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ASSESSING WINTER SURVIVAL OF THE APHID PATHOGENIC FUNGUS *PANDORA NEOAPHIDIS* AND IMPLICATIONS FOR CONSERVATION BIOLOGICAL CONTROL

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In all things of nature there is something of the marvelous.

Aristotle (384 BC - 322 BC)

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Summary

Aphids are major agricultural pests worldwide with significant economic impact on agriculture, forestry, and horticulture. They damage plants by direct feeding on phloem, transmitting plant viruses, and depositing honeydew, which can affect crop quality. Proliferation and great dispersal power are two features that make these insects such redoubtable crop pests. Chemical insecticides have been extensively used against aphids, however, due to major concerns about environmental sustainability and public health there has been an increasing interest for environmentally friendly alternatives such as biological control.

Pandora neoaphidis is one of the most important fungal pathogen of aphids and has a great potential in biological control of aphids. Augmentation biological control approaches have been investigated in various studies, but they have shown limited effectiveness. Recently, conservation biological control (CBC), where the environment is modified to enhance specific natural enemies, has been favoured as an alternative approach for use of *P. neoaphidis* in biological control. Implementation of such a strategy requires a profound knowledge on all aspects of the ecology of the natural enemy. However, for *P. neoaphidis* many of the involved aspects are still not known including for instance its population structure and its overwinter and migration strategies.

In this thesis, new powerful tools were developed that allow accurate and rapid monitoring of *P. neoaphidis* in the environment. They enable the specific detection of *P. neoaphidis* in diverse environmental samples types by using end-point PCR; as well as the quantification of genetic material in soil using a combination of

quantitative PCR and bioassay approaches. Moreover, a SNP genotyping assay was developed, which allows for discrimination of *P. neoaphidis* strains and their cultivation-independent monitoring in the environment. The monitoring tools were applied in field experiments to investigate key aspects of the *P. neoaphidis* ecology and relevant insights were gained, especially into the winter persistence of the fungus in the environment as well as its dispersal and transmission. Results have indicated that *P. neoaphidis* may overwinter in the soil of semi-natural nettle patch habitats, however, not in field plots cultivated with lucerne plants. Moreover, dispersal and transmission capacity of *P. neoaphidis* was demonstrated, as the fungus was able to re-colonize field plots in spring after having disappeared from these plots during winter. Finally, the importance of the time of occurrence of *P. neoaphidis* and of its aphid hosts as well as the amount of *P. neoaphidis* inoculum present was shown, as the fungus could control aphids in field plots in which it was introduced early enough but not in plots in which the natural infection occurred later. The results obtained in this thesis provide a significant step forward in the understanding of the ecology of *P. neoaphidis* and the monitoring tools developed will be helpful to further investigate remaining questions.

Résumé

Les pucerons sont des parasites majeurs des végétaux dans le monde, avec des conséquences économiques négatives sur l'agriculture, les forêts et l'horticulture. Ils causent des dommages aux plantes par la ponction directe de la sève, la transmission de virus, et le dépôt de miellat pouvant affecter la qualité des récoltes. Leur capacité de prolifération et de dispersion sont les deux caractéristiques qui les rendent si redoutables pour les récoltes. Des insecticides chimiques ont été utilisés abondamment contre les pucerons, cependant à cause de leurs impacts sur l'environnement et sur la santé humaine, un intérêt croissant se manifeste actuellement pour des alternatives basées sur le contrôle biologique de ces parasites.

Pandora neoaphidis est un des plus importants champignons pathogènes s'attaquant aux pucerons et présente de ce fait un grand potentiel dans la lutte biologique contre ces derniers. Des approches utilisant la lutte biologique par augmentation ont été testées dans de nombreuses études, cependant avec une efficacité très limitée à ce jour. Récemment, des approches alternatives de lutte biologique par conservation ont été préférentiellement considérées pour l'usage de *P. neoaphidis* en vue d'un contrôle efficace des pucerons. Ces approches sont basées sur la stratégie de modification de l'environnement afin de favoriser spécifiquement certains ennemis naturels. La mise en place d'une telle stratégie requiert une connaissance approfondie de tous les aspects de l'écologie des ennemis naturels. Cependant, pour *P. neoaphidis*, plusieurs des paramètres qui rentrent en ligne de compte ne sont pas encore connus, comme par exemple, la structure des populations, les stratégies de survie hivernale et les stratégies migration.

Dans la présente thèse, des nouvelles et puissantes méthodes de monitoring qui permettent un suivi rapide et précis de *P. neoaphidis* dans l'environnement ont été développées. Ces méthodes permettent la détection spécifique de *P. neoaphidis* dans divers types d'échantillons environnementaux en utilisant une technique de 'end-point PCR', ainsi que la quantification de matériel génétique se trouvant dans les sols en utilisant une combinaison de PCR quantitative et de bioessais. De plus, une méthode de genotyping basée sur l'analyse de SNPs a été développée, permettant la discrimination des différentes lignées de *P. neoaphidis* et leur suivi directement à partir d'échantillons environnementaux, sans devoir recourir à des étapes de culture. Ces méthodes ont été appliquées à des expériences en champ afin d'investiguer des paramètres clés de l'écologie de *P. neoaphidis.* Des données nouvelles ont été obtenues, spécialement en ce qui concerne la persistance hivernale du champignon dans l'environnement, ainsi que sa dispersion et transmission. Les résultats obtenus ont montré que *P. neoaphidis* pouvait survivre l'hiver dans le sol d'un habitat semi-naturel tel qu'un groupe d'orties, mais pas dans le sol de plots en champ contenant de la luzerne. De plus, après la disparition du champignon durant l'hiver, la recolonisation des plots au printemps a été démontrée. Finalement, l'importance du moment d'apparition de *P. neoaphidis* en relation avec la quantité d'inoculum du champignon et sa capacité à infecter les pucerons a été démontrée. Introduit suffisamment tôt, *P. neoaphidis* empêche le développement des pucerons, mais pas si l'introduction est tardive. Les résultats obtenus constituent une avancée significative dans la compréhension de l'écologie de *P. neoaphidis*. Les méthodes de monitoring qui ont été développées dans cette thèse représentent des outils performants pour de futures études.

Chapter 1:

General Introduction

1. General Introduction

1.1. Aphids pest in the agriculture

Aphids (family Aphididae, order Hemiptera) are major agricultural pests in the world, causing substantial crop damage in the order of hundreds of millions of dollars in production losses each year (Oerke, 1994). From the approximately 4700 aphid species known worldwide (Remaudière and Remaudière, 1997), about 100 are considered as crop pests with a significant economic impact on agriculture, forestry, and horticulture (Blackman and Eastop, 2007). Most of these species are found in temperate regions (Blackman and Eastop, 2007). Aphids feed on plant sap by directly inserting their maxillary stylets into the phloem. They damage plants by direct feeding (Quisenberry and Ni, 2007), transmitting plant viruses (Katis et al., 2007), and depositing honeydew, which can affect crop quality (Katis et al., 2007). Proliferation and great dispersal power are two features that make aphids such redoubtable crop pests (Williams and Dixon, 2007). It has been suggested that temperature increase due to global climate change might have a great impact on aphid population dynamics (Bale et al., 2002). Different models are suggesting that high winter temperatures in Europe increase aphid survival and allow early aphid population build-up, early flights, and early colonization of crops (Bale et al., 2002; Harrington et al., 2007). Therefore the importance of aphids as crop pest may become even more significant in the future.

Chemical insecticides have been extensively used for aphid control (Nielsen, 2002). However, major concerns are associated with the use of pesticides including

environmental pollution, adverse effects on non-target organisms such as beneficial organisms and humans, as well as development of resistance to insecticides (Foster et al., 2007). This has led to an increasing interest and need for alternatives that may be more environmentally friendly such as biological control strategies (Hajek, 1997).

1.1.1. Aphid life cycles

Life cycles of aphids are diverse and complex (Williams and Dixon, 2007). Typically, during aphid life cycles different morphs occur, each with specific functions such as sexual reproduction, multiplication, dispersal, and survival. Aphids propagate either anholocyclic, which is a parthenogenetic reproduction without a sexual generation or holocyclic, where parthenogenetic reproduction is interrupted with egg producing sexual generations.

Some holocyclic aphid species are heteroecious, which means they alternate between a winter host (primary host), usually a woody plant on which fertilised eggs are deposited and hibernate before hatching in spring, and a summer host (secondary host), which is usually a herbaceous plant. Other holocyclic species are monoecious, remaining either on the same plant species or migrate between closely related species throughout the year (Nielsen, 2002; Williams and Dixon, 2007). Pest aphids include species that are monoecious holocyclic, e.g. *Sitobion avenae*; dioecious holocyclic, e.g. *Rhopalosiphum padi*; or anholocyclic, e.g. *Elatobium abietinum* (Nielsen, 2002). Furthermore, some aphid species can have variable life cycles, depending on the environmental conditions (Williams and Dixon, 2007). For example, in temperate climates, life cycle of the aphid *Myzus persicae* (Sulzer) can switch from holocyclic to anholocyclic during mild winters (Blackman, 1971).

1.2. Biological control of aphids

Biological control has been described by Eilenberg et al. (2001) as: '*The use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging that it would otherwise be*'. The natural enemies (antagonists) that are used to control invertebrate pests are diverse and include the functional groups of predators, parasitoids, and pathogens (Hajek, 2004). Antagonists that are used for biological control are found in a broad range of taxonomic groups ranging from fish to insects, mites, nematodes, and microorganisms, including bacteria, viruses, fungi, and protozoa (Hajek, 2004).

According to Van Driesche and Bellows (1996), there are three main strategies for biological pest control: (1) 'Classical' biological control, involving the long-term introduction of antagonists in new geographic areas usually to control introduced pest species; (2) 'Augmentation', relying on mass-rearing and release of antagonists that exist in the system but not in sufficient amount to control the target pest. Augmentation can be achieved through 'inoculative' release, in which introduced antagonists are expected to multiply, or 'inundative' release, in which introduced antagonists are not expected to multiply; and (3) 'Conservation biological control' (CBC), aiming to enhance naturally occurring antagonists by means of habitat management, e.g. establishment of field margins, reduced pesticide application, reduced ploughing, or irrigation.

1.2.1. Aphid antagonists

Commercial use of aphid antagonists is widespread in greenhouse crop production but remains a challenge for field applications (Powell and Pell, 2007). Until recently, costs associated with mass-rearing and release of aphid antagonists have been a

drawback for implementing augmentation biological control strategies in the field. However, the situation is changing due to technological advances, and to a growing number of aphid antagonist species are that commercially available for use in greenhouse and field crop protection. There are three groups of aphid antagonists that are used as biological control agents: 1) predators, such as ladybirds (Coccinellidae), midges (Itonididae), hover flies (Syrphidae), lace wings (Chrysopoidae), and various predatory bugs; 2) hymenopteran parasitoids, such as various braconid and aphelinid parasitoids; and 3) various fungal pathogens (for review, see Powell and Pell, 2007). So far, most success has been achieved with greenhouse crops with several hymenopteran parasitoids and with the predatory midge *Aphidoletes aphidimyza* especially (Powell and Pell, 2007).

1.2.2. Fungal pathogens

Aphids have a reduced risk for ingesting insect pathogens such as viruses, bacteria, and protozoa that are present on plant surfaces, as they exclusively feed by directly inserting their maxillary stylets into the plant phloem. Fungi, however can infect aphids directly through their integument and are therefore the most important microbial pathogens of aphids (Steinkraus, 2006). Aphid pathogenic fungal species belong either to the order Hypocreales (Ascomycota) such as the genera *Beauveria, Metharizium, Paecilomyces*, and *Lecanicillium* or to the order Entomophthorales (Entomophthoromycotina) (Powell and Pell, 2007). Hypocrealean species have been mass-produced, formulated, and applied against aphids (Wraight et al., 2001; Jackson et al., 2003; Powell and Pell, 2007). Species such as *Beauveria bassiana*, *Paecilomyces fumosoroseus*, and *Lecanicillium spp*. are commercially available for use in innondative augmentation strategies against aphids (Völkl et al., 2007). In

contrast, it is difficult and in some cases impossible to mass-produce entomophthoralean fungi (Powell and Pell, 2007). Moreover, issues regarding their formulation and application remain largely unresolved (Pell et al., 2001). Nevertheless, Entomophthorales have some important advantages over Hypocreales, which make them great candidates for use in biological control of aphids. Unlike most Hypocreales, Entomophthorales have a very narrow host range, are highly infectious, and tend to cause epizootics that can erase aphid populations (Evans, 1989; Keller, 1998; Pell et al., 2001). More than 20 species of Entomophthorales are known to infect aphids (Keller, 1987, 1991) and four of them, i.e. *Pandora neoaphidis, Conidiobolus obscurus, Entomophthora planchoniana* and *Neozygites fresenii*, dominate among aphids of agricultural importance in Switzerland (Keller and Suter, 1980).

Pandora neoaphidis is considered as one of the most important fungal pathogens of aphids (Ekesi et al., 2005; Steinkraus, 2006). This fungus has a worldwide distribution (Pell et al., 2001), is aphid specific (Pell et al., 2001; Völkl et al., 2007), infects more than 70 aphid species (Pell et al., 2001), and can cause epizootics that dramatically reduce host aphid populations in natural ecosystems as well as in field crops (Keller and Suter, 1980; Feng et al., 1991; Steenberg and Eilenberg, 1995).

1.3. *Pandora neoaphidis*

1.3.1. Life cycle of *P. neoaphidis*

The life cycle of *P. neoaphidis* corresponds to the generalized life cycle of Entomophthorales (Figure 1.1), except that resting spores (zygospores or azygospores) produced by various entomophthoralean species to survive hostile

conditions have never been documented for this species. Infective propagules, the conidia, are actively discharged from the conidiophores by hydrostatic pressure (Papierok and Hajek, 1997; Völkl et al., 2007). Discharged airborne conidia (called primary conidia; Figure 1.2) that land on a suitable host surface germinate and their germ-tubes penetrate through the host cuticule. Upon entering the aphid, the fungus multiplies in form of hyphal bodies and colonizes the entire insect body (Keller, 2007; Völkl et al., 2007).

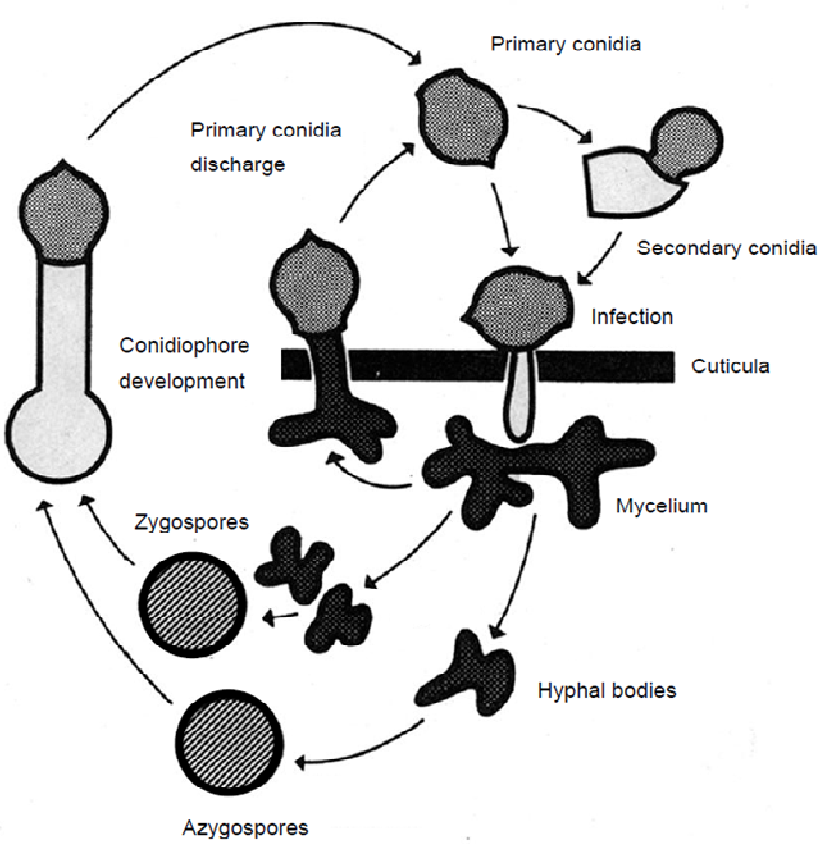

Figure 1.1. Generalized life cycle of the Entomophthorales (modified from Eilenberg, 1987).

Alternatively, if primary conidia land on unsuitable surfaces they germinate and differentiate into secondary conidia, which are actively discharged and are as infective as primary conidia (Glare and Milner, 1991; Morgan et al., 1992). Infected

host aphids die once the fungus has spread throughout the insect and consumed the nutrients available (Shah and Pell, 2003).

At room temperature, death usually occurs approximately 5 days after infection. Cadavers are fixed on plant surfaces by fungal rhizoids emerging on the ventral surface, ensuring that the fungus remains in position for optimal discharge of the conidia (Keller, 2007; Völkl et al., 2007). In suitable conditions, such as high humidity, the conidiophores develop on dead host surfaces from hyphal bodies penetrating and elongating through the cuticule (Papierok and Hajek, 1997) and form a new generation of conidia. Thousands of conidia can be produced and discharged from a single infected aphid cadaver (Hemmati et al., 2001a).

Figure 1.2. Primary conidida of *Pandora neoaphidis* (left); sporulating *Pandora neoaphidis* infected *Acyrthosiphon pisum* cadaver (center); epizootics caused by *Pandora neoaphidis* in a population of *Acyrthosiphon pisum* on *Vicia faba. Left picture,* by A. Fournier. Center and right picture by S. Keller.

1.3.2. Genotyping of *P. neoaphidis*

Identification of species within the entomophthoralean genera has been based on the knowledge of the host insect species and the fungal structures (Humber, 1989; Keller, 1991; Keller, 2007).The most important fungal structures in respect to the identification are: hyphal bodies, conidiophores, primary and secondary conidia,

resting spores, and the number of nuclei (Keller 2007). However, characterization methods based on morphology and pathobiology have low resolution and do not allow differentiation at strain levels (Nielsen et al., 2001). Molecular techniques have provided powerful tools to investigate inter- and intraspecific fungal variations. These variations have been assessed in the entomophthoralean genera *Pandora*, *Zoophthora*, *Entomophaga* and *Entomophthora* using genotyping methods such as ribosomal internal transcribed spacer (ITS; Rohel et al., 1997; Nielsen et al., 2001; Francis et al., 2004; Tymon et al., 2004), randomly amplified polymorphic DNA (RAPD; Hodge et al., 1995; Hajek et al., 1996; Rohel et al., 1997; Sierotzki et al., 2000; Nielsen et al., 2001; Tymon and Pell, 2005), enterobacterial repetitive intergenic consensus (ERIC; Tymon and Pell, 2005), and inter-simple-sequence repeat (ISSR; Tymon and Pell, 2005) analyses.

Different genotyping approaches have been used to investigate variations in the ITS of *P. neoaphidis*, including size, restriction, and sequence analyses (Rohel et al., 1997; Nielsen et al., 2001; Tymon et al., 2004). However, *P. neoaphidis* could not be consistently discriminated using ITS analysis, as the level of intraspecific variation was to low. On the other hand, approaches such as RAPD, ERIC and ISSR analyses that are based on analysis of anonymous loci, which do not provide sequence information of the polymorphic site (Enkerli et al., 2007), have allowed for detection of intraspecific variations among *P. neoaphidis* isolates. For instance, Tymon and Pell (2005) have been able to distinguish all 30 *P. neoaphidis* isolates of a worldwide collection using the combination of RAPD, ERIC and ISSR approaches. Moreover, the RAPD approach has allowed to distinguish between all *P. neoaphidis* isolates analyzed in the different studies except those isolated from the same epizootics (Rohel et al., 1997; Sierotzki et al., 2000; Nielsen et al., 2001; Tymon and Pell, 2005).

As the primers used in analyses of anonymous loci are not species-specific, isolation and cultivation of *P. neoaphidis* is required prior analyses with such methods. Hence, until now, there was no cultivation-independent genotyping methods available allowing discrimination of *P. neoaphidis* strains directly in environmental samples like aphids or soil.

1.3.3. Overwintering of *P. neoaphidis*

Most aphid-pathogenic entomophthoralean fungi produce resting spores (zygospores or azygospores) to survive hostile conditions such as winter when larval or adult aphids are not present or only at low densities (Humber, 1989; Hajek and Wheeler, 1994). These spores are resistant to abiotic stress due to their thick double walls. Dead aphids that contain resting spores may remain attached on leaves or bark or may fall on the soil. In the soil, spores can remain dormant for a long period of time before germinating and infecting new hosts. However, resting spores have never been documented for *P. neoaphidis* and very little is known about the winter survival mechanisms of this obligate aphid pathogen. In cold temperate areas like Switzerland, where most aphid species are holocyclic (Keller and Suter, 1980; Blackman and Eastop, 2007), *P. neoaphidis* may have to survive winter in absence of larval or adult aphid hosts. The fungus may survive as specialized hyphal bodies within infected aphid cadavers attached on plant substrates (Feng et al., 1992) or as dormant loricoconidia in top soil (Nielsen et al., 2003). Alternatively, it has been suggested that *P. neoaphidis* does not survive winter in cold areas but repopulates such areas in spring by co-migrating with alate aphid flights that originate from other climatic zones where aphids are always present (Feng et al., 2004; Feng et al., 2007).

1.3.4. *P. neoaphidis* in augmentation biological control strategies

Several attempts have been made to use *P. neoaphidis* for inoculation biological control of aphids (Wilding, 1981; Latgé et al., 1983; Latteur and Godefroid, 1983; Wilding et al., 1986; Sylvie et al., 1990; Wilding et al., 1990; Shah et al., 2000). Trials have been performed to control several aphid species in glasshouse and field conditions. Most of these trials have resulted in inconsistent data that did not allow to draw general conclusions. The experiments have been conducted under highly variable conditions and the experimental setups did not allow to distinguish applied strains from naturally occurring ones (Shah et al., 2000; Nielsen, 2002).

In these experiments, *P. neoaphidis* has been applied by dispersing sporulating cadavers (Wilding et al., 1986; Wilding et al., 1990) or living infected aphids (Wilding, 1981; Wilding et al., 1986) as well as by spraying *in vitro* produced mycelium preparations (Latgé et al., 1983; Latteur and Godefroid, 1983; Sylvie et al., 1990) and hyphal bodies encapsulated into sodium alginate beads (Shah et al., 2000). Although the preparations were generally effective as they produced conidia and infect aphids, however, aphid population suppression has never been achieved. It is suggested that factors such as environmental conditions and aphid density has limited the efficacy of these approaches (Milner, 1997). Moreover, difficulties of mass producing entomophthoralean fungi, problems of storage, and the need for large amounts of mycelium make this strategies unlikely to be cost efficient unless there are some technological breakthroughs (Milner, 1997).

1.3.5. *P. neoaphidis* in conservation biological control (CBC) strategies

During the last decade, there has been an increasing interest for using *P. neoaphidis* in conservation biological control (CBC) approaches as alternative to augmentation

approaches (Pell et al., 2001; Ekesi et al., 2005; Powell and Pell, 2007). CBC aims at increasing the occurrence and impact of naturally occurring antagonist populations on pests by modifying the environment and existing practices (Eilenberg et al., 2001). A major problem connected with natural epizootics of *P. neoaphidis* is that in some years infections occur early enough to control pest populations, whereas in other years they are too late and weak to reduce the host populations below the damage threshold (Keller, 1998; Pell et al., 2001). Although the mechanisms behind host-pathogen relations that result in epizootics are largely unknown (Meyling and Hajek, 2010). It has been suggested that the regularity and the intensity of natural *P. neoaphidis* epizootics could be improved through appropriate CBC strategies resulting in an efficient control of aphids in the crop. Pell et al. (2001) have suggested several CBC management practices that could have a beneficial impact on entomopthoralean fungi, including irrigation (increased moisture), less spraying of pesticides, and provision of overwintering sites for alternative hosts. An example of a successful implementation of such management practices is the use of irrigation in spinach plants in the USA, which significantly increased the impact of naturally occurring *P. neoaphidis* populations on the pest aphid *M. persicae* (McLeod and Steinkraus, 1999).

Several characteristics of *P. neoaphidis* make it a perfect candidate for implementation in CBC strategies: 1) it is aphid-specific (Keller, 1991); 2) it causes epizootics able to suppress entire aphid populations (Keller and Suter, 1980; Feng *et al.*, 1991; Steinkraus, 2006); 3) it infects numerous aphid species, which occur in field crops and non-cropped areas (Shah et al., 2004; Ekesi et al., 2005); 4) it can survive in the environment in the absence of larval or adult host stages (Nielsen et al., 2003; Baverstock et al., 2008); and 5) it interacts with various predatory or non-predatory

insect species, which enhance its transmission (Ekesi et al., 2005; Baverstock et al., 2008). In particular, there is a growing interest for using *P. neoaphidis* in CBC strategies that are based on management of natural or semi-natural areas adjacent to crop fields (Keller and Suter, 1980; Barta and Cagan, 2003; Shah et al., 2004; Ekesi et al., 2005; Baverstock et al., 2008). The following 'migration model' has been suggested (Figure 1.3): natural and semi-natural areas, including field margins, natural meadows, and nettle patches provide refuges for *P. neoaphidis* to survive advert conditions such as winter when no hosts are present in the crops.

Figure 1.3. Functions of the semi-natural and natural areas in the 'migration model'. These areas may serve for the winter survival, the population build up, and the dispersal of *P. neoaphidis* into the agro-system.

The idea is that such landscape elements contain alternative aphid hosts or economically important aphids on primary host plants in which the fungus could remain active and/or they provide an environment, which allows fungal structures to survive outside of the host. This may include e.g. appropriate humididity and UV light conditions, no tiling, and no pesticide applications. After surviving the winter in such

refuge areas, *P. neoaphidis* rapidly becomes active in spring and establishes itself in the local aphid populations in natural areas at the start of the population build up. From about mid-May to mid-June the aphid species of economic importance colonize specific crops and build up populations. At this time point, *P. neoaphidis* disperses and rapidly establishes itself into the adjacent crop fields along with the aphids migrating form the natural and semi-natural areas (from the primary to the secondary host plants). Due to its early and strong presence in the crops, the fungus can control the economically important aphid populations below damage threshold. To demonstrate the validity of this model as well as to integrate it into practical CBC management applications, various aspects of *P. neoaphidis* ecology remain to be elucidated, including the genetic diversity of this fungus, the diversity comparison between populations in natural or semi-natural areas and to those in arable crops, the determination of genotypes responsible for the epizootics among economically important aphids, the possible overwintering sites, and the ways of dissemination. A critical factor for attempting to elucidate such complex aspects of *P. neoaphidis* ecology is the availability of appropriate monitoring tools.

1.3.6. Monitoring of *P. neoaphidis* in the environment

Traditionally, monitoring of entomophthoralean fungi has relied on cultivation, followed by morphological (e.g. Keller, 1987, 1991), biochemical (e.g. Glare et al., 1989; Wilding et al., 1993), or molecular (Rohel et al., 1997; Nielsen et al., 2001) analyses. However, the cultivation steps that are required make these approaches laborious and time-consuming.

So far, two approaches have been used for monitoring *P. neoaphidis* in the environment without prior isolation of the fungus. The first approach is a bioassay. It

consists of placing aphids on top of collected soil samples for a certain period of time and by monitoring the resulting infections (Latteur, 1980; Nielsen et al., 2003; Baverstock *et al.*, 2008). Although bioassays provide direct information on the viability and virulence of the detected fungal material, they are labour intensive and may lack robustness, as the factors involved in the infection process are not yet fully understood (Nielsen et al., 2003; Baverstock et al., 2008; Nielsen et al., 2008). The second approach relies on the cultivation-independent detection of *P. neoaphidis* in infected aphid cadavers. This approach uses conventional end-point PCR with primers that target the ribosomal internal transcribed spacer (ITS) and are specificity to *P. neoaphidis* (Tymon et al., 2004). This method, however, does not allow to discriminate strains of *P. neoaphidis.*

A better understanding of the life cycle and ecology of *P. neoaphidis* is a prerequisite for the implementation of efficient CBC strategies using this fungus. This requires the implementation of new powerful monitoring tools that allow for: 1) rapid cultivation-independent detection of the fungus in the environment, 2) quantification of its inoculum or genetic material present in the soil, and 3) genotyping the fungus at strain level. Such tools are essential for monitoring individual strains in the environment and for assessing the population structure of the fungus. Therefore, specific strains could be assessed for their potential as biological control agents by investigating e.g. their virulence, their persistence, or their specificity to host aphid species. In particular, strains of *P. neoaphidis* present in natural and semi-natural areas could be identified and their potential dispersal to adjacent crop could be monitored to validate the ‘migration model’. In addition, the host specificity of different strains could be investigated.

1.4. Objectives and outline of the thesis

1.4.1. Objectives

The first specific objective of this thesis was to develop methods that would allow accurate and rapid monitoring of *P. neoaphidis* inoculum in the environment. As *P. neoaphidis* is difficult to isolate and to cultivate, focus was set on developing bioassays and PCR-based methods that obviate the need of time-consuming cultivation steps and allow cultivation-independent detection, quantification, and genotyping of *P. neoaphidis* inoculum in various types of environments.

The second objective of this thesis was to apply the developed monitoring tools in field experiments to investigate key aspects of the 'migration model' of *P. neoaphidis* such as the role of natural and semi-natural areas as refuge for *P. neoaphidis* during winter, and the role of soil as winter survival matrix of the fungus when no hosts are present. The aim was to gain insights into the ecology of *P. neoaphidis*, particularly in the topics of persistence, dispersal, and transmission of this fungus, which could contribute to the efficient implementation of CBC strategies.

1.4.2. Outline

Chapter 1 is a general introduction which describes the damages caused by aphid pests on crops and the challenges involved in their management. The concept of biological control is introduced and the strategies that are applicable against aphid pests are presented. Focus is put on *P. neoaphidis*, one of the most important pathogens of aphids and on its potential for use in CBC strategies. Finally, the different monitoring approaches used to detect *P. neoaphidis* are introduced.

Chapter 2 describes the development of a cultivation-independent PCR-based detection tool relying on two primer pairs specific to *P. neoaphidis* that target sequences in the ribosomal RNA gene cluster. The specificity of both primer pairs was demonstrated using strains of *P. neoaphidis* and various close entomophthoralean relatives. Single amplicons of expected sizes were obtained with both primer pairs from various environmental sample types, including aphid cadavers, plant material, and soil. The detection tool was applied to investigate the persistence of *P. neoaphidis* in soil samples of a nettle patch during winter.

Chapter 3 describes the development of a genotyping assay targeting single nucleotide polymorphisms (SNPs) distributed among different genomic regions of *P. neoaphidis* that allow high-resolution monitoring of target strains in the environment. The assay was applied to DNA extracts obtained from *P. neoaphidis* infected aphids to investigate its applicability for the cultivation-independent detection of *P. neoaphidis* in aphids collected from the environment.

Chapter 4 presents a field experiment, which aimed to investigate the winter survival of *P. neoaphidis* in topsoil layers of lucerne field plots. *P. neoaphidis* was released to induce epizootics in the aphid populations present in the plots. Abundance and activity of *P. neoaphidis* material in topsoil layers of the plots were monitored using bioassay approaches and the developed quantitative PCR method. The fate of the release strain as well as from the other strains present in the plots was monitored using the SNP genotyping assay developed in Chapter 3. The genotyping assay was directly applied to DNA extracted from infected aphid cadavers.

Chapter 5 provides a thorough discussion of the data obtained and includes an assessment of the applicability of the methods developed in this thesis. The contribution of this thesis to an improved aphid management is discussed in detail

and the perspectives on future applications of the methods developed and evaluated in the frame of this thesis are outlined.

Chapter 2:

A PCR-based tool for the cultivation-independent monitoring of *Pandora neoaphidis*

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2. A PCR-based tool for the cultivation-independent monitoring of *Pandora neoaphidis*

2.1. Abstract

Pandora neoaphidis is one of the most important fungal pathogens of aphids and has a great potential for use in biocontrol. Little is known on how this fungus persists in an area and in particular on its overwintering strategies. It is hypothesized that natural areas play an important role for survival and that soil may serve as a source of inoculum for new aphid populations in spring. To test these hypotheses, a cultivation-independent PCR-based diagnostic tool was developed, that allows the detection of *P. neoaphidis* in the environment. Two *P. neoaphidis* specific PCR primer pairs were designed, targeting sequences in the ribosomal RNA gene cluster. Specificity of both primer pairs was demonstrated with *P. neoaphidis* and non-target close entomophthoralean relatives. Moreover, single amplicons of expected sizes were obtained with both primer pairs from various environmental sample types, including aphid cadavers, plant material, and soil. The PCR-based diagnostic tool was applied to investigate the persistence of *P. neoaphidis* in soil samples obtained in 2004/2005 from a nettle field harbouring infected aphids in fall 2004. *P. neoaphidis* was detected in every sample collected in November 2004 and March 2005, suggesting an overwintering stage of *P. neoaphidis* in top soil layers. The developed cultivation-independent PCR-based tool will be valuable for further investigation of the ecology of *P. neoaphidis* and for the development and future implementation of management strategies against aphids involving conservation biocontrol.

2.2. Introduction

Pandora neoaphidis (Remaudière and Hennebert; Zygomycota, Entomophthorales) is one of the most important fungal pathogens of aphids (Hemiptera: Aphidoidea) in temperate areas (Pell et al., 2001; Steinkraus, 2006; Baverstock et al., 2008). This aphid-specific fungal pathogen has been reported to cause natural epizootics, which can dramatically reduce host populations, and thereby reduce or prevent serious damage to crops (e.g. Keller and Suter, 1980; Feng et al., 1991; Steenberg and Eilenberg, 1995). Therefore, *P. neoaphidis* has a great potential for use in biological control of aphids. Two approaches, i.e. inundation and inoculation biocontrol, have been investigated in various studies, but have shown limited effectiveness (Powell and Pell, 2007). Conservation biocontrol, which is defined as "*modification of the environment or existing practices to protect and enhance specific natural enemies or other organisms to reduce the effect of pests*" (Eilenberg et al., 2001), is a promising third approach for the control of aphids with *P. neoaphidis*. However, for implementation of such an approach, profound knowledge on life cycle and ecology of the biocontrol organism is a prerequisite.

Natural and semi-natural landscape elements such as field margins, nettle patches, and natural meadows have been suggested to serve as reservoir for aphid-pathogenic entomophthoralean fungi (Keller and Suter, 1980; Barta and Cagan, 2003; Shah et al., 2004; Ekesi et al., 2005; Baverstock et al., 2008). It is hypothesized that after overwintering, new infection cycles are initiated in natural and semi-natural areas, from where fungal inoculum may disseminate to adjacent agricultural land and subsequently infect and control aphid populations developing in crops.

However, knowledge on the winter survival strategies of entomophthoralean fungi and the following initiation of infection in spring is very limited. As most aphid species are holocyclic in cold temperate areas, like Switzerland (Keller and Suter, 1980; Blackman and Eastop, 2007), obligate aphid-pathogenic fungi such as *P. neoaphidis* may have to survive during winter in absence of larval or adult host stages, and/or to co-migrate with migratory alate aphids originating from other climatic zones (Feng et al., 2004; Feng et al., 2007). Some aphid-pathogenic entomophthoralean fungi have been reported to produce resting spores (zygospores or azygospores) in order to survive hostile conditions such as winter (Humber, 1989; Hajek and Wheeler, 1994). However, except for loricoconidia (Nielsen et al., 2003), which are thick-walled conidia no such survival structures have been reported for *P. neoaphidis*. Furthermore, the environment in which *P. neoaphidis* may overwinter is still not well defined. The fungus has been reported to survive on plant material (Feng et al., 1992), as well as in top soil layers (Nielsen et al., 2003; Baverstock et al., 2008).

Recent advances in molecular techniques have enabled the use of cultivation-independent approaches that no longer require isolation of fungi from the environment prior to analysis (Anderson and Cairney, 2004). These techniques rely on extraction of total DNA from environmental samples, and on subsequent use of PCR-based detection methods. Various PCR primers, most of them targeting sequences in the ribosomal RNA gene cluster, have been developed that allow for amplification of wide taxonomic ranges of fungi, or of more specific fungal groups at a genus, species, or even subspecies level. Such cultivation-independent PCR-based techniques have been applied for analysing structures and/or dynamics of fungal communities in different ecosystems (e.g. Schwarzenbach et al., 2007a), as well as for detecting specific fungal groups belonging for instance to mycorrhizal fungi (e.g.

Filion et al., 2003), phytopathogenic fungi (e.g. Cullen et al., 2005), nematophagous fungi (e.g. Atkins et al., 2005), or entomopathogenic fungi (e.g. Schwarzenbach et al., 2007b). For Entomophthorales, such cultivation-independant PCR-based approaches have been applied for detection of the fungi in insect cadavers, using primers with specificity to the order Entomophthorales (Jensen and Eilenberg, 2001), to the genus *Entomophthora* (Thomsen and Jensen, 2002), or to *Pandora kondoiensis* and *P. neoaphidis* (Tymon et al., 2004). Recently, Castrillo et al. (2007) reported the development of a PCR assay for specific detection and quantification of *Entomophaga maimaiga* resting spores in soil.

The aim of the present study was to develop a cultivation-independent PCR-based diagnostic tool for specific detection of *P. neoaphidis* in various environmental sample types. The new tool was applied to investigate presence of overwintering material of *P. neoaphidis* in top soil samples in a natural nettle plant patch.

2.3. Materials and methods

2.3.1. Field sites and sample collection

Field investigations were performed in a 100 m^2 stinging nettle (*Urtica dioica* L.) patch in the region of Klettgau, Schaffhausen, Switzerland, in the years 2004 and 2005. In June 2004, density of the common nettle aphid (*Microlophium carnosum* Buckton) was visually estimated to 10^4 individuals per square meter in this nettle patch. For assessing infection with *P. neoaphidis*, 30 aphids were collected from each of three nettle plants and incubated for 1 day in three closed plastic boxes containing one nettle leaf. On the average, ten percent of the aphids were infected with *P. neoaphidis*. In November 2004, estimated aphid density was less than five living

aphids per square meter. Prevalence of infection was not determined at that time, however, *P. neoaphidis*-infected aphid cadavers were observed at an estimated density of one per square meter. No aphid populations or *P. neoaphidis*-infected aphid cadavers were observed in the nettle patch between March and August 2005 (investigated once per month).

In November 2004, three individual plants were identified within the nettle patch that bared a *P. neoaphidis*-infected *M. carnosum* cadaver. Plants were labelled 1 to 3 and marked with a ribbon. The three plants were situated about 4.5 m from each other. Four soil samples consisting of 25 cm^2 of top soil layer (top 1 cm) were collected within a radius of 50 cm around each of the three marked nettle plants in November 2004, March 2005, and June 2005.

In addition, top soil samples were collected in May 2005 around the nettle patch: 2 samples, at the border of a forest that was adjacent to the nettle patch; 3 samples, from the forest approximately 30 m from the nettle patch; 9 samples, from surrounding fallow fields within a radius of 40 m around the nettle patch; and 3 samples from a neighbouring wheat field 30 to 100 m from the nettle patch. All samples were stored at -80 °C before processing.

2.3.2. Fungal strains and cultivation techniques

Entomophthoralean strains were either cultivated in the laboratory or extracted genomic DNA was obtained from different sources (Table 2.1). Except for Strongwellsea castrans, the mycelium of all strains was produced as follows: 1) The strains were grown in Petri dishes on Sabouraud-dextrose agar supplemented with egg yolk (SDAEY; Keller, 1991) and maintained in closed plastic boxes at 20 °C, 100 % RH, in the dark. 2) Liquid precultures were initiated by dispensing 25 ml of semi-

synthetic complete medium (CM; Riba and Ravelojoana, 1984) in 100 ml conical flasks and by inoculating the medium with eight 10 mm diameter plugs cut from the growing edges of 2 to 3 weeks old agar plate cultures. Liquid precultures were incubated for 3-4 days, at 20 °C, 180 rpm, in the dark. 3) Seven milliliters of preculture were added to 50 ml fresh liquid CM in 200 ml conical flasks and incubated under the same conditions for 5-7 days. *S. castrans* mycelium was produced in GLEN liquid medium (Beauvais and Latgé, 1988) in Petri dishes at 20 °C, in the dark. Every 2 weeks, the mycelium mats were fragmented and transferred to new dishes (Eilenberg et al., 1992). Mycelium from all liquid cultures was harvested by filtration (Schleicher & Schuell, No. 595), frozen in liquid nitrogen, and stored at -80 °C.

Table 2.1. Entomophthoralean strains used in this study.

Fungal strain[a]	Species	Family	Origin
IESR 04:427[c]	*Conidiobolus coronatus*	Ancylistaceae	UK
KVL 04-94[b]	*Conidiobolus obscurus*	Ancylistaceae	Denmark
ARSEF 387	*Entomophthora culisis*	Entomophthoraceae	Switzerland
KVL 99-11[b]	*Entomophthora muscae*	Entomophthoraceae	Denmark
ARSEF 6252[b]	*planchoniana*	Entomophthoraceae	Denmark
910	*Erynia dipterigena*	Entomophthoraceae	Switzerland
ARSEF 1141	*Erynia rhizospora*	Entomophthoraceae	UK
ARSEF 1339	*Furia ithacensis*	Entomophthoraceae	Poland
ARSEF 1145	*Furia neopyralidarum*	Entomophthoraceae	Japan
301	*Neozygites parvispora*	Neozygitaceae	Switzerland
ARSEF 217	*Pandora blunckii*	Entomophthoraceae	Germany
ARSEF 828[b]	*Pandora kondoiensis*	Entomophthoraceae	Australia
ARSEF 7939	*Pandora neoaphidis*	Entomophthoraceae	Switzerland
ARSEF 1607	*Pandora neoaphidis*	Entomophthoraceae	France
ARSEF 199[b]	*Pandora nouryi*	Entomophthoraceae	USA
ARSEF 3485	*Strongwellsea castrans*	Entomophthoraceae	Denmark
ARSEF 388	*Zoophtora radicans*	Entomopht horaceae	Switzerland

[a] ARSEF: ARS Collection of Entomopathogenic Fungi, Ithaca, USA; KVL: Culture Collection of Fungi at the Royal Veterinary and Agricultural University, Copenhagen, DK; IESR, strain from the Collection of Microorganisms, Institute of Environment and Sustainability Research, Staffordshire University; UK. Strains 301 and 910 have been obtained from the Institute of Microbiology, ETH, Zurich, CH.

[b] DNA was received from A. Jensen and C. Nielsen, Royal Veterinary and Agricultural University, Frederiksberg, DK.

[c] DNA was received from W. Wieloch, Institute of Parasitology, Polish Academy of Sciences, Warsaw, PL.

2.3.3. DNA extraction

Genomic DNA of aphids, fungal cultures, and plant leaf samples was extracted using DNeasy Plant Mini Kit (Qiagen, Basel, Switzerland). Sample types were processed differently prior to extraction: 1) single frozen aphids were directly transferred into 600 µl lysis buffer and disrupted with a spatula; 2) 0.2 g of filtered mycelium was lyophilised and ground with a spatula prior to addition of 600 µl lysis buffer; 3) 0.15 g (fresh weight) plant leaf material was added to 500 mg glass beads of 0.10 mm diameter (B. Braun Biotech International, Melsungen, Germany), suspended in 600 µl lysis buffer, and subjected to mechanical disruption using a bead beater FastPrep FP120 (Savant Instruments, Farmingdale, USA) at 5.5 m s^{-1} (approx. 6800 rpm) for 45 s.

Bulk soil DNA was extracted from 500 mg fresh soil according to Hartmann et al. (2005) using a bead beating procedure. Extracted DNA was suspended in TE (10 mM Tris-HCl, 1mM EDTA, pH 8) at 1 ml TE per gram dry weight equivalent of extracted soil. To remove PCR inhibiting agents, 25 µl of each extract was purified using NucleoSpin Extract II kit (Macherey & Nagel, Düren, Germany) and eluted in 200 µl elution buffer (Macherey & Nagel). DNA content was quantified fluorometrically (Sandaa et al., 1998) using PicoGreen (Molecular Probes, Eugene, USA) and a luminescence spectrometer LS30 (Perkin Elmer; Buckinghamshire, England). Herring sperm DNA (Promega, Madison, USA) was used as DNA concentration standard.

2.3.4. Primer design

Two sequence alignments were generated with entomophthoralean sequences retrieved from GenBank (Table 2.2) using CLUSTAL W (Thompson et al., 1994) and edited manually with the software BioEdit v. 7.0.1 (Hall, 1999). The first alignment consisted of 18 intergenic transcribed spacer (ITS) sequences. The second alignment consisted of 39 small-subunit (SSU) rRNA gene sequences. Potential target regions for PCR primers were manually determined from both alignments.

Specificity of designed primers for *P. neoaphidis* was evaluated in 3 steps: 1) BLAST homology searches in the nonredundant (nr) nucleotide database of GenBank (Altschul et al., 1990); 2) PCR on genomic DNA of fungal strains (Table 2.1); and 3) PCR on DNA from soil, aphid, or plant leaf samples. Soil samples consisted of one top soil layer sample, as well as of one lower soil layer sample (25 cm^2, at 25 cm to 26 cm depth), both collected in the nettle patch in November 2004. Aphid samples consisted of one of the *P. neoaphidis*-infected aphid cadavers identified in the nettle patch in November 2004, and of an uninfected pea aphid (*Acyrthosiphon pisum* Harris) from a laboratory culture. Plant leaf samples consisted of the nettle leaf from which the *P. neoaphidis*-infected cadaver was removed; a nettle leaf without infected aphid, but collected 50 cm apart from a leaf bearing an infected aphid; and a nettle leaf washed with a solution containing 0.1 % SDS and 0.1 % Tween 80, to remove possibly adhering *P. neoaphidis* material.

Table 2.2. List of the 18 ITS sequences and the 39 SSU rRNA gene sequences retrieved from GenBank and used to generate sequence alignments.

Species	Strain	Accession number
ITS sequences		
Basidiobolus ranarum	MCCL W6	AY211271
Conidiobolus coronatus	P1	AJ 345094
Entomophaga aulicae	FPMI 646	U35394
Pandora kondoiensis	ARSEF 828	AF543199
Pandora kondoiensis	ARSEF 5707	AF543200
Pandora kondoiensis	ARSEF 5708	AF543201
Pandora neoaphidis	NW 343	AF543202
Pandora neoaphidis	NW 356	AF543203
Pandora neoaphidis	NW 195	AF543204
Pandora neoaphidis	NW 283	AF543205
Pandora neoaphidis	NW 316	AF543206
Pandora neoaphidis	NW 327	AF543207
Pandora neoaphidis	NW 415	AF543208
Pandora neoaphidis	ARSEF 835	AF543209
Pandora neoaphidis	ARSEF 1609	AF543210
Pandora neoaphidis	ARSEF 5374	AF543211
Zoophthora radicans	NW 250	AF548394
Zoophthora radicans	NW 325	AF548395
SSU rRNA gene sequences		
Basidiobolus haptosporus	ARSEF 263	AF368504
Basidiobolus microsporus	ARSEF 265	AF368505
Basidiobolus ranarum	NRRL 20525	AF113414
Conidiobolus brefeldianus	ARSEF 452	AF368506
Conidiobolus coronatus	unknown	AF296753
Conidiobolus firmipilleus	ARSEF 2731	AF368507
Conidiobolus lamprauges	NRRL 28637	AF113420
Conidiobolus obscurus	ARSEF 3088	AF368508
Conidiobolus osmodes	ARSEF 79	AF368510
Conidiobolus pumilus	ARSEF 453	AF368511
Conidiobolus rhysosporus	ARSEF 448	AF368512
Conidiobolus thromboides	ARSEF 115	AF052401
Entomophaga aulicae	FPMI 646	U35394
Entomophaga conglomerata	ARSEF 2273	AF368509
Entomophtho planchoniana	ARSEF 6251	AF353723
Entomophthora chromaphidis	ARSEF 1860	AF353725
Entomophthora culicis	ARSEF 387	AF368516
Entomophthora muscae	F1020	D29948
Entomophthora schizophora	ARSEF 2541	AF052402
Entomophthora thripidum	ARSEF 6518	AF296755
Erynia conica	ARSEF 1439	AF368513
Erynia rhizospora	ARSEF 1441	AF368514
Erynia sciarae	ARSEF 1870	AF368515
Eryniopsis caroliniana	ARSEF 641	AF368517
Eryniopsis ptycopterae	ARSEF 2671	AF052403
Furia ithacensis	ARSEF 1339	AF351134
Furia neopyralidarum	ARSEF 1145	AF368518
Furia pieris	ARSEF 781	AF368519
Neozygites floridana	315	AF296758
Pandora blunckii	ARSEF 217	AF368520
Pandora delphacis	ARSEF 459	AF368521
Pandora dipterigena	ARSEF 397	AF368522
Pandora kondoiensis	ARSEF 825	AF351133
Pandora neoaphidis	ARSEF 5403	AF052405
Schizangiella serpentis	ARSEF 203	AF368523
Strongwellsea castrans	unknown	AF052406
Zoophthora anglica	ARSEF 396	AF368524
Zoophthora occidentalis	ARSEF 3073	AF368525
Zoophthora radicans	F853	D61381

2.3.5. PCR amplification

PCR of 20 µl contained either 1 ng genomic DNA of fungal strains, 2.5 ng aphid DNA, 20 ng soil DNA, or 20 ng plant DNA, as well as 0.5 µM of each primer (Table 2.3), 200 µM of each dNTP, 0.6 mg/ml bovine serum albumin (BSA), 0.4 units of Phusion Hot Start DNA polymerase (Finnzymes, Espoo, Finland) in either 1 x Phusion HF buffer (for primer pairs PnITSf/PnITSr, and NS1/ITS4), or 1 x Phusion GC buffer (for primer pair PnSSUf/PnITSr). Cycling conditions were 30 s of initial denaturation at 98 °C, followed by 30 cycles (fungal or aphid samples) or 40 cycles (plant or soil samples) of denaturation at 98 °C for 30 s, annealing for 20 s, and extension at 72 °C for 2 min. Final extension was performed at 72 °C for 7 min. Annealing temperatures were 63 °C for primer pair PnSSUf/PnITSr, 61 °C for PnITSf/PnITSr, and 59 °C for NS1/ITS4. Four microliters PCR products were analyzed by agarose gel electrophoresis (1.5 % wt/vol).

2.3.6. PCR inhibition test

PCR inhibition was tested by amplification of 10^6 copies of a plasmid containg a *Beauveria brongniartii* microsatellite insert (clone Bb5F4, Enkerli et al., 2001; Schwarzenbach et al., 2007b) in presence of 20 ng soil DNA. PCR was performed with plasmid-targeting M13 primers (Table 2.3) for 25 cycles, as described above, and with an annealing temperature of 59 °C. PCR products were quantified by gel electrophoresis using a GelDoc XRS gel imager (BioRad Laboratories), 1kb DNA size standard (Invitrogen) and the software Quantity One v. 4.6 (BioRad Laboratories). Analysis of variance (ANOVA) was performed using the software SAS statistical v. 9.1 (SAS Institute, Cary, USA).

Table 2.3. Oligonucleotide primers used in this study for PCR and sequencing.

Primer name	Direction	Sequence	Target region	Reference
PnITSf[a]	forward	5'-GAATAGATTGTCTTTATAACTACGTGTAGA	ITS 1	this study
PnITSr[a]	reverse	5'-ACCAGAGTACCAGCATATCC	ITS 2	this study
PnSSUf[a]	forward	5'-GTCTTTGGGCTTAGTTGGTACT	SSU	this study
NS1[a]	forward	5'-GTAGTCATATGCTTGTCTC	SSU	White et al., 1990
ITS4[a]	reverse	5'-TCCTCCGCTTATTGATATGC	LSU	White et al., 1990
M13 forward[a]	forward	5'-TGTAAAACGACGGCCAGT	pGEM-T Easy	Promega Technical Manual 042
M13 reverse[a]	reverse	5'-CAGGAAACAGCTATGACC	pGEM-T Easy	Promega Technical Manual 042
Eneo2[b]	reverse	5'-TAGAGCACTTACTATCACTCAAGAATAG	ITS 2	this study
Eneo3[b]	reverse	5'-AAGCTTCAGGTATCAGTTTTACC	ITS 2	this study
Eneo4[b]	forward	5'-CAACTTTTTCTTAGCAACATTTTG	ITS 1	this study
R12_2[b]	forward	5'-TCATAGTAAACTTACAGATCGTTG	SSU	this study
EF3[b]	reverse	5'-TCCTCTAAATGACCAAGTTTG	SSU	Smit et al., 1999
FF390[b]	forward	5'-CGATAACGAACGAGACCT	SSU	Smit et al., 1999
FF390-rev[b,c]	reverse	5'-AGGTCTCGTTCGTTATCG	SSU	Smit et al., 1999
fRISArev[b,d]	reverse	5'-ATATGCTTAAGTTCAGCGGGT	LSU	Sequerra et al., 1997
ITS1-E432[b]	forward	5'-CTATCTGCGTTATAACTTGCCA	ITS 1	Tymon et al., 2004
ITS5[b]	forward	5'-GGAAGTAAAAGTCGTAACAAGG	SSU	White et al., 1990
Nu-5.8-5'[b]	forward	5'-TCATCGATGAAGAACGTAGT	5.8S	Jensen and Eilenberg, 2001
Nu-LSU-0018-5'[b]	forward	5'-GTAGTTATTCAAATCAAGCAAG	LSU	Jensen and Eilenberg, 2001
PN3[b]	forward	5'-CCGTTGGTGAACCAGCGGAGGGATC	SSU	Neuvéglise et al., 1994
SSU-Uni-511[b,e]	reverse	5'-TACCGCGGCKGCTGGCA	SSU	Pesaro and Widmer, 2006
UNI-b-for[b]	forward	5'-TGCCAGCMGCCGCGGTA	SSU	Pesaro and Widmer, 2006
UNI-b-rev[b]	reverse	5'-GACGGGCGGTGTGT(A/G)CAA	SSU	Bundt et al., 2001

[a] Primers used for PCR and sequencing.
[b] Primers used for sequencing.
[c] Reverse of FF390.
[d] Also named 3126T (Sequerra et al., 1997).
[e] Reverse of UNI-b-for.

2.3.7. Restriction fragment length polymorphism (RFLP) analysis

Restriction endonuclease *Mbo*I (Promega) was selected for RFLP analysis based on results from *in silico* restriction analysis of SSU rRNA gene sequences of *P. neoaphidis* and its close relatives: *F. ithacensis*, *P. blunckii*, *F. neopyralidarum, P. kondoiensis, S. castrans* (Table 2.2). The close relatives of *P. neoaphidis* were defined as the entomophthoralean species that clustered with this species in the phylogenetic analysis presented by Jensen et al. (2007). The *Mbo*I *in silico* RFLP pattern of the PnSSUf/PnITSr sequence of *P. neoaphidis* consisted of eight fragments of 21 bp, 57 bp, 65 bp, 291 bp, 277 bp, 278 bp, 415 bp, and 695 bp in

length. This pattern was distinct from the ones of all of *P. neoaphidis* close relatives, allowing therefore to discriminate this species from the other species.

Aliquots of 5 µl of each PnSSUf/PnITSr PCR product were used for restriction digestion in 20 µl containing 2 units *Mbo*I and 1 x reaction buffer. Digests were incubated at 37 °C for 10 h. Restriction fragments were analyzed by gel electrophoresis in 3 % (wt/vol) MetaPhor agarose (BioWhittaker Molecular Applications, Rockland, USA).

2.3.8. Cloning and Sequencing

Blunt-ended PCR products obtained with Phusion Hot Start DNA polymerase were purified with QIAquick PCR Purification Kit (Qiagen), A-tailed with *Taq* DNA Polymerase (Invitrogen, Carlsbad, USA), according to Promega Technical Manual 042, and cloned using the pGEM-T Easy Vector System (Promega), according to the manufacturer's recommendations. Sequencing reactions were performed using the BigDye Terminator v. 1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA). Primers used for sequencing are listed in Table 2.3. Sequencing reactions were analyzed on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems) and sequences were assembled with the software Vector NTI v. 10.3 (Invitrogen).

2.3.9. Sequence analysis

An alignment of 1032 bp length was generated using SSU rRNA gene sequences (corresponding to *P. neoaphidis* sequence AF052405, position 714 bp to 1742 bp) of *P. neoaphidis* and of its close relatives. Sequence identities were calculated using the software TREECON v. 1.3b (Van de Peer and De Wachter, 1994).

2.3.10. Nucleotide sequence accession numbers

Nucleotide sequences determined in this study are deposited in GenBank under accession numbers EU267188 for clone *pn*1607-1, EU267192 for clone *pn*7939-1, and EU267193 for clone *pn*7939-3.

2.4. Results

2.4.1. Primer design and evaluation of their specificities

In the ITS region, target regions for *P. neoaphidis* PCR primers (PnITSf and PnITSr) were identified at locations 127 to 156 and 963 to 985 of the *P. neoaphidis* sequence AF543202. In the SSU rRNA gene region, a target region for a PCR primer (PnSSUf) was identified at location 679 to 700 of the *P. neoaphidis* sequence AF052405. BLAST searches performed in GeneBank with the sequences of primers PnITSf and PnITSr revealed exact matches exclusively with ITS sequences of *P. neoaphidis* (AF543202 to AF543211). BLAST searches performed with the sequence of primer PnSSUf revealed exact matches with SSU rRNA gene sequences of *P. neoaphidis* (AF052405), *Pandora blunckii* (AF368520), *Furia neopyralidarum* (AF368518), *Furia ithacensis* (AF351134), and *Pandora kondoiensis* (AF351133). PCR with both primer pairs resulted in single amplicons of expected sizes (856 bp for PnITSf/PnITSr, and 2099 bp for PnSSUf/PnITSr) for *P. neoaphidis* ARSEF 1607 (Figure 2.1, lane 1); whereas no amplification products were obtained with the 15 entomophthoralean relatives screened (data not shown). Positive PCR results were obtained with both primer pairs from top soil layer sample, infected aphid cadaver, and nettle leaves collected in the nettle patch (Figure 2.1, lanes 2i, 3i, 4i, 4ii). In contrast, no

amplification products were obtained from the lower soil layer sample, uninfected aphid, and washed nettle leaf sample (Figure 2.1, lanes 2ii, 3ii, 4iii).

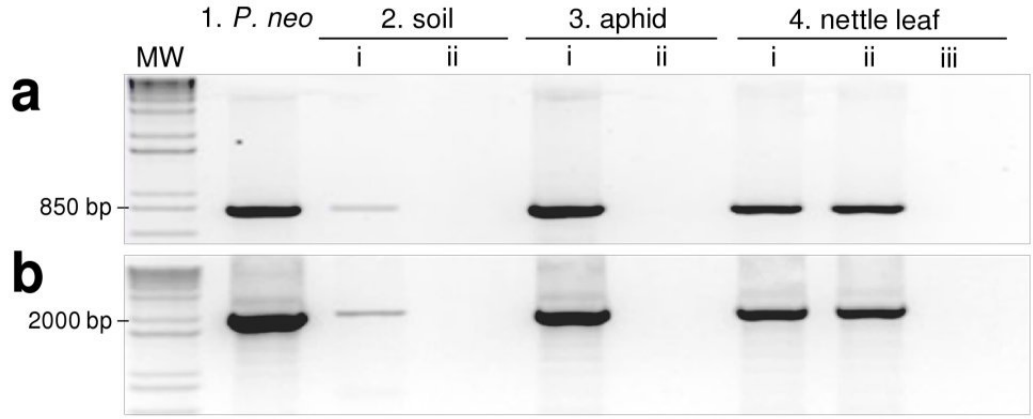

Figure 2.1. PCR amplification of *P. neoaphidis* marker DNA from various sample types. Two *P. neoaphidis* specific PCR primer pairs were used: a) PnITSf/PnITSr, and b) PnSSUf/PnITSr. Sample types were 1) *P. neoaphidis* strain ARSEF 1607, 2) soil samples from an aphid-infested nettle patch (i: top layer; ii: lower layer), 3) aphid samples (i: *P. neoaphidis*-infected aphid cadaver; ii: uninfected aphid), and 4) nettle leaf samples (i: nettle leaf from which a *P. neoaphidis*-infected aphid cadaver was removed; ii: nettle leaf without infected aphid, but collected 50 cm apart from a leaf bearing an infected aphid; iii: washed nettle leaf). The 850 bp and 2000 bp fragments of the 1 Kb+ molecular weight marker (MW) are indicated on the left margin of the gel.

2.4.2. Detection of *P. neoaphidis* in soil within and around a nettle patch

Both diagnostic primer pairs PnITSf/PnITSr and PnSSUf/PnITSr were applied to determine the presence of *P. neoaphidis* in the top soil layer of a nettle patch, in which 4 soil samples were collected around each of 3 plants in November 2004, as well as in March, and June 2005. PCR products of the expected sizes were obtained from all November 2004 and March 2005 samples with both primer pairs (Figure 2.2). In the June 2005 samples, amplicons were obtained from 6 (3 strong and 3 weak) out of 12 samples with primer pair PnITSf/PnITSr, and from 3 out 12 samples with primer pair PnSSUf/PnITSr. A PCR Inhibition test showed no significant difference (P =

0.6616) between the mean product quantities obtained from amplification of the Bb5F4 plasmid insert in presence of 20 ng soil DNA for the 3 time points: November 2004 (7.7 ng/µl ± 0.9 ng/µl), March 2005 (8.1 ng/µl ± 0.9 ng/µl), and June 2005 (7.8 ng/µl ± 1.1 ng/µl).

In addition, the presence of *P. neoaphidis* material was investigated in 17 top soil layer samples collected in Mai 2005. However, the fungus was not detected in any of the samples with both primer pairs (data not shown).

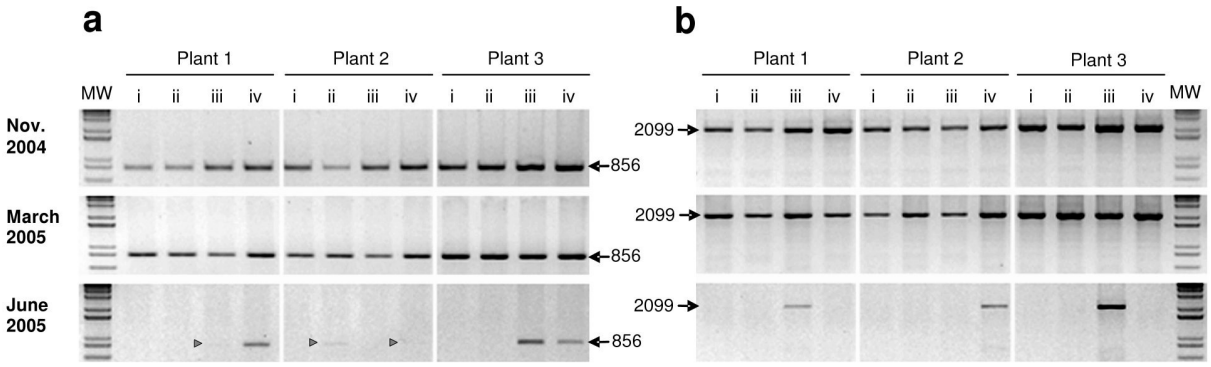

Figure 2.2. PCR amplification of *P. neoaphidis* marker DNA from top soil layer of the nettle patch. Two *P. neoaphidis* specific PCR primer pairs were used: a) PnITSf/PnITSr, and b) PnSSUf/PnITSr. Soil samples were collected around each of three single nettle plants (plants 1 to 3, situated about 4.5 m from each other) in November 2004, March 2005, and June 2005. The four replicates collected around the 3 plants at each time point are indicated with i, ii, iii, and iv. Three weak PCR signals are indicated with grey arrowheads. The 1 Kb+ molecular weight marker (MW) was used as size standard. The expected PCR fragment sizes of 856 bp and 2099 bp are indicated.

2.4.3. Analysis of PnSSUf/PnITSr PCR amplicons

RFLP analysis was applied to verify the identity of the PnSSUf/PnITSr PCR amplicons that were obtained from all environmental samples. *Mbo*I restriction of the PnSSUf/PnITSr PCR amplicon obtained from strain ARSEF 1607 (Figure 2.3 a, lane 1) revealed the predicted pattern for the detectable 5 largest fragments of which two comigrated (i.e. the fragments of 277 bp and 278 bp) in this gel electrophoretic

analysis. A distinct *Mbo*I restriction pattern with an additional fragment of approximately 555 bp was observed for all the positive environmental samples, e.g. the top soil layer from the nettle patch (Figure 2.3 a, lane 2). Furthermore, *Mbo*I restriction analysis of the PnSSUf/PnITSr amplicon obtained from *P. neoaphidis* strain ARSEF 7939 originating from the same region, also revealed the additional 555 bp fragment (Figure 2.3 a, lane 3).

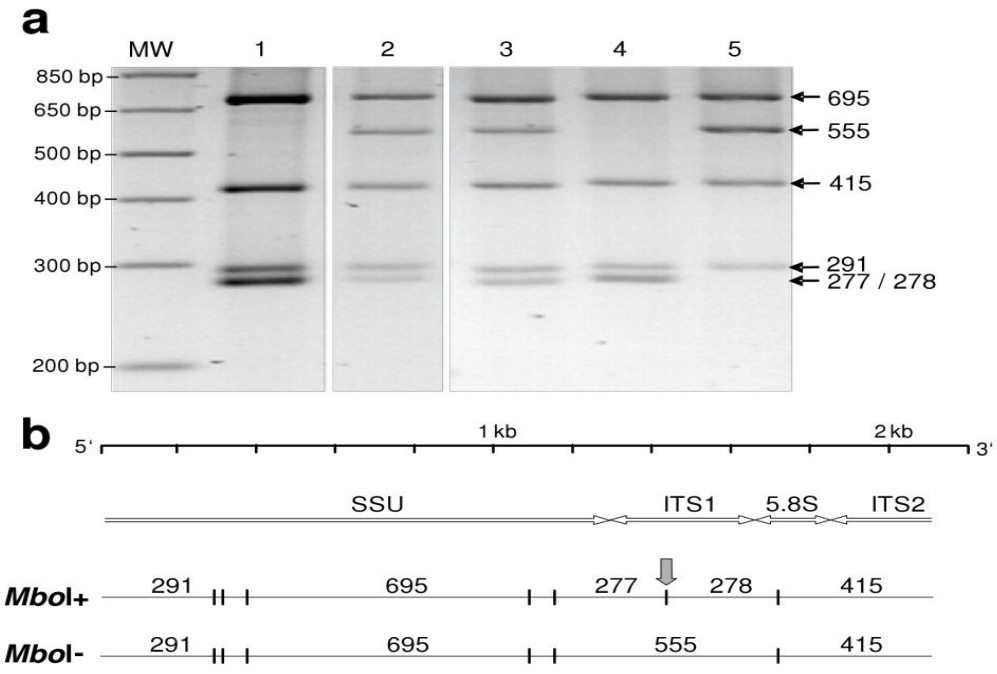

Figure 2.3. *Mbo*I restriction patterns of the 2099 bp PnSSUf/PnITSr DNA fragment. a) *Mbo*I restriction patterns of the PnSSUf/PnITSr PCR amplicons obtained from 1) *P. neoaphidis* strain ARSEF 1607; 2) top soil layer from the nettle patch; 3) *P. neoaphidis* strain ARSEF 7939; 4) ARSEF 7939-clone *pn*7939-1 that lacks the 555 bp *Mbo*I fragment; and 5) ARSEF 7939-clone *pn*7939-3 that displays the 555 bp fragment, but lacks the 277/278 bp fragments. The 1 Kb+ molecular weight marker (MW) was used as size standard. Sizes of the restriction fragments are indicated on the right margin of the gel. b) *Mbo*I restriction maps of PnSSUf/PnITSr sequences. The *Mbo*I+ pattern consists of 8 restriction fragments (21 bp, 57 bp, 65 bp, 291 bp, 277 bp, 278 bp, 415 bp, and 695 bp) and the *MboI*- pattern consists of 7 restriction fragments (21 bp, 57 bp, 65 bp, 291 bp, 415 bp, 555 bp, and 695 bp). The *Mbo*I restriction site missing in the *Mbo*I- pattern (at position 1405-1408) is indicated with a grey arrow. Sizes of the fragments smaller than 100 bp are not shown. The different rRNA operon regions are represented with open arrows. The scale represents sizes in kilobases (kb).

The NS1/IT4 PCR fragments obtained from strains ARSEF 1607 and ARSEF 7939 were cloned. Seven clones from each strain (*pn*1607-1 to *pn*1607-7; and *pn*7939-1 to *pn*7939-7) were analysed by PCR with PnSSUf/PnITSr primers and subsequent *Mbo*I restriction analysis. All seven ARSEF 1607-clones, as well as two ARSEF 7939-clones (*pn*7939-1 and *pn*7939-2) displayed the *Mbo*I restriction pattern without the 555 bp fragment (Figure 2.3 a, lane 4). The restriction patterns of the remaining five ARSEF 7939-clones (*pn*7939-3 to *pn*7939-7) displayed the 555 bp fragment, but did lack the 277/278 bp fragments (Figure 2.3 a, lane 5). Sequencing revealed the presence of a *Mbo*I restriction site at position 1405-1408 of the PnSSUf/PnITSr fragment of clones *pn*7939-1 and *pn*7939-2 as well as of all ARSEF 1607-clones, and absence of the site in clones *pn*7939-3 to *pn*7939-7. Therefore, the *Mbo*I pattern obtained from the clones *pn*7939-1 and *pn*7939-2 and from all the ARSEF1607-clones was denoted as *Mbo*I+ (Figure 2.3 b), whereas the pattern obtained from the clones *pn*7939-3 to *pn*7939-7 was denoted as *Mbo*I-.

Sequence analysis was performed to verify species allocation of strains ARSEF 1607 (GeneBank accession number EU267188) and ARSEF 7939 (GeneBank accession numbers EU267192 and EU267193) and to assess the relationship with the close relatives of *P. neoaphidis.* All *P. neoaphidis* sequences shared 100 % identity, whereas they shared identities of 98.7 % with *P. blunckii,* 98.6 % with *F. ithacensis*, 98.3 % with *P. kondoiensis,* 98 % with *F. neopyralidarum,* and 95.9 % with *S. castrans*.

2.5. Discussion

In this study, we developed and tested a cultivation-independent PCR-based approach to detect *P. neoaphidis* in the environment. The approach is based on the

use of two specific PCR primer pairs, i.e. PnITSf/PnITSr and PnSSUf/PnITSr. The specificity of both primer pairs was demonstrated as PCR amplification products were obtained exclusively from *P. neoaphidis* but not from non-target species, including close relatives of *P. neoaphidis*. Moreover, single amplicons of the expected sizes were obtained with both primer pairs from various environmental sample types including aphid cadavers, plant material, and soil (Figures 2.1 and 2.2), and restriction patterns of the amplicons were consistent with those expected for *P. neoaphidis* (Figure 2.3). *P. neoaphidis* could be specifically detected in environmental samples, despite the great complexity and quantity of DNA from other microorganisms. Moreover, no signals were detected in the negative controls that would indicate a lack of specificity of the assay. The results obtained validated the robustness of both primers pairs to specifically detect *P. neoaphidis* in complex environmental samples. Tymon et al. (2004) have reported the use of the ITS1-E_{432} primer in combination with the fungal universal primer ITS4 to selectively detect *P. neoaphidis* in infected aphid cadavers. We tested this primer pair for detection of *P. neoaphidis* in top soil samples, however, observed unspecific PCR amplicons in addition to the expected ca. 650 bp fragment (data not shown). Therefore, unlike PnITSf/PnITSr and PnSSUf/PnITSr, ITS1-E_{432}/ITS4 primer pair may not be suitable for the detection of *P. neoaphidis* in highly complex substrates such as the soil samples analyzed in the present study.

PCR-RFLP analyses as well as sequencing of NS1/ITS4 clones revealed that the *Mbo*I restriction patterns of the PnSSUf/PnITSr PCR fragments obtained from ARSEF 7939 DNA and from all environmental samples were mixes of *Mbo*I+ and *Mbo*I- patterns. By performing a sequence analysis we demonstrated that the sequences corresponding to both pattern types *Mbo*I+ and *Mbo*I- were belonging to

P. neoaphidis and that they were distinct from the sequences of *P. neoaphidis* close relatives. Furthermore, our results indicate that DNA extracts of ARSEF 7939 and of all environmental sample contained more than one type of ITS sequence of *P. neoaphidis*. Several hypotheses may explain this heterogeneity: 1) Culture and environmental samples may contain more than one strain of the fungus (mixed strains). *In vitro* cultures of *P. neoaphidis* are typically not derived from single spores but from a “shower” of conidia produced by the fungus sporulating on the surface of aphid cadavers. Such cadavers may be infected by more than one strain of *P. neoaphidis* (mixed infection). 2) Even though the asexual propagules of *P. neoaphidis* are haploid uninucleate conidia, the ITS heterogeneity observed in this fungus may be due to the presence of several nuclear variants in a given mycelium resulting from the formation of vegetative heterokaryons within infected insects. Vegetative heterokaryosis can arise due to spontaneous mutations (Maheshwari, 2005) or to hyphal fusion (anastomosis; Leslie, 1993), which has been reported to occur widely in Ascomycetes (Leslie, 1993; Glass et al., 2000), in some Basidiomycetes (e.g. Ainsworth and Rayner, 1986; McCabe et al., 1999) and Glomeromycetes (e.g. Giovannetti et al., 1999 ; De la Providencia et al., 2005). However, until now, anastomosis has never been observed in entomophthoralean fungi. 3) A further possibility to consider is the coexistence of more than one ITS form within a single genome. For instance, such intragenomic rDNA heterogeneity has been observed for Glomeromycetes and Ascomycetes in ITS (Pawlowska and Taylor, 2004) and in 5S rRNA gene (Rooney and Ward, 2005) regions. Pawlowska and Taylor (2004) hypothesized that the observed rDNA heterogeneity in *Glomus etunicatum* may be an expression of genome polyploidization, which would explain the large genome size of Glomerales. The same might be true for *P. neoaphidis*,

which in analogy to *Entomophaga aulicae* may have a genome that is two order of magnitudes greater than average fungal genomes (Butt and Beckett, 1984; Murrin et al., 1986). Further studies are needed to determine the nature of the ITS heterogeneity occurring in *P. neoaphidis*.

Pandora neoaphidis was detected in all top soil samples collected in November 2004 and March 2005 (Figure 2.2); whereas, mostly faint or no PCR signals were obtained from the June 2005 samples. Moreover, PCR results from June 2005 samples revealed variation among the replicates (i to iv), as well as between both primer pairs. The overall weak PCR signal and the reduced reproducibility suggest that the amount of *P. neoaphidis* present in the samples of June 2005 was close to the stochastic limit of detection of the assay. The fact that no significant differences were found with the PCR inhibition test allowed to exclude inhibitory substances as cause for decreasing *P. neoaphidis* detectability. Disappearance of *P. neoaphidis* material from top soil layers during spring is consistent with previous findings, which have shown that environmental factors, such as increasing temperature and solar radiation can have a negative impact on conidia production and survival, and initiation of infection of entomopathogenic fungi (Morgan et al., 1992; Fargues et al., 1996; Nielsen *et al.*, 2003; Steinkraus, 2006; Baverstock et al., 2008).

The absence of aphids in the nettle patch between spring and summer 2005 prevented the production and accumulation of new *P. neoaphidis* inoculum on the soil. This allowed to monitor the persistence of the fungal inoculum that was deposited in fall 2004. The fact that *P. neoaphidis* was not detectable either from any of the samples collected around the nettle patch in May 2005 or from the lower soil layers, indicated that no *P. neoaphidis* inoculum was originating from the surrounding areas at least until May 2005 and that the fungus was only present on the soil

surface. *P. neoaphidis* detected in November 2004 as well as in March 2005 suggested a survival stage of the fungus in top soil during winter. This result supported several laboratory and field studies that have shown that *P. neoaphidis* inoculum deposited on soil remains active and is able to infect aphids for up to 4 months when maintained at temperatures between 4 °C and 10 °C (Morgan et al., 1992; Nielsen et al., 2003; Baverstock et al., 2008).

PCR-based approaches rely on the detection of DNA molecules, and therefore do not provide information on viability and virulence of the detected material. Nevertheless, such a tool offers a powerful complementation of bioassays for monitoring of *P. neoaphidis* in the environment. A bioassay may lack robustness and therefore produce false-negative results, as the factors required for the inoculum to infect aphids are not yet elucidated (Nielsen et al., 2003; Baverstock et al., 2008). In contrast, the use of PCR-based approach is simpler and more reliable, as it is based on well established standard protocols for DNA extraction and PCR amplification from various environmental sample types. Therefore, bioassay and PCR-based approaches have both advantages and disadvantages and may be complementary.

We have developed a cultivation-independent PCR-based assay for specific and rapid detection of *P. neoaphidis* in the environment. The tool allows to screen directly for the presence of *P. neoaphidis,* not only in infected aphid cadavers and plant material but also in complex substrate such as soil. This approach will be useful to further investigate the ecology of *P. neoaphidis,* by allowing for efficient monitoring of the fungus in the agroecosystem. This will help to gain a more detailed knowledge on the ecology of *P. neoaphidis*, which is a requirement for future implementation of efficient management strategies against aphids involving conservation biological control.

2.6. Acknowledgments

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Chapter 3:

Development of a single nucleotide polymorphism (SNP) assay for genotyping of *Pandora neoaphidis*

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3. Development of a single-nucleotide polymorphism (SNP) assay for genotyping of *Pandora neoaphidis*

3.1. Abstract

Pandora neoaphidis (Entomophthoromycotina, Entomophthorales) is one of the most important fungal pathogens of aphids with great potential as a biological control agent. Development of tools that allow high-resolution monitoring of *P. neoaphidis* in the environment is a prerequisite for the successful implementation of biological control strategies. In this study, a single-nucleotide polymorphism (SNP) assay was developed. The assay targets 13 SNPs identified in 6 genomic regions including the largest subunit of nuclear RNA polymerase II (RPB1) gene, the second-largest subunit of nuclear RNA polymerase II (RPB2) gene, the β-tubulin (BTUB) gene, the elongation factor 1α-like (EFL) gene, the large subunit (LSU) rRNA gene, and the small subunit (SSU) rRNA gene together with the internal transcribed spacer (ITS). The assay allowed the discrimination of 15 different SNP profiles among 19 *P. neoaphidis* isolates and 4 *P. neoaphidis*-infected cadavers. Results showed that the assay is applicable to DNA extracted from infected aphids allowing genotyping of the fungus without cultivation. The SNP assay provides an efficient tool for investigation of population structures and dynamics of *P. neoaphidis*, as well as its persistence and epidemiology in agro-ecosystems. Furthermore, it constitutes a powerful approach for monitoring potential biological control strains of *P. neoaphidis* in the environment.

3.2. Introduction

The entomopathogenic fungus *Pandora neoaphidis* (Entomophthoromycotina, Entomophthorales) is one of the most common pathogens of aphids (Powell and Pell, 2007). It occurs in temperate regions worldwide and has great potential for use in biological control strategies against pest aphids (Chapter 2; Baverstock et al., 2008; Powell and Pell, 2007; Shah and Pell, 2003). Availability of genotyping tools that allow discrimination of isolates, assessment of their distribution in different ecosystems, as well as monitoring of selected strains represent important requirements when developing and implementing such strategies.

Several PCR-based genotyping approaches have been investigated for their ability to discriminate isolates of *P. neoaphidis*. Techniques such as randomly amplified polymorphic DNA (RAPD, Williams et al., 1990), enterobacterial repetitive intergenic consensus PCR (ERIC)-PCR (Versalovic et al., 1991), and inter-simple-sequence repeat (ISSR; Zietkiewicz et al., 1994) analyses have allowed for detection of intraspecific variation among *P. neoaphidis* isolates (Nielsen et al., 2001; Rohel et al., 1997; Tymon and Pell, 2005). However, several problems are typically associated with these latter approaches that are based on analyses of anonymous loci: 1) lack of reproducibility among laboratories (Enkerli et al., 2007); 2) risk of misinterpretation of results, e.g., due to size homoplasy (Rieseberg, 1996); and 3) impossibility of applying these methods directly to complex DNA extracts obtained from environmental samples, due to the lack of target specificity of the primers used. Therefore, a laborious and time-consuming isolation and cultivation of the organism of interest is required. In contrast, approaches based on analyses of characterized loci such as the ribosomal small subunit (SSU) rRNA gene or the internal transcribed spacer (ITS) can be applied to pure cultures as well as complex DNA extracts as

primers specific for the target organism can be used (Chapter 2; Tymon et al., 2004). Size, restriction, and sequence analyses of SSU and ITS regions have been used to investigate and discriminate *P. neoaphidis* strains and isolates in various studies (Chapter 2; Nielsen et al., 2001; Rohel et al., 1997; Tymon et al., 2004). However, these approaches have not allowed consistent discrimination of *P. neoaphidis* at an intraspecific level. For instance Tymon et al. (2004) performed restriction fragment length polymorphism (RFLP) analysis of ITS regions amplified from DNA extracts from pure *P. neoaphidis* cultures. Even though analyses were based on six restriction enzymes they failed to distinguish genotypes. In contrast, Fournier et al. (Chapter 2) applied the same approach to DNA extracts from pure cultures as well as from topsoil layers and were able to discriminate *P. neoaphidis* genotypes based on MboI restriction. In the same study the RFLP approach not only allowed detection of intraspecific variation but it also revealed the presence of two alleles (mixed alleles) at a specific *Mbo*I restriction site in single *P. neoaphidis* isolates. However, whether this ITS heterogeneity reflected intragenomic variety or a heterokaryotic stage of these isolates and whether this represents a common phenomenon in *P. neoaphidis* remains to be elucidated.

Sequence analyses of genes encoding for instance rRNA and β-tubulin are widely used for phylogenetic studies (James et al., 2006). They are also applied in multi locus sequence typing (MLST) approaches that have emerged as a reliable typing technique for high-resolution characterization of bacteria (Maiden, 2006) and fungi (e.g. Bain et al., 2007; Jacobsen et al., 2007). MLST is based on the comparison of DNA sequences derived from various genes. In the project 'Assembling the Fungal Tree of Life' (AFTOL; James et al., 2006), genes like the largest subunit of nuclear RNA polymerase II (RPB1) gene, the second-largest subunit of nuclear RNA

polymerase II (RPB2) gene, the elongation factor 1α-like gene (EFL; James et al., 2006; Keeling and Inagaki, 2004), the large subunit (LSU) and the SSU rRNA genes, and their ITS were sequenced and analyzed. These genes provide particularly interesting targets for MLST approaches in fungi because a large number of sequences are available in public databases and PCR amplification has been established across most fungal taxa. Data derived from MLST are usually robust, reproducible, and can easily be compared among laboratories (Maiden, 2006; Urwin and Maiden, 2003). However, MLST procedures may be costly and laborious for large-scale analyses because of the large amount of sequencing and sequence analyses required (Hommais et al., 2005; Olive and Bean, 1999; Scott et al., 2007). In two recent studies on *Escherichia coli* (Hommais et al., 2005) and *Brucella* sp. (Scott et al., 2007), intraspecific single-nucleotide polymorphisms (SNPs) have been identified from sequences of several genes and used to develop SNP assays. Such a strategy does not depend on sequencing and provides a rapid and simple approach for characterizing microbial isolates. SNPs have been characterized in many species (Morin et al., 2004), including fungi such as *Fusarium* spp. (Kristensen et al., 2007), *Cercospora beticola* (Groenewald et al., 2007), and *Coccidioides immitis* (Fisher et al., 1999). SNPs are stable with low rates of recurrent mutations (Sachidanandam et al., 2001) and, therefore, are ideal for diagnostic purposes. The typically binary nature of this marker (Brookes 1999) and the commercially available kits make the analyses simple and compatible with high-throughput systems (Brumfield et al., 2003; Kruglyak 1997). The commonly applied approach to assay SNPs relies on single-base extension of oligonucleotide primers immediately adjacent to the SNP sites (Morin et al., 2004). Resulting fluorescently labelled extension products are subsequently visualized and sized on standard capillary electrophoresis equipment.

The aim of the present study was to develop a SNP-based cultivation-independent tool that allows detection and high-resolution characterization of *P. neoaphidis* in infected aphids, as well as to investigate whether mixed alleles as observed in the ITS region of *P. neoaphidis* (Chapter 2) may be detectable in other genomic regions. For this purpose, SNPs were identified in six genomic regions of *P. neoaphidis* and a SNP assay was developed and validated. Based on the AFTOL (James et al., 2006) project, RPB1, RPB2, EFL, LSU, and SSU/ITS regions were chosen as target regions. In addition, β-tubulin (BTUB) was included in the analysis, as this gene has been extensively investigated in several phylogenetic studies of fungi (e.g. Keeling, 2003; Moon et al., 2002).

3.3. Material and methods

3.3.1. Fungal isolates, aphid culture, and DNA extraction

Nineteen *in vitro* cultivated *Pandora neoaphidis* isolates were either maintained in the laboratory or extracted genomic DNA was obtained from different sources (Table 3.1). Mycelium was produced in liquid cultures and harvested as described by Fournier et al. (Chapter 2).

Uninfected pea aphids (*Acyrthosiphon pisum* Harris) were obtained from a laboratory clonal culture maintained on broad bean plants (*Vicia faba* L., cultivar: *Sirocco*) at 18 °C, 16:8 h light:dark. Two *P. neoaphidis*-infected pea aphid cadavers (I and II) were collected from lucerne (*Medicago sativa*) fields located less than 2 km from Agroscope Reckenholz-Tänikon Research Station in Switzerland in summer/fall 2006. Two *P. neoaphidis*-infected pea aphid cadavers (III and IV) were collected from different generations of an *in vivo* culture originally obtained from a cadaver of

Microlophium carnosum Buckton collected from a nettle plant in 2005 in Klettgau, Schaffhausen. The *in vivo* culture was maintained by periodic infections of cohorts of the laboratory *A. pisum* culture using cadavers of the preceding generation. Infections were performed by applying the conidia shower procedure described by Papierok and Hajek (1997). Infected aphids were incubated on broad bean plants as described above. Aphid and cadaver samples collected were stored at -20 °C.

Genomic DNA of fungal isolates as well as uninfected and infected aphids was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Fungal DNA was extracted from 0.2 g fresh mycelium, which was lyophilized and disrupted with a sterile spatula prior to addition of 600 µl lysis buffer. DNA of uninfected and infected aphids was extracted by placing the aphids directly into 600 µl lysis buffer and disrupting them with a sterile spatula. DNA quality was gel electrophoretically verified and DNA quantity was estimated and adjusted to desired concentration.

Table 3.1. The *19 Pandora neoaphidis* strains used in this study.

Isolate [a]	Host species	Host plant species	Collection site	Year
ARSEF1985 [b]	Aphis fabae	no data	La Minière, France	1985
ARSEF2018 [b]	Acyrthosiphon pisum	Pisum sativum	Voyvodina, Yugoslavia	1985
ARSEF5372	Brevicoryne brassicae	no data	Hegnstrup, Denmark	1996
ARSEF5995	Aphis fabae	Euonymus europae	Copenhagen, Denmark	1998
ARSEF639 [b]	Aphis fabae	no data	Szczecin, Poland	1981
ARSEF7937	Acyrthosiphon pisum	Trifolium repens	Katzensee, Zurich, Switzerland	1998
ARSEF7939	Microlophium carnosum	Urtica dioica	Katzensee, Zurich, Switzerland	1998
127	Acyrthosiphon pisum	Trifolium repens	Katzensee, Zurich, Switzerland	no data
107	Microlophium carnosum	Urtica dioica	Klettgau, Schaffhausen, Switzerland	1996
ARTpn2	Acyrthosiphon pisum	Vicia ssp., Pisum sativum	Klettgau, Schaffhausen, Switzerland	2003
ARTpn3	Acyrthosiphon pisum	Medicago sativa	Klettgau, Schaffhausen, Switzerland	2003
ARTpn4	Acyrthosiphon pisum	Medicago sativa	Klettgau, Schaffhausen, Switzerland	2003
ARTpn5	Acyrthosiphon pisum	Vicia ssp., Pisum sativum	Klettgau, Schaffhausen, Switzerland	2003
ARTpn6	Acyrthosiphon pisum	Vicia ssp., Pisum sativum	Klettgau, Schaffhausen, Switzerland	2003
ARTpn7	Acyrthosiphon pisum	Vicia ssp.	Klettgau, Schaffhausen, Switzerland	2003
145	Macrosiphum rosae	Dipsacus silvester	Frauenfeld, Thurgau, Switzerland	1996
158	Macrosiphum rosae	Dipsacus silvester	Frauenfeld, Thurgau, Switzerland	1996
176	Macrosiphum rosae	Dipsacus silvester	Frauenfeld, Thurgau, Switzerland	no data
171	Myzus persicae	Lactuca sativa	Stetten, Argau, Switzerland	1997

[a] Isolates ARSEF1985 to ARSEF7939 were obtained from the ARS Collection of Entomopathogenic Fungi, Ithaca, USA; isolates 107 to 171 were obtained from the Institute of Microbiology, ETH, Zurich, Switzerland; isolates ARTpn2 to ARTpn7 were isolated from infected aphids collected in the field.

[b] DNA was received from A. Jensen and C. Nielsen, Royal Veterinary and Agricultural University, Frederiksberg, Denmark.

3.3.2. Primer design

DNA fragments of the six target regions RPB1, RPB2, BTUB, EFL, LSU, and SSU/ITS were PCR amplified using either published primers or primers that were designed in the frame of this study (Table 3.2). These primers as well as the primers used for sequencing (Tables 3.2 and 3.3) and SNP analysis (Table 3.4) were developed based on alignments of entomophthoralean DNA sequences obtained from GenBank and/or on *Pandora neoaphidis* sequences generated by applying a gene walking approach. Sequence alignments were generated with the software Clustal W (Thompson et al., 1994) and edited manually with the software BioEdit v. 7.0.1 (Hall, 1999).

Table 3.2. Oligonucleotide primer pairs used for PCR amplification of the target regions.

Primer name	Target region [b]	PCR fragment approx. size [bp]	Direction	Sequence	Annealing [°C]	Reference
gRPB1-Af [a]	RPB1	800	forward	GAKTGTCCKGGWCATTTTGG	62	Stiller and Hall,1997
PnRPB1-r7 [a]			reverse	GGAACACCCACTTCGTTGATCG		this study [d]
bRPB2-3.1F	RPB2	2100	forward	ATYGCYCAAGARMGNATGGC	56	http://www.aftol.org/
PnRPB2-r1			reverse	AAGTGCCATGCCGTAATCGGAGC		this study [d]
Beta-Tub1-for [a]	BTUB	1300	forward	GCCTGCAGGNCARTGYGGNAAYCA	57	Keeling at al., 1998
Beta-Tub1-rev			reverse	GGCCTCAGTRAAYTCCATYTCRTCCAT		Keeling at al., 2000
PnEFL-f1 [a]	EFL	500	forward	AGAACATGATTTCCGGWGCCGC	65	this study [c]
PnEFL-r1 [a]			reverse	ATACCGGAAACGGGGAGACGC		this study [c]
LR0R-ento	LSU	2700	forward	ACCCGCTGAAYTTAAGC	60	this study [c]
PnLSU-r1			reverse	AAAGGATCGATAGGCCACAC		this study [c]
PnSSUf	SSU/ITS	2100	forward	GTCTTTGGGCTTAGTTGGTACT	63	Chapter 2
PnITSr			reverse	ACCAGAGTACCAGCATATCC		Chapter 2

[a] Primers that were also used for sequencing (see Table 3.3).

[b] RPB1: largest subunit of nuclear RNA polymerase II gene; RPB2: second-largest subunit of nuclear RNA polymerase II gene; BTUB: β-tubulin gene; EFL: elongation factor 1α-like gene ; LSU: large subunit of rRNA gene; SSU: small subunit of rRNA gene, and ITS: internal transcribed spacer.

[c] Primers designed based on alignments of entomophthoralean sequences retrieved from GeneBank.

[d] Primers designed based on P. neoaphidis sequence information generated in this study.

Table 3.3. Oligonucleotide primers used for sequencing.

Primer name	Target region [a]	Direction	Sequence	Reference
RPB1-Afor	RPB1	forward	GARTGYCCDGGDCAYTTYGG	Liu et al., 2006
PnRPB1-r6	RPB1	reverse	GGGATCACCAGTAATAACGGTACGAG	this study [c]
PnRPB1-r9	RPB1	reverse	AHCATCCAKTCDGGWCKRGCRAA	this study [b]
PnRPB2-f1	RPB2	forward	TCYYTKGCKACWGGHAACTGGGGYG	this study [b]
PnRPB2-r7	RPB2	reverse	CCTTCAGGAGTCTCDGCAGGRCADAC	this study [b]
PnRPB2-r8	RPB2	reverse	CRCCCCAGTTDCCWGTMGCMARRGA	this study [b]
PnRPB2-r9	RPB2	reverse	CCAATAGGTGTGTTACAACGACGC	this study [c]
PnBTUB-r1	BTUB	reverse	GCACCTTCRGTRTARTAACCYTTDGC	this study [b]
PnBTUB-r3	BTUB	reverse	CCARTTRTTWCCDGCWCCRGATTG	this study [b]
PnEFL-f2	EFL	forward	CCGGWGCCGCYCARGCTGATG	this study [b]
PnEFL-r2	EFL	reverse	AAACGGGGAGACGCATRGGRGC	this study [b]
NL2f-Pn	LSU	forward	GTGAAATTGTTATAAGGAAAGC	this study [c]
CTW13	LSU	forward	CGTCTTGAAACACGGACC	http://plantbio.berkeley.edu/~bruns/
PnLSU-r3	LSU	reverse	TGTTTCAAGACGGGTCGC	this study [c]
TW14	LSU	reverse	GCTATCCTGAGGGAAACTTC	http://plantbio.berkeley.edu/~bruns/

[a] RPB1: largest subunit of nuclear RNA polymerase II gene; RPB2: second-largest subunit of nuclear RNA polymerase II gene; BTUB: β-tubulin gene; EFL: elongation factor 1α-like gene ; LSU: large subunit of rRNA gene; SSU: small subunit of rRNA gene, and ITS: internal transcribed spacer.

[b] Primers designed based on alignments of entomophthoralean sequences retrieved from GeneBank.

[c] Primers designed based on P. neoaphidis sequence information generated in this study.

Table 3.4. SNP primers developed in this study.

SNP primer name	Target gene [a]	Direction	Sequence	SNP [b]
PnSNP1	RPB1	reverse	GAGCAGATAATCCCATGATTTCCAGATC	A/G
PnSNP2	RPB2	reverse	TCCTAAACAATAGGGTTCGGTAGG	A/G
PnSNP3	RPB2	forward	GGAAGAGACGGAAAGATTGC	A/G
PnSNP4	BTUB	forward	GCGACGGCCGTTATGT	A/G
PnSNP5	BTUB	forward	ATTTTTATTATTATTTCACTCTTTTTTTCAA	T/C
PnSNP6	EFL	reverse	TCTTGTTAATACCAATGATAAGTTGTTT	A/G
PnSNP7	EFL	reverse	GATCAACATATTGCGCATTTCATC	T/A
PnSNP8	EFL	forward	CGCAATATGTTGATCAAGGT	T/C
PnSNP9	EFL	forward	GAAGAAAGAATTCATTGAGCAAAA	T/C
PnSNP10	LSU	reverse	CCGAGCAATAGTCACCCA	T/C
PnSNP11	LSU	reverse	GTTAGACCGAAATCCAACTTACCTTACT	T/C
PnSNP12	SSU	reverse	CCCGAGGGAAATGACTTA	T/C
PnSNP13	ITS	reverse	CAATTTTACAACTACATTAAGA	T/A

[a] RPB1: largest subunit of nuclear RNA polymerase II gene; RPB2: second-largest subunit of nuclear RNA polymerase II gene; BTUB: β-tubulin gene; EFL: elongation factor 1α-like gene; LSU: large subunit of rRNA gene; SSU: small subunit of rRNA gene, and ITS: internal transcribed spacer.

[b] The two possible alleles are indicated for each SNP.

The following entomophthoralean sequences were retrieved from GenBank: 1) RPB1 gene sequences: EF014382 (*Basidiobolus ranarum*), DQ294591 (*Conidiobolus coronatus*), DQ294590 (*Entomophthora muscae*); 2) RPB2 gene sequences: DQ302777 (*B. ranarum*), DQ302779 (*C. coronatus*), DQ302778 (*E. muscae*); 3) BTUB gene sequences: AY138794 (*Conidiobolus lamprauges*), AY138793 (*Entomophaga destruens*), AF162062 (*Entomophaga maimaiga*), AY944832 (*Entomophthora blunckii*); 4) for EFL gene sequences: DQ275340 (*B. ranarum*), DQ275337 (*C. coronatus*), DQ275343 (*E. muscae*); and 5) LSU gene sequences: DQ273807 (*B. ranarum*), AY546691 (*C. coronatus*), EAU35394 (*Entomophaga aulicae*), DQ273772 (*E. muscae*).

3.3.3. PCR amplification of target regions

Amplification of the six target regions was performed in reaction mixtures of 20 µl containing 0.5 µM of each primer (Table 3.2), 200 µM of each dNTP, 1 Phusion GC buffer, 0.4 units of Phusion Hot Start DNA polymerase (Finnzymes, Espoo, Finland), and either 1) approx. 5 ng DNA of an uninfected aphid, 2) 5 ng DNA of *Pandora neoaphidis* isolate 107, 3) approx. 5 ng DNA of an uninfected aphid spiked with 5 ng DNA of *P. neoaphidis* isolate 107, or 4) approx. 5 ng DNA of an infected aphid cadaver collected from the field. Cycling conditions were 1 min of initial denaturation at 98 °C, followed by 40 cycles of denaturation at 98 °C for 10 s, annealing at 56-65 °C (Table 3.2) for 20 s, extension at 72 °C for 2 min, and a final extension at 72 °C for 7 min. Five microliters of each PCR product were analyzed by agarose gel electrophoresis (1.5 % wt/vol). PCR products used for sequencing and SNP analysis were treated with ExoSAP-IT (GE Healthcare Bio-Sciences Corp., Piscataway, USA)

according to the manufacturer's recommendations, to degrade unincorporated PCR primers and dNTPs.

3.3.4. Direct sequencing of target regions

Partial sequencing of PCR product was performed with the sequencing primers (Tables 3.2 and 3.3) using the BigDye Terminator v. 1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) according to the manufacturer's recommendations. Sequencing products were purified using the Montage SEQ96 Sequencing Reaction Cleanup Kit (Millipore Corporation, Billerica, USA), denaturated at 95 °C for 5 min, and resolved on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, USA) equipped with a 36-cm capillary array and POP7 polymer. Results were analyzed with the software Vector NTI v. 10.3 (Invitrogen, Carlsbad, USA).

3.3.5. SNP assay

SNP analyses were performed using the SNaPshot Multiplex Kit (Applied Biosystems) according to the manufacturer's recommendations in volumes of 10 µl containing 5 µl of SNaPshot Multiplex Ready Reaction Mix, 0.2 µM of SNP primer (Table 3.4), and 1 µl PCR product. Cycling conditions were 25 cycles of denaturation at 96 °C for 10 s, annealing at 50 °C for 5 s, and extension at 60 °C for 30 s. Subsequently, unincorporated fluorescent ddNTPs were inactivated in volumes of 10 µl containing 8 µl of SNaPshot reaction, 1 µl of 10 x reaction buffer, and 1 µl of Antarctic Phosphatase (New England Biolabs, Beverly, USA). Reaction conditions were 60 min at 37 °C followed by 5 min at 65 °C.

A volume of 0.5 µl per inactivated SNapPshot reaction was mixed with 9 µl HiDi formamide and 0.5 µl of GeneScan-120 LIZ Size Standard (Applied Biosystems), denaturated at 95 °C for 5 min, and resolved on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems) using a 36-cm capillary array and POP7 polymer. Results were analyzed with the GeneMapper software v. 4.0 (Applied Biosystems). For each fungal isolate and aphid a SNP profile was generated by concatenating the alleles of the SNPs. Two SNP profiles were considered different from each other as soon as there was a variation in one of their alleles. Mixed alleles were considered different from both single alleles that constituted them.

3.4. Results

3.4.1. Sequencing and genotyping of *Pandora neoaphidis* isolates

PCR amplifications of all 6 target regions from the 19 *Pandora neoaphidis* isolates consistently yielded single bands of the expected sizes (Table 3.2). PCR fragments obtained from RPB1, RPB2, BTUB, EFL, and LSU rRNA genes were partially sequenced and 11 single-nucleotide polymorphisms (SNPs) were identified in the generated alignments (Supplement 1), i.e., one SNP in the RPB1 gene, two in the RPB2 gene, two in the BTUB gene, four in the EFL gene, and two in the LSU rRNA gene (Table 3.5). Alignment and assessment of 6 previously published *P. neoaphidis* sequences of the ribosomal SSU/ITS region (GenBank sequences EU267188 to EU267193, Chapter 2) allowed identification of two additional SNPs (Supplement 1), i.e., PnSNP12 in the SSU gene and PnSNP13 in the ITS gene (Table 3.5). SNP analysis for all 13 developed SNP primers (Table 3.4) was validated using the 19 *P.*

neoaphidis isolates. It revealed 100 % concordance with the corresponding sequencing information (Figure 3.1).

Table 3.5. Sequence analysis of the 6 target regions.

Target region [a]	Fragment size (bp)	Accession number [b]	SNP position in the GenBank sequences [c]
RPB1	697	GU132803 to GU132821	PnSNP1: 380
RPB2	695	GU132822 to GU132840	PnSNP2: 9, PnSNP3: 684
BTUB	77-116	GU132746 to GU132764	PnSNP4: 73, PnSNP5: 112
EFL	289	GU132765 to GU132783	PnSNP6: 63, PnSNP7: 141, PnSNP8: 171, PnSNP9: 201
LSU	539	GU132784 to GU132802	PnSNP10: 17, PnSNP11: 58
SSU/ITS	2894-2897	EU267188 to EU267193	PnSNP12: 731, PnSNP13: 2102

[a] RPB1: largest subunit of nuclear RNA polymerase II gene; RPB2: second-largest subunit of nuclear RNA polymerase II gene; BTUB: β-tubulin gene; EFL: elongation factor 1α-like gene ; LSU: large subunit of rRNA gene; SSU: small subunit of rRNA gene, and ITS: internal transcribed spacer.

[b] Sequences generated in this study, except EU267188 to EU267193 (Chapter 2).

[c] Positions based on sequence GU132803 for RPB1, GU132822 for RPB2, GU132746 for BTUB, GU132765 for EFL, GU132784 for LSU, and on EU267188 for SSU/ITS.

Isolate				PnSNP													SNP profile number
Name	Host	Origin	Date	1	2	3	4	5	6	7	8	9	10	11	12	13	
				RPB1	RPB2		BTUB		EFL				LSU		SSU/ITS		
ARTpn2	A. pisum	Klettgau, CH	2003	C	T	C	G	T	C	T	Y	C	R	G	Y	A	1*
ARTpn6	A. pisum	Klettgau, CH	2003	C	T	C	G	T	C	T	Y	C	R	G	Y	A	1*
ARTpn3	A. pisum	Klettgau, CH	2003	C	T	Y	G	Y	C	W	Y	Y	R	R	Y	A	2
ARTpn5	A. pisum	Klettgau, CH	2003	T	T	T	G	T	C	W	Y	Y	R	G	Y	A	3
ARTpn4	A. pisum	Klettgau, CH	2003	C	T	T	G	T	C	T	T	C	R	R	Y	A	4
ARSEF 5372	B. brassicae	Hegnstrup, DK	1996	C	Y	T	A	T	C	T	T	C	G	G	Y	A	5*
ARSEF 5995	A. fabae	Hegnstrup, DK	1998	C	Y	T	A	T	C	T	T	C	G	G	Y	A	5*
ARSEF 639	A. fabae	Szczecin, PL	1981	C	C	T	G	Y	C	T	T	C	G	G	Y	A	6
ARSEF 7939	M. carnosum	Katzensee, CH	1998	T	T	T	G	T	T	A	C	T	G	G	T	W	7
107	M. carnosum	Klettgau, CH	1996	T	T	T	G	T	T	A	C	T	G	G	Y	W	8
145	M. rosae	Frauenfeld, CH	1996	C	T	T	G	T	T	A	C	T	G	G	Y	A	9*
158	M. rosae	Frauenfeld, CH	1996	C	T	T	G	T	T	A	C	T	G	G	Y	A	9*
ARSEF 1985	A. fabae	La Minière, F	1985	C	Y	T	G	C	C	T	C	T	R	R	Y	A	10*
ARSEF 2018	A. pisum	Voyvodina, YU	1985	C	Y	T	G	C	C	T	C	T	R	R	Y	A	10*
171	M. persicae	Stetten, CH	1997	Y	Y	T	G	C	C	T	C	T	R	R	Y	A	11
ARTpn7	A. pisum	Klettgau, CH	2003	C	Y	T	R	Y	C	T	C	T	R	R	Y	A	12*
ARSEF 7937	A. pisum	Katzensee, CH	1998	C	Y	T	R	Y	C	T	C	T	R	R	Y	A	12*
127	A. pisum	Katzensee, CH	ND	C	T	T	R	Y	C	T	C	T	R	R	Y	A	13
176	M. rosae	Frauenfeld, CH	ND	Y	T	T	R	Y	C	T	C	T	R	R	Y	A	14

Figure 3.1. SNP profiles consisting of 13 SNP alleles detected in six genomic regions RPB1, RPB2, BTUB, EFL, LSU, and SSU/ITS (full names in Table 3.2) in 19 *Pandora neoaphidis* isolates. SNP profiles present in more than one isolate are indicated with the same profile number and an asterisk. Alleles of the SNPs are displayed as follows: light grey squares for G and T; dark grey squares for C and A, white squares for the mixed alleles. Letter Y indicates T/C mixed alleles, letter R indicates G/A mixed alleles, and letter W indicates T/A mixed alleles. 'ND' means 'no data'.

Fourteen different SNP profiles were detected among the 19 *P. neoaphidis* isolates (Figure 3.1). Nine SNP profiles were detected in single *P. neoaphidis* isolates, whereas 5 profiles were detected in two isolates each, i.e., profile 1 in *ARTpn2* and *ARTpn6*, profile 5 in ARSEF 5372 and ARSEF 5995, profile 9 in 145 and 158, profile 10 in ARSEF1985 and ARSEF 2018, and profile 12 in *ARTpn7* and ARSEF 7937 (asterisks in Figure 3.1). All SNPs were bi-allelic, and except for PnSNP6, they simultaneously displayed both alleles (mixed alleles) in at least one *P. neoaphidis* isolate. The SNPs of RPB1, RPB2, BTUB, and EFL displayed both single alleles in addition to the mixed alleles, whereas the SNPs in the rRNA gene cluster (LSU, SSU, ITS) exclusively displayed one single allele in addition to the mixed alleles. All *P. neoaphidis* isolates contained at least one SNP displaying a mixed allele. The number of mixed alleles varied greatly among the fungal isolates and among the SNPs, i.e., isolates ARSEF7939, 145, and 158 contained one mixed allele, while isolate *ARTpn3* contained 8 mixed alleles. Locus PnSNP3 displayed a mixed allele in one isolate, whereas, PnSNP11, PnSNP10, and PnSNP12 displayed mixed alleles in 9, 12, and 18 isolates, respectively. In total, 28 % of the SNPs analyzed in the 19 *P. neoaphidis* isolates were displaying mixed alleles. All the alleles identified in this study represented synonymous mutations for the corresponding loci and the corresponding amino acid sequences for all *P. neoaphidis* isolates were identical.

3.4.2. Cultivation-independent SNP analysis

No signals were detected from the uninfected aphid DNA (Figure 3.2), while signals for each SNP of *Pandora neoaphidis* isolate 107 were detected from the spiked aphid DNA. Different profiles were obtained from the two *P. neoaphidis*-infected aphid cadavers collected in the field, i.e., profile 5 from cadaver I and profile 12 from

cadaver II. Profile 5 was also detected in isolates ARSEF 5372 and ARSEF 5995, whereas profile 12 was also detected in isolates *ARTpn7* and ARSEF 7937 (Figure 3.1). Profiles obtained from cadavers III and IV, which originated from different generations of an *in vivo* culture of a *P. neoaphidis* field isolate were identical (profile 15) and distinct from all other profiles.

Template DNA	PnSNP													SNP profile number
	1	2	3	4	5	6	7	8	9	10	11	12	13	
	RPB1	RPB2		BTUB		EFL				LSU		SSU/ITS		
uninfected aphid	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. neoaphidis isolate 107	T	T	T	G	T	T	A	C	T	G	G	Y	W	8
uninfected aphid spiked with isolate 107	T	T	T	G	T	T	A	C	T	G	G	Y	W	8
infected aphid cadaver I	C	Y	T	A	T	C	T	T	C	G	G	Y	A	5
infected aphid cadaver II	C	Y	T	R	Y	C	T	C	T	R	R	Y	A	12
infected aphid cadaver III	Y	T	T	G	T	T	A	C	T	G	G	Y	W	15
infected aphid cadaver IV	Y	T	T	G	T	T	A	C	T	G	G	Y	W	15

Figure 3.2. Cultivation-independent SNP analysis of DNA samples obtained from 1) one uninfected pea aphid, 2) *Pandora neoaphidis* isolate 107, 3) one uninfected pea aphid spiked with *P. neoaphidis* isolate 107 DNA, and 4) four *P. neoaphidis*-infected pea aphid cadavers. Cadavers I and II were collected from the field and cadavers III and IV originated from different generations of an in vivo culture of a *P. neoaphidis* field isolate. SNP profiles consist of 13 SNP alleles detected in six genomic regions RPB1, RPB2, BTUB, EFL, LSU, and SSU/ITS (full names in Table 3.2). Alleles of the SNPs are displayed as follows: light grey squares for G and T; dark grey squares for C and A, white squares for the mixed alleles. Letter Y indicates T/C mixed alleles, letter R indicates G/A mixed alleles, and letter W indicates T/A mixed alleles.

3.5. Discussion

The SNP assay developed in this study allowed to distinguish *Pandora neoaphidis* isolates at high resolution. Results of the SNP assay were identical to those of sequence analysis, and all 13 SNP primers specifically detected the corresponding target locus (Figure 3.1). The fact that 15 distinct SNP profiles were obtained from the 19 *P. neoaphidis* isolates and the 4 *P. neoaphidis*-infected cadavers indicated a high level of intraspecific genetic diversity within this fungal species. This supports the results of Tymon and Pell (2005), who have also detected high levels of intraspecific variation in *P. neoaphidis*, distinguishing all 30 isolates of a worldwide collection using approaches such as ISSR, ERIC, and RAPD fingerprinting. Moreover, results showed that the new SNP tool is applicable to DNA extracts of infected aphids (Figure 3.2), allowing the characterization and the discrimination of *P. neoaphidis* genotypes directly in infected aphid cadavers, and therefore obviating the time, cost, and labour intensive cultivation steps necessary with approaches based on analyses of anonymous loci. Analyses may be performed within one week from the collection date, whereas the standard cultivation-dependent approach may require 4 to 6 weeks. Applicability of the assay to DNA extracted from other complex samples including plant and soil requires further assessment.

The 13 SNPs were recovered by sequencing approximately 5200 bp from the 6 target regions from each of the 19 *P. neoaphidis* isolates. About 0.25 % of the analyzed nucleotide positions revealed informative polymorphisms. This is rather low compared to the percentages of polymorphic positions detected in other fungal species, ranging from 1.35 % in *Aspergillus fumigatus* (Bain et al., 2007) to 6.3 % in *Candida tropicalis* (Tavanti et al., 2005). The lower rate of mutation of *P. neoaphidis*

DNA may indicate that this fungus is a recently evolved species in comparison to other fungal species.

Unlike other approaches that were used to detect genetic variability within *P. neoaphidis* (Tymon and Pell, 2005), the SNP assay directly provided sequence information of the polymorphic site. Due to this feature a number of mixed alleles (27 % of all data points) have been unambiguously identified in the 19 *P. neoaphidis* isolates and the 4 *P. neoaphidis*-infected cadavers. They were present in 12 of the 13 SNPs (except PnSNP6 in EFL) and in all DNA samples analyzed. Mixed alleles have been observed for the first time in *P. neoaphidis* by performing RFLP analysis on the ITS region of this fungus (Chapter 2). The presence of mixed alleles implies that the samples investigated contained more than one allele of the target loci and therefore multiple gene copies. This may be explained with the presence of multiple genotypes within single aphid cadavers. To test such hypotheses isolates of *P. neoaphidis* would have to be grown from single conidia. Single conidium isolation for *P. neoaphidis* has been described (Gray et al., 1991), however, it is very difficult to perform and has rarely been done. Alternatively, detection of more than one allele at a specific locus may indicate either the presence of several nuclear variants in a given mycelium resulting from the formation of vegetative heterokaryons within infected insects, or the occurrence of variations within a single genome (Chapter 2). Recent data have shown that genomes of diverse organisms including fungi can contain multiple copies of rRNA- and protein-coding genes with sequences that may differ from each other (e.g. Dujon et al., 2004; Harris and Crandall, 2000; Keeling et al., 2000; Luo et al., 2007; Moon et al., 2004; Oxelman and Bremer, 2000; Pawlowska and Taylor, 2004; Rooney and Ward, 2005; Wörheide et al., 2004).

The number of mixed alleles in the 15 SNP profiles varied greatly among the SNPs and ranged from zero for PnSNP6 to 14 for PnSNP12. This could be explained by a correlation between the frequency of mixed alleles for a given SNP and the number of copies of the corresponding gene per genome. However, this is invalidated by the fact that the ITS region, which is present in multiple copies in fungal genomes contains a SNP (PnSNP13) that displayed three mixed alleles only. Even within the same gene the number of mixed alleles per locus varied, i.e., for RPB2, locus PnSNP3 displayed only one mixed allele, whereas locus PnSNP2 displayed 4 mixed alleles. Nevertheless, the LSU and SSU rRNA genes which are known to be present in multiple copies displayed the highest number of mixed alleles. Unlike all other SNPs, the ones located on the rRNA gene cluster (LSU, SSU, ITS) displayed exclusively one specific allele in addition to the mixed allele.

Despite the presence of mixed alleles, the SNP profiles obtained from the different isolates and *P. neoaphidis*-infected aphid cadavers provided insights on the diversity of *P. neoaphidis* (Figure 3.1). Data obtained with the *ARTpn* isolates showed that although these isolates were all collected from one host species, at the same date and location, their SNP profiles were distinct from each other except for *ARTpn2* and *ARTpn6*. This result demonstrates the discriminatory power of the SNP assay and its promising potential for identifying *P. neoaphidis* genotypes in the environment.

One great advantage of using SNP-based typing method is the ease of constantly updating the dataset with new sequences, allowing the discovery of additional SNPs, and therefore improving the resolution of the assay. SNP profiles of isolates ARSEF1985 and ARSEF 2018 were identical although these two isolates could be differentiated with RAPD analysis (Rohel et al., 1997). Possibly, by sequencing

further genomic regions one may identify additional SNPs that would allow to differentiate both isolates.

With the SNP assay developed in this study, it is now possible to rapidly and robustly detect intraspecific variations in *P. neoaphidis*. Despite the presence of mixed alleles that remain to be further elucidated, 15 different SNP profiles could be detected among 19 isolates and 4 *P. neoaphidis*-infected cadavers, showing the high-resolution power of the approach. Results showed that the assay may be implemented using DNA extracts from pure cultures or from infected aphid cadavers collected in the environment, overcoming laborious isolation and cultivation steps. This tool will allow investigation of the population structure and dynamics of *P. neoaphidis*, as well as its persistence and epidemiology in agro-ecosystems. It will also constitute a powerful approach for monitoring potential biological control strains of *P. neoaphidis* in the environment.

3.6. Acknowledgments

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Chapter 4:

Assessing winter-survival of *Pandora neoaphidis* in soil with bioassays and molecular approaches

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4

4. Assessing winter-survival of *Pandora neoaphidis* in soil with bioassays and molecular approaches

4.1. Abstract

Pandora neoaphidis (Entomophthoromycotina, Entomophthorales) is a specific pathogen of aphids with a great potential for use in biological control. The development of effective biological control strategies requires detailed knowledge of its biology and ecology. However, little is known on the overwintering strategies of this fungus. It is believed that natural areas may play an important role for survival and soil may serve as an inoculum source for new populations in spring. This study aimed to investigate winter-survival of *P. neoaphidis* in topsoil layers in a field experiment by assessing fungal persistence and potential to infect and control pea aphid (*Acyrthosiphon pisum*) populations in spring. For this purpose, a selected *P. neoaphidis* strain was introduced in the form of living infected pea aphids into caged plots containing defined pea aphid populations. Within 3 weeks the aphid populations decreased massively and a significant amount of *P. neoaphidis* inoculum accumulated on the soil providing optimal conditions for investigating the winter-survival of this fungus. Prevalence and pathogenicity of *P. neoaphidis* in plot soils were assessed from fall 2006 until spring 2007 using a combination of bioassay, quantitative PCR, and single nucleotide polymorphism genotyping approaches. Results indicated that the introduced strain did not survive the winter on the soil and that the strains present in the plots in the following spring were derived from outside the plots. However, the introduced strain had a beneficial effect on plant survival. The

tools developed and applied in this study proved powerful and reliable for tracking specific target strains of *P. neoaphidis* in the environment.

4.2. Introduction

Aphids can cause severe crop losses by inducing feeding injuries on plants (Quisenberry and Ni, 2007) and by transmitting plant viruses (Katis et al., 2007). *Pandora neoaphidis* (Remaudière and Hennebert) Humber (Entomophthoromycotina, Entomophthorales) is an important fungal pathogen of aphids in temperate regions and it has great potential for use in biological control. It is aphid-specific (Keller, 1991), infects more than 70 aphid species (Pell et al., 2001), and can cause epizootics that dramatically reduce aphid populations (Feng et al., 1991; Keller and Suter, 1980; Steenberg and Eilenberg, 1995). *In vitro* cultivation of this fungus is difficult and large scale production of biological control strains has not been successful so far (Shah and Pell, 2003). During the last decades, interest in the use of *P. neoaphidis* in conservation biological control strategies has grown (Chapter 2; Barta and Cagan, 2003; Baverstock et al., 2008; Ekesi et al., 2005; Keller, 1998; Pell et al., 2001; Powell and Pell, 2007). The general aim of this strategy is to modify habitats to increase the occurrence, and therefore the efficacy of biocontrol organisms to control pests (Eilenberg et al., 2001). It has been suggested that management practices such as irrigation (increased moisture), reduced pesticide applications, and establishment of overwintering sites for possible alternative hosts may enhance abundance of entomophthoralean fungi for biological control of aphids (Pell et al., 2001). However, successful use of *P. neoaphidis* in such strategies requires a thorough knowledge of its biology and the environmental factors influencing its presence and survival (Nielsen et al., 2003).

Many aspects of the life cycle of *P. neoaphidis* are only poorly understood. In particular, knowledge of overwintering stages and sites as well as the factors that trigger initiation of infection in spring is very limited (Nielsen et al., 2008). It has been suggested that soil may play an important role in winter-survival of *P. neoaphidis* and may serve as the inoculum source for new aphid populations in spring (Chapter 2; Baverstock et al., 2008; Keller, 1998; Nielsen et al., 2003). Furthermore, it has been proposed that natural and semi-natural landscape elements such as field margins, nettle patches, and natural meadows may provide overwintering sites and reservoirs for aphid-pathogenic entomophthoralean fungi (Barta and Cagan, 2003; Baverstock et al., 2008; Ekesi et al., 2005; Keller and Suter, 1980; Shah et al., 2004). However, detailed knowledge of the overwintering ecology of *P. neoaphidis* is still missing.

The development of biological control agents (BCAs) including application and quality control strategies requires tools to detect, genotype, and quantify the BCAs. Two approaches have been used for detecting *P. neoaphidis* in soil. One approach is based on baiting the fungus using bioassays (Baverstock et al., 2008; Latteur, 1980; Nielsen et al., 2003) and the other approach relies on detection with cultivation-independent conventional end-point PCR (Chapter 2; Tymon et al., 2004). Both approaches have specific advantages and disadvantages, which make them complementary. On the one hand, bioassays provide information on viability and pathogenicity of the detected fungi. On the other hand, they are labor intensive and may lack robustness as the factors involved in the infection process are not yet fully understood (Baverstock et al., 2008; Nielsen et al., 2003, 2008). The PCR-based approach is faster and more robust as it is based on well-established protocols for DNA extraction and PCR amplification. However, DNA-based cultivation-independent tools do not provide information on viability and pathogenicity of the detected

organisms (Enkerli and Widmer, 2010; Nielsen et al., 2008). Both detection methods have been applied to investigate overwintering of *P. neoaphidis* and have suggested survival of the fungus in topsoil layers (Chapter 2; Baverstock et al., 2008; Nielsen et al., 2008).

Genotyping tools allow investigation of population structures in the environment and therefore may help to provide basic information on the biology and ecology of a potential BCA (Anderson and Cairney, 2004). Moreover, such tools may be crucial for characterization of specific BCA strains when assessing specificity and virulence during strain selection processes (Bidochka, 2001) or when investigating persistence of specific strains in the environment (Enkerli et al., 2004; Schwarzenbach et al., 2007b). Various genetic tools have been applied to characterize *P. neoaphidis* strains, including randomly amplified polymorphic DNA (Williams et al., 1990), enterobacterial repetitive intergenic consensus PCR (Versalovic et al., 1991), and inter-simple-sequence repeat PCR (Zietkiewicz et al., 1994). Although these methods have allowed detection of intraspecific variation among *P. neoaphidis* isolates (Nielsen et al., 2001; Rohel et al., 1997; Tymon and Pell, 2005; Tymon et al., 2004), they cannot be applied directly to complex DNA extracts obtained from environmental samples due to the lack of target specificity of the primers. Therefore, laborious and time-consuming isolation and cultivation of the organisms of interest are required. Approaches based on ribosomal internal transcribed spacer (ITS) size, restriction, and sequence analyses have been applied in various studies and can be used without prior cultivation steps (Chapter 2; Nielsen et al., 2001; Rohel et al., 1997; Tymon et al., 2004). However, these approaches have not allowed to consistently discriminate *P. neoaphidis* at the intraspecific level. Recently, a molecular assay has been developed that targets single nucleotide polymorphisms (SNPs) distributed

among different genes or genomic regions of *P. neoaphidis* (Chapter 3). SNPs are commonly detected by single-base extension of oligonucleotide primers adjacent to the SNP sites, and they typically display two alleles (Brookes, 1999). This genotyping tool has been reported as powerful for typing *P. neoaphidis* isolates (Chapter 3). Application of the SNP assay has allowed discrimination of *P. neoaphidis* strains with high resolution. It is applicable to DNA extracts obtained from *P. neoaphidis* cultures, as well as from fungal-killed aphid cadavers, and therefore allows for cultivation-independent genotyping of *P. neoaphidis* in the environment. Fournier et al. (Chapter 3) have observed that at a given locus the two alleles can be present simultaneously in *P. neoaphidis*, which they referred to as mixed alleles.

Quantification of fungal species in the environment has traditionally relied on the use of selective media (Lievens et al., 2005). This approach does not allow for quantification of organisms that cannot be cultivated or are difficult to cultivate as in the case for *P. neoaphidis* (Papierok and Hajek, 1997). Quantification techniques that are based on quantitative PCR (Heid et al., 1996) allow to circumvent this problem as no cultivation step of the fungus is required prior to quantification (Bustin, 2004). Approaches based on quantitative PCR have successfully been implemented to quantify entomopathogenic fungal species such as *Beauveria brongniartii* (Saccardo) Petch (Schwarzenbach et al., 2009) and *Entomophaga maimaiga* Humber, Shimazu, & Soper (Castrillo et al., 2007), or specific strains of *Beauveria bassiana* (Balsamo) Vuillemin (Bell et al., 2009; Castrillo et al., 2008) and *Metarhizium anisopliae* var. *acridum* Driver and Milner (Bell et al., 2009) in host insects or soil. Such approaches for quantification of *P. neoaphidis* in the environment have not been established yet.

The goal of our study was to determine the ability of *P. neoaphidis* for winter-survival in topsoil layers and to assess its potential to infect and control aphid populations in a

field experiment. A *P. neoaphidis* strain was applied in caged field plots containing healthy aphids in fall 2006. Abundance and infection rate of the inoculum were monitored until spring 2007 using a bioassay, SNP-based genotyping, and a quantitative PCR approach developed in this study.

4.3. Material and methods

4.3.1. Plant culture

Lucerne plants (*Medicago sativa* Linnaeus, cv. Sanditi) used in the field experiment were grown from seeds germinated between layers of water-saturated filter paper in 16 h light and 8 h dark at 20 °C. Seedlings were planted in pots (9 cm diameter, 7 cm deep) containing commercial turf soil (Ricoter Erdaufbereitung AG, Aarberg, Switzerland) and grown in the greenhouse for 3 weeks in 16 h light at 17 °C and 8 h dark at 13 °C. Subsequently, the lucerne plants were acclimatized to outdoor conditions for 2 weeks in the shade before transplanting them into the field plots.

Broad bean plants (*Vicia faba* Linnaeus, cv. Sirocco) were grown from seeds in an incubation chamber in 16 h light and 8 h dark, at 18 °C with 65-75 % humidity in 35 x 22 x 5 cm seed trays (50 seeds/tray) filled with autoclaved (121 °C, 25 min) multi-purpose compost (Obi-Ter, Märwil, Switzerland). Two- to three-week-old plants were transplanted into either ‘large pots’ (13 cm diameter, 11 cm deep) or ‘small pots’ (8 cm diameter, 8 cm deep). The ‘large pots’ were used for propagation of aphids for field release. They were filled with autoclaved multi-purpose compost and planted with six *V. faba* plants. The ‘small pots’ were used for monitoring aphid infection and production of infected aphid cadavers. They were prepared by pouring a 5-cm layer of 1 % agar into the pot and transplanting one individual *V. faba* plant into a hole (1

cm diameter, 5 cm deep) that was made in the center of the solidified agar piece of each pot. Twenty milliliters of water were poured into the hole and a disc of parafilm (8 cm diameter, with a 5-mm hole in the center for the plant) was placed on the top of the agar before adding a 0.5-cm layer of sieved (2 mm) and gamma-sterilized soil (40 kGy, Studer Hard, Däniken, Switzerland). This setup allowed plant maintenance for 1 week without watering and therefore to circumvent disturbance and contamination of the aphids placed on the plants.

4.3.2. Aphid culture

Four clonal cultures of pea aphids (*Acyrthosiphon pisum* Harris, Homoptera: Aphididae) were used. One culture had been maintained for more than 20 years in the laboratory (culture 1) and three cultures (cultures 2-4) were started in summer 2006, each from a single aphid collected in a field at Agroscope Reckenholz-Tänikon. All cultures were maintained on *V. faba* plants in the 'large pots'. Aphid propagation for field release was performed by placing 40 healthy adults on 3-week-old *V. faba* plants transplanted into 'large pots'. The pots were incubated for 12 days as described below to obtain a population of approximately 1000 individuals/ pot. Two types of aphid populations were prepared: 'mixed populations' and 'laboratory culture populations'. The 'mixed populations' were initiated by placing 10 adults of each of the four aphid monoclonal cultures (cultures 1-4) on *V. faba* plants (40 aphids/pot); whereas the 'laboratory culture populations' were initiated by placing 40 adult aphids of laboratory culture 1 on *V. faba* plants.

As soon as aphids were placed on newly transplanted *V. faba* plants, the pots ('large pots' or 'small pots') were covered with cellophane bags (Celloclair AG, Liestal, Switzerland) and sealed with a rubber band around the pot rim. Incubation conditions

of aphid-infested *V. faba* plants were 16 h light and 8 h dark, at 18 °C with 65-75 % humidity.

4.3.3. Fungal culture

Pandora neoaphidis strain *ARTpn1* was used for the field experiment. This strain was isolated from a cadaver of Microlophium carnosum Buckton (Homoptera: Aphididae) collected from a nettle plant (*Urtica dioica* Linnaeus) in 2005 in Klettgau, Schaffhausen, Switzerland. The fungal culture was maintained with periodic passages using the laboratory aphid culture. Aphids were infected with *ARTpn1* using a conidia shower procedure modified from Papierok and Hajek (1997). Briefly, a 1-cm layer of 1% agar was poured into plastic cups (8 cm in diameter and height). The agar surface was covered with *V. faba* leaves, and five aphid cadavers with sporulating *ARTpn1* were placed on three layers of wet filter paper fixed to cup lids. Approximately 500 aphids from the laboratory culture were transferred to each of the cups, which were then closed with the lids and kept in the dark for 8 h at 20 °C with 100% humidity. After incubation, the lids were removed and the cups were sealed with cellophane and incubated in the dark for 16 h at 18 °C with 65-75 % humidity. Subsequently, the aphids were either transferred to *V. faba* plants in 'large pots' and incubated for 3 days before field release or transferred to individual *V. faba* plants in 'small pots' and incubated for 5 days to produce cadavers of fungus-killed aphids. These cadavers were used in experiments, as well as to maintain the fungal strain. They were stored in the dark at 4 °C with 20 % humidity for a maximum of 3 months.

Three *P. neoaphidis*-infected pea aphid cadavers were collected in July 2006 in lucerne fields located less than 2 km from the experimental field. The three cadavers

were used to assess genetic variability among native *P. neoaphidis* isolates and to identify informative SNP.

4.3.4. Experimental design and plot preparation

The field experiment was conducted in a grass-clover field (47 25'52''N, 8 31'19''E) at Agroscope Reckenholz-Tänikon in summer 2006. The experimental layout consisted of 32 square plots (0.16 m^2) separated by 1.5 m and arranged in a complete randomized block design with eight replicated blocks of four treatments. The treatments consisted of various combinations of the organisms; i.e. (1) plants, aphids and fungus (*paf*); (2) plants and aphids (*pa*); (3) plants (*p*); (4) a bare soil control (*bs*).

The plots were prepared by clearing all vegetation using the herbicide Roundup (Syngenta, Basel, Switzerland) on June 26, 2006 and transplanting sixteen 6-week-old acclimatized lucerne plantlets into each plot of the plant containing treatments on August 11, 2006. These plots were caged (40 cm x 40 cm x 40 cm) with a 200-µm Nitext mesh fabric (Sefar, Heiden, Switzerland) to avoid insect infestation. The *bs* plots were covered with a weed barrier fabric ('GrowStop', Windhager, Thalgau, Austria). Transplanted lucerne plants were cut 10 cm above ground on September 8, 2006 and the plant-cuttings were removed from the plots. In order to prevent contamination by native aphid populations present prior to setting up the cages 0.05% of Pyrethrum FS (Andermatt Biocontrol AG, Grossdietwil, Switzerland) was sprayed into the cages at three different time points, i.e., September 13, 25, 28, 2006. Approximately 1000 aphids from the 'mixed populations' of *A. pisum* were released on October 2, 2006 in the *paf* and *pa* treatments by placing five aphid-infested plant-cuttings from 'large pots' per plot. 'Laboratory culture populations' of

approximately 1000 aphids exposed to conidia showers on October 6, 2006, and with a mean prevalence of *P. neoaphidis* infection of 14.2 ± 3.2 %, were released on plant-cuttings in each of the *paf* plots on October 10, 2006. At the same time, uninfected laboratory culture populations of approximately 1000 healthy aphids were released in each of the *pa* plots.

4.3.5. Estimation of aphid populations, prevalence of *P. neoaphidis* infection, and plant survival

The mean aphid population per plot type at a given time point was estimated by counting all aphids present on one plant in each replicate plot, extrapolating to 16 plants/plot, and calculating the mean number of aphids per plot type (n = 8). The mean prevalence of *P. neoaphidis* infection per plot type at a given time point was estimated by collecting 100 3rd to 4th instar aphid nymphs from each plot that were not displaying infection signs or symptoms, incubating them for 5 days on individual *V. faba* plants in 'small pots', counting the number of *P. neoaphidis*-infected aphid cadavers, and calculating the mean per plot type (n = 8). One aphid cadaver per plot (*paf* and *pa*) and time point (October 22 and 29) was stored for subsequent genotyping of the fungus. The mean number of surviving plants per plot type at a given time point was determined by counting the number of surviving plants (plants with green leaves) in each plot and calculating the mean per plot type (n = 8).

4.3.6. Soil sample collection

Soil samples were collected from the plots at five different time points in 2006 and 2007. These collection times were (1) on October 2, 2006, just before releasing the aphids into the *pa* and *paf* plots; (2) on November 2, after high levels of infection with

P. neoaphidis were observed; (3) on November 21, after night temperatures fell below 0 °C; (4) on December 13, when no more living aphids were observed in the *paf* and *pa* plots; (5) after the winter on April 11, 2007 at the start of aphid population build-up in neighboring fields. From each plot and at each time point, four soil surface samples potentially bearing the *P. neoaphidis* inoculum were collected. Each sample consisted of 25 cm^2 topsoil (1 cm depth) collected within a radius of 15 cm around the center of each plot. The four samples per plot were transferred to a 10 cm Petri dish with a spoon without disturbing the soil structure and sample surface. The samples were subsequently submitted to laboratory bioassays, DNA extraction, and quantitative PCR analyses.

4.3.7. Laboratory and field bioassay

Laboratory bioassays were performed with the collected topsoil samples in Petri dishes, which were pre-incubated for 24 h in 16 h light and 8 h dark, at 18 °C with 100 % humidity. Approximately 100 aphids from the laboratory culture of all developmental stages were added to each soil sample (Petri dish). After incubation for 14 h in the dark at 18 °C with 100 % humidity, 20 3rd to 4th instar nymphs/sample were randomly collected and transferred to single *V. faba* plants in 'small pots'. After 7 days, the number of aphid cadavers was determined. Mortality was calculated according to Feng et al. (1991); i.e., mortality (%) = [(aphid cadavers)/(living aphids + aphid cadavers)] x 100.

Field bioassays were performed by inoculating the *paf* and *p* plots with approximately 1000 healthy aphids of the 'mixed populations' on April 17, 2007 as described above. The mean aphid population and prevalence of *P. neoaphidis* infection per plot were determined on April 24, May 1, 8, 15, and June 13, 2007. The mean values were

calculated from the eight experimental replicates for each of the two plot types and time point. One infected aphid cadaver per plot and time point was stored for subsequent genotyping of the fungus.

4.3.8. Quantitative PCR

Five hundred milligrams of soil were retrieved from each topsoil sample, and bulk soil DNA was extracted and quantified as described by Fournier et al. (Chapter 2). Quantitative real-time PCR (qPCR) detection of *P. neoaphidis* in soil was performed using the primer pair PnITSf/PnITSr, which specifically targets sequences in the rRNA gene cluster of *P. neoaphidis* (Chapter 2) and amplifies a fragment of 856 bp in length. Aliquots of soil DNA extracts were adjusted to a concentration of 2 ng μl^{-1} and bovine serum albumin (BSA) was added to a final concentration of 3 µg μl^{-1}. The aliquots were heated for 5 min at 95 °C to bind PCR-inhibiting substances like humic acids (Hartmann et al., 2005). Quantitative PCR amplification was performed in 25 µl reaction volumes containing 12.5 µl 2 x iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 300 nM of each primer, and 10 ng bulk soil DNA, representing 0.08-0.65 mg soil (dry weight equivalent). Cycling conditions consisted of 3 min initial denaturation at 95 °C, followed by 45 cycles with denaturation for 40 s at 94 °C, annealing for 40 s at 60 °C and extension for 80 s at 72 °C, and a final extension for 5 min at 72 °C. Tenfold serial dilutions with 10^1-10^7 copies of the plasmid *pn*1607-1 containing the qPCR target (Chapter 2) were used as quantification standard. Quantitative PCR was performed in three analytical replicates using an iCycler IQ Quantitative PCR Detection System with software version 3.1 (Bio-Rad Laboratories). For each time point, the means were calculated from the eight experimental replicates of each plot type. The detection limit for the

qPCR assay was 7 x 10^4 gene copies/g soil and PCR inhibition was tested according to Fournier et al. (Chapter 2).

4.3.9. Genotyping of *P. neoaphidis* in collected aphid samples

DNA from infected aphid cadavers was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The cadavers were placed directly into 600 µl lysis buffer, disrupted with a spatula, and DNA was extracted according to the manufacturer's protocol. DNA quality was gel electrophoretically verified and concentrations were adjusted. Twelve single nucleotide polymorphisms (SNPs) were detected according to Fournier et al. (Chapter 3), targeting the seven loci: the largest subunit of nuclear RNA polymerase II (RPB1) gene, the second largest subunit of nuclear RNA polymerase II (RPB2) gene, the elongation factor 1α-like (EFL) gene, the β-tubulin gene (BTUB), the large subunit (LSU) rRNA gene, the small subunit (SSU) rRNA gene, and the internal transcribed spacer (ITS). Briefly, the loci were amplified from approximately 5 ng template DNA using locus-specific PCR primer pairs in PCR volumes of 20 µl. To remove unincorporated PCR primers and dNTPs, PCR products were treated with ExoSAP-IT according to the manufacturer's recommendations (GE Healthcare Bio-Sciences Corp., Piscataway, USA). SNPs were analyzed in the PCR-amplified target regions using the SNaPshot Multiplex Kit (Applied Biosystems) and the corresponding SNP primers (Chapter 3). Reactions were performed according to the manufacturer's recommendations in volumes of 10 µl. Unincorporated fluorescent ddNTPs were inactivated with an Antarctic Phosphatase treatment (New England Biolabs, Beverly, USA) as suggested by the protocol of the SNaPshot Multiplex Kit. SNaPshot reaction products were resolved on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems) using a 36-cm capillary

array and POP7 polymer. Results were analyzed with the GeneMapper software version 4.0 (Applied Biosystems). SNP profiles were generated by concatenating the alleles of the SNPs analyzed for each sample. SNPs displaying simultaneously two alleles were referred to as 'mixed alleles' (Chapter 3). Mixed alleles were considered different from each single alleles that constituted them.

4.3.10. Statistical analysis

Differences between samples were assessed with the SAS statistical software version 9.1 (SAS Institute, Cary, NC, USA). Analysis of variance (ANOVA) was used to test for PCR inhibition. Wilcoxon/Kruskal-Wallis test was applied to assess the differences in aphid numbers, prevalence of infection, aphid mortality, gene copy numbers, and plant survival. Correlation analyses were performed using the ''Corr procedure" of SAS. Differences were considered significant at P values < 0.05.

4.4. Results

4.4.1. Aphid population sizes and prevalence of *P. neoaphidis* infection on plants

No aphids were detected in any plots 1 day prior to release of approximately 1000 healthy aphids into the *paf* and *pa* plots on October 2, 2006 (Table 4.1). Mean aphid population size in the *paf* plots peaked 12 days after inoculation with *P. neoaphidis* strain *ARTpn1*. The *paf* plots remained free of aphids from December 10, 2006 until the start of the field bioassay on April 17, 2007. Prevalence of *P. neoaphidis* infection in the *paf* plots was high on October 22 and 29 and could not be detected thereafter as no living aphids were present in the *paf* plots after the temperature dropped below

0 °C at night. In the *pa* plots mean aphid population size reached its peak by October 29. The *pa* plots also remained free of aphids from December 10, 2006 until the end of the experiment in June 2007. Even though not inoculated, *P. neoaphidis* infections were detected in the *pa* plots reaching their maximum on October 29. As for the *paf* plots, the prevalence of infection could not be determined thereafter as frost at night has killed the aphids. Aphids were never observed in the plots in which they were not released, i.e., in the *p* plots between October 2006 and April 17, 2007 (start of field bioassay); and in the *bs* plots throughout the entire duration of the experiment (October 2006 to June 2007).

Table 4.1. Aphid population size and prevalence of *Pandora neoaphidis* infection in the plots after release of uninfected aphids in the paf and pa plots on October 2, 2006 and of infected aphids in the paf plots on October 10, 2006.

Date	Mean number of aphids per plot (x 10^3)[a]				Prevalence of infection per plot (%)[a]			
	paf[b]	*pa*[b]	*p*[b]	*bs*[b]	*paf*[b]	*pa*[b]	*p*[b]	*b*[b]
October 1, 2006	0	0	0	0	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]
October 22, 2006	6.7 ± 0.6 *a*	8.2 ± 1.1*a*	0	0	87.7 ± 5.2*a*	16.2 ± 10.5*a*	n.d.[d]	n.d.[d]
October 29, 2006	0.9 ± 0.2 *b*	11.6 ± 1.0*b*	0	0	90.3 ± 8.4*a*	68.0 ± 7.1*b*	n.d.[d]	n.d.[d]
December 10, 2006	0	0	0	0	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]
April 17, 2007	0[c]	0[c]	0[c]	0[c]	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]
June 13, 2007	n.d.[e]	0	n.d.[e]	0	n.d.[e]	n.d.[d]	n.d.[e]	n.d.[d]

[a] Means of the eight replicates per plot type (plants, aphids, and fungus (*paf*); plants and aphids (*pa*); plants (*p*); bare soil (*bs*)) and time point ± SD.

[b] Within columns, values followed by a different letter are significantly different ($P < 0.05$).

[c] Values that were recorded just before release of 1000 uninfected aphids per plot to perform the field bioassay.

[d] No data available, as no aphid were present in the plots.

[e] No data available, as 1000 uninfected aphids were released into the *paf* and *p* plots on April 17, 2007 to perform the field bioassay.

4.4.2. *Pandora neoaphidis* prevalence and density in soil

Aphid mortality as determined with the laboratory bioassay progressed simultaneously in the soils of the *paf* and *pa* plots (Figure 4.1A).

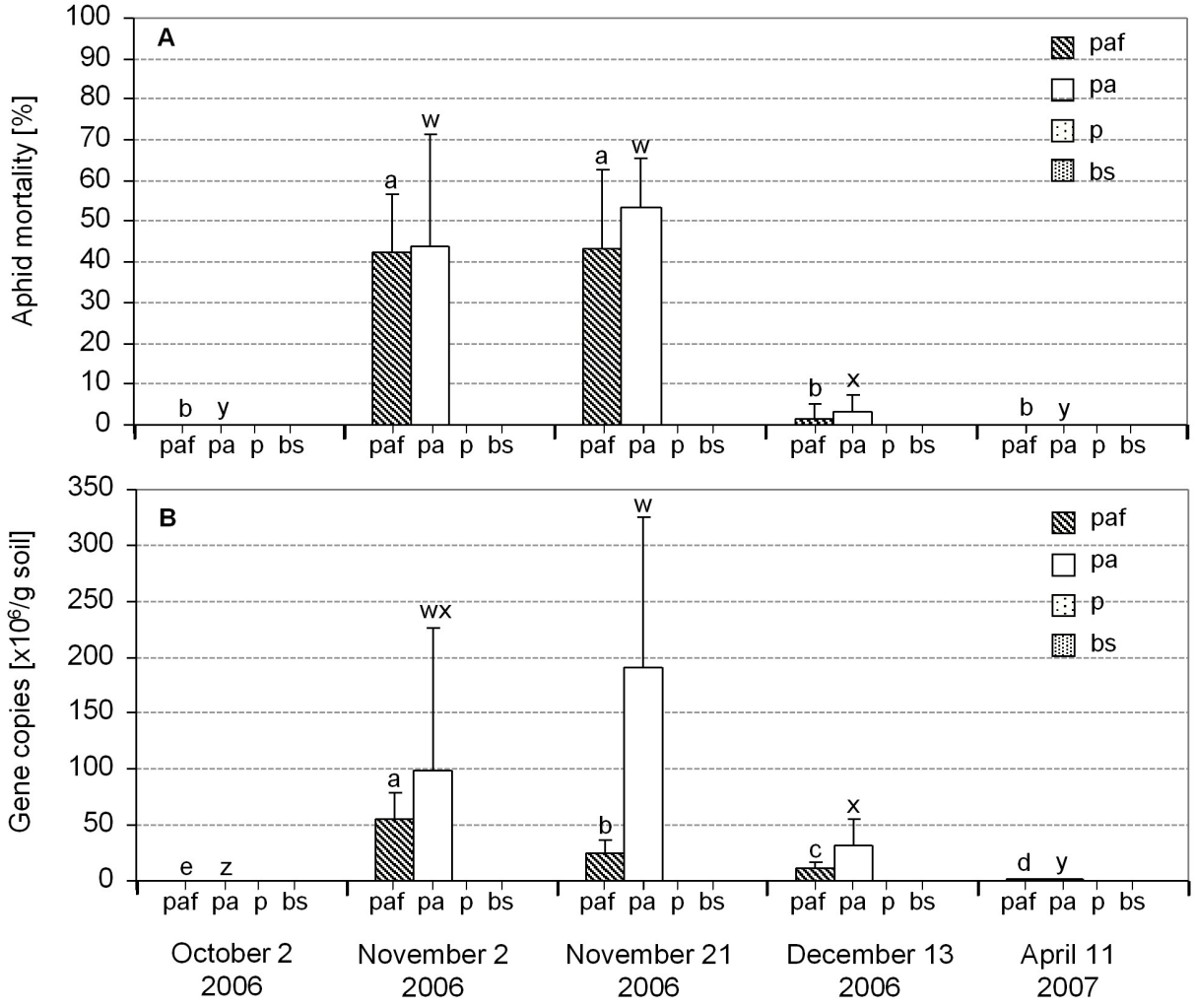

Figure 4.1. Detection of *Pandora neoaphidis* prevalence and density in soil samples collected from plots containing plants, aphids and fungus (*paf*); plants and aphids (*pa*); plants (*p*); or bare soil (*bs*) between fall 2006 and spring 2007. (A) Aphid mortality determined with the laboratory bioassay. Columns represent mean percent aphid mortality due to *P. neoaphidis* infection treatment type and time point (n = 8). (B) Gene copy numbers detected by quantitative real-time PCR quantification of the ITS target sequence of *P. neoaphidis*. Columns represent the mean gene copy numbers per gram soil treatment and time point (n = 8). Columns labeled with identical letters are not significantly different ($P < 0.05$) among *paf* (a, b, c, d, and e) or *pa* (w, x, y, and z) plots at different time points.

No mortality was detected 8 days prior to *P. neoaphidis* inoculation in the *paf* plots. It reached maximal values on November 2 and 21, decreased during the winter and was not detectable by April 11, 2007 for both plot types.

Quantitative real-time PCR did not detect *P. neoaphidis* in the *paf* as well as in the *pa* plots on October 2 (Figure 4.1B). The number of detected gene copies in the *paf* plots reached its peak on November 2, while in the *pa* plots it peaked on November 21. In both plot types the number of gene copies per gram soil decreased during the winter, reaching values of $1.1 \times 10^6 \pm 0.9 \times 10^6$ and $1.4 \times 10^6 \pm 1.1 \times 10^6$ in the *paf* and *pa* plots by April 11, 2007. Gene copies correlated significantly with the mortality values obtained with the laboratory bioassay ($P < 0.05$). The Spearman coefficient was 0.794 for the data of the *paf* plots and 0.750 for those of the *pa* plots. *P. neoaphidis* was not detected in any of the *bs* and *p* soil samples neither with the laboratory bioassay nor with quantitative PCR. The PCR Inhibition test showed no significant ($P > 0.05$) inhibition in the presence of 10 ng soil DNA for the different plot types and time points.

4.4.3. Detection of *P. neoaphidis* in the field in spring 2007

No aphids were present on April 17, 2007 prior to the release of 1000 healthy aphids for the field bioassay, which was performed in the *paf* and *p* plots (Table 4.2). In both plot types, mean aphid populations increased consistently and significantly until May 15, 2007 and reached numbers, which were more than 20-fold larger than those of the released populations. By June 13, 2007 the population sizes had dropped to less than 500 individuals per plot in both plot types. Prevalence of infection was 0 % during the first month of the experiment and reached 46.9 % in the *paf* plots and 19.8% in the *p* plots at the last sampling on June 13, 2007 (Table 4.2).

Table 4.2. Aphid population size and prevalence of *Pandora neoaphidis* infection in the *paf* and *p* plots during the field bioassay performed in spring 2007.

Date	Mean number of aphids (x 10^3)[a]		Prevalence of infection (%)[a]	
	paf[b]	*p*[b]	*paf*[b]	*p*[b]
April 17	approx. 1.0[d]	approx. 1.0[d]	0*a*	0*a*
April 24	4.0 ± 0.4*b*	4.0 ± 0.7*b*	0*a*	0*a*
Mai 1	5.8 ± 0.3*c*	5.7 ± 0.6*c*	0*a*	0*a*
Mai 8	11.5 ± 0.8*d*	11.0 ± 0.6*d*	0*a*	0*a*
Mai 15	21.6 ± 1.7*e*	22.3 ± 1.7*e*	0*a*	0*a*
June 13	0.4 ± 0.2*a*	0.3 ± 0.1*a*	46.9 ± 23.6*b*[c]	19.8 ± 16.5*b*[c]

[a] Means of the eight replicates per plot type ± SD (plants, aphids, and fungus (*paf*); plants (*p*)).
[b] Within columns, values followed by a different letter are significantly different ($P < 0.05$).
[c] Values that are significantly different within a row ($P < 0.05$).
[d] Values indicate the approximate number of aphids released in the *paf* and *p* plots on April 17, 2007.

4.4.4. Plant survival in the plots

The beneficial effect on plant survival obtained from the artificial introduction of *P. neoaphidis* in the *paf* plots was assessed by determining the number of surviving plants in each plot on February 26, 2007. In the *paf* and *p* plots all plants survived while in the *pa* plots 87.5 % ± 10.02 % survived, which was significantly lower than in the *paf* and *p* plots ($P < 0.05$).

4.4.5. Genotyping *P. neoaphidis* in infected aphids

An *ARTpn1*-infected cadaver and three *P. neoaphidis*-infected cadavers collected in surrounding fields were genotyped based on 12 SNPs to characterize the infecting *P. neoaphidis* strains (Figure 4.2). The four SNP profiles (Figure 4.2, profiles a-d) were distinct from each other based on allelic differences at eight of the 12 SNPs. Profile ‘a’ of *ARTpn1* differed at seven SNPs from the other profiles, whereas differences among profiles ‘b’, ‘c’, and ‘d’ derived from allele differences at PnSNP1 and PnSNP2. Based on these data, six informative SNPs, i.e., PnSNP1, PnSNP2, PnSNP4, PnSNP7, PnSNP10, and PnSNP13, were selected each located in a

different target locus. They were used to analyze *P. neoaphidis* strains in 32 infected aphid cadavers (eight from each treatment) collected from the *paf* and *pa* plots on October 29, 2006, and from the *paf* and *p* plots on June 13, 2007 (Figure 4.3). Nine different SNP profiles were detected, i.e., profiles 'a', 'c', 'e', 'f', 'g', 'h', 'i', 'j', and 'k'. Profiles 'a' and 'c' (Figure 4.3) were consistent with the profiles 'a' and 'c' in Figure 4.2. Profile 'a', characteristic for strain *ARTpn1*, was detected in six of the eight cadavers collected in the *paf* plots on October 29, 2006. It was not detected in any other sample type. Profile 'f' was detected in one to four cadavers at both time points and all the plot types investigated. The remaining seven profiles were detected in one to three of the four sample types. Profile 'e' was exclusively detected in a *paf* plot in 2006, while profile 'h' was exclusively detected in a *pa* plot in 2006. Profile 'k' was exclusively detected in a *p* plot in 2007, and profile 'j' was detected in a *paf* and a *p* plot in 2007.

sample type	PnSNP												SNP profile type
	1*	2*	3	4*	5	7*	8	9	10*	11	12	13*	
	RPB1	RPB2		BTUB		EFL			LSU		SSU	ITS	
ARTpn1	Y	T	T	G	T	A	C	T	G	G	Y	W	a
cadaver 1	C	T	T	R	Y	T	C	T	R	R	Y	A	b
cadaver 2	C	Y	T	R	Y	T	C	T	R	R	Y	A	c
cadaver 3	T	Y	T	R	Y	T	C	T	R	R	Y	A	d

Figure 4.2. Alleles of 12 SNPs and resulting SNP profiles detected in an *ARTpn1*-infected aphid cadaver and three cadavers collected around the experimental field. Alleles G and T are displayed in light grey squares; alleles C and A are displayed in dark grey squares, and the mixed alleles are displayed in white squares. Letter Y indicates T/C mixed alleles, letter R indicates G/A mixed alleles, and letter W indicates T/A mixed alleles. The six SNPs selected for analyzing *Pandora neoaphidis* strains in infected aphid cadavers collected from the plots are indicated with *.

sample type	PnSNP						SNP profile type
	1	2	4	7	10	13	
	RPB1	RPB2	BTUB	EFL	LSU	ITS	
ARTpn1	Y	T	G	A	G	W	a
paf plots - October 06	Y	T	G	A	G	W	a
	Y	T	G	A	G	W	a
	Y	T	G	A	G	W	a
	Y	T	G	A	G	W	a
	Y	T	G	A	G	W	a
	Y	n.d.	G	A	G	W	(a)
	Y	Y	R	W	G	W	e
	C	Y	A	T	G	A	f
pa plots - October 06	C	Y	A	T	G	A	f
	C	Y	A	T	G	A	f
	C	Y	G	T	R	A	g
	C	Y	G	T	R	A	g
	C	Y	R	T	R	A	c
	C	Y	R	T	R	A	c
	C	Y	R	T	G	A	h
	C	C	A	T	G	A	i
paf plots - June 07	C	Y	A	T	G	A	f
	C	Y	A	T	G	A	f
	C	Y	A	T	G	A	f
	C	Y	A	T	G	A	f
	C	Y	R	T	R	A	c
	C	C	A	T	G	A	i
	C	C	A	T	G	A	i
	C	T	A	T	R	A	j
p plots - June 07	C	Y	A	T	G	A	f
	C	Y	A	T	G	A	f
	C	Y	G	T	R	A	g
	C	Y	G	T	R	A	g
	C	Y	G	T	R	A	g
	C	C	A	T	G	A	i
	C	T	A	T	R	A	j
	C	Y	A	T	R	A	k

Figure 4.3. Alleles of six SNPs and resulting SNP profiles detected in *Pandora neoaphidis* infected aphid cadavers collected in the *paf* (plants, aphids and fungus), *pa* (plants and aphids), *p* (plants) plots at different time points (*paf* and *pa* plots in October 2006; *paf* and *p* plots in June 2007). Alleles G and T are displayed in light grey squares; alleles C and A are displayed in dark grey squares, and the mixed alleles are displayed in white squares. Letter Y indicates T/C mixed alleles, letter R indicates G/A mixed alleles, and letter W indicates T/A mixed alleles. Profiles ‘‘a” and ‘‘c” are consistent with profiles ‘‘a” and ‘‘c” in Figure 4.2 but based only on the six SNPs used in this analysis: PnSNP1, PnSNP2, PnSNP4, PnSNP7, PnSNP10, and PnSNP13. Profile ‘‘a” is consistent with profile ‘‘a” except for PnSNP2 for which no data (n.d.) is available.

4.5. Discussion

After introduction into the *paf* plots in fall 2006, strain *ARTpn1* established rapidly and within 3 weeks aphid populations in these plots decreased. At the same time, a considerable amount of *P. neoaphidis* inoculum accumulated on the soil and provided optimal conditions for investigating the winter-survival of this fungus in topsoil. The dynamics of fungal infection and aphid populations in the *pa* plots, in which *P. neoaphidis* naturally established, was different than the dynamics observed in the *paf* plots, in which the fungus was introduced (Table 4.1). SNP analyses indicated that fungal strains detected in the *pa* plots in fall 2006 represented naturally occurring strains, as none of their SNP profiles (f, g, c, h, and i) matched profile 'a', which is characteristic for strain *ARTpn1* (Figure 4.3). Airborne conidia of *P. neoaphidis* measuring less than 30 µm (Keller, 1991) could easily penetrate the 200 µm mesh fabric covering the plots. Therefore, cross-contamination from the *paf* to the *pa* plots was a possible reason for fungal infections observed in the *pa* plots. However, the genotyping results clearly ruled out the possibility of cross-contamination. Particularly, PnSNP7 unequivocally distinguished the naturally occurring *P. neoaphidis* strains from the inoculated strain *ARTpn1* (Figure 4.3). The allele 'A' of PnSNP7 was characteristic of *ARTpn1*, whereas the allele 'T' was characteristic of the naturally occurring strains. One sample only displayed simultaneously both alleles, i.e., mixed alleles (Chapter 3).

The presence of mixed alleles has been reported previously and implies the presence of multiple copies of the target loci (Chapter 2). It has been speculated that this could be due to a heterokaryotic stage of the fungus, variations within a single genome or the presence of multiple strains within one single aphid cadaver.

However, the genetic background for this observation is still unclear and awaits further investigation. In our study, mixed alleles were observed in 27 % of the SNP data obtained from the field experiment. They were present in all six investigated SNPs, and in eight of the nine profiles identified from the 32 aphid cadavers that were collected in October 2006 and June 2007.

Even though prevalence of *P. neoaphidis* infection rapidly increased in the *pa* plots, the efficacy of the fungus was not sufficient to reduce the aphid populations as observed in the *paf* plots (Table 4.1). This may be a result of the delayed onset of natural *P. neoaphidis* infections in the *pa* plots. As a consequence, aphid populations continuously increased reaching more than 10,000 individuals per plot by the end of October. However, this population increase also represented an improved host availability for *P. neoaphidis*, which may have resulted in increased infection and inoculum production and may explain the generally higher inoculum concentration in the soil of the *pa* than in the *paf* plots in November (Figure 4.1). Results indicate that time point and severity of *P. neoaphidis* infection appear to be critical for successful aphid control. The importance of the coincidence of sufficient numbers of aphids and sufficient amounts of fungal inoculum has previously been suggested as a critical factor for initiation of epizootics (Barta and Cagan, 2003; Wilding et al., 1986). The fast and efficient aphid control in the *paf* plots resulted in less damage to the host plants, demonstrating the potential of *P. neoaphidis* in plant protection strategies against aphids. Such positive effects on plant fitness have also been observed by Wilding (1981). He reported that yield in bean plots infested with aphids was two times lower than in plots in which entomophthoralean species, including *P. neoaphidis*, were introduced in the form of living infected aphids or fungal-killed cadavers. However, when applying the same strategy in two successive years no

difference in bean yield was observed even though fungus applications reduced the aphid populations (Wilding, 1981; Wilding et al., 1986).

Monitoring of *P. neoaphidis* concentrations in topsoil layers of the *paf* and *pa* plots revealed a significant decrease during winter and spring in both plot types (Figure 4.1). In spring 2007, a very low level of *P. neoaphidis* was detected with the quantitative PCR approach, whereas with the bioassay the fungus was not detected anymore. The field bioassay initiated in spring 2007 showed the establishment of new *P. neoaphidis* infections that might suggest overwintering of strain *ARTpn1* in the *paf* plots. However, infections were also detected in the *p* plots, which had not been inoculated in 2006. In addition, aphid populations released in the bioassay developed and remained uninfected for 2 months, which implies that no inoculum was present in the plots at the initiation of the bioassay. Furthermore, SNP analysis performed on aphid cadavers from the field bioassay experiment revealed the presence of exogenous *P. neoaphidis* strains, which clearly differed from the released strain *ARTpn1*. In combination, these data indicated that strain *ARTpn1*, even though present with high inoculum concentration in the topsoil in fall 2006, did not survive the winter in the experimental plots and that infections observed in 2007 were derived from naturally occurring strains.

Only few studies have evaluated overwintering of *P. neoaphidis* in field soil. Fournier et al. (Chapter 2) detected overwintering *P. neoaphidis* genetic material in soil of a nettle patch using a PCR-based approach. However, this method does not provide information on the viability and pathogenicity of the material detected. On the other hand, bioassays as performed by Baverstock et al. (2008) and Nielsen et al. (2003) provide information on viability and activity of *P. neoaphidis* inoculum present on the soil. Baverstock et al. (2008) have shown that *P. neoaphidis* may completely lose its

activity in field margin soils after a 49-day incubation during winter. This supports the data obtained in our study that suggested the disappearance of *P. neoaphidis* from soil during winter. In contrast, Nielsen et al. (2003) detected active *P. neoaphidis* inoculum in spring, which was able to infect aphids after overwintering on soils of agricultural fields that had been cultivated with organically grown winter wheat during the previous season, as well as on soils collected in permanent grass areas and underneath bird cherry trees (*Prunus padus* L.). Moreover, it has been reported that under controlled laboratory conditions, *P. neoaphidis* inoculum that has been deposited on the soil and maintained at 5 or 4 C has remained active and has retained infectivity for aphids for up to 64 days (Nielsen et al., 2003) and 80 days (Baverstock et al., 2008), respectively.

Several factors may be responsible for the discrepancies observed between the different laboratory and field studies that have investigated winter-survival of *P. neoaphidis* in soil. As suggested by Baverstock et al. (2008), it is likely that experiments performed under controlled conditions resulted in over-estimation of the activity of *P. neoaphidis* propagules present in soil, as they were not exposed to the negative effects of abiotic factors, such as high temperatures, high solar radiation, and low humidity (Baverstock et al., 2008; Fargues et al., 1996; Nielsen et al., 2003; Steinkraus, 2006). Field experiments performed in the different studies contained different plant communities, which may have resulted in different microclimatic conditions. For instance, the plant canopy of dense nettle plant stands (Chapter 2) may allow higher humidity and better protection from radiation than the loose stands of lucerne plants investigated in the current study. Furthermore, the effects of the overall climatic conditions on winter-survival of *P. neoaphidis* are not known and have not been assessed in the different studies. For instance, the field experiments

performed in this study took place during the exceptional period of fall 2006 to spring 2007, one of the warmest on record in Switzerland (Beniston, 2007; Luterbacher et al., 2007; Rutishauser et al., 2008). These exceptional environmental conditions may have had a negative impact on the survival of *P. neoaphidis*. Although not yet assessed, another factor to be considered is a possible variation among individual strains in their ability to survive the winter in the absence of their hosts. Strain *ARTpn1* was isolated from an infected aphid and selected without prior assessment of any physiological characteristics; e.g., its potential to survive low temperatures. It was maintained exclusively by regular passages through aphids to maintain its virulence. Cultivation on artificial media was avoided as it has been reported as a cause of loss of virulence of entomopathogenic fungi (Butt et al., 2006; Hajek et al., 1990; Shah et al., 2007).

Four different SNP profiles (c, f, i, and j) were detected in the infected cadavers collected from the field bioassay in the *paf* plots in June 2007. Among these profiles, profile 'f' was the only one, which was also detected in one of the *paf* plots in fall 2006. Therefore, this strain may have survived the winter in the plot. However, in fall the fungus was not detected in the topsoil of the *p* plots (Figure 4.1) and in spring 2007 five different profiles (f, g, i, j, and k) were obtained in these plots (Figure 4.3). These findings suggested that the inoculum detected in 2007 with the field bioassay in the *p* plots and likewise in the *paf* plots represents inoculum that originated from outside of the plots and penetrated the 200 µm mesh fabric covering the plots. Propagation of *P. neoaphidis* infection through airborne conidia is a well-known phenomenon, in which conidia are actively discharged from infected insect cadavers to break through the boundary layer of the plant canopy and enter the airstream (Hemmati et al., 2001b; Steinkraus et al., 1993, 1996). As suggested by Baverstock

et al. (2008), *P. neoaphidis* may remain active locally through the combination of continuous infections of overwintering aphid hosts present for example in natural areas, and the activity of fungal inoculum deposited on substrates such as soil, on which it may remain active for a certain period of time. Inoculum can be disseminated through wind, or can be carried by infected aphids, or co-occurring predators carrying conidia (Roy et al., 2001). Alternatively, *P. neoaphidis* strains may originate from distant regions where milder climatic conditions allow a constant presence of aphid populations and therefore also of *P. neoaphidis* populations. For example, Feng et al. (2007) have shown that *P. neoaphidis* can disseminate through migratory flight of infected alate aphids over distances that exceed 1000 km.

Quantitative PCR, as well as the laboratory bioassay, allowed reliable detection of *P. neoaphidis* abundance in fall 2006, as revealed by the positive correlation between the data derived from both methods. However, in spring 2007, when *P. neoaphidis* abundance was low, the fungus was detected with PCR but not with the laboratory bioassay. This discrepancy may be due to less sensitivity of the laboratory bioassay approach compared to the PCR approach. On the one hand, the bioassay may be less robust as the factors required for the inoculum to infect aphids are not yet well-understood and controlled (Chapter 2; Baverstock et al., 2008; Nielsen et al., 2003, 2008). On the other hand, PCR detects not only virulent but also non-virulent and possibly dead or dormant fungal material. Furthermore, handling of the topsoil may have affected accessibility of the inoculum in the bioassay while this aspect may not be relevant in the PCR approach, which is based on soil homogenization and bulk DNA extracts.

The controlled field experiment in combination with molecular tools and bioassays demonstrated that artificial introduction of *P. neoaphidis* can result in efficient aphid

population control and can be beneficial for host plant survival. However, results indicated that the introduced strain *ARTpn1* did not survive the winter in soil and that the strains present in the following spring were derived from outside the plots. Further experiments are required to assess the role of soil characteristics and natural factors on the survival mechanisms of *P. neoaphidis* and to investigate whether strain-specific aspects affect winter-survival.

4.6. Acknowledgments

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Chapter 5:

General Discussion

5. General Discussion

Conservation biological control (CBC), which is defined as 'modification of the environment or existing practices to protect and enhance specific natural enemies or other organisms to reduce the effect of pests' (Eilenberg et al., 2001) is a promising strategy for the control of aphids with *P. neoaphidis*. Natural and semi-natural landscape elements such as field margins, natural meadows, and nettle patches have generated interest for their roles as overwintering sites and reservoirs for aphid-pathogenic entomophthoralean fungi (Keller and Suter, 1980; Barta and Cagan, 2003; Shah et al., 2004; Ekesi et al., 2005; Baverstock et al., 2008). The 'migration model' suggests that after overwintering in natural and semi-natural areas, new infection cycles of *P. neoaphidis* are initiated in such areas and subsequently spread into pest aphid populations in adjacent crop fields. To demonstrate the validity of the 'migration model' as well as to integrate such a model into practical CBC management applications, various aspects of *P. neoaphidis* ecology remain to be elucidated, particularly its overwintering strategies and subsequent dissemination in spring.

A substantial part of the work presented in this thesis was dedicated to the development and validation of tools that aim for efficient monitoring of *P. neoaphidis* in the environment (Chapters 2, 3, 4). The developed monitoring tools which can be categorized as detection, quantification, and genotyping tools were implemented for investigating the winter persistence of *P. neoaphidis* in topsoils of a natural nettle patch (Chapter 2) and of field plots (Chapter 4). This discussion reviews the most important features of the monitoring tools that were developed and focuses on the

new insights that were obtained into the ecology of *P. neoaphidis*, including its persistence, dispersal, and transmission.

5.1. Tools for monitoring of *P. neoaphidis* in the environment

5.1.1. Detection of *P. neoaphidis* in the environment

A cultivation-independent PCR approach has been developed by Tymon (2004) to monitor *P. neoaphidis* in the environment. This approach was based on a *P. neoaphidis*-specific primer combined with a universal fungal primer for the specific detection of *P. neoaphidis* in infected host cadavers. This primer pair was assessed for applicability to detect *P. neoaphidis* in soil DNA extracts (Chapter 2). However, its specificity was not sufficient as unspecific PCR amplicons were obtained. In Chapter 2, a new cultivation-independent PCR-based approach was developed that relies on primer pairs that specifically target the ribosomal RNA (rRNA) gene cluster of *P. neoaphidis*. The specificity of these primers was high enough to allow specific detection of *P. neoaphidis* in various environmental sample types such as aphid cadavers, plant material, and complex substrates such as soil that contains DNA from a wide variety of microorganisms. PCR results obtained in Chapter 2 suggested that material of *P. neoaphidis* was present in the soil of a nettle patch from fall 2004 until spring 2005. Although the PCR-based approach allowed specific and rapid detection of *P. neoaphidis* DNA, it did not provide information on the viability and infectivity of the overwintering fungal material detected in spring. To complement this approach, a biossay was developed that allowed the assessment of the infectivity of the fungal material present in soil (Chapter 4).

5.1.2. Quantification of *P. neoaphidis* in soil

Performing bioassays is currently the only approach available for quantifying the infectivity of *P. neoaphidis* in the environment (Chapter 4). However, it is labour intensive and may lack robustness as the factors involved in the infection process are not yet fully understood (Nielsen et al., 2003; Baverstock et al., 2008; Nielsen et al., 2008). A PCR-based approach is simpler and more reliable as it is based on well-established protocols for DNA extraction and PCR amplification. Therefore, a quantitative PCR (qPCR) approach was developed based on the *P. neoaphidis* specific primers designed in Chapter 2, to allow quantification of *P. neoaphidis* genetic material present in soil (Chapter 4). The qPCR allowed reliable quantification of *P. neoaphidis* in the topsoil of the plot experiment and it revealed the decrease of *P. neoaphidis* abundance from fall 2006 until spring 2007. It correlated positively with the laboratory bioassay indicating robustness of both approaches.

The estimated rRNA gene cluster copy number in fungi has been estimated to be in the range of 50 to 200 copies per genome (Garber et al., 1988; Maleszka and Clark-Walker, 1992; Tsuchiya and Taga, 2001; Atkins et al., 2003; James et al., 2009). However, it has not been determined yet for *P. neoaphidis* or for other entomophthoralean fungi. Thus, the qPCR-based detection system developed in Chapter 4 allows only relative comparisons of fungal population sizes among different samples or time points. In a next step, soil samples could be inoculated with defined amounts of conidia and subsequently analyzed with the qPCR approach. In this way, the data obtained with qPCR, expressed in number of gene copies per gram soil could be correlated to the number of conidia present in the sample. As *P. neoaphidis* conidia contain one nucleus per conidia, the number of gene copies per genome could be determined.

5.1.3. Cultivation-independent genotyping of *P. neoaphidis*

A key requirement for assessing the performance of specific biological control agents in the environment is the development of genotyping tools that allow the differentiation between strains (Avis et al., 2001; Hermosa et al., 2001). With such tools, specific strains can be characterized and monitored in the environment and used to explore so far unknown stages of their life cycles. Population structures of the organism can be analyzed, which may contribute to a better understanding of its ecology. Preferably, such genotyping tools are cultivation-independent to allow efficient analysis of the organisms without tedious cultivations steps. Various genotyping tools have been applied to *P. neoaphidis*, however, they were either not discriminative enough (Chapter 2; Rohel et al., 1997; Nielsen et al., 2001; Tymon et al., 2004) or they were not applicable for the cultivation-independent detection of the fungus (Rohel et al., 1997; Sierotzki et al., 2000; Nielsen et al., 2001; Tymon et al., 2004; Tymon and Pell, 2005).

Simple sequence repeat (SSR) genotyping approach allows high-resolution identification at strain level and can be used for cultivation-independent applications. Therefore, the development of SSR markers was initiated for *P. neoaphidis* in the frame of this thesis (Fournier, unpublished). Four types of SSR-enriched clone libraries (GAA, CAA, GA, CA) were constructed and 21 SSR clones were isolated. However, no polymorphisms were detected among the different *P. neoaphidis* strains tested. Three additional clone libraries (CA, TAC, GATA) were constructed and screened by a specialized company (Ecogenics GmbH, Zurich, Switzerland) but no markers that were reliably amplifiable by PCR were identified. SSR markers have been reported to be more difficult to isolate from fungi then from other organisms, however the reason for this discrepancy is not known (Dutech et al., 2007). As

alternative to SSR markers, 13 single nucleotide polymorphisms (SNP) markers were isolated from 6 (RPB1, RPB2, EFL, LSU, SSU, ITS) loci that are investigated in the frame of the project 'Assembling the Fungal Tree of Life' (AFTOL; James et al., 2006), as well as from the β-tubulin gene which is not part of the AFTOL project but has been investigated in several fungal phylogenetic studies (e.g. Moon et al., 2002; Keeling, 2003).

PCR-RFLP analyses (Chapter 2) and SNP analyses (Chapters 3 and 4) performed on DNA extracted from fungal cultures (Chapters 2 and 3) and from environmental samples including plant (Chapter 2), soil (Chapters 2 to 4), and infected aphid cadavers (Chapter 2 to 4) all revealed the presence of SNPs displaying single alleles but also two alleles simultaneously. The simultaneous presence of two alleles has been referred to as 'mixed alleles'. Mixed alleles were found in all seven loci in 12 out of 13 SNPs that were analyzed. They were present in the 19 *P. neoaphidis* strains used to develop the SNP assay (Chapter 3), and in 28 out of 32 infected aphid cadavers collected from the field experiment (Chapter 4). On average, they represented 27% of the SNP data obtained in both studies. The presence of mixed alleles implies that the samples investigated contained more than one copy of the target loci, despite the fact that *P. neoaphidis* is haploid. As described in Chapters 2 and 3, several hypotheses may explain the occurrence of mixed alleles including e.g. the presence of multiple strains within single aphid cadavers, the presence of several nuclear variants in a given mycelium resulting from the formation of vegetative heterokaryons within infected insects, or the occurrence of multiple copies of the same locus, as reported for instance for the rRNA gene cluster (Pawlowska and Taylor, 2004; Rooney and Ward, 2005). Prior to the studies presented in Chapters 2, 3, 4, mixed alleles have never been documented for this fungus. This is due to the

fact that the genotyping approaches that have been used including RAPD, ERIC-PCR, and ISSR-PCR were not sequence-based, and therefore, did not allow the detection of mixed alleles.

Although the occurrence of allele mixes needs to be carefully investigated, the SNP genotyping approach is currently the best available tool for investigating *P. neoaphidis* ecology, as it allows discrimination of *P. neoaphidis* strains and their cultivation-independent monitoring in the environment. For instance, the resolution of the genotyping assay allowed the discrimination of 15 SNP profiles in a worldwide collection of 19 strains (Chapter 3) and of 9 SNP profiles in a collection of 32 infected aphids cadavers collected within one field (Chapter 4). This monitoring tool will be of great use for assessing the 'migration model', as it will allow detection and monitoring of specific strains in the different locations and host species.

5.2. New insights into the ecology of *P. neoaphidis*

5.2.1. Persistence of *P. neoaphidis* in soil

Unlike for hypocrealean fungi such as *Beauveria* spp. and *Metarhizium* spp., there are no reports on soil serving as habitat for *P. neoaphidis* and other entomopthoralean fungi, which are generally infecting foliar insects (Pell et al., 2010). It seems that soil is rather an environment in which entomophthoralean fungi persist for a certain period of time for example when their hosts are absent (Nielsen et al., 2008; Pell et al., 2010). Soil could serve as reservoir in which fungal inoculum is picked up from aphids and their predators and brought to the leave areas in which most of the aphid hosts are located (Nielsen et al., 2008; Baverstock et al., 2008; Pell et al., 2010).

An important aspect of this thesis was the assessment of soil as a possible overwintering site for *P. neoaphidis* inoculum. Several ecological studies have indicated that soil may play a role in the survival of *P. neoaphidis* during winter when host aphid populations are generally absent (Chapter 1; Nielsen et al., 2003; Baverstock et al., 2008; Nielsen et al., 2008). Therefore, soil might be an important factor to consider for designing future CBC strategies that promote the occurrence of *P. neoaphidis.* Overwintering material of *P. neoaphidis* was detected in the soil of a natural nettle patch (Chapter 2) but not in the soils of the plot field experiment (Chapter 4), even though these soils were inoculated with massive amounts of fungal inoculum in fall. The biological activity and virulence of *P. neoaphidis* material detected in the soil of the nettle patch was not assessed, as the bioassay was not available at that time (Chapter 2). Therefore, it is not know whether the material detected with PCR in the soil of the nettle patch was pathogenic and viable. On the other hand, the fungal inoculum present in the soil of the nettle patch might have persisted during winter, whereas the one present in the soil of the field plot did not survive. The discrepancy in *P. neoaphidis* survival observed between both habitats might fit to the 'migration model', in which beneficial organisms may not overwinter in the field but in neighbouring natural and semi-natural areas. It is possible that the plot design in the field experiment was not appropriate for the survival of the fungus in the soil (Chapter 4). For instance, plant coverage (16 lucerne plants per 0.16 m^2) may have been too sparse to provide optimal conditions for the survival of fungal inoculum. Therefore, fungal material might have been directly exposed to UV radiations and dry conditions which are known to have a negative impact on the survival of entomopathogenic fungi (Fargues et al., 1996; Nielsen et al., 2003; Steinkraus, 2006; Baverstock et al., 2008). Reducing plant spacing might have

created more favorable microclimatic conditions due to increased plant canopy cover. This might have improved the protection against UV and increased the ambient humidity. Unlike the plants in the field plots, nettle plants were high and close to each other. Moreover, plant debris that are accumulating on the soil of the nettle patch may provide a direct protection against solar radiations and help to maintain a certain humidity in the topsoil level. Another characteristic of nettle patches that may be beneficial for survival of *P. neoaphidis* is the fact that they host the aphid species *Microlophium carnosum*, which is susceptible to several entomophthoralean diseases including *P. neoaphidis* (Keller, 1991; Barta and Cagan, 2003) and therefore may serve as a reservoir for the fungal inoculum.

As discussed in Chapter 4, the period of fall 2006 to spring 2007 was one of the warmest on record in Switzerland (Beniston, 2007; Luterbacher et al., 2007; Rutishauser et al., 2008). This may have had a negative impact on the survival of *P. neoaphidis* on soil. The winter 2006 to 2007 was so mild that anholocyclic populations of aphids have been observed on crops in Switzerland. For instance, *Sitobion avenae, Rhopalosiphum padi* and *Metopolophium ssp.* have been detected on barley (personal communication, Gabriel Goy, Agroscope Changins-Wädenswil). This is rather unusual as these species normally overwinter as fertilized egg (holocyclic) in temperate regions with cold winters like Switzerland (personal communication, Gabriel Goy). Survival strategies of *P. neoaphidis* might possibly vary depending on the environmental conditions. Possibly, in cold winter conditions when no aphid hosts are present, survival of *P. neoaphidis* might only be possible on soil, whereas under milder conditions when aphid populations may overwinter as larval, nymphal, or adult stages, *P. neoaphidis* may survive by continuous infections. Baverstock et al. (2008) suggested that *P. neoaphidis* could remain active through

the combination of both continuous infection of aphid hosts and as inoculum deposited on soil. Such overwintering strategies need to be further investigated to allow development of CBC management approaches against aphids.

5.2.2. Dispersal and transmission of *P. neoaphidis*

A crucial point to consider when developing CBC strategies in which natural or semi-natural habitats are used as reservoir of antaganonists is the capacity of these organisms to disperse into adjacent crops and to be transmitted to the pest insects present in the crops (Pell et al., 2010). It is known that inoculum of *P. neoaphidis* can efficiently disperse and be transmitted through several vectors including wind (e.g. Steinkraus et al., 1993; Steinkraus et al., 1996; Hemmati et al., 2001b), alate and apterous infected aphids (e.g. Feng et al., 2004; Feng et al., 2007), and co-occurring predators contaminated with conidia (e.g. Roy et al., 2001; Baverstock et al., 2008). Moreover, several studies have demonstrated that *P. neoaphidis* can be transmitted from one aphid species to the other (Shah et al., 2004; Ekesi et al., 2005). However, despite these data, there is no specific evidence for the ability of *P. neoaphidis* to disperse from aphids present in natural and semi-natural areas into pest aphid populations in adjacent crops. One of the main reasons this aspect has not been investigated was the lack of appropriate monitoring tools. In order to investigate the dispersal strategy of the fungus, it is necessary to monitor the fate of individual strains in the environment. The SNP genotyping assay that was developed in Chapter 4 fulfils this requirement and will be of great help in further studies addressing the issue of dispersal and transmission between different habitats.

The results obtained with the field experiment performed in Chapter 4 indicated that the strains detected in spring 2007 in the *paf* and *p* plots were originating from

outside the plots as five out of six strains were not detected in these plots in fall 2006. In this case, dispersal of *P. neoaphidis* in the plots has happened without insect vectors as the 200 µm mesh fabric covering the plots prevented any insect transit. However, airborne conidia of *P. neoaphidis* measuring less than 30 µm (Keller, 1991) can easily penetrate the 200 µm mesh fabric covering the plots and infect aphids present in the plots. These results clearly indicate the dispersal capacity of *P. neoaphidis*, which is essential for future use of this fungus in CBC strategies. The fact that local populations of *P. neoaphidis* became extinct in the plots during the winter and that the plots were subsequently re-colonized by other strains in spring might fit with the 'migration model' in which population developing in refuge areas spread into neighboring fields in spring. This might indicate the existence of metapopulations of *P. neoaphidis*. A metapopulation is defined as a 'set of local populations, which interact via individuals moving among populations' (Hanski, 1991). Metapopulation dynamics is typically characterized by interplays between local population extinction and re-colonization through dispersal (Hanski, 1991). Moreover, there was a great diversity in the strains that disseminated into the plot in spring 2007, as six different strains could be detected in 16 plots. However, the experimental setup used in the field study presented in Chapter 4 did not allow to determine the origins of the strains. Further studies are needed in which the SNP genotyping assay would be implemented to characterize and compare *P. neoaphidis* populations present in natural and semi-natural areas as well as in the adjacent crops, and to monitor the movement of single strains in the agro-ecosystem in order to assess whether these strains are able to disseminate among the different habitats.

Aside of the spatial aspects of dispersal and transmission, the temporal aspects are critical as well (Powell and Pell, 2007). For instance, an effective use of natural and

semi-natural refuges as reservoir for fungal inoculum would only be achieved if a good synchronisation exists between the dispersal of the aphids present in the refuge areas in spring (infection donors) and the occurrence of the pest aphids in the crops (infection recipient). Ideal timing would be reached if aphid population development in refuge areas would take place ahead of the development of pest aphid population development in crops. In this way, *P. neoaphidis* infection would rapidly get established in the refuge areas, and spread into the crops early enough to control aphid species of economic importance. The importance of the factors such as the 'temporal factor' and of the amount of inoculum present in the development of biological control strategies against aphids was demonstrated in the field experiment presented in Chapter 4. Less than 3 weeks after the artificial release of *P. neoaphidis* in the treated plots (*paf* plots), the aphid populations have collapsed to less than 1/10 of the populations of the control plots (*pa* plots), in which the fungus established naturally. *P. neoaphidis* took more time to establish in the *pa* plots and therefore was not able control the aphid populations in these plots and to prevent plant damage. On the other hand, the fast and efficient aphid control in the *paf* plots prevented damage of the host plants, demonstrating the potential of *P. neoaphidis* in plant protection strategies against aphids. This shows the importance for aphid control measures to be implemented early enough to have a significant impact on aphid populations. If the 'window of opportunity' is missed, aphid populations would continue to increase as observed in the *pa* control plots (Chapter 4).

5.3. Conclusions

There was a general need for efficient PCR-based tools to monitor *P. neoaphidis* in the environment. For instance, two recent studies, which have focused on assessing

the potential of *P. neoaphidis* in CBC strategies have pointed out that molecular techniques are required to understand the spatial structure and the dispersal characteristics of *P. neoaphidis* in the ecosystem (Ekesi et al., 2005) and to further assess the role of field margins on the overwintering mechanisms of *P. neoaphidis* (Baverstock et al., 2008). The monitoring tools developed in this thesis will allow the investigation of such open questions. The developed tools enable the specific detection of *P. neoaphidis* in diverse types of environmental samples by using end-point PCR with species-specific primers targeting the ribosomal RNA (rRNA) gene cluster of this fungus (Chapter 2); as well as the quantification of inoculum or genetic material in soil using the combination of quantitative PCR and bioassay approaches (Chapter 4); finally the SNP genotyping assay that was developed in Chapter 3 enables the discrimination of *P. neoaphidis* strains and their cultivation-independent monitoring in the environment (Chapters 3 and 4). Due to these features, the SNP genotyping assay is currently the best available tool for elucidating fundamental aspects of *P. neoaphidis* ecology.

The developed monitoring tools were implemented to investigate different aspects *of P. neoaphidis* ecology, including the role of natural and semi-natural areas as refuges for *P. neoaphidis* during winter, and the role of soil as winter survival matrix of the fungus when no hosts are present. The understanding of such processes is essential for validating the “migration model” and for assisting the future development and implementation of successful CBC strategies. Although a lot of work remains to be done on the topic, relevant insights were gained into the ecology of *P. neoaphidis*, especially about the persistence of the fungus in the environment as well as about its dispersal and transmission. For instance, the field experiments performed in Chapter 2 and 4 revealed discrepant results about the capacity of *P. neoaphidis* to overwinter

on soil depending on the type of habitat, as overwintering material was detected in the soil of the nettle patch habitat but not in the soil of the field plots. This discrepancy in *P. neoaphidis* survival might fit to the 'migration model' indicating that unlike field habitats, semi-natural nettle patch habitats might serve as refuge for *P. neoaphidis* in winter when conditions in the field are not favourable. Moreover, the results obtained in Chapter 4 also demonstrated the capacity of *P. neoaphidis* for dispersal and transmission, as this fungus was able to recolonize the field plots from outside the plots in spring after having disappeared during winter. The capacity to disperse from reservoir into adjacent crops constitutes an essential aspect of the "migration model", as without efficient dispersal from one area to an other, CBC strategies based on the use of reservoirs of fungal inoculum would not be applicable. The results obtained in Chapter 4 also show that not only spatial aspects of dispersal are critical for the controlling of aphids with *P. neoaphidis* but temporal aspects too. The importance of timing of *P. neoaphidis* infection as well as of the amount of inoculum present was demonstrated as the fungus could control the aphids in the treated plots (the *paf* plots) in which it was introduced early enough but not in the untreated plots (the *pa* plots) in which the natural infection occurred latter and could not control the growth of the aphid populations.

In this thesis, key aspects of the 'migration model' were investigated such as the persistence in soil, the ability to disperse, and the timing of *P. neoaphidis* infection. Many questions remain to be addressed about the life cycle and the ecology of *P. neoaphidis*, however, this thesis has provided powerful tools that will contribute to answer these questions by allowing accurate and rapid monitoring of *P. neoaphidis* in the environment.

5.4. Perspectives

By enabling cultivation-independent detection, quantification, and genotyping of *P. neoaphidis* inoculum in the environment, the monitoring tools developed in this thesis will allow investigation of fundamental questions about *P. neoaphidis* ecology. An important point that remains to be elucidated is the occurrence of mixed alleles found in all types of samples that were analyzed including plant and soil samples, infected aphid cadavers, and culture extracts (Chapters 2, 3, and 4). As *P. neoaphidis* is haploid, the presence of mixed alleles implies that the samples investigated contained more than one allele of the target loci and therefore multiple gene copies. A first step to perform would be to test whether the occurrence of mixed alleles is due to the presence of more than one strain in a single sample. This will require the development of a procedure to establish single spore cultures of *P. neoaphidis*.

In this thesis a quantitative PCR approach was developed which allows the rapid assessment of the amount of genetic material present in soil (Chapter 4). Due to its simplicity, this approach could be used to screen large numbers of samples, determining the amount of *P. neoaphidis* genetic material present before performing bioassay with the samples which contain significant amounts of the fungus. In this way, the number of samples to be analyzed with the bioassay, which is labor intensive, could be reduced. Therefore, by using the combination of both approaches, it would be possible to screen large numbers of soil samples for the presence and activity of *P. neoaphidis* material, improving the knowledge about soil as overwintering matrix for this fungus.

The fact that the SNP genotyping assay developed in Chapter 3 allows discrimination of *P. neoaphidis* strains and their cultivation independent monitoring, makes this tool particularly promising for the investigation and elucidation of fundamental questions

about the ecology of *P. neoaphidis*. Using this monitoring tool, it would be possible to conduct field studies to determine the genetic diversity of *P. neoaphidis* and the spatial and temporal distribution of the strains in the environment. Moreover, the pathogenicity of specific strains could be assessed by performing bioassays. The strains which would be particularly pathogenic and therefore possibly suitable for biological control applications could be identified and monitored in the environment without the requirement for cultivation steps. Moreover, experiments could be designed, which would investigate the persistence of specific strains of *P. neoaphidis* in the agro-ecosystem in order to understand the actual mechanisms of survival of this fungus. For this purpose, different potential habitats including natural and semi-natural areas as well as crop fields could be investigated by collecting infected aphid cadavers and/or directly environmental samples such as soil or plant material. By identifying the strains present in the different habitats, it would be possible to assess whether these strains are able to disseminate between habitats.

Although CBC strategies could be implemented as ‘stand alone’ strategies it would be certainly more efficient to combine them with other management strategies such as augmentation. For instance, in the field study described in Chapter 4 an augmentation strategy was used successfully for introducing *P. neoaphidis* in the field plots using living infected aphids. One would have to investigate how to use both approaches in conjunction.

A lot more work is still needed to understand the factors governing the survival and spread of *P. neoaphidis* in the environment and to use this knowledge for implementing CBC strategies against aphids. However, the monitoring tools developed in this thesis as well as the insights into *P. neoaphidis* ecology that were

gained by validating the tools make a substantial contribution to the development of efficient CBC strategies against aphids.

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Curriculum vitae

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Publications and Presentations

Publications

A.B. Jensen, A. Fournier, F. Widmer, S. Keller, J. Eilenberg, J. Enkerli., 2007. Molecular tools to study natural occurrence, ecology and phylogeny of Entomophthorales. IOBC WPRS Bulletin 30, 99-105.

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Fournier, A., Enkerli, J., Widmer, F., Keller, S., 2005. Molecular studies of the aphid pathogenic fungus *Erynia neoaphidis*: population diversity and ecology. 2nd Annual Symposium of the PhD Program in Sustainable Agriculture (ASPSA), November 4, Zurich, Switzerland. [Poster presentation]

Fournier, A., Widmer, F., Keller, S., Enkerli, J., 2007. Assessing the potential of *Pandora neoaphidis* for winter survival in soil applying soil baiting and molecular approaches. 3rd Annual Symposium of the PhD Program in Sustainable Agriculture (ASPSA), Zurich, Switzerland. [Oral presentation]

Fournier, A., Widmer, F., Keller, Enkerli, J., 2007. Assessing winter survival of *Pandora neoaphidis* in soil applying bioassay and molecular approaches. 11th European Meeting of the IOBC WPRS, working group 'Insect Pathogens and Insect Parasitic Nematodes', June 3-7, Ales, France. [Oral Presentation]

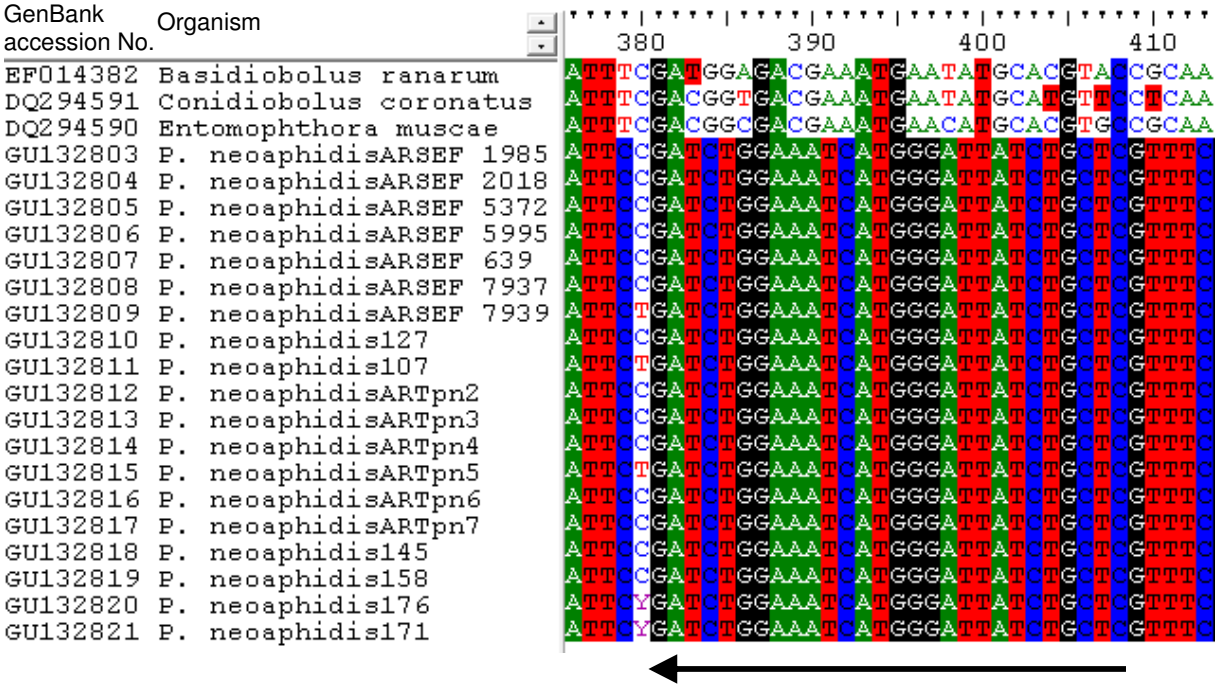
Fournier, A., Widmer, F., Keller, S., Enkerli, J., 2008. Monitoring of the aphid pathogenic fungus *Pandora neoaphidis* in the environment. MykologischesKolloquium beider Hochschulen, January 17, ETH Zurich, Zurich, Switzerland. [Oral presentation]

Fournier, A., Widmer, F., Keller, S., Enkerli, J., 2009. Winter survival of the entomopathogenic fungus *Pandora neoaphidis* in soil. 3rd Swiss Microbial Ecology (SME) Meeting, January 28-30, Einsiedeln, Switzerland. [Oral presentation]

Supplement

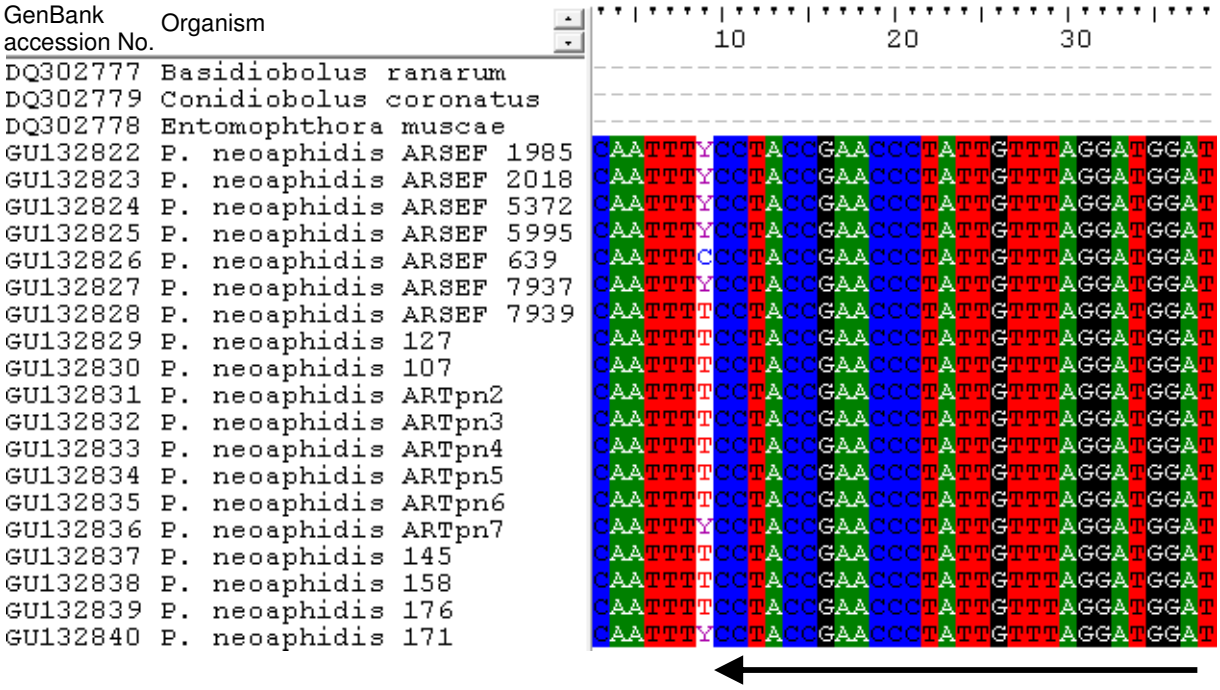
PnSNP1

SNP at position 380 of the largest subunit of nuclear RNA polymerase II (RPB1) sequence GU132803. Arrow indicates orientation and position of SNP primer PnSNP1.



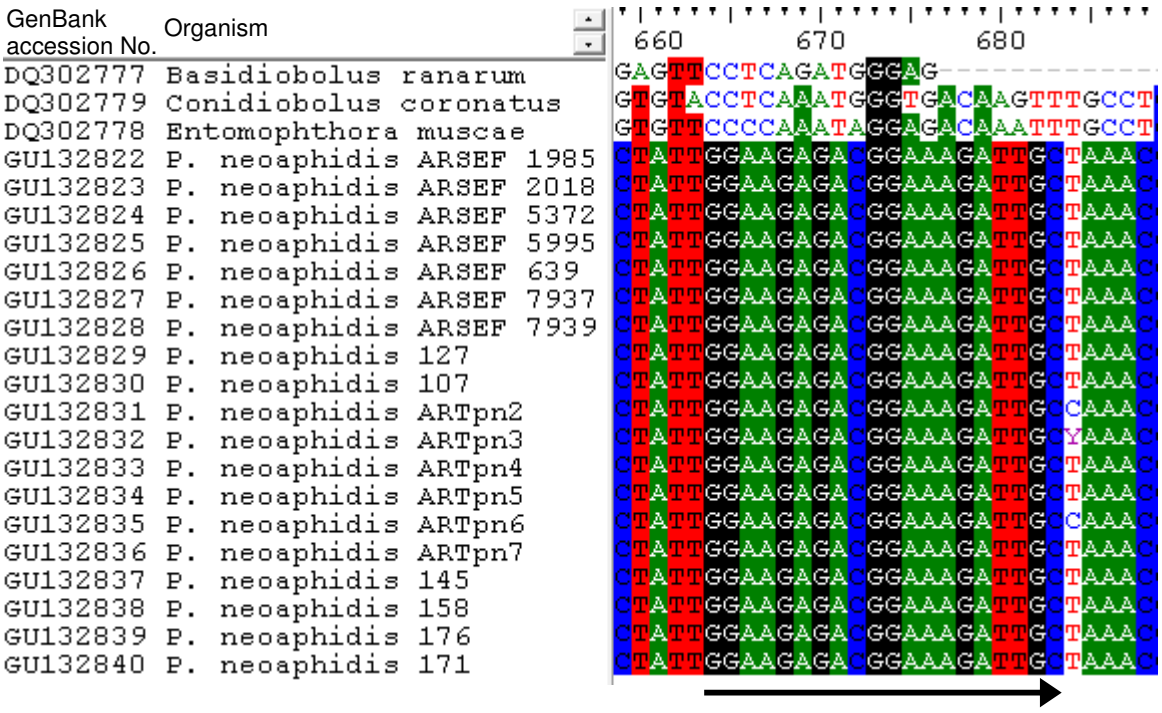
PnSNP2

SNP at position 9 of the second-largest subunit of nuclear RNA polymerase II (RPB2) sequence GU132822. Arrow indicates orientation and position of SNP primer PnSNP2.



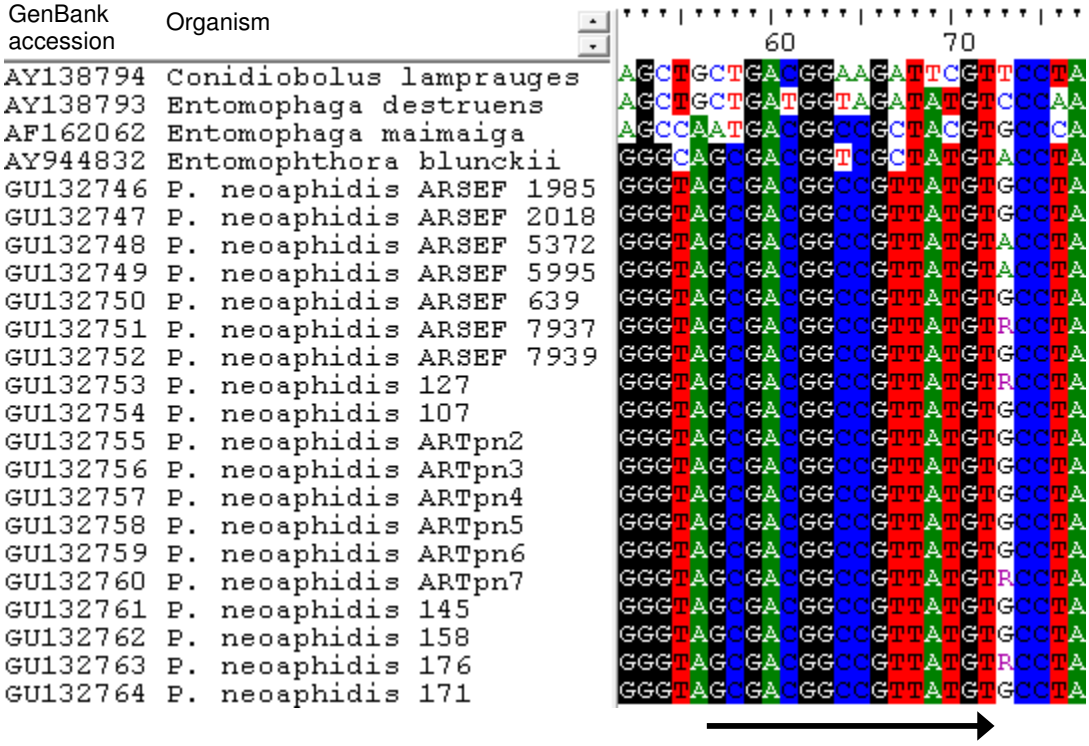
PnSNP3

SNP at position 684 of the second-largest subunit of nuclear RNA polymerase II (RPB2) sequence GU132822. Arrow indicates orientation and position of SNP primer PnSNP3.



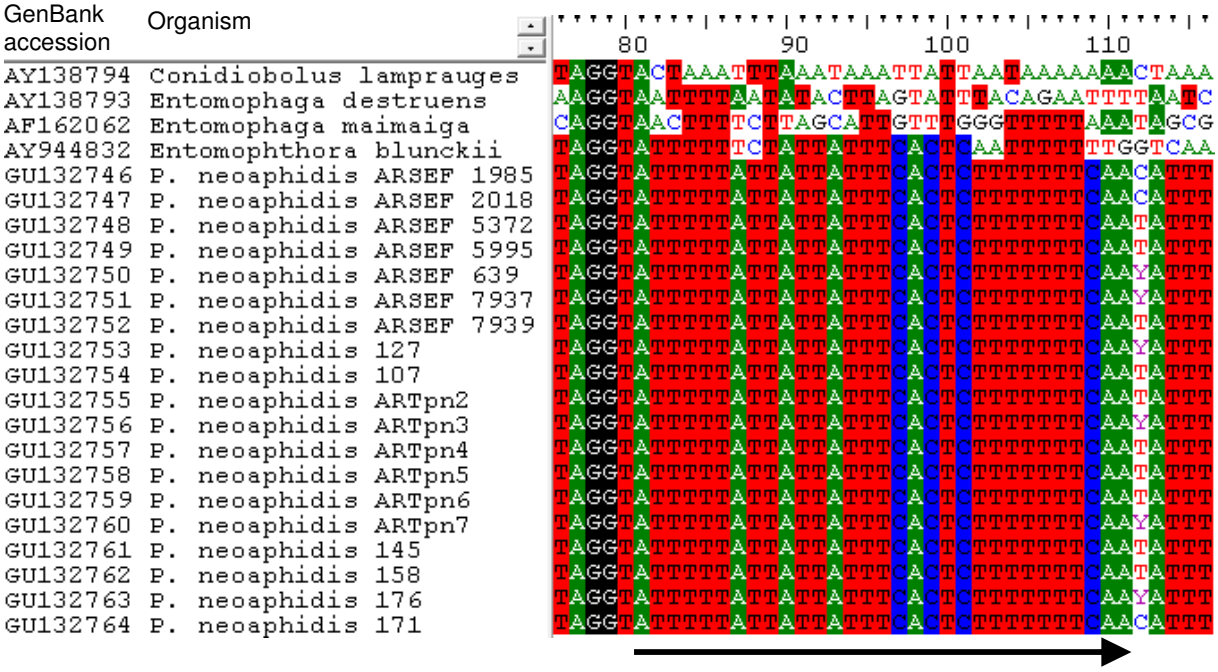
PnSNP4

SNP at position 73 of the β-tubulin (BTUB) sequence GU132746. Arrow indicates orientation and position of SNP primer PnSNP4.



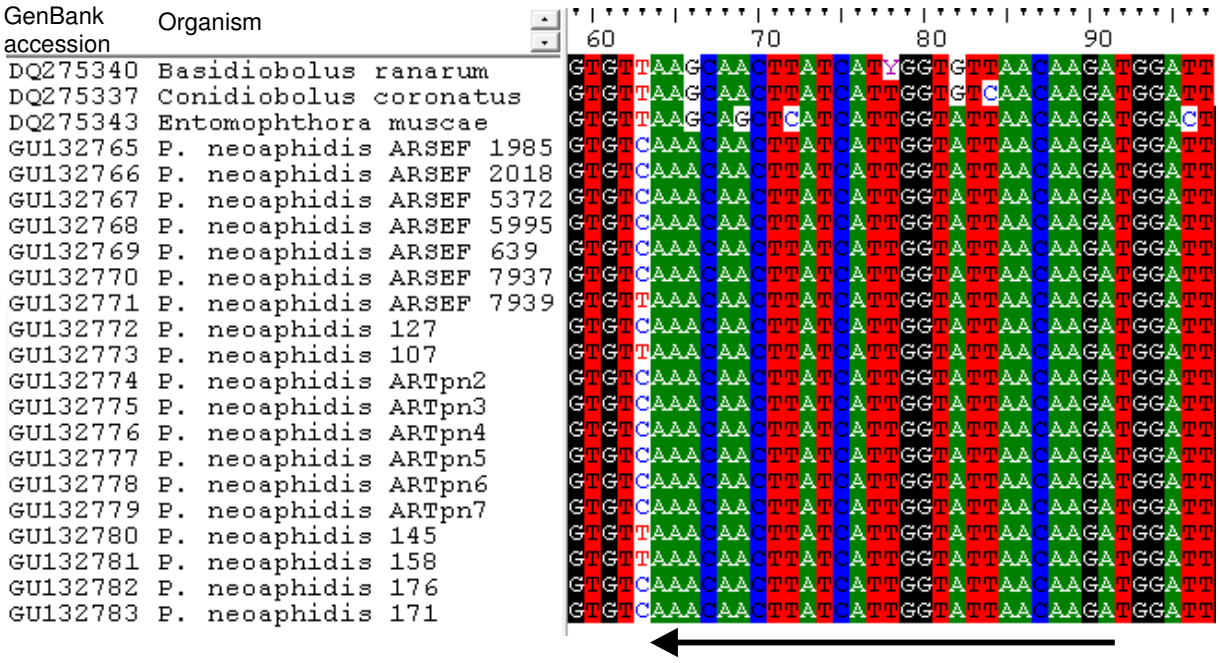
PnSNP5

SNP at position 112 of the β-tubulin (BTUB) sequence GU132746. Arrow indicates orientation and position of SNP primer PnSNP5.



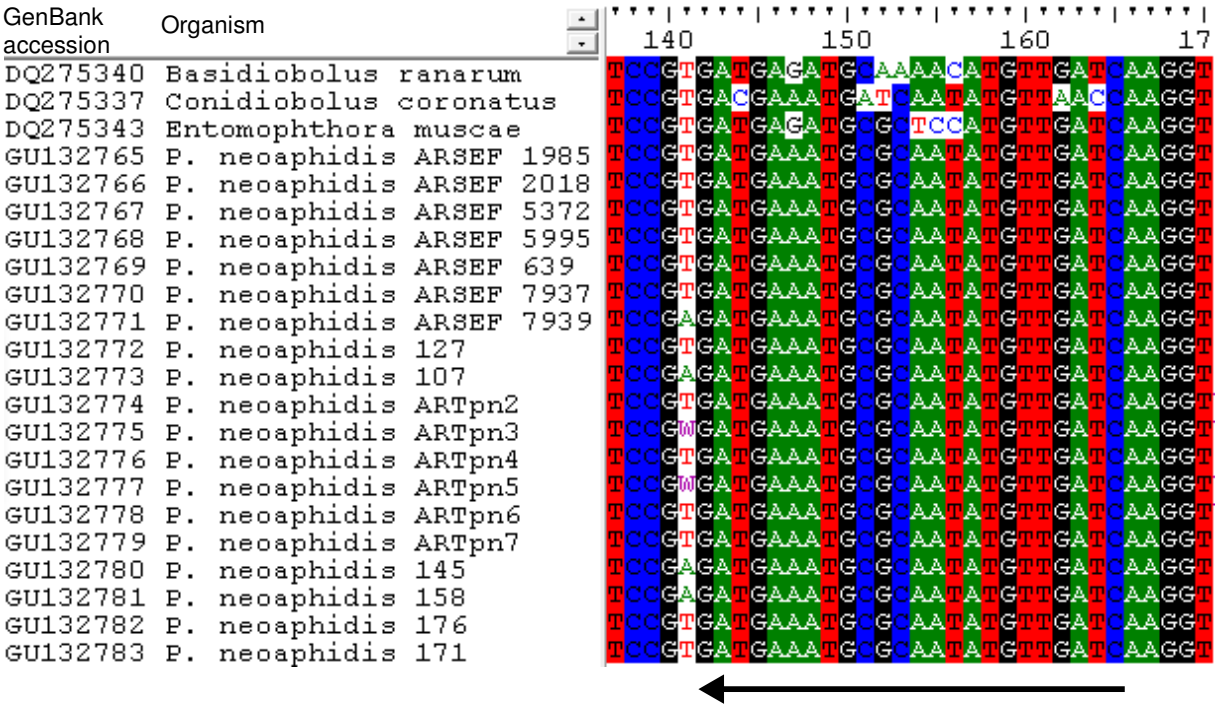
PnSNP6

SNP at position 63 of the elongation factor 1α-like (EFL) sequence GU132765. Arrow indicates orientation and position of SNP primer PnSNP6.



PnSNP7

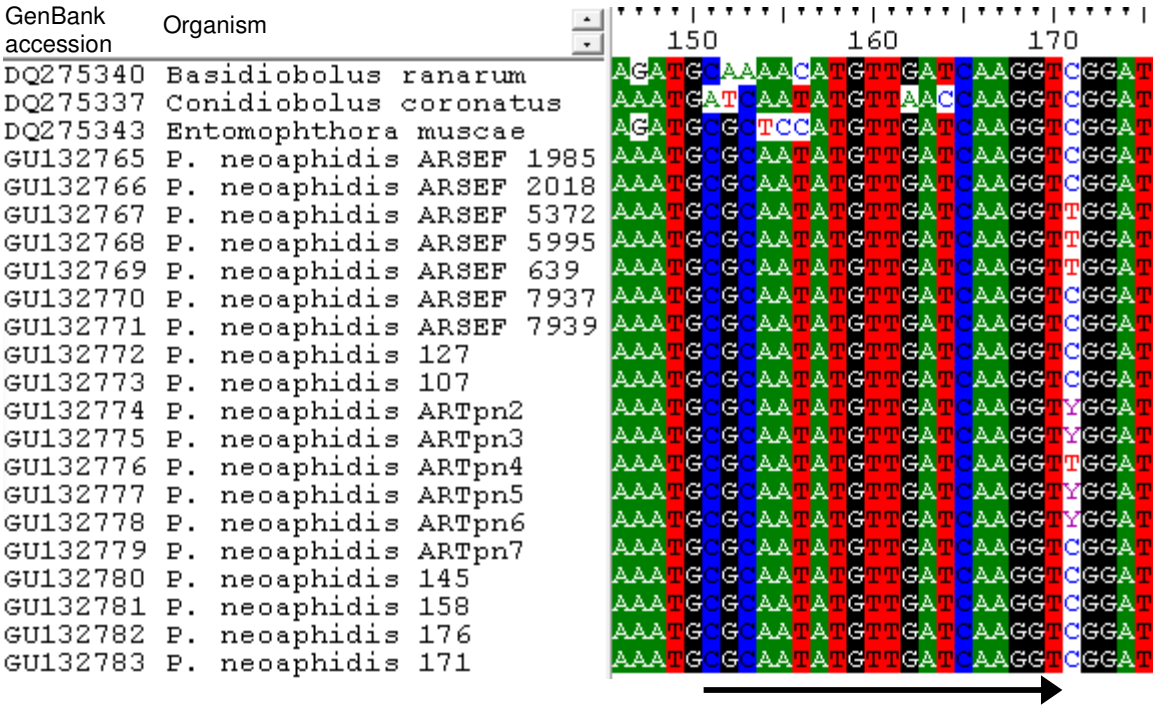
SNP at position 141 of the elongation factor 1α-like (EFL) sequence GU132765. Arrow indicates orientation and position of SNP primer PnSNP7.



PnSNP8

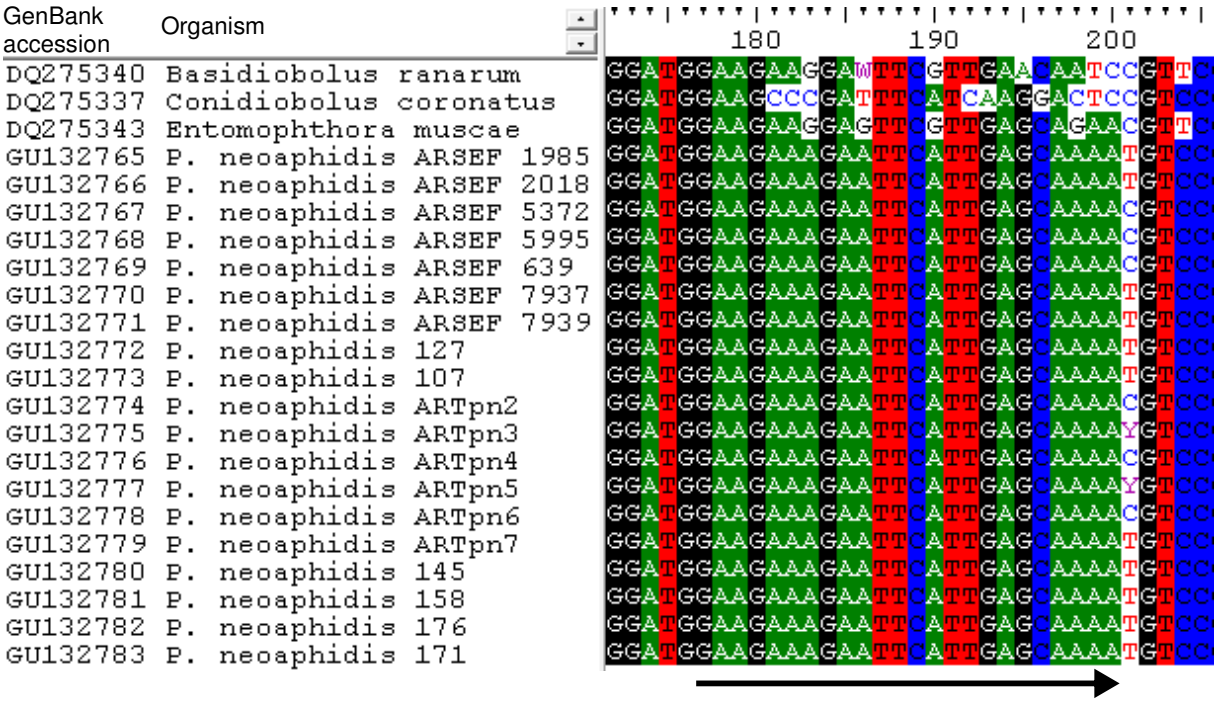
SNP at position 171 of the elongation factor 1α-like (EFL) sequence GU132765.

Arrow indicates orientation and position of SNP primer PnSNP8.



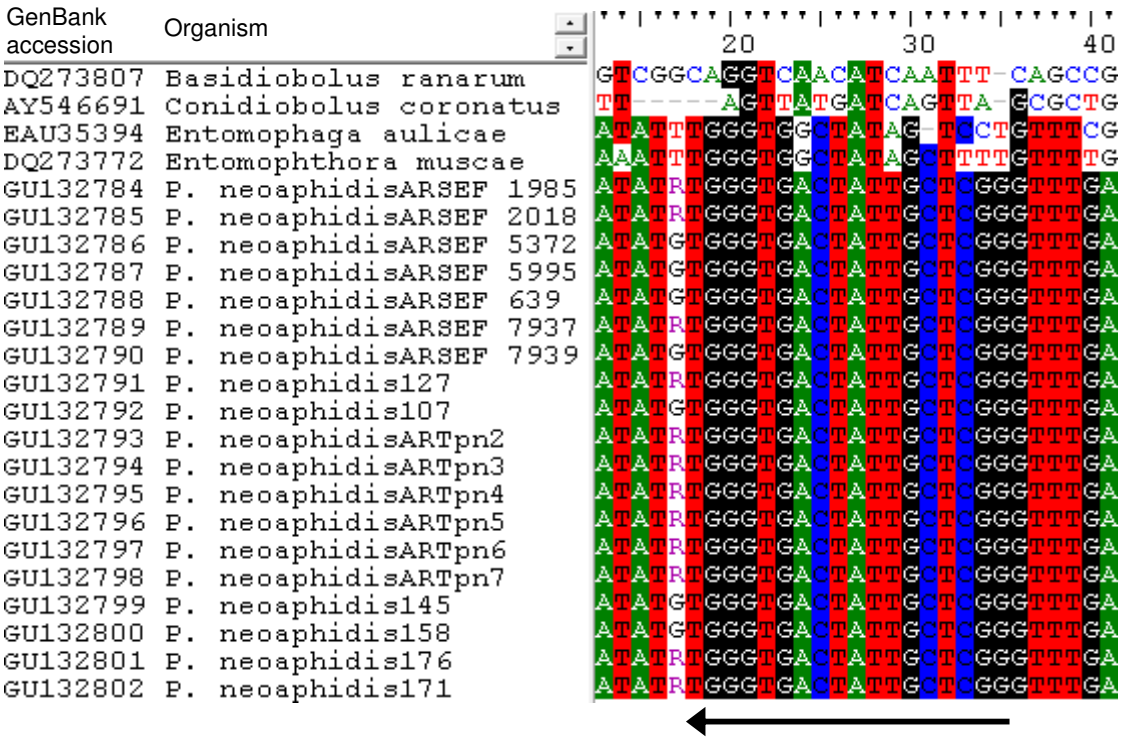
PnSNP9

SNP at position 201 of the elongation factor 1α-like (EFL) sequence GU132765. Arrow indicates orientation and position of SNP primer PnSNP9.



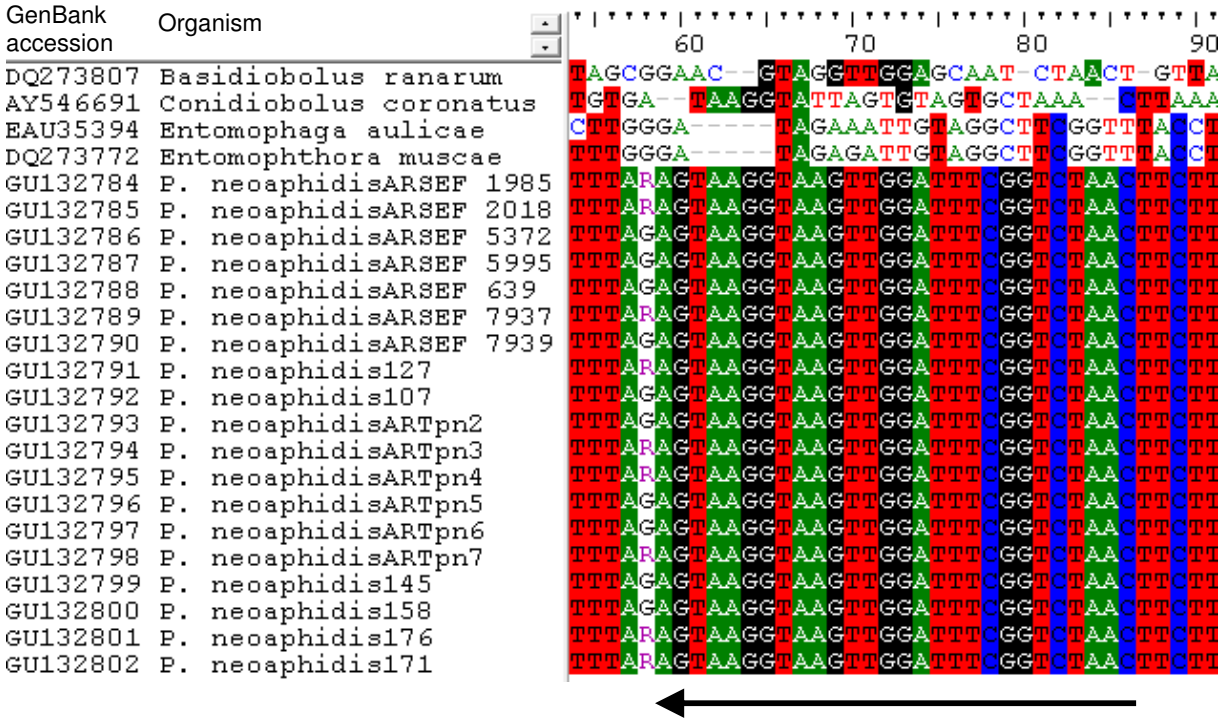
PnSNP10

SNP at position 17 of the large subunit rRNA (LSU) sequence GU132784. Arrow indicates orientation and position of SNP primer PnSNP10.



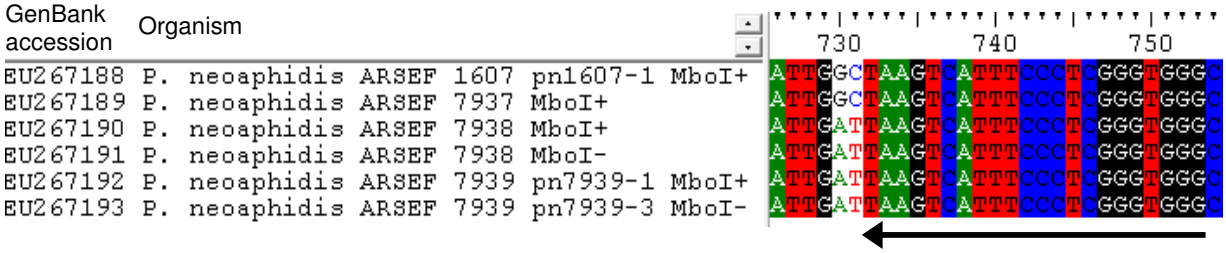
PnSNP11

SNP at position 58 of the large subunit rRNA (LSU) sequence GU132784. Arrow indicates orientation and position of SNP primer PnSNP11.



PnSNP12

SNP at position 731 of the small subunit / internal transcribed spacer (SSU / ITS) sequence EU267188. Arrow indicates orientation and position of SNP primer PnSNP12.



PnSNP13

SNP at position 2102 of the small subunit / internal transcribed spacer (SSU / ITS) sequence EU267188. Arrow indicates orientation and position of SNP primer PnSNP13.