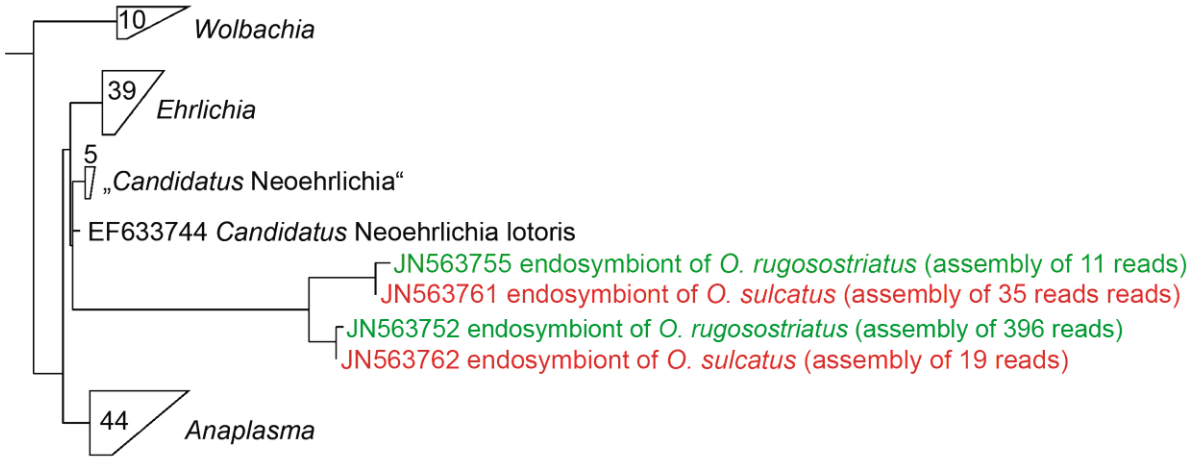

Figure 21: Phylogenetic analysis of endosymbionts under "*Candidatus* Neoehrlichia" subregion in *Otiorhynchus* spp. The tree represents the "*Candidatus* Neoehrlichia" subregion of the complete tree (see appendix, additional file 1: 16S rDNA gene-based phylogeny of endosymbionts in four different *Otiorhynchus* spp. larvae) and was constructed by using parsimony algorithm. Sequences obtained in the present study are coloured. The amount of sequences included in the groups of *Wolbachia*, *Ehrlichia*, „*Candidatus* Neoehrlichia" and *Anaplasma* are indicated by numbers.

8.4.3 Phylogenetic analysis and putative biological function of *Rickettsia* endosymbionts

In the parthenogenetically reproducing species *O. sulcatus* and *O. rugosostriatus, Rickettsia* endosymbionts were the most dominant group found via 454 pyrosequencing. By using *Rickettsia* specific primers for the 16S rDNA and the coxA gene these results were strengthened, however, a fragment of the *Rickettsia* specific coxA gene was also amplified in *O. armadillo* and *O. salicicola,* respectively, while 454 pyrosequencing previously indicated that these two species were missing *Rickettsia* endosymbionts (Table 6). Yet, at the same time it was not possible to amplify the *Rickettsia* specific 16S rDNA fragment in the same two species. We thus suppose that the coxA gene sequence is rather conserved among bacteria and may not be adequate for precise species determination. Supplementary sequence analysis of a range of additional bacterial genes may resolve this issue.

Phylogenetic analysis of the *Rickettsia* endosymbiontic 16S rDNA and coxA gene fragments amplified from *Otiorhynchus* spp. revealed the relatedness to the rhizobius and/or adalia *Rickettsia* group as defined by Weinert et al. (2009). These subgroups

contain *Rickettsia* bacteria identified in various beetles, including members of the Curculionidae (Weinert et al. 2009). *Rickettsia* endosymbionts act as male-killing agents in leaf mining beetles and ladybirds (Majerus and Hurst 1997, Fukatsu and Shimada 1999) and play an essential role in the early development of the oocyte and egg production in parthenogenetic book lice (Yusuf and Turner 2004, Perotti et al. 2006). Thus it could be speculated that *Rickettsia* endosymbionts may also manipulate host reproduction in *Otiorhynchus* species.

8.4.4 Phylogenetic analysis and putative biological function of "*Candidatus* Nardonella" endosymbionts

454 pyrosequencing detected endosymbionts similar to "*Candidatus* Blochmannia" and bacterial endosymbionts of the lice *Pedicinus obtusus* and *P. badii* in *O. armadillo*, *O. salicicola* and to a lesser extent in *O. rugosostriatus*. The presence of these putative "*Candidatus* Blochmannia" like bacteria was verified in these species by using primers specific for the "*Candidatus* Blochmannia" 16S rDNA (Wernegreen et al. 2009), which indicated that the obtained sequences are similar to "*Candidatus* Nardonella". In addition, a fragment of the same size and sequence was also amplified in *O. sulcatus*, even though 454 pyrosequencing did not reveal the presence of these bacteria in this weevil species (Table 6). "*Candidatus* Nardonella" bacteria are often localized in the bacteriome whereas *Rickettsia* endosymbionts may infect as well different tissues. As we used whole larvae for DNA extraction, the amount of overall isolated DNA might have been lower for "*Candidatus* Nardonella" than for *Rickettsia*. Therefore we assume that respective bacterial DNA might have not been amplified in *O. sulcatus* with the universal primers used for 454 pyrosequencing due to competition for PCR reagents with taxa such as *Rickettsia*, having a higher template abundance (Amend et al. 2010). However, these results also demonstrate that studies using 454 pyrosequencing can be regarded as a first step towards identifying respective endosymbiotic species in insects, but that for a detailed phylogeny and a more comprehensive insight into endosymbiont-insect-associations, the amplification of specific gene regions is still indispensable.

Phylogenetic analysis of the putative "*Candidatus* Blochmannia" specific 16S rDNA sequence amplified from the four studied *Otiorhynchus* weevils showed a close

relatedness of these bacteria to the genus "*Candidatus* Nardonella". Sequences generated in the present study build a separate branch next to endosymbionts from molytine, cryptorhynchine and dryophthorid weevils (Lefèvre et al. 2004, Conord et al. 2008, Hosokawa and Fukatsu 2010) (Figure 20). The biological function of "*Candidatus* Nardonella" endosymbionts in their host weevils is unknown so far, except for the cryptorhynchine West Indian sweet potato weevil, *Euscepes postfasciatus.* Within this species "*Candidatus* Nardonella*"* endosymbionts are involved in growth and development of the host weevil (Kuriwada et al. 2010).

8.4.5 Implications and future directions of endosymbiosis in different *Otiorhynchus* species

For several *Otiorhynchus* species, an association with bacteria of the genus *Wolbachia* has been proven in previous studies (Stenberg and Lundmark 2004, Son et al. 2008, Lachowska et al. 2010). *Wolbachia* cause several reproductive alterations in insects, including cytoplasmic incompatibility, feminization of genetic males or parthenogenesis (Werren et al. 2008). In *Otiorhynchus* species *Wolbachia* are assumed to rather play a role in normal development of e.g. *O. sulcatus* eggs (Son et al. 2008) rather than in the evolution of parthenogenesis or polyploidy (Stenberg et al. 2003, Stenberg and Lundmark 2004, Lachowska et al. 2010). Unexpectedly, in the present 454 pyrosequencing approach, none of the bacterial sequence reads obtained from four different *Otiorhynchus* spp. weevil larvae corresponded to *Wolbachia.* Instead, bacterial sequences similar to "*Candidatus* Neoehrlichia", a close relative to *Wolbachia*, were found in however low frequencies in *O. sulcatus* (~1% of the total reads) and *O. rugosostriatus* (~5% of the total reads) (Table 6, Figure 21). Species of that genus are known as tick-borne bacterial pathogens (Fehr et al. 2010) and have been isolated from raccoons and rats (Kawahara et al. 2004, Yabsley et al. 2008) but their biological function in insects is unclear so far. As the presence of different *Wolbachia* strains may differ within a given species between geographical regions (Arthofer et al. 2009) further studies are required using *Wolbachia* specific PCR primers to shed light on the prevalence and distribution of *Wolbachia* within *Otiorhynchus* species and between populations, respectively.

Recent microbiological characterization of bacterial endosymbionts in the Curculionoidea of the family Molytinae and Dryophthoridae has demonstrated that endosymbiosis with "*Candidatus* Nardonella" bacteria is ~125 Myr old in curculionids and is most of the times evolutionary stable, except for a few clades where respective endosymbionts have been lost and were replaced by different microbes during evolution (endosymbiont replacement; Conord et al. 2008). Our study broadens the range of weevils associated with "*Candidatus* Nardonella" endosymbionts and indicates a benefit for *Otiorhynchus* weevils due to the long-lasting bacterial inheritance.

In a number of weevil species it has been shown that endosymbionts are frequently found within specialized host cells (so-called bacteriocytes) sometimes forming a distinctive organ, the bacteriome, which is often associated with the larval midgut (Lefèvre et al. 2004, Anselme et al. 2006, Nardon 2006, Conord et al. 2008, Toju et al. 2010). As Buchner (1953) has described a bacteriome in *Otiorhynchus* spp., we assume that the four *Otiorhynchus* species analysed in the present study also harbour their endosymbiotic bacteria intracellularly in a bacteriome. However, this assumption has to be confirmed via microscopic examinations of the respective organs.

For a couple of insects and their associated microorganisms it has been shown, that endosymbiotic bacteria are known to be involved in protecting their host insect against natural antagonists such as predators and pathogens or are even implicated in insecticide resistance mechanisms (for a review see Zindel et al. 2011). Moreover, particularly obligatory endosymbionts are essential for central functions of their host insect (Moya et al. 2008). Accordingly, endosymbiotic bacteria are an interesting target for direct or indirect manipulation, thus offering new possibilities for designing insect control strategies (Hurst and Jiggins 2000, Zabalou et al. 2004, Zindel et al. 2011). Identification of respective endosymbiotic organisms of the target insect is an important step in exploring these associations for potential use in insect pest control. Thanks to the agar-based artificial diet for rearing of *O. sulcatus* (Fisher and Bruck 2004), physiological, nutritional and reproductive studies will be carried out to analyse the respective effects of symbionts on the host development and reproduction.

8.5 Conclusion

In this study, endosymbiotic bacterial diversity in weevil larvae was assessed via multitag 454 pyrosequencing of a bacterial 16S rRNA fragment. Pyrosequencing is therefore a promising, fast and economic alternative to other culture-independent methods in metagenomics like DGGE (denaturing gradient gel electrophoresis) or SSCP (single-strand conformation polymorphism), which have been used in bacterial community studies of the red turpentine beetle (Adams et al. 2010) or for diversity assessment of gut microbiota in bees (Mohr and Tebbe 2006), respectively. However, as 454 pyrosequencing generates only quite short sequences, results of such studies can just be regarded as a first step towards identifying respective endosymbiotic species in insects. Accordingly, a subsequent analysis of sequences of specific gene regions of selected endosymbiont genera detected via 454 pyrosequencing revealed the presence of endosymbionts of the genera *Rickettsia* and "*Candidatus* Nardonella" in *Otiorhynchus* spp. Further studies are now required to clarify the biological function of these endosymbiotic bacteria in *Otiorhynchus* spp. and their potential as novel targets for weevil pest control.

9 Conclusion and prospects

The weevil genus *Otiorhynchus* contains several species which are serious pests in horticulture. However, currently there are no satisfying biological or chemical management strategies for the control of *Otiorhynchus* pests. Therefore, the development of novel environmentally friendly control strategies is needed. This chapter intends to summarize the most important results gained in the present thesis and to outline promising research approaches, which may help to manage *Otiorhynchus* weevils as pests in horticulture.

The prime example of *Otiorhynchus* pests is the black vine weevil *Otiorhynchus sulcatus*. In recent years however, the number of documented records of damage inflicted by other *Otiorhynchus* species has increased and/or has been given more attention (Collman 2001, Sprick 2009). As different *Otiorhynchus* species show a different phenology (Sprick 2009), continuous monitoring is a prerequisite for the successful application of biological or chemical plant protection products. Traditionally, wet pitfall traps have been used for monitoring of weevils. However, a mark-release-recapture study performed in a peony plantation near Wiesbaden (Germany) showed that *Otiorhynchus* weevils may be effectively captured with dry pitfall traps (Reineke et al. 2011). These traps are described in chapter 6 and have also been applied for mass trapping of adult weevils in the present thesis (chapter 5 and 6). Reineke et al. (2011) assume that dry pitfall traps already containing living beetles are attractive for other weevils, probably due to aggregation pheromones. Therefore, for horticultural practice, replacing traps containing liquids with dry pitfall traps for continuous monitoring of *Otiorhynchus* weevils is recommended.

The fact that other *Otiorhynchus* species, aside from *O. sulcatus*, may occur in horticultural crops (Sprick 2009), reflects first of all the necessity for a correct species identification prior to the development of pest management strategies. Although adult *Otiorhynchus* weevils are morphologically distinguishable by skilled personnel, there is high potential for misidentification of cryptic weevil larvae. Therefore, a diagnostic molecular fingerprint, based on PCR-RFLP technique, was developed, which offers

species determination of weevils in all developmental stages (chapter 4). This method allows the identification of 21 economically important weevil species of the 31, which were found in tree nurseries and horticultural and hop cultivation farms in 2008 across Germany (Sprick 2009). The molecular fingerprint, which is indispensable for correct determination of weevil larvae, may be complemented in the near future by a digital illustrated key for the identification of adult *Otiorhynchus* weevils. This determination key is currently being developed by the Curculio Institute (http://www.curci.de/). Hence both methods could be used in future as complementary strategies for species determination of *Otiorhynchus* weevils.

Entomopathogenic fungi (EPF) have shown a high potential for biological insect pest control in numerous studies (Hajek and Delalibera 2010). In the present thesis, the EPF *B. bassiana* (Naturalis®), *I. fumosorosea* (PreFeRal® WG) and *M. anisopliae* strain Ma43 were used in laboratory trails against different developmental stages of *Otiorhynchus* spp. Both *B. bassiana* (Naturalis®) and *M. anisopliae* strain Ma43 showed good effectiveness against adults of *O. sulcatus* (chapter 5). However, adults of *O. raucus* and *O. dieckmanni* were only susceptible to *B. bassiana* (Naturalis®) (chapter 5). The fact that different *Otiorhynchus* species in the adult stage show a different susceptibility to various EPF reflects furthermore that species identification is an indispensable prerequisite before applying plant protection products. In addition, due to the varying susceptibility of adult weevils, the effectiveness of both fungi, *B. bassiana* (Naturalis®) and *M. anisopliae* strain Ma43 in a combined application, may be in future a promising strategy for *Otiorhynchus* pest control, especially if several weevil species are present at a site.

Entomopathogenic fungi are often poured as aqueous solutions onto pots containing plants infested with *Otiorhynchus* larvae, or they are directly incorporated into the potting media at the time of planting (Bruck and Donahue 2007). In the present thesis, infection experiments revealed that *B. bassiana* (Naturalis®) may infect eggs and larvae of *Otiorhynchus* spp. (chapter 5). As *B. bassiana* (Naturalis®) may persist in different potting media (Bischoff-Schaefer 2010) and is still infectious for insect larvae six weeks post-treatment (Gottwald 2011), pouring *B. bassiana* (Naturalis®) onto pots or mixing the fungus into the potting media may be promising strategies for

Otiorhynchus pest control. However, incorporating *B. bassiana* (Naturalis®) into the potting media at the time of planting appears to be preferable. When *B. bassiana* (Naturalis®) is poured onto pots containing ED 73 planting substrate, fungal propagules remain in the upper soil layers (Gottwald 2011). Consequently, large parts of the root are unprotected - which is not the case when *B. bassiana* (Naturalis®) is homogeneously mixed into the soil - and might subsequently be attacked by weevil larvae. However, Beck (1996) documented that immature adults of *O. sulcatus* may be infected with EPF in the upper soil layers, when they emerge from the pupa stage. So far it is unclear if freshly emerged adults of *Otiorhynchus* spp. are susceptible to *B. bassiana* (Naturalis®). In future, both strategies, pouring *B. bassiana* (Naturalis®) onto pots and mixing it into the soil, should be tested against different developmental stages of several *Otiorhynchus* species.

In the present thesis, a method to quantify the efficiency of EPF against adult *Otiorhynchus* weevils under field conditions was established (chapter 6). In 2010 and 2011, this method was used to measure the success of an application of *B. bassiana* (Naturalis®) against adults of *Otiorhynchus* spp. in a peony plantation. According to the present results, it is assumed that at least ~6.9 x 10^8 conidia/m² of *B. bassiana* (Naturalis®) are necessary to control adult weevils in the field. However, as *Otiorhynchus* weevils are flightless, quite sedentary and show the tendency to aggregate, which may be recognized e.g. by cumulative feeding traces on infested plants, it might be sufficient and cost-saving to apply biocontrol agents to the infested plants and the immediate vicinity, instead of treating the whole area (Reineke et al. 2011).

The fate and potential effect of an application of *B. bassiana* strain ITCC 4688 on the indigenous soil fungal community of an agricultural field in India was analysed (chapter 7). As a result, both 454 pyrosequencing and SSR marker analysis revealed that *B. bassiana* strain ITCC 4688 became established within the field. In addition, a natural spread and no effect on the present soil fungal diversity was detected. For registration of plant protection products based on EPF within the EU, information on the effect of EPF on non-targets including soil microorganisms is required (Scheepmaker and Butt 2010). Microsatellite markers for strain-specific identification of *B. bassiana* strain ATCC 74040 (Naturalis®) are available and have been

successfully applied to detect *B. bassiana* strain ATCC 74040 in diffferent bulk soil DNA preparations (Sandhya et al. 2011, Bischoff-Schaefer 2010). Therefore, it is suggested that the above mentioned experiment is repeated with an application of *B. bassiana* strain ATCC 74040 (Naturalis®) onto the soil of a horticultural field under moderate climate conditions, in order to investigate the effect of *B. bassiana* strain ATCC 74040 on non-target soil microorganisms. Such a study may be helpful to speed up the national registration process of Naturalis® for application in open fields.

The association of insects and endosymbiotic microorganisms harbours enormous potential for the design of novel pest management strategies. Therefore, first of all a comprehensive picture of the diversity of endosymbionts present inside the respective pest insect is needed. In the present thesis, 49 different operational taxonomic units (OTUs) were detected in the four *Otiorhynchus* species studied. For a more detailed phylogeny of some selected OTUs, specific gene regions were analysed and a new clade of "*Candidatus* Nardonella" and *Rickettsia* endosymbionts in weevils of the genus *Otiorhynchus* were identified. So far, the biological function of detected bacteria in *Otiorhynchus* spp. is unclear. *Rickettsia* endosymbionts are known to cause male killing and parthenogenetic reproduction in several insect species (Majerus and Hurst 1997, Lawson et al. 2001, Schulenburg et al. 2001, Hagimori et al. 2006, Giorgini et al. 2010). Therefore, it could be assumed that *Rickettsia* bacteria may be also involved in manipulation of host reproduction in *Otiorhynchus* weevils. Bacteria of the genus "*Candidatus* Nardonella" are the most widespread and ancient endosymbiont lineage within weevils and for instance, removal of these bacteria may result in the West Indian sweet potato weevil in negative fitness effects (Kuriwada et al. 2010). The next challenge would be to elucidate the function of "*Candidatus* Nardonella" and *Rickettsia* bacteria within *Otiorhynchus* weevils. Therefore, additional different *Otiorhynchus* species should be analysed in order to investigate whether there is are correlation between the occurrence of specific endosymbionts and the mode of reproduction of the respective weevil species. Moreover, in situ hybridization targeting the 16S rRNA of specific endosymbionts, as performed for endosymbionts in weevils of the genus *Curculio* (Toju et al. 2010), may give valuable insight into the localization of these bacteria and may show their potential mode of transmission. Moreover, generating aposymbiotic weevils, as done for developing

Wolbachia-free lines in *O. sulcatus* (Son et al. 2008) and *Nardonella*-free West Indian sweet potato weevils (Kuriwada et al. 2010), may help to determine the biological function of "*Candidatus* Nardonella" and *Rickettsia* bacteria in *Otiorhynchus* weevils. Subsequently this knowledge may be used for the development of new management strategies to control *Otiorhynchus* pests in horticulture.

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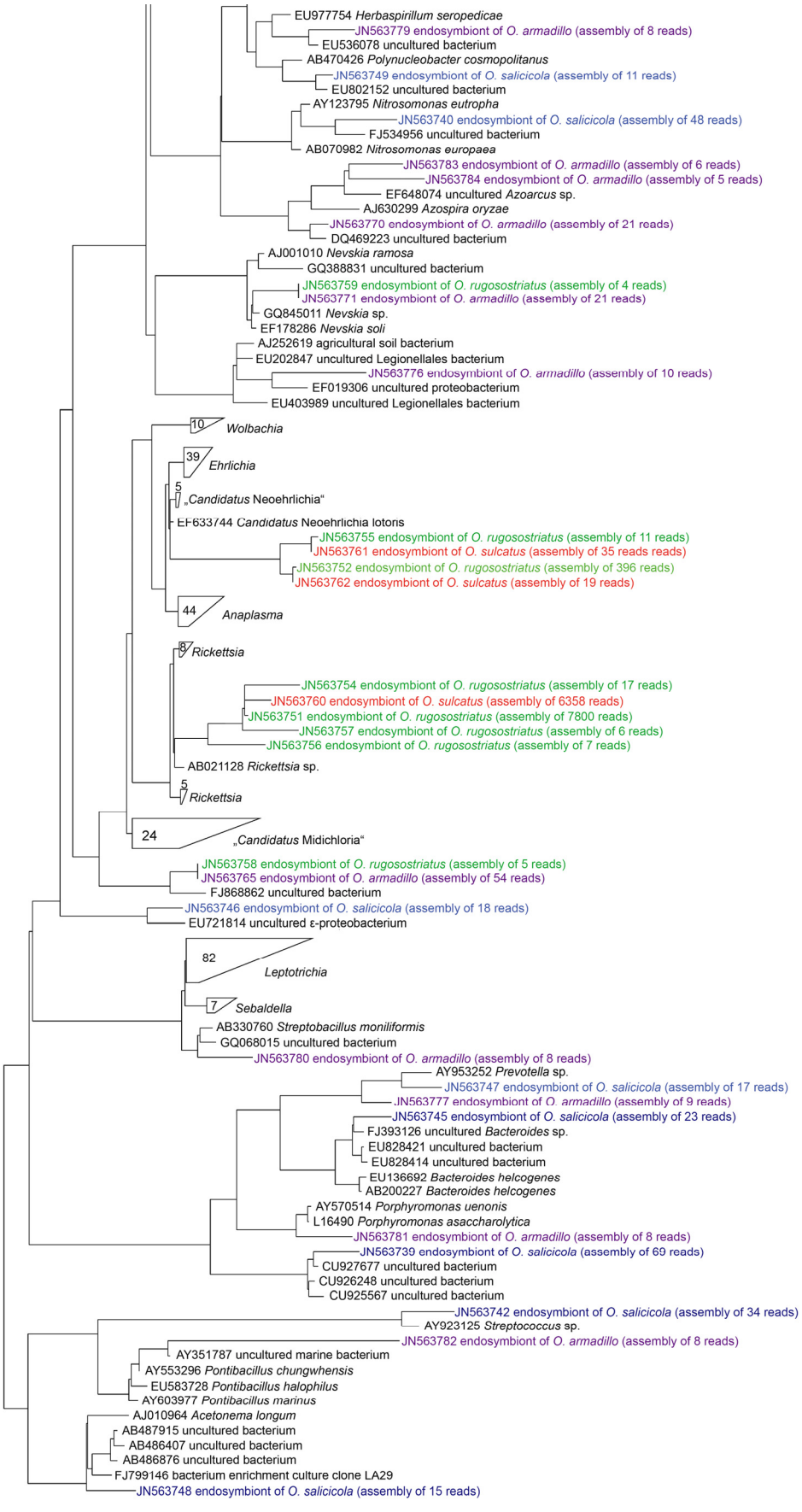
11 Appendix

11.1 Additional file 1

16S rDNA gene-based phylogeny of endosymbionts in four different *Otiorhynchus* spp. larvae which were analysed in chapter 8. Sequences obtained in the present thesis (chapter 8) are coloured and accession numbers of 16S rDNA sequences are shown for related bacterial species. More distantly related or unrelated sequences are not shown. Sequences from this work (chapter 8) were added using the parsimony algorithm. This tree results from a phylogenetic calculation including more than than 260000 bacterial 16S rDNA sequences. Only the nearest relatives are shown in this tree.

Please note, phylogenetic tree continues on next page.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EU536078 uncultured bacterium
AB470426 Polynucleobacter cosmopolitanus
JN563749 endosymbiont of O. salicicola (assembly of 11 reads)
EU802152 uncultured bacterium
AY123795 Nitrosomonas eutropha
JN563740 endosymbiont of O. salicicola (assembly of 48 reads)
FJ534956 uncultured bacterium
AB070982 Nitrosomonas europaea
JN563783 endosymbiont of O. armadillo (assembly of 6 reads)
JN563784 endosymbiont of O. armadillo (assembly of 5 reads)
EF648074 uncultured Azoarcus sp.
AJ630299 Azospira oryzae
JN563770 endosymbiont of O. armadillo (assembly of 21 reads)
DQ469223 uncultured bacterium
AJ001010 Nevskia ramosa
GQ388831 uncultured bacterium
JN563759 endosymbiont of O. rugosostriatus (assembly of 4 reads)
JN563771 endosymbiont of O. armadillo (assembly of 21 reads)
GQ845011 Nevskia sp.
EF178286 Nevskia soli
AJ252619 agricultural soil bacterium
EU202847 uncultured Legionellales bacterium
JN563776 endosymbiont of O. armadillo (assembly of 10 reads)
EF019306 uncultured proteobacterium
EU403989 uncultured Legionellales bacterium
10
Wolbachia
39
Ehrlichia
5
„Candidatus Neoehrlichia“
EF633744 Candidatus Neoehrlichia lotoris
JN563755 endosymbiont of O. rugosostriatus (assembly of 11 reads)
JN563761 endosymbiont of O. sulcatus (assembly of 35 reads reads)
JN563752 endosymbiont of O. rugosostriatus (assembly of 396 reads)
JN563762 endosymbiont of O. sulcatus (assembly of 19 reads)
44
Anaplasma
8
Rickettsia
JN563754 endosymbiont of O. rugosostriatus (assembly of 17 reads)
JN563760 endosymbiont of O. sulcatus (assembly of 6358 reads)
JN563751 endosymbiont of O. rugosostriatus (assembly of 7800 reads)
JN563757 endosymbiont of O. rugosostriatus (assembly of 6 reads)
JN563756 endosymbiont of O. rugosostriatus (assembly of 7 reads)
AB021128 Rickettsia sp.
5
Rickettsia
24
„Candidatus Midichloria“
JN563758 endosymbiont of O. rugosostriatus (assembly of 5 reads)
JN563765 endosymbiont of O. armadillo (assembly of 54 reads)
FJ868862 uncultured bacterium
JN563746 endosymbiont of O. salicicola (assembly of 18 reads)
EU721814 uncultured ε-proteobacterium
82
Leptotrichia
7
Sebaldella
AB330760 Streptobacillus moniliformis
GQ068015 uncultured bacterium
JN563780 endosymbiont of O. armadillo (assembly of 8 reads)
AY953252 Prevotella sp.
JN563747 endosymbiont of O. salicicola (assembly of 17 reads)
JN563777 endosymbiont of O. armadillo (assembly of 9 reads)
JN563745 endosymbiont of O. salicicola (assembly of 23 reads)
FJ393126 uncultured Bacteroides sp.
EU828421 uncultured bacterium
EU828414 uncultured bacterium
EU136692 Bacteroides helcogenes
AB200227 Bacteroides helcogenes
AY570514 Porphyromonas uenonis
L16490 Porphyromonas asaccharolytica
JN563781 endosymbiont of O. armadillo (assembly of 8 reads)
JN563739 endosymbiont of O. salicicola (assembly of 69 reads)
CU927677 uncultured bacterium
CU926248 uncultured bacterium
CU925567 uncultured bacterium
JN563742 endosymbiont of O. salicicola (assembly of 34 reads)
AY923125 Streptococcus sp.
JN563782 endosymbiont of O. armadillo (assembly of 8 reads)
AY351787 uncultured marine bacterium
AY553296 Pontibacillus chungwhensis
EU583728 Pontibacillus halophilus
AY603977 Pontibacillus marinus
AJ010964 Acetonema longum
AB487915 uncultured bacterium
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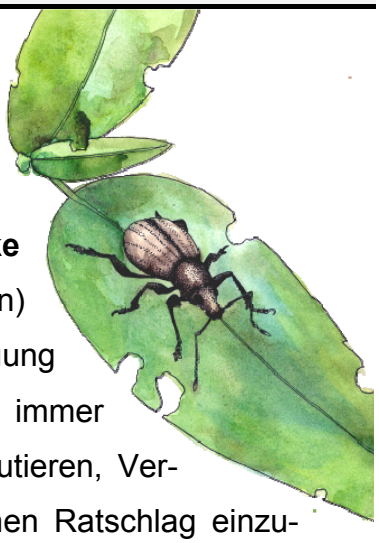
1.00



12 Danksagung

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(*Otiorhynchus sulcatus*) in Gartenbaukulturen unter besonderer Berücksichtigung des Einsatzes und dauerhaften Etablierung entomopathogener Pilze im Bestand“, welches Teil des BLE (Bundesanstalt für Landwirtschaft und Ernährung)-Verbundvorhabens „Erarbeitung von integrierten Pflanzenschutzverfahren gegen Bodenschädlinge“ (Förderkennzeichen: 06HS018/4) war. An dieser Stelle sei auch **allen Verbundpartnern** sowie allen **Mitgliedern des Curculio Institute** (insbesondere **Dr. Peter Sprick** und **Dr. Peter Stüben**) gedankt, die mich im Laufe der Promotion unterstützt haben.

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