Analysis of the Association Between *Pristionchus* and Beetles with Emphasis on the Special Case of *P. uniformis*

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***Pristionchus uniformis*, should I stay or should I go? Recent host range expansion in a European nematode**

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PREFACE

Before to go into the technical details of my phD work, I would like to give a personal interpretation of what this thesis is. I personally think that we understand and therefore appreciate something if we enjoy it. Enjoying understanding is one of the main reasons, I hope true for all scientists, we choose to challenge our brain so as to better understand what surrounds us. Being curious is our favourite hobby, therefore I decided to try and stimulate the reader's curiosity with a short story. Every scientific paper that we read may well be a source of information but the final goal of a scientific paper is surely not just to be a cold table of results but to generate thoughts and reactions based on these data interpretations. Sometimes papers can motivate our curiosity if we read them like a story.
The following lines have the arrogant claim of seeking to discourage any possible thought like "let's see what this student achieved" and instead inspire questions such as "let's see why this thesis could be interesting".

Once upon a time there was a worm, a special worm called Pristionchus uniformis. *That little worm always wanted to discover new worlds and have new adventures. It was bored by life in the colourless, lazy scarabs and always wandered around our old, small Europe.*
Suddenly one day a colourful foreign insect arrived and upset the quiet European lands and something started to change for P. uniformis *too. This was more or less the time when the easiest way to move goods from one Continent to another was by sea. Together with some plants or soil a little unexpected guest travelled across the Atlantic Ocean from the west coast of the USA to one big European harbour. The little passenger soon recognized how nice the old world was with its abundance of nice and delicious crops and climate so much more hospitable than in some North American states. This is most likely how the Colorado potato beetle arrived and spread throughout Western Europe as an invasive species at the beginning of the XX century.*

At that time, the main character of this story, P. uniformis*, was, in all probability, already present and spread across Europe. Like most of its cousins belonging to the* Pristionchus *genus, it used to travel and lazily wait to reproduce on some scarab beetle. As usual* P. uniformis *used to feed on the detritivores bacteria developed on the beetle carcass it was sitting on. After feeding enough and developing,* P. uniformis *males were ready to look for a* P. uniformis *female. The lucky ones would find each other already on the beetle carcass or on the surroundings. Following the fast nematode rhythms, the mother then was ready to lay her eggs very soon. She may leave her progeny in the soil and sometimes far away from a nice food source. Thus after hatching, the brothers and sisters would often all have to take the same path. Enter into dauers! Yes, better to stop developing and keep the energy until they can attach to a passing beetle and wait until it "transforms" into a suitable food supply therefore continuing life over and over!*

The problem with the scarabs, the Pristionchus*' beloved beetle group, is that they can live "forever" (from a worm's point of view), it takes up to three-four years until they finally die and can ultimately provide some bacteria meal. That is probably also one of the reasons why* P. uniformis *could not ignore the opportunity and decided to try and jump on the dangerous Colorado potato beetle! Once, or twice or more, nobody can really know,* P. uniformis *dauer did not attach to the usual scarab beetle but chose instead a potato beetle. And why not? Nothing seems to be wrong with this more colourful host. In some ways it's even better than the usual host. It dies much faster, food is available much earlier and* P. uniformis *only has to be patient for a few weeks! Additionally, when the beetle dies,* P. uniformis *does not have to eat fast and compete with other worms considering that nobody else around! No other worms can stand the blood of such a toxic insect. Even better, no birds or other predator seems to like it, either. Nobody seems to like this nice little leaf beetle but* P. uniformis*. Not yet satisfied, having discovered a new host,* P. uniformis *decided to cross the Ocean from Europe to the USA and try American insects so now* P. uniformis *can be considered a happy, satisfied worm that has courageously explored new hosts and new lands!*

SUMMARY

Pristionchus nematodes serve as a model in evolutionary biology. In particular, *P. pacificus* became a well-established model in evolutionary developmental biology and for comparison with *Caenorhabditis elegans*. In nature, *Pristionchus* associates with insect hosts, predominately from the Scarabaeidae group. Under unfavourable environmental conditions, *Pristionchus* nematodes follow an indirect life cycle, during which development is arrested by forming a non-feeding, resistant stage, the *dauer* larva. Dauer larvae serve as a dispersal stage to locate and attach to the beetle host. *Pristionchus* dauer larvae wait until the host dies to continue development, mature and reproduce, feeding on the microbes on the decaying carcass. In this thesis, I present new insights into nematode and beetle associations in the *Pristionchus* genus. I show the results that I obtained investigating *P. pacificus* host finding behaviour illustrating that *P. pacificus* attaches to hosts through a dauer-specific standing behaviour (nictation). *P. pacificus* mutant strains lacking this peculiar nictation behaviour were unsuccessful in reaching the beetle host. In addition, the analyses of *P. pacificus* strains that show significantly higher nictation behaviour, when compared to the wild-type strain, provide evidence for a positive correlation between nictation behaviour and host attachment ability.

In the context of *Pristionchus* nematode ecology, I also initiated the analysis of the unique *P. uniformis* beetle association. Unlike most *Pristionchus* species, *P. uniformis* has been found in association with two biologically and ecologically unrelated hosts, scarabs and the chrysomelid *Leptinotarsa decemlineata* (Colorado potato beetle). Using olfactory tests, I show that *P. uniformis* has a species-specific preference for its beetle hosts' odor profiles and is able to discriminate the chemoattractive profiles of these two hosts. By genetic analysis of the mitochondrial marker *nd2* in 81 strains isolated in Europe and North America, I provide evidence for a European origin of *P. uniformis*. The *P. uniformis* phylogeny reconstructed based on the *nd2* marker shows the absence of clear separation between strains associated with scarab or chrysomelid beetles, supporting the hypothesis that *P. uniformis* expanded its host range in Europe and North America by host-switching events.

ZUSAMMENFASSUNG

Nematoden der Gattung *Pristionchus* dienen als Modell in der Evolutionsbiologie. Insbesondere *P. pacificus* wurde als Modell in der evolutionären Entwicklungsbiologie und zum Vergleich mit *Caenorhabditis elegans* etabliert. In der Natur ist *Pristionchus* mit Insektenwirten assoziiert, vorwiegend aus der Gruppe der Blatthornkäfer (Scarabaeidae). Unter ungünstigen Umweltbedingungen folgen Nematoden der Gattung *Pristionchus* einem indirekten Lebenszyklus, bei dem die Entwicklung angehalten wird und ein sich nicht ernährendes, widerstandsfähiges Stadium, die Dauerlarve, entsteht. Dauerlarven dienen als Verbreitungsstadium um den Käferwirt zu finden und sich an ihn anzuheften. *Pristionchus* Dauerlarven warten, bis der Wirt stirbt, setzen dann ihre Entwicklung fort und ernähren sich von den Mikroorganismen auf der verwesenden Leiche. In dieser Arbeit präsentiere ich neue Erkenntnisse der Nematoden- und Käfer-Assoziationen der Gattung *Pristionchus*. Ich untersuchte das Verhalten von *P. pacificus* bei der Wirtssuche und meine Ergebnisse zeigen, dass *P. pacificus* sich an Wirte durch ein dauer-spezifisches Winkenverhalten (Niktation) anlagert. *P. pacificus* Mutanten, Niktations-defekt sind, konnten ihren Käferwirt nicht erreichen. Außerdem liefern Untersuchungen von *P. pacificus* Stämmen, die ein signifikant höheres Niktationsverhalten im Vergleich zum Wildtyp-Stamm zeigen, den Beweis für eine positive Korrelation zwischen Niktationsverhalten und der Fähigkeit, sich an einen Wirt anzulagern.

Im Rahmen der Ökologie der Nematoden der Gattung *Pristionchus* untersuchte ich die einzigartige Assoziation von *P. uniformis* mit Käfern. Im Gegensatz zu den meisten *Pristionchus* Arten wurde *P. uniformis* in Assoziation mit zwei biologisch und ökologisch verschiedenen Wirten, Blatthornkäfern sowie dem Kartoffelkäfer *Leptinotarsa decemlineata* (Chrysomelidae), gefunden. Mittels olfaktorischer Tests zeige ich, dass *P. uniformis* eine artspezifische Vorliebe für die Geruchsprofile seiner Käferwirte hat und zwischen den chemoattraktiven Profilen dieser zwei Wirte unterscheiden kann. Durch die genetische Analyse des mitochondrialen Markers *nd2* in 81 Stämmen aus Europa und Nord Amerika liefere ich den Beweis für eine europäische Herkunft von *P. uniformis*. Die Phylogenie von *P. uniformis*, die unter Anwendung des *nd2* Markers rekonstruiert wurde, zeigt die Abwesenheit einer klaren Trennung von Stämmen, die mit Blatthornkäfern oder Kartoffelkäfer assoziiert sind. Dies unterstützt die Hypothese, dass *P. uniformis* seine Wirtswahl in Europa und Nord Amerika durch Wirtswechsel-Ereignisse ausgedehnt hat.

1. INTRODUCTION

Denn die Wahrheiten der organischen Natur sind von liebenswürdiger und ehrfurchtgebietender Schönheit, und sie werden immer schöner, je tiefer man in ihre Einzelheiten und Besonderheiten eindringt. Es ist unsinnig zu meinen, die Sachlichkeit der Forschung, das Wissen, die Kenntnis der natürlichen Zusammenhänge schmälerten die Freude am Wunderbaren der Natur. Im Gegenteil: Der Mensch wird um so tiefer und nachhaltiger von der lebendigen Wirklichkeit der Natur bewegt werden, je mehr er über sie weiß.

Le verità dell'universo organico si impongono sempre più al nostro amore e alla nostra ammirazione e divengono sempre più belle quanto più profondamente si penetra in ogni loro peculiarità, ed è proprio insensato credere che l'oggettività della ricerca, il sapere, la conoscenza dei fenomeni naturali, possano far diminuire la gioia procurataci dalle meraviglie della natura. Anzi, quanto più l'uomo impara a conoscere la natura, tanto più viene preso profondamente e tenacemente dalla sua viva realtà.

Increasingly the truths of the organic universe are the subject of our love and admiration becoming more and more beautiful the more we understand their peculiarities. This means there's simply no truth in the belief that the objectivity of research and knowledge together with an increased awareness of natural phenomena reduces the joy of nature's wonders. Indeed the more man understands nature, the more deeply and tenaciously he feels his own true existence.

KONRAD LORENZ, 1949

1.1 Why study nematodes? *Caenorhabditis elegans* and *Pristionchus pacificus* two important cases

The diplogasterinae nematode is a large group, which biologically as well structurally may be called extraordinarily interesting (Bovien 1937). This could already be a sufficient reason to attract a student to dedicate a doctoral thesis to one species of the Diplogasteridae group. Much more knowledge from the nineteenth century up to now has been accumulated. Nematoda (derived from νῆμα - nema gr., thread and εἶδος – eidos gr., shape) is one of the most generous phylum among animals. Indeed nematodes are the most numerous, ubiquitous and diverse multicellular animals on Earth (Platt et al. 1984; Lambshead 1993, 2004; Blaxter et al. 1998). They are found in fresh water, salt water, and soil, ranging from hot springs to icy polar regions, and from ocean depths to mountain tops. A handful of soil will contain thousands of free living nematodes, while many other nematode species live as parasites of insects, plants or animals. Yet the vast majority of species encountered are poorly understood biologically. If this animal group is so diffuse and apparently so successful during the evolution of life on Earth, both scientists and lay people, will very easily start to ask many questions about the strategies that allowed nematodes to be so widespread and abundant. Evolutionary biology is the scientific challenge to explain the origin and pattern of this diversity.

Some of these questions have been addressed through the investigation of a single representative of the Nematoda group. Most of the biological knowledge about nematode and other multicellular organisms raised from the deep investigation of the “nice” (*elegans,* lat.) nematode that “looks like a rod” (*Caenorhabditis:* καινός – kainos, gr., recent and ῥάβδος - rhabdos, gr., rod), a free-living nematode that during the last sixty years expanded its geographic range in and outside the laboratory of developmental biology, genetics, physiology, neurobiology and many other area of investigation (Brenner 1974; Riddle 1997; The *C. elegans* Research Community 2011). *C. elegans* conquered all these territories thanks to its extremely easy and safe handling in research. Roughly one millimetre long, it is already able to reproduce after three days from hatching and four molts. The *C. elegans* body is transparent with all cells visible through a microscope and it is easy to culture, making it an ideal candidate for developmental and genome studies. It reproduces predominately by self-fertilization, a hermaphroditic mode of reproduction. *C. elegans* has two sexes: males and selfing hermaphrodites, which first produce a limited number of sperm and then switch to egg production. The sperm are used for self-fertilization. However, outcrossing can occur when hermaphrodites mate with a rare male. Males are hemizygous for the X chromosome (XO), and can arise from X-nondisjunction at meiosis, from mating an XO male with an XX hermaphrodite (producing up to 50% male progeny), or possibly from the loss of an X chromosome during the development of XX cross progeny under particular environmental conditions (Hodgkin & Brenner 1977).

The genome of *C. elegans* is completely sequenced and relatively small, with only about 100 Megabases (*C. elegans* Sequencing Consortium 1998). The estimated number of around 20,000 genes is arranged on six chromosomes (*C. elegans* Sequencing Consortium 1998). Ever since the sequencing of the *C. elegans* genome in 1998, “omics” approaches have been used to study the trascriptoms, proteome and metabolome in detail (Joyce & Palsson 2006). While all of this makes *C. elegans* one of best characterized metazoan organisms, very little is known about its ecology, natural history, or life span in the wild. The strain *N2* is the standard wild-type *C. elegans* and arose from the progeny of a single hermaphrodite (Hansen et al. 1960). Under favourable conditions, the animals will complete their life cycle in less than four days going through a “direct” developing cycle, reaching adulthood throughout four larval stages (L1-L4) (figure 1) (Cassada & Russell 1975). At the end of the L2 stage animals may enter a developmentally arrested state called the *dauer* (derived from German: enduring) larva, if the environmental conditions are not favourable for further growth. Environmental factors, such as the presence of a pheromone as indicator of population density, the absence of food, and high temperature can act as signals that trigger formation of this morphologically distinct L2 dauer larva (Cassada & Russell 1975). The dauer state ends when the animal experiences favourable conditions (Riddle 1988; Hu 2007).

The habitats, in which *Caenorhabditis* species can be found are very diverse, ranging from compost heaps to invertebrates. Dauer juveniles of many *Caenorhabditis* species were shown to be associated with snails (e.g. *Helix aspersa*), some isopod species or millipedes (e.g. *Glomeris* spp.)

(Barrière & Félix 2005; Chen et al. 2006; Kiontke & Sudhaus 2006). This type of association with invertebrates has been defined either *phoresy* (for transport to a new habitat) or *necromeny* (to secure the body of the associated animal as a future food source) (Kiontke & Sudhaus 2006; see also paragraph 1.2). *C. elegans* itself is commonly found in nutrient and bacteria-rich substrates and, like all *Caenorhabditis* species currently in culture, in laboratory conditions is fed with the gram-negative bacterium *Escherichia coli*. However, *E. coli* is clearly an unnatural food source. Indeed, several *Caenorhabditis* species are now studied in laboratories in great detail, but the knowledge of the ecology of most *Caenorhabditis* species is scarce and, their exact natural habitat is unknown.

The study of this single nematode model organism provided detailed insight to many basic biological phenomena commonly present in many animal taxa. However, it was not sufficient to fully investigate the question discussed above: how can we comprehend and explain the extraordinary nematode diversity? More recently, the exploration of genetics, developmental biology and ecology has been enriched with another nematode model organism: the worm with the shark (πρίστις – pristis, gr.) hook (ὄγκος – ogchos, gr.) (Kreis 1932) from the American western coast, *Pristionchus pacificus* (Sommer et al. 1996). *P. pacificus* has been selected as a model system since it shares many laboratory facilitations with *C. elegans*.

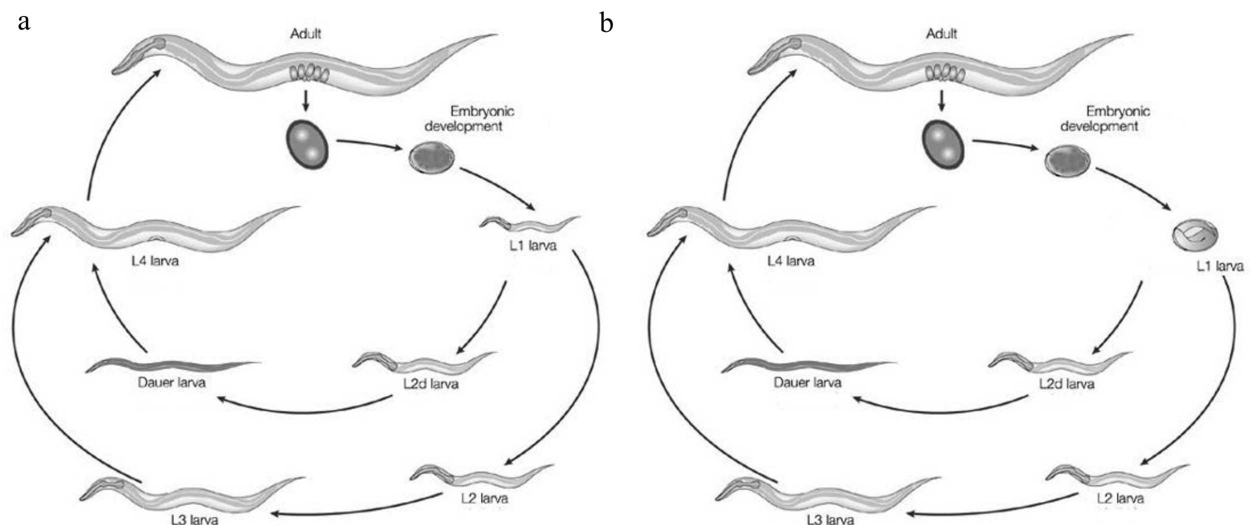

Figure 1. Life cycle of *Caenorhabditis elegans* (a) and *Pristionchus pacificus* (b) (modified after: Altun, Z. F. and Hall, D. H. 2005. Handbook of *C. elegans* Anatomy. In *WormAtlas*).

Originally, *P. pacificus* was investigated and compared with *C. elegans* largely focusing on vulva development. Generally, studies in evolutionary developmental biology (*evo-devo*) deals with queries such as: how can distinct pathways lead to the same morphological structure? In the last twenty years, several questions in this context have been answered. Evo-devo studies in *P. pacificus* aimed to unveil mechanistic changes in development that give rise to evolutionary alterations and novelty in part by studying vulva development (Sommer 2009). The vulva is the egg-laying and copulatory organ of

nematodes and *C. elegans* vulva development is one of the best understood processes in animal developmental biology (Wang & Sternberg 2001; Rudel & Sommer 2003). The *P. pacificus* vulva is morphologically and functionally similar to the *C. elegans* vulva. Nonetheless, cell ablation and forward genetic experiments have shown that the underlying developmental programme is rather different (Sommer & Sternberg 1996; Eizinger & Sommer 1997; Zheng et al. 2005). Differences involve the genetic and molecular pathways in vulva signalling (Tian et al. 2008), but also the domain structure of individual signalling components (Wang & Sommer 2011).

Evolutionary biology has been significantly enriched by gene conservation studies, expression pattern analyses and evo-devo approaches (Raff 2000; Carroll 2008). Nevertheless, evolutionary biology needs to look at natural phenomena from very diverse perspectives to arrive at a complete and integrated understanding. It could be argued that one of the fundamental questions in evolutionary biology is "the understanding of the genetic changes that affect morphological change in and among species". Nonetheless, such a mechanistic description (*how question*) of a phenomenon, is not fully sufficient to understand the role of this phenomenon in the organism's life (*why question*). It is in fact necessary to take into consideration that the phenotype is not merely the unrolling of genotype (Gilbert & Epel 2009). Coupling of evolutionary theory and mechanistic biology studies started in the early Twentieth century, when evolutionary biology studies began to investigate the genetic basis of natural selection within species and populations. This resulted in what is now called the "modern synthesis or evolutionary synthesis", the fusion of the Darwinian idea of natural selection with the revelation of the gene as the agent of heredity. The new synthesis arose from the integration of palaeontology (Simpson 1944), systematic (Mayr 1942), morphology (Rensch 1947), and genetics (Dobzhansky 1937).

However, also this synthesis had its limitations. The modern synthesis focussed on genes themselves as evolutionary units, rather than on the phenotype as a whole (Sapp 1987; Sultan 1992). The revival of evo-devo since 1980s can be considered as a response to these limitations (Minelli 2003; Amundson 2005). Bringing development close to evolution (evo-devo) fulfilled an important task. Nevertheless, it still missed consideration of ecology. Individual capacity for flexible response to the environment, or adaptive phenotypic plasticity was clearly neglected (Schoener 2011). Nowadays, Modern Synthesis has also been extremely successful in showing how environmental pressures influence the survival and propagation of a subset of variations. As consequences, there have been recent attempts to include ecological factors in the study of evolutionary processes and biological diversity. One of these is "ecological developmental biology", which aims to investigate the interactions between developing organisms and their environmental contexts (Gilbert 2001). It has also been identified as "integrative biology", synthesizing development, ecology and evolution (Wake 2008). Organisms evolve in the context of their physical environments and of the other living "things" with which they interact (Dusheck 2002). Therefore, it also became crucial to ask how developmental processes evolve under changing environmental conditions. Ecological developmental biology claims that development encompasses the use of environmental signals to produce distinct phenotypes (West-Eberhard 2003). Although biologists

have always been aware that organisms develop differently in different conditions, environmental effects on phenotype were regarded as uninformative "noise" obscuring the "true" expression of the genotype (Sultan 1992; Schlichting & Pigliucci 1998; Pigliucci 2005). Finally, it should be stated that the inclusion of the environment is not a contemporary innovation, but rather a restoration of an earlier, more holistic approach to understanding individual development (Sultan 2003). Indeed, already Charles Darwin in a letter to Moritz Wagner in 1876 wrote:

> *"In my opinion the greatest error which I have committed, has been not allowing sufficient weight to the direct action of the environment, i.e. food, climate, etc., independently of natural selection. Modifications thus caused, which are neither of advantage nor disadvantage to the modified organism, would be especially favoured, as I can now see chiefly through your observations, by isolation in a small area, where only a few individuals lived under nearly uniform conditions."*

One of the most important phenomena showing the relationship between development, evolution and ecology is phenotypic plasticity. Plasticity in response to a changing environment has been well documented in physiology, development, behaviour in plants and animals (Barata et al. 2001; Griffith & Sheldon 2001; Hammond et al. 2001; Sultan 2003). Phenotypic plasticity has long been proposed as a key strategy that enable organism to face changing environmental conditions and "it may serve as facilitator in the evolution of morphological novelties" (Sommer & Ogawa 2011).

To study environment-dependent phenotypic expression it is often necessary to investigate the influence of environmental factors by "dissecting" them under laboratory conditions. The most common way to access nature and grasp some of its fascinating riddles is to bring part of it inside the laboratory, "sectioning it into small pieces". A complex natural phenomenon indeed needs to be analyzed in its single parts. However, it has often been observed that results obtained from *in vitro* experiments do not always reflect what happens in nature (Mead & Epel 1995). Organisms in their natural habitats have a complex set of challenges, cues and behaviours that are normally not considered in the laboratory setting. An "expanded" investigation can provide precise answers to questions about the developmental, physiological and behavioural processes that lead to adaptive and maladaptive phenotypes in specific environments (Sultan 2007). This would challenge the experimental convention of a „controlled' environment. Instead, plant and animal biologists are becoming aware that the environment, very often variable, multifaceted, and stressful, is a source of specific, essential regulatory information. It is then necessary to "*study evolutionary processes and outcomes in realistic ranges of conditions that accurately recreate these regulatory elements*" (Sultan 2010). In the investigation of the *Pristionchus* model nematode, these conditions can now start to be accomplished by enclosing information of its beetle host association.

1.2 Nematode ecology and their association with beetle

A major question regarding nematode diversity is the relative importance of associations with other organisms, in particular animals or plants (Giblin-Davis et al. 2004). Nematodes are best known as parasites, but the phylum has an abundant and diverse free-living fauna with global distribution in soils and sediments (Lambshead & Boucher 2003). During their life cycle, individuals of a given species of nematodes may inhabit different substrates and niches and this might include free-living and parasitic species. Nematodes can reside in non-living substrates (water sediments, mineral soil, organic layers, plant residues or animal carcasses) or in living substrates such as plant tissues, invertebrates or vertebrates (Yeates & Boag 2004). Consequently the food sources used by nematodes vary considerably, ranging from bacterial, algal and fungal feeding, to different levels of utilization of plant or animal tissues. Nematodes that inhabit such diverse ecological niches colonize them through dispersal. Dispersal is a biological phenomenon utilized by the majority of living organisms and is often defined as movement of organisms from their location of birth to other locations at which they reproduce (Futuyma 1997). Through a broad range of strategies and adaptations, plants, animals, fungi, bacteria and viruses, try to conquer new areas, new niches, and new life opportunities. Very often, dispersion is crucial for the survival of the organisms (Di Domenico 2008).

Dispersal is constrained by the morphology, physiology, ecology and behaviour of the organism. Nematodes, with the exception of tylenchids, disperse predominately through the dauer stage (see paragraph 1.1) often by using insects or other invertebrates as host. There are millions of insects in the world with projections of greater than five million species and nematode evolved a complex of adaptations to live in patchily distributed habitats, including associations with insects present in them (Giblin-Davis et al. 2004). Many of these insects spend most or part of their life cycles in environments favourable to nematode attachment, growth and reproduction (Giblin-Davis et al. 2004). In fact, the insect itself represents a potential for food and shelter for nematodes. Nematodes that develop associations with insects stand to benefit from more predictable resource availability to an easy dispersal mechanism. Fuchs (1915) proposed the term “Wohnungseinmieter” (derived from *Wohnung*: flat and *Mieter*: tenat, ger.) to describe the nematodes that associate with insects. One insect group that is commonly associated with nematodes and that has been well investigated is the order of the Coleoptera. The biological relationship between nematodes and beetles, defined also as “Oikophilie” (derived from οἶκος - oikos: home and φίλία - philia: love, gr.) (Fuchs 1915; Bovien 1937) can have different degrees. Under these circumstances, nematode survival is highly or little dependent on its host and more or less harmful for it.

In cases, in which the host is not damaged by the nematodes, the insect can be just a carrier (phoretic association). Alternatively, it might represent a transporter and food source, when the insect finally dies (necromeny). In detail, phoresy is a prerequisite for nematode to colonize a patchily distributed ephemeral substrate. In nematodes, phoresy (derived from φορέω – phoreo, gr.: bearing)

means the use of an insect (as carrier) during a special stage in the life cycle to reach a new habitat (Farish & Axtell 1971; Kiontke 1996; Timper & Davies 2004). Phoretic relationships can be either facultative or obligate; they are very often obligate when the nematode is adapted to ephemeral resources (Timper & Davies 2004). For example, *Pelodera coarctata* is a nematode that is commonly found living in cow dung. When the conditions in the dung deteriorate and become inhospitable for the nematode, it attaches itself to the legs of *Aphodius* spp., dung beetles which will carry it to a new fresh dung pat (Sudhaus 1976). Likewise, to colonize a new dung pat, the phoretic nematode *Diplogaster coprophila* is carried by flies (Sepsidae, Diptera) and rarely also by *Cercyon* species (Hydrophilidae, Coleoptera), an unusual case for nematode dauers (Bovien 1937; Sudhaus & Kuhne 1989; Kiontke 1996). Phoresy is a common process also in the non-nematode world, for example with bird lice that “hitchhike” phoretically on hippoboscid flies to move between species of hosts (Couch 1962; Johnson & Clayton 2003) or various mites on insects (Farish & Axtell 1971).

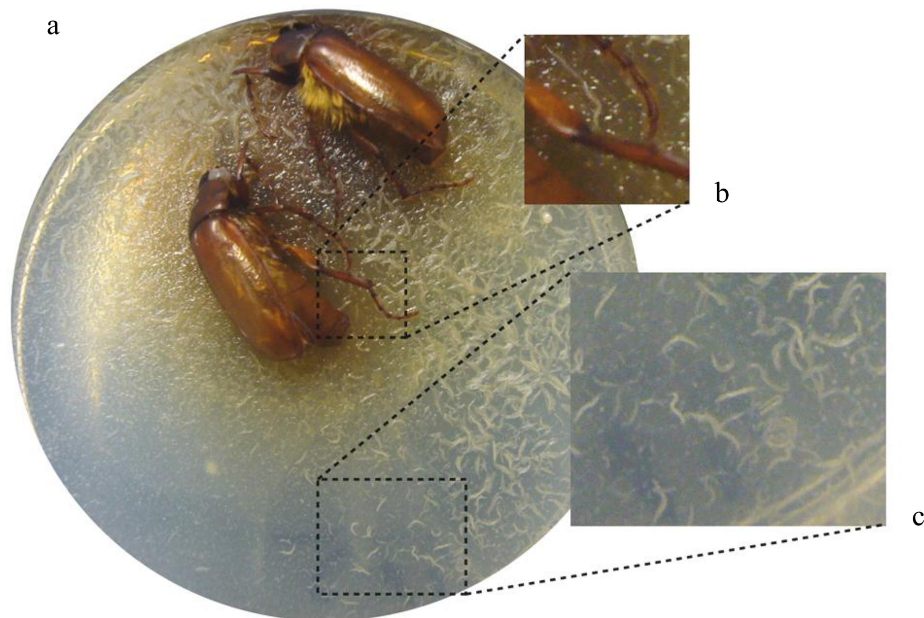

Figure 2. *Pristionchus* and beetle association. Few-days-old beetle carcass dissected on NGM agar Petri dish (a). Magnified adult nematodes feeding on detritivores bacteria (b and c).

A key preadaptation for phoresy in nematodes is the formation of dauer larvae (Sudhaus 1976; Maggenti 1981; Kiontke 1996). Dauers are the dispersal forms for many nematode species. They are resistant to a variety of conditions, like desiccation, and can survive for months without feeding (see paragraph 1.1). During a phoretic association, the carried organism (dauer larva) can be transported in different ways, inside the carrier (endo-phoresy) or outside the carrier (ecto-phoresy), or a combination of both (Timper & Davies 2004). Endo-phoresy has also been considered as a preadaptation for other associations like entoecy, necromeny, entomopathogeny (Sudhaus 2008). Entoecy literally refers to the

colonization of another organism's open cavities or orifices (Sudhaus 2008). It takes place inside the host's body, without the existence of a food relationship between the guest and its host. These commensal nematodes complete their life cycles in hollow spaces of the host, without obtaining nutrients from the host and without living at the host's expense (Sudhaus 2010). For example *Rhabditis adenobia* lives and reproduces in the accessory glands of the female genitalia of dynastid beetles (*Oryctes* spp.) (Poinar 1971; Sudhaus 2008). Unfortunately, nothing is known about the functional aspects of these interactions.

In the past, nematodes that fed on bacteria or by-products of bacterial metabolism were named "saprobiotic" (Sudhaus & Kuhne 1989), "saprophytic" or "saprozoic" (Chantanao & Jensen 1969). Nowadays the special type of phoresy, in which dauers colonize a living organism and wait until the host dies is commonly defined as necromeny (derived from νεκρός – necros, gr.: dead body) . The dauer larvae enter the host or are ingested by it but never leave or reach maturity and complete their life cycles while the host is alive. Instead, they wait until the host dies to continue development, mature and reproduce, feeding on the bacteria of the decaying carcass (Sudhaus 2008). The feeding behaviour of *Pristionchus* nematodes fits to this description. In fact, field studies have revealed that *Pristionchus* nematodes have a necromenic association with their beetle hosts (Herrmann *et al*. 2006a, Weller *et al.* 2010) (figure 2). Several *Pristionchus*-beetle associations have been characterized, such as the oriental beetle (*Exomala orientalis*) with *P. pacificus*, the cockchafer (*Melolontha melolontha*) with *Pristionchus maupasi* and dung beetles (*Geotrupes* spp.) associated with *Pristionchus entomophagus* (Herrmann et al. 2006a). Only one *Pristionchus* species could also be frequently found in association with a non-scarab beetle. *Pristionchus uniformis* has been isolated from the chrysomelid *Leptinotarsa decemlineata*, also known as the Colorado potato beetle (Herrmann et al. 2006a, 2006b; D'Anna & Sommer 2011) (figure 3). Interestingly, *P. uniformis* could also be isolated from scarab beetles species (see paragraphs 2.2 and 2.5).

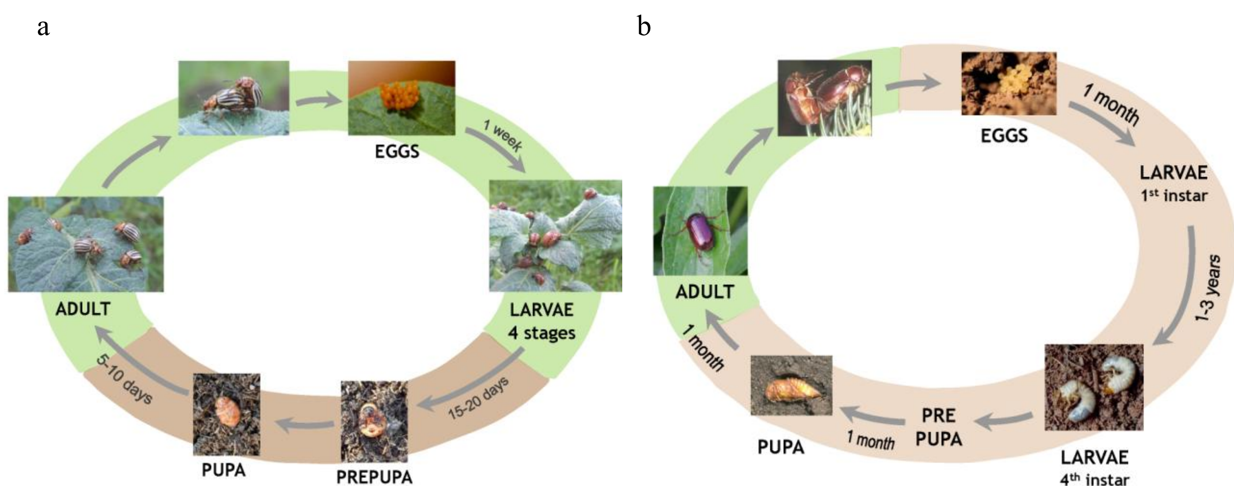

Figure 3. *P. uniformis*' hosts life cycle comparison. The chrysomelid Colorado potato beetle (*Leptinotarsa decemlineata*) in (a) and *Phyllophaga* spp. (Scarabaeidae; May-June beetle) in (b).

The vast majority of *Pristionchus* nematodes have been found in association with beetle hosts that were collected healthy and alive (Herrmann et al. 2006a, 2006b, 2007, 2010; Weller et al. 2010; D'Anna & Sommer 2011). These insect samples contained a variable number of nematode species, in some cases predominantly *Pristionchus* spp. (see paragraph 2.3). Investigation of the dauer behaviour inside the beetle host is still on-going and so far we could never find any evidence of harmful effects of *Pristionchus* on their beetle hosts (Weller et al. 2010; Herrmann & Sommer personal communication).

True host-parasite interactions are instead the preferred subject of most of nematode ecology literature. Nematodes in this case are dependent on their animal or plant host. The feeding behaviour of zoo-parasitic nematode ranges from blood (e.g. *Haemonchus* spp.) to tissue ingestion (e.g. *Capillaria* spp.). Nematodes feeding on plants are obligate parasites with a diet that ranges from living cells of root to leaf tissues (Yeates et al. 1993; Bilgrami & Gaugler 2004). Some nematodes species, like *Steinernema* spp or *Heterorhabdtis* spp., feed on living insects and have a specialized entomopathogenic larval stage (infective). The infective larvae of entomopathogenic nematodes are non-feeding, free-living stages that carry symbiotic bacteria (e.g. *Xenorhabdus* spp and *Photorhabdus* spp.) within their gut, which are released into the insect shortly after host penetration (Forst et al. 1997; Griffin et al. 2005). The bacteria will kill the host usually within one or two days and the infective nematode larva will resumes development to start feeding on bacteria and continue development (Bird & Akhurst 1983; Bilgrami & Gaugler 2004). Eggs are laid and hatch in the host carcass and reproduction continues until resources in the cadaver are depleted (Gaugler 2002). According to Sudhaus (2010), entomopathogenic nematodes cannot be considered as real parasites because they live at the expense of a host with the aid of insect-pathogenic bacteria, so that in this respect they behave like parasitoids. Therefore, he defines the commonly known entomopathogenic as "entomoparasitoids".

1.2.1 Nematode life style evolution

When describing associations between nematodes and insects we must be aware that a comprehensive description is often difficult. Furthermore, these different types of associations cannot be precisely defined; categories "*are arbitrary and without exact boundaries because there is a continuous spectrum of relationship linked by intermediates*" (Maggenti 1981; Sudhaus 2008). It is instead more interesting to try to reconstruct the evolution of these insect-nematode associations. Although nematode parasites are generally assumed to have evolved from free-living ancestors, absence of informative fossil records make it difficult to reconstruct the precise evolutionary history of the nematode insect relationship (Clark 1994; Blaxter et al. 1998).

Osche (1956) was the first author that claimed that parasitism in nematode is a derived state. All nematode parasitism had its developmental origin terrestrially (Maggenti 1981), whereas there are almost no parasitic nematodes that originated in the marine environment (Osche 1966). This observation can be explained by the scarcity, in marine habitats, of conditions that normally would function as "incentives"

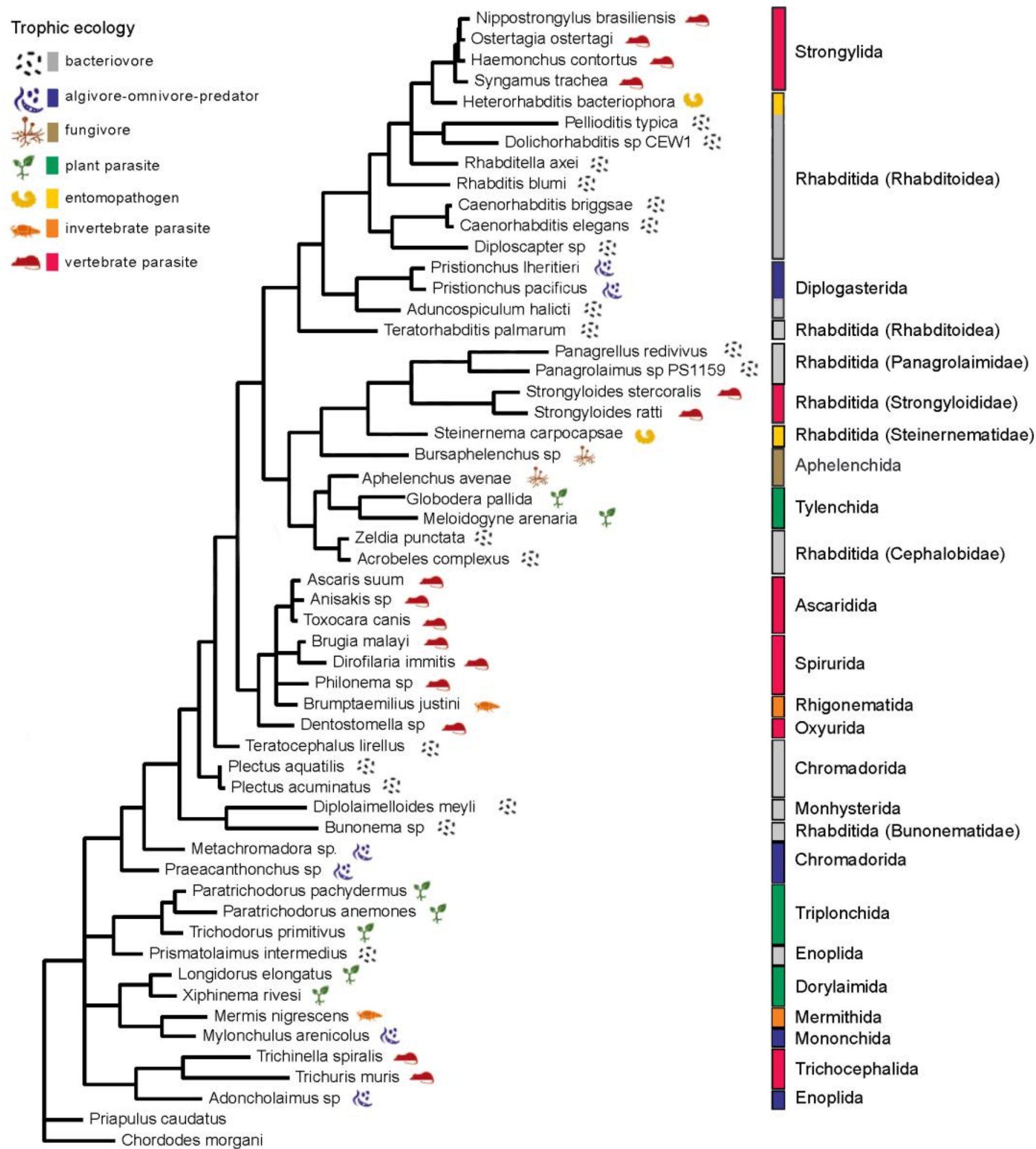

Figure 4. Feeding strategy evolution among nematodes (figure adapted from Blaxter et al. 1998).

to the evolution of the parasitic life style, such as ephemeral and uncertain habitats (Sudhaus 2008). Blaxter's (1998) molecular phylogenetic analysis predicts multiple origins of animal-parasites within the phylum Nematoda and a minimum of four independent origins of vertebrate parasites is supported (figure 4). The beginning of parasitism cannot be considered as clear cut, since there is a continuum from free-living existence via different "preaparasitic contacts" to accidental and obligate parasitism (Giblin-Davis et al. 2004). This suggests that many expedients need to "show up" in a free-living nematode that attempts to behave like a parasite. For example, the nematode has to overcome strong metabolic and physiological stresses (e.g. in the host gut or immune system). Also, it has to be well adapted to oxygen and osmotic changes. For example, the nematode's strong cuticle can efficiently offer protection from exo-enzymes.

Flexible behaviour may also play an important role on the evolution of feeding strategies and might facilitate the emergence of new ecological life styles. For example, a phoretic dauer might have achieved necromenic behaviour by extending its time on the carrier and not leaving it until the host death has become a suitable habitat to complete the nematode's development and life cycle. *"Phoretic and especially ento-phoretic behaviour was one of the key innovations that opened the door for different types of association with insects"* (Sudhaus 2008). According to this hypothesis, the host tenant (*wohnungseinmieter*) might be considered as a transitional stage to actual parasitism (Sudhaus 2008, 2010; Clark 1994; Osche 1956). Already Osche has formulated the theory that infective juveniles of parasitic nematodes have evolved from the dauer stage of free living species.

It is easy to find similarities between the free-living dauer and the parasitic infective larva. For instance, both have a specialized resistant cuticle, dauer larvae and parasitic infective stage are almost always third stage larva. In addition both long-lived larvae resume development (to a fourth larval stage) after encountering favourable conditions (available food or suitable host). Dauers have been defined also as a phoretic stage that in parasitic nematodes becomes the infective stage, one of the key steps in the evolution of nematode parasitism (Ogawa et al. 2009; Viney 2009).

Recent studies have indeed indentified that free-living and parasitic nematodes share molecular and genetic control of their development (Ogawa et al. 2009). In the non-parasitic species *C. elegans* and *P. pacificus,* the steroid hormone dafachronic acid (DA) and the nuclear hormone receptor encoded by the gene *daf-12* represent an enocrine-signaling module strictly required for dauer development. The presence of Δ7−DA steroid hormone prevents dauer formation in these two non-parasitic nematodes (Vowels & Thomas 1992; Motola et al. 2006; Ogawa et al. 2009). Δ7−DA completely inhibits the development of parasitic forms also in the animal parasite *Strongyloides papillosus* that retains a developmental choice between the development of infective larvae or free-living forms (Ogawa 2009). Similar findings have been made in other parasitic nematodes (Wang et al. 2009) The conservation of Δ7−DA function suggests that the dauer and infective larvae indeed share a common regulatory basis (Sommer & Ogawa 2011).

Such features have been defined as "preadaptations" or "preconditions" to parasitism (Osche 1956; Maggenti 1981; Poulin 2007; Sudhaus 2008, 2010). These authors also describe other intermediate steps towards true parasitism, e.g. the entoecy life-style in some Thelastomatoidea (order Oxyurida). Thelastomatoidea nematodes are parasites or commensals of saprophytic terrestrial arthropods. They live within the hindgut of the host and usually feed on the bacterial microfauna found there (Jex et al. 2005).

1.2.2 How to find the host: first smell it, then attach to it

Life in association with a host is, of course, highly dependent on finding this host. In phoretic species, host seeking behaviour is crucial in facing a constantly ephemeral habitat (beetle host). The dauer larva need to detect the potential host and therefore, must be able to respond to different stimuli,

like those provided by the carrier and those provided by fresh substratum of a new habitat after transportation. Nematodes that have different degrees of association with the host evolved two different strategies to detect and attach the potential new host. Nematology literature classified predatory and parasitic species into two broad host-finding strategies: cruisers and ambushers (Pianka 1966; Schoener 1971; Campbell & Gaugler 1997). Cruisers are in constant movement and actively search for sedentary hosts, possibly following volatile cues (high energy cost) (Lewis 2002). In contrast, ambushers tend to stand still, and wait for their host to approach and attach to it through body waving movements (nictation, see paragraph 1.2.2.2) and jumps (low energy cost) (Lewis 2002; Yeates 2004). *Heterorhabdtis bacteriophora* and *Steinernema carpocapsae* are the two most famous representatives of these two categories and described as the opposite extremes of the continuum of foraging strategies among entomopathogenic nematodes (Grewal et al. 1994; Lewis & Gaugler 1994; Robinson 2002). These two broad categories represent end points on a continuum (Huey & Pianka 1981; Pianka 1966; Schoener 1971). For example, the genus *Steinernema* carries an interesting interspecific behavioural variation in the host finding strategy (Campbell et al. 2003). Originally it was assumed that ambush foragers were incapable of a cruise foragers-like response to chemical cues. By now, it has since become apparent that they do respond to chemical cues, but their response is fundamentally different from cruise foragers. Lewis et al. (1995) proposed that responses in ambusher species can be triggered only by an appropriate stimuli sequence. Certain entomopathogenic species, such as *Steinernema feltiae* and *S. riobrave,* have adapted a strategy between ambush and cruise strategies called an intermediate strategy that enables attachment to both mobile and sedentary hosts (Lewis et al. 2006).

1.2.2.1 Detecting the host: chemotaxis behaviour

Nematodes can detect their environment via specialized behaviours, such as chemoreception, thermoreception, mechanoreception and orientation to magnetic and electric currents (Lee 2002; Riga 2004). Nematodes have a variety of sensory receptors (Jones 2002), with which they perceive cues from their environment. The main sensory organs are located anteriorly and consist of paired amphids (Wright 1983). The amphids and inner sensilla are open to the environment and function mostly in chemoreception. Ultrastructural studies, especially on *C. elegans* showed that the amphids are primarily chemosensory structures, although there is no evidence indicating that they may serve additional functions (Bargmann et al. 1990).

Chemosensation is crucial for nematode host finding, reproduction and orientation (Bilgrami and Gaugler 2004). Indeed, several nematode species are able to detect their ecological niche through chemotaxis triggered by host plant exudates, beetle pheromone or food stimulants (Hong & Sommer 2006). For example, many plant parasitic nematode responds to root diffusates (Wallace 1968; Perry 1997).

Signals perceived by nematodes are suggested to be non-volatile or volatile. Non-volatile are likely to be water soluble, facilitating the establishment of concentration gradients in soil water (Perry & Maule 2004). However, most authors have studied volatiles components thought to be important for long distance orientation towards the plant or animal host (Robinson 2002; Wright & Perry 2002). Nematode chemotaxis responses are often triggered by various, mostly volatile, known and unknown substances in insect blood (Khlibsuwan et al. 1992), excreta (Grewal et al. 1993) and aqueous external washing. Interestingly, Fairbaim et al. (1999) observed that insects already infected by nematodes, result to be less attractive than uninfected insects.

It has been observed that carbon dioxide (CO_2) is also a universal nematode attractant (Bird 1959; Johnson & Viglierchio 1961; Hallem & Sternberg 2008). Today however, the central ecological question remains still unanswered: how does the same respiratory gas effectively guide bacterial feeder, insect parasites, plant parasites, free-living marine nematodes and vertebrate parasites (Robinson, 2004)? CO_2 is a metabolic by-product secreted by most organisms. It has received much attention as potential kairomone mediating nematode food-searching behaviour (Barbercheck & Duncan 2004). For instance, CO_2 can induce eggs hatching in *Ascaris* species and human breath stimulates directional movement of animal-parasites *Ancylostoma caninum* and *Strongyloides stercoralis*. It has been shown that CO_2 can also induce nictation (Sciacca et al. 2002).

Chemoreception is also essential for inter and intraspecific communications. In this case chemicals that cause interactions between organisms are called semiochemicals, a term that includes allelochemicals, mediating interspecific responses, and pheromones, mediating intraspecific responses (Bargmann & Horvitz 1991; Bargmann & Mori 1997; Riddle & Albert 1997). Pheromones in nematodes other than *C. elegans* are largely uncharacterized. Experimental evidence of pheromone activity in nematodes was first seen in *Panagrolaimus rigidus* (Greet 1964). However, chemotaxis behaviour in nematodes has been studied predominantly in *C. elegans*. Extensive genetic analyses have also helped to identify the molecular mechanisms underlying the chemosensory pathways (Jovelin et al. 2003; Troemel 1999). It has been observed that *C. elegans* is able to discriminate between a wide range of volatile and non-volatile chemicals, both attractive and repellents (Bargmann & Horvitz 1991; Bargmann & Mori 1997; Riddle & Albert 1997). For instance, adult *C. elegans* males are attracted to hermaphrodites (Simon & Sternberg 2002; White et al. 2007). *C. elegans* hermaphrodites produce a chemical mating signal that consists of a synergistic blend of several ascarosides (Srinivasan et al. 2008). Interestingly, the same compounds are also used as pheromone for induction of dauer larvae (Butcher et al. 2007; 2008). Indeed, population density is monitored through specific glycosides of the dideoxysugar ascarylose (the ‚ascarosides' (-)-6-(3,5-dihydroxy-6-methyltetrahydropyran-2-yloxy) heptanoic acid) that induce entry into the dauer (Jeong et al. 2005; Golden & Riddle 1982; 1984). These results indicate that ascarosides regulate both dauer formation and male attraction in *C. elegans*.

Also in *P. pacificus* and other *Pristionchus* nematodes, interception of the chemical communication system of the insect is likely to be involved in host preferences (Hong et al. 2008).

Laboratory experiments show that *Pristionchus* species have the ability to recognize and move towards host-associated volatiles by chemotaxis (Herrmann et al. 2007; Hong et al. 2008; D'Anna and Sommer 2011).

1.2.2.2 Host attachment: nictation behaviour

Dauer larvae and infective juveniles of some nematode species show a specific search behaviour, in which the animals stand on their tail and wave in order to increase their chance of attaching to the potential host. This standing behaviour has been termed „winken' (Völk, 1950), „nictation' (Croll & Matthews 1977; Ishibashi & Kondo 1990; Campbell & Gaugler 1993), „standing' (Campbell & Kaya 1999; 2000a; 2000b), and most recently, „body waving' (Kruitbos, Heritage, Hapca, & Wilson, 2009). For a complete revision about the terminology of this behaviour see Kruitbos and Wilson (2010); for the sake of simplicity I refer to this behaviour as „nictation'. Nictation consists of raising the anterior and middle body regions of the juvenile off the ground, supported only by the tip of its tail (Reed & Wallace 1965; Ishibashi & Kondo 1990). In different species of nictating nematodes, juveniles can either stay in an erect pose or wave their bodies in three dimensional spirals and loops (Reed & Wallace 1965; Croll & Matthews 1977; Ishibashi & Kondo 1990). In the laboratory, nictation behaviour is only observed when the nematodes are exposed to irregular substrates (Kruitbos *et al.* 2009). As mentioned above, CO_2 can evoke multiple host-seeking behaviours. Certain entomopathogenic species are attracted to it, while others start nictating. In the parasitic *Steinernema carpocapsae* CO_2 first stimulates nictation and then a peculiar jumping behaviour (Gaugler et al. 1980; Hallem et al. 2011).

Nictation and jumping have been proven to be able to increase the probabilities of coming into contact with a potential carrier (Barr & Hu 2004; Kiontke & Sudhaus 2006; Brown et al. 2011). Nictation has been observed also in phoretic nematodes and is considered as an adaptation that facilitates phoresy (Timper & Davies 2004). The molecular bases for this peculiar attaching behaviour have been investigated in nematode species, in which genetic tools are available such as *C. elegans* and *P. pacificus.* In both species, forward genetics allowed identification of mutant strains with deficient nictation behaviour (Jorgensen & Mango 2002; Brown et al. 2011).

1.3 Aim of the thesis

This PhD thesis aimed to provide novel insights into the ecology of the *Pristionchus* genus. I intended to delve into nematode life history traits correlated with *Pristionchus* beetle associations. Specifically, I investigated the strategies that *Pristionchus* nematodes use to specifically perceive and to attach to their host. To accomplish these purposes, I investigated the role of nictation behaviour, commonly present across the *Pristionchus* genus, using the model *P. pacificus*. The study of *P. uniformis* aimed then to unveil the behavioural, physiological and genetic basis of a unique nematode-beetle association. Based on the well develop set of molecular and genetic information available for the model organism *P. pacificus*, I initiated the first genetics investigation in a dioceous *Pristionchus* species, *P. uniformis*.

2. RESULTS

2.1 Host-finding behaviour in the nematode *Pristionchus pacificus*

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*Authors contributed equally
Proceedings of the Royal Society Biological Science 2011 278: 3260-3269.

2.1.1 Synopsis

In this study we investigated the function of nictation behaviour (see introduction paragraph 1.2.2.2) by relating the occurrence of nictation in *Pristionchus pacificus* dauer juveniles to the ability to attach to the laboratory host *Galleria mellonella*. The *P. pacificus* community has up-to-date genetic and genomic tools available as well as transgenic techniques and the nematode is also amenable to studies of behaviour and neurobiology due to the relative simplicity and detailed description of its nervous system. Therefore, *P. pacificus* allows to address the question whether nictation behaviour provides a selective advantage for nematode-host associations.

To test the hypothesis that nictation behaviour affects attachment abilities for host association, we used forward genetics to generate strains lacking this behaviour in the laboratory strain *P. pacificus* PS312. In a mutant screen we identified nictation-defective mutants. These mutants were used during experiments designed to check the ability to attach to an insect host. This kind of host attachment trial were done in parallel using *P. pacificus* PS312 and *P. pacificus* strains recently isolated in the wild (characterized by an higher proportion of nictating individuals than *P. pacificus* PS312). For the latter, we found a proportional increase in the attachment to the host, which is directly related to the increase in nictation behaviour previously recorded for the different strains. The combination of genetic analyses with natural variation studies presents a novel approach to study the ecological relevance of behavioural traits. It is tempting to speculate that nictation or nictation-like host finding behaviours are crucial for the initial steps of the evolution of parasitism.

Our investigation showed that nictation behaviour in *P. pacificus* serves as host finding-behaviour. We could find a positive correlation between nictation frequencies and host attachment in these strains.

2.1.2 Contributions

This paper included nictation quantification experiments, and screens for nictation defective mutants, which were carried out by Federico D. Brown. I designed, performed and analysed the data on host attachment experiments with Federico D. Brown. These experiments provided the ecological context of the nictation behaviour analyses. The manuscript was written by Federico D. Brown, myself and Ralf J. Sommer.

2.2 *Pristionchus uniformis,* should I stay or should I go? Recent host range expansion in a European nematode

Isabella D'Anna and Ralf J. Sommer

Ecology and Evolution. doi: 10.1002/ece3.28 (Article first published online: 11 November 2011)

2.2.1 Synopsis

Pristionchus nematodes are thought to preferentially establish a necromenic association with scarab beetles (Herrmann et al. 2006a, 2006b). One interesting exception is *Pristionchus uniformis,* one of a few *Pristionchus* species that frequently associate with a non-scarab beetle. In particular, *P. uniformis* is observed on both, the chrysomelid *Leptinotarsa decemlineata* (Colorado potato beetle) and scarab beetles in Europe and North America. This unusual pattern of association with two unrelated groups of beetles on two continents requires the involvement of geographical and host range expansion events. We used population genetic and phylogenetic analyses of the mitochondrial gene *nd2* to reconstruct the genetic history and analyzed the directionality of both, biological invasion and host-switch during geographic and host range expansion events.

Phylogenetic analyses of 81 *P. uniformis* strains provided evidence for host range expansion through host-switching events in Europe, where *P. uniformis* was originally associated with scarab beetles. Additionally, the population structure of *P. uniformis* strains shows a much higher genetic diversity than American strains, arguing for an invasion from Europe to North America.

P. uniformis insect association is also interesting because the two preferred hosts are both biologically and ecologically highly distinct (figure 3). Beetles from the Scarabaeidae group are generally long-lived (one to three year life cycle) whereas the leaf beetle *Leptinotarsa decemlineata* is characterized by a much shorter life cycle (around 30 days). They also occupy totally different ecological niches; scarabs spend most of their life under the soil, while all but one Colorado potato beetle life stages develop on the host plant. Despite these differences in this study we observed that *P. uniformis* can easily discriminate between both insect groups. Olfactory tests on beetle chemical extracts showed that *P. uniformis* has a unique chemoattractive profile toward its beetle hosts.

Our results provide evidence for host range expansion through host-switching events in Europe where *P. uniformis* was originally associated with scarab beetles and the nematode's subsequent invasion of North America. So far *P. uniformis* is the only *Pristionchus* species that is found consistently on disparate families of hosts among nearly 30 species collected worldwide (Mayer et al. 2009). We deduced that *P. uniformis* might easily conquer new ecological niches (geographical and host). For example, successful association with the “new” host, the Colorado potato beetle, may have been facilitated by the

lack of competition in this unwelcome beetle, and by the faster life cycle of this host but also by the flexible host seeking behaviour of *P. uniformis*.

2.2.2 Contributions

I designed and performed all the experiments and, together with Ralf J. Sommer, wrote the manuscript.

2.3 Scarabaeidae vs. chrysomelidae *Pristionchus* residents

2.3.1 Field nematode survey in North America

2.3.1.1 The scarab case

Numerous field trips to locations in Western Europe, North America, South-Africa and Japan, revealed that *Pristionchus* nematodes associates in a necromenic manner with beetle hosts (Herrmann et al., 2006a, 2006b, 2007) (figure 5). Particularly interesting scenarios in nematode – beetle and nematode – nematode interactions emerged from a general field collection in 2006 in North America (Herrmann et al. 2006b). In one of the most widely distributed Scarabaeidae species in North America, the *Phyllophaga* spp. (commonly known as the May-June beetle), four novel *Pristionchus* species were discovered. Among all the 14 different scarab species collected, *Phyllophaga* spp. was seen to be the most appreciated host for *Pristionchus* species.

All four novel *Pristionchus* species were gonochoristic, phylogenetically related and morphologically nearly indistinguishable. Molecular analyses revealed that two of these novel species, *P. aerivorus* and *P. pseudaerivorus* showed a pairwise molecular distance of only 1.3 % (Herrmann et al. 2006b). Additionally, cross-species mating experiments between *P. aerivorus* and *P. pseudaerivorus* revealed a scarcity or absence of male offspring (the heterogametic sex), a scenario that according to "Haldane's rule" proves that the two species are diverging (Haldane 1922).

The high association rate of *Pristionchus* in the widespread *Phyllophaga* beetle and the peculiar scenario depicted by *P. aerivorus* and *P. pseudaerivorus* species encouraged more exhaustive investigations on nematode population associated with *Phyllophaga* spp.

2.3.1.2 A chrysomelid example, the Colorado potato beetle

In contrast to beetles belonging to the Scarabaeidae group, very few *Pristionchus* samples could be obtained from Chrysomelidae beetles (Herrmann et al. 2006b). Only in one chrysomelid species, *Leptinotarsa decemlineata* (commonly known as Colorado potato beetle), could few *Pristionchus* nematodes be isolated. Also, only one *Pristionchus* species was frequently recovered from both potato beetles collected in North America and Europe, *P. uniformis* (Herrmann et al. 2006a, 2006b and see also paragraph 2.2). Investigation of *P. uniformis* biogeography would benefit from similar numbers of European and North American *P. uniformis* strains. However, when this thesis work started, the number of European strains was much higher. Therefore, it was necessary to balance the study on *P. uniformis* populations by increasing the number of North American isolates.

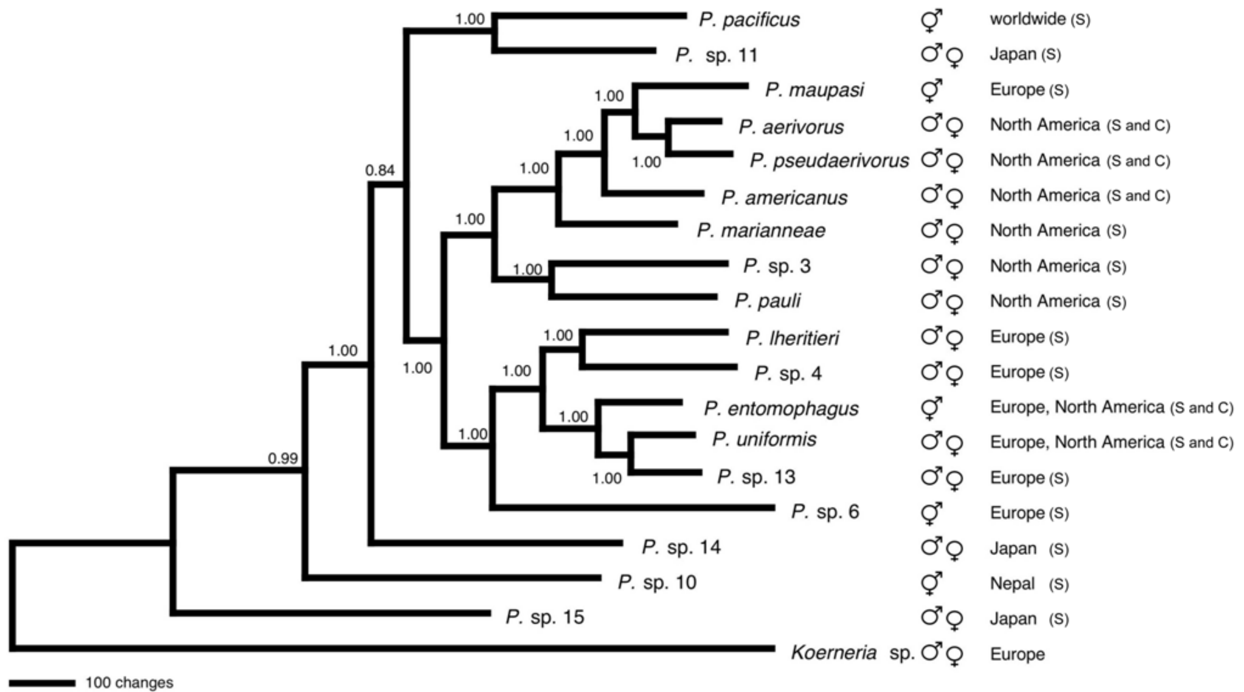

Figure 5. Phylogenetic relationships of *Pristionchus* species based on ribosomal protein genes. Hermaphroditic and gonochoristic (male/female) species are marked. The geographic origins of the species are indicated. (S) designates an association with beetles from the scarab group and (C) indicates that the *Pristionchus* species were isolated from the chrysomelid Colorado potato beetle. Modified after Mayer et al. 2007.

2.3.2 Methods

2.3.2.1 *Phyllophaga* beetle collection

I sampled *Phyllophaga* spp. in locations selected from areas where historical information indicated the occurrence of this scarab species (table 1 and figure 7). Adult beetles were captured using either UV light traps or various blends of L-valine methyl ester and L-isoleucine methyl ester. *Phyllophaga* spp. female sex pheromone is in fact a blend of the methyl esters of these two amino acids (Zhang et al. 1997; Robbins et al. 2006). Lures were prepared by Dr. Paul Robbins (Cornell Univ., NY, USA) and placed in the field vane traps (Robbins et al. 2006). *Phyllophaga* species identifications were assigned under consultation with Dr. Paul Robbins and Dr. Matt Paulsen (UNSM, NE, USA), and by comparison with the *Phyllophaga* specimen in Cornell University and in the University of Nebraska State Museum, and verification from the literature of Lunginbill and Painter (1953). The nematode lines were obtained using the standard procedure to isolate *Pristionchus* nematode from the field (D'Anna and Sommer, 2011; Herrmann et al. 2006a).

2.3.2.2 Colorado potato beetle sampling

Field collected Colorado potato beetles (*Leptinotarsa decemlineata*) were sent to our Institute in September and October 2008. Sampling localities are listed in table 2.

Together with Andreas Weller, beetles were dissected in agar plates and the nematode lines were obtained using the standard procedure to isolate *Pristionchus* nematode from the beetles (D'Anna and Sommer, 2011; Herrmann et al. 2006a).

2.3.2.3 Molecular species identification

For species identification, DNA was prepared from single individual nematodes and species identity was assessed by their having identical small subunit ribosomal RNA (SSU) sequences as described in Herrmann *et al* (2006a).

Table 1. *Phyllophaga* field collection locations in USA and type of beetle trap used

state	location	trap used	date
New York (NY)	Waterloo (1)	Pheromone (L-valine ME and L-isoleucine ME)	May, 2007
New York (NY)	Geneva (2)	Pheromone (L-valine ME and L-isoleucine ME)	May, 2007
New York (NY)	Franklinville (3)	Pheromone (L-valine ME and L-isoleucine ME)	May, 2007
Massachusetts (MA)	Cape Cod (4)	Pheromone (blend: 50% L-valine ME plus 50% L-isoleucine ME)	May, 2007
Nebraska (NE)	Bennet (Lincoln) (5)	Pheromone (various blend of L-valine ME and L-isoleucine ME: 100/0, 90/10, 60/40 and 0/100)	May, 2008
Nebraska (NE)	Nine Mile prairie (Lincoln) (6)	Pheromone (various blend of L-valine ME and L-isoleucine ME: 100/0, 90/10, 60/40 and 0/100)	May, 2008
Nebraska (NE)	Wilderness Park (Lincoln) (7)	UV-light	May, 2008
Nebraska (NE)	Indian cave state park (8)	Pheromone (various blend of L-valine ME and L-isoleucine ME: 100/0, 90/10, 60/40 and 0/100) and UV-light	May, 2008
Kansas (KS)	Scenic (Manhattan) (9)	Pheromone (various blend of L-valine ME and L-isoleucine ME: 100/0, 90/10, 60/40 and 0/100)	May, 2008
Kansas (KS)	Wildcat Park (Manhattan) (10)	Pheromone (various blend of L-valine ME and L-isoleucine ME: 100/0, 90/10, 60/40 and 0/100)	May, 2008
Kansas (KS)	River Pond State Park (Manhattan)	Pheromone (various blend of L-valine ME and L-isoleucine ME: 100/0, 90/10, 60/40 and 0/100)	May, 2008
Kansas (KS)	Whichita (12)	UV-light (from Dr. Mary Liz Jameson)	May, 2008

(ME) indicate methyl ester. The numbers associated with the localities refer to the numbers in figure 7

Table 2. Colorado potato beetle (*Leptinotarsa decemlineata*) field collection locations in USA and Canada

state	location	sender	date
Maryland (MD)	Petersburg (1)	Dr. Ami Miller	August 2008
Maryland (MD)	East New Market (2)	Dr. Ami Miller	August 2008
Maryland (MD)	Upper Marlboro (3)	Dr. Ami Miller	August 2008
Wisconsin (WI)	Madison (4)	Dr. Ami Miller	August 2008
Virginia (VA)	Painter (5)	Dr. Ami Miller	August 2008
Maine (ME)	Fryeburg (6)	Dr. Ami Miller	August 2008
Maine (ME)	Bridgewater (7)	Dr. Ami Miller	August 2008
Delaware (DE)	Newark (8)	Dr. Ami Miller	August 2008
Minnesota (MN)	Becker (9)	Dr. Ami Miller	August 2008
Ohio (OH)	Woostar (10)	Dr. Casey Hoy	September 2008
Idaho (ID)	Kimberly (11)	Dr. Ami Miller	August 2008
Canada	Fredericton	Dr. MacKinley Pamela	September 2008
Canada	Lanoraie, Quebec	Dr. Gary Sewell	September 2008
Canada	Lanoraie, Quebec	Dr. Ami Miller	August 2008

The numbers associated with the localities refer to the numbers in figure 8.

Figure 6. Summary of the *Phyllophaga* and Colorado potato beetle sampling sites in USA. White dots indicate the surveyed States (map modified after http://www.remax.com).

Figure 7. Collection sites in USA. Numbers indicate places where *Phyllophaga* samples were trapped (see table 1).

Figure 8. Sampling sites in USA. Numbers indicate places where Colorado potato beetle samples were collected (see table 2).

2.3.3 Results

2.3.3.1 *Phyllophaga* and *Pristionchus* association in North America

I could confirm that *Phyllophaga* beetles carry numerous *Pristionchus* nematodes. Interestingly, beetles collected in the states New York and Massachusetts showed a lower *Pristionchus* variability when compared to the sampling in Nebraska and Kansas. In general, 50 to 80 % of *Phyllophaga* beetles were positive for *Pristionchus* nematodes. The details are presented in table 3 and figure 9.

Table 3. Summary of *Phyllophaga* beetles collected and frequencies of *Pristionchus* isolated in each *Phyllophaga* species

Phyllophaga species	n°. of individuals collected	n°. of individuals positive for *Pristionchus*	percentage of individuals positive for *Pristionchus*
P. anxia (2007)	493	218	44.22
P. bipartita	7	4	57.14
P. congrua	55	42	76.36
P. crassissima	22	18	81.82
P. fusca	86	56	65.12
P .futilis	181	130	71.82
P. futilis (2007)	261	77	29.50
P. hirticula	1	1	100.00
P. implicita	18	18	100.00
P. inversa	4	3	75.00
P. praeterissima	1	0	0.00
P. rubiginosa	21	20	95.24
P. tristis	1	0	0.00
P. vehemens	47	37	78.72

When not differently specified, beetles were collected in 2008, in Nebraska and Kansas.

When possible, I isolated approximately 10 *Pristionchus* lines from each positive beetle. We observed that *P. pseudaerivorus* and *P. aerivorus* are the most common *Pristionchus* species on *Phyllophaga* beetles (figure 9).

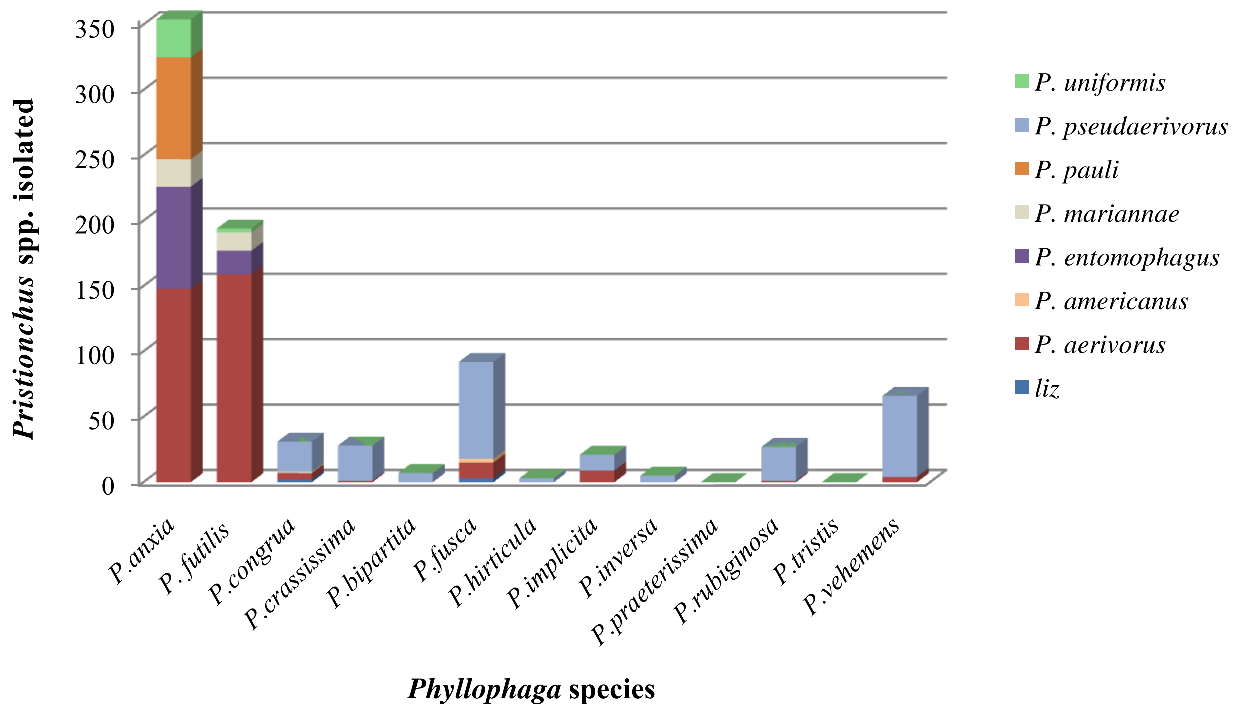

Figure 9. Frequencies of *Pristionchus* species isolated in different *Phyllophaga* species.

2.3.3.2 *P. aerivorus* and P. pseudaerivorus cohabitation

Herrmann (2006b) observed that from crosses between *P. aerivorus* and *P. pseudaerivorus* the majority of F1 were females, the homogametic sex in *Pristionchus*. This observation coincided with the scenario described by Haldane for species that are diverging. Additionally, during the laboratory crosses between *P. aerivorus* and *P. pseudaerivorus*, certain F1 SSU sequences showed a *P. aerivorus/s P. pseudaerivorus* mixed profile (Herrmann et al. 2006b).

One of my purposes during the field trip collection in 2007 and 2008 was to test whether these kinds of interbreeding cases could also happen in nature; specifically, inside the ecological niche that both *Pristionchus* species share, the *Phyllophaga* beetles. Only from the 2008 survey I could collect beetles, from which I isolated both *P. aerivorus* (figure 10a) and *P. pseudaerivorus* (figure 10b). Four *Phyllophaga futilis* and one *P. fusca* isolates were inhabitated at the same time by *P. aerivorus* and *P. pseudaerivorus.* Interestingly, some nematodes showed mixed or very similar *P. aerivorus* and *P. pseudaerivorus* SSU profiles (figure 10). In detail, 45 nematodes showed a *P. aerivorus/ P. pseudaerivorus* mix SSU sequence profile (figure 10c,d). Six individuals, denoted as "*liz*", showed to have a single nucleotide difference from the SSU sequence profile of *P. pseudaerivorus* (figure 10e). I could also detect 15 lines that resulted in a mixture of the *P. pseudaerivorus* and the "*liz*" SSU profile

(figure 10f). This result can be seen as a first indication that hybridization of *P. aerivorus* and *P. pseudaerivorus* does occur in nature.

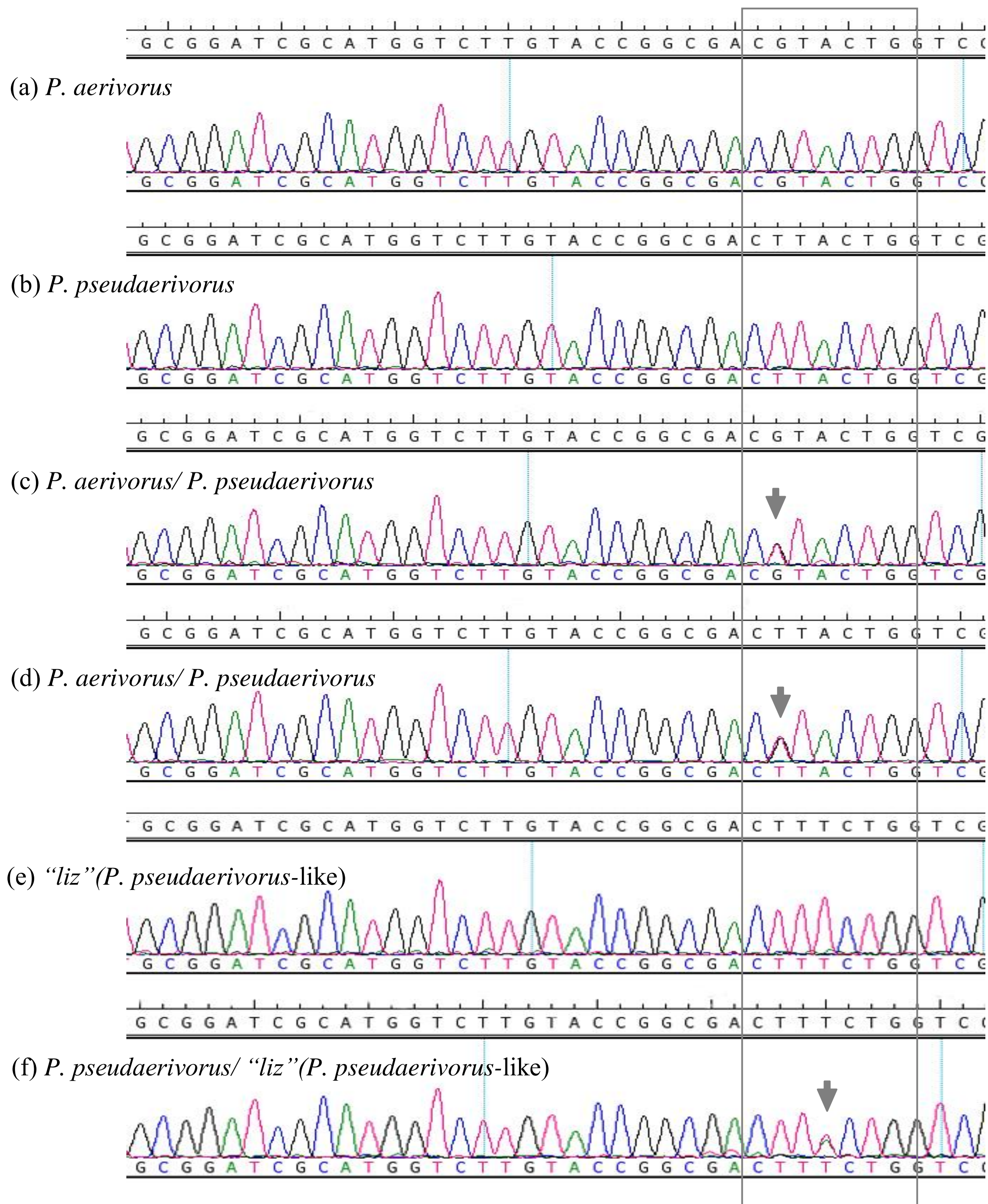

Figure 10. SSU sequence profiles of *P. aerivorus*, *P. pseudaerivorus* and some "mixed" profiles isolated from *Phyllophaga* spp.

2.3.3.3 Colorado potato beetle carries less *Pristionchus*

When compared to the *Phyllophaga – Pristionchus* association, the probability to isolate *Pristionchus* nematodes from the Colorado potato beetle is considerably reduced. In the 2008 survey from North America, we received and analysed 1021 *L. decemlineata* individuals. In only 160 (15 %) of them we could observe nematodes or nematode tracks. In figure 11 I present the nematode species from which I could establish stable lines and analyse the SSU species identification profile.

It is very important to note that, in contrast to scarab beetles, no other nematode species could be isolated from these sampled potato beetles. This low amount of nematodes that associates with *L. decemlineata* could be explained by the high level of toxicity present in this beetle haemolymph (Hsiao and Fraenkel 1969 and paragraph 2.5).

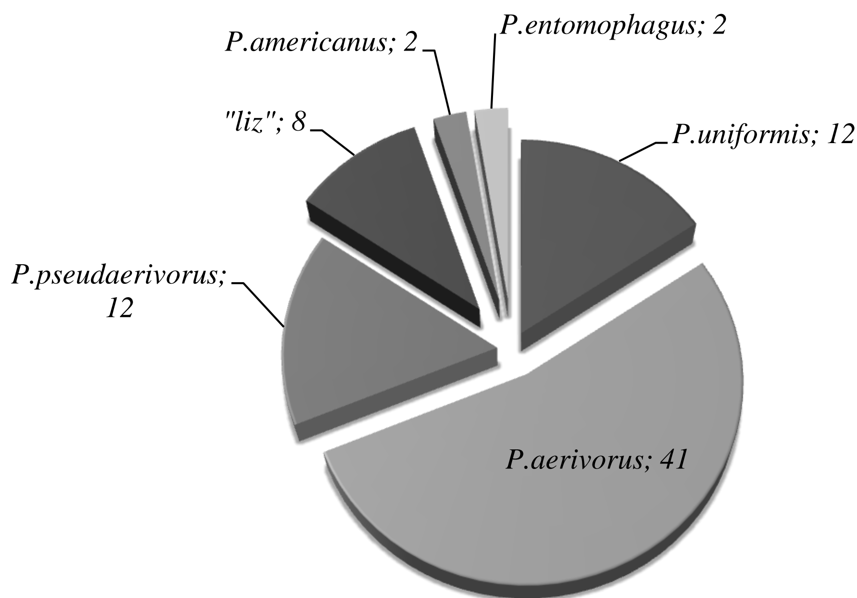

Figure 11. *Pristionchus* frequency on *Leptinotarsa decemlineata* (Colorado potato beetle).

2.4 Genetics of *P. uniformis*, a first approach

2.4.1 Background: interconnecting ecology and genetics

Early observations on multi-faceted biodiversity have already raised the question about the origin, structure, and evolution of genetic diversity. After Darwin and Mendel it becomes clear that "*an underlying reservoir of innate and heritable genetic possibilities delineated the options for the growth, development, and reproduction of organisms at both the individual and population levels*" (McCouch, 2001). Nowadays the genetic characterization of species and populations has become almost routine (Freeland 2005). Genetics and molecular biology can provide novel data and insights into the ecology and evolution of living organisms. For instance, molecular markers allow to quantify genetic diversity, measure inbreeding, characterize new species or follow species' biogeography changes. To deepen the understanding of evolutionary biology, an admixture of "exterior" (ecology) and "interior" (genetics) observations of organisms is necessary.

The merging of molecular and ecological studies were initially started in the hermaphroditic species *P. pacificus*. However, an exhaustive understanding of the evolutionary history of the *Pristionchus* genus can be fulfilled by including a dioceous (gonochoristic) species in the research project. Indeed, the vast majority of nematodes are gonochoristic and it has been already proven that gonochorism most likely was the "ancestral state" in nematode evolutionary history (Kiontke et al.2004; Haag 2005). In the Diplogasteridae group hermaphrodites are described in only two genera, *Diplogasteroides* and *Pristionchus* (Mayer et al. 2007).

During my doctoral work, while studying *P. uniformis* beetle association, I started to build tools that allow a genetic approach to this gonochoristic species investigation. Genetic knowledge of *P. uniformis* might help to identify the genetic basis of the unique association between the nematode *P. uniformis* and the Colorado potato beetle and possibly to facilitate the understanding of the genetic mechanisms that underlie organismal responses to their environment, such as the possible toxicity of the Colorado potato beetle.

Acquired information about *P. uniformis* genome can also serve to start a comparative analysis with the model *P. pacificus*. Comparative genomics can facilitate not only the investigation of specific evolutionary questions, such as the mechanism behind the beetle-nematodes association, but also the study of chromosome evolution (Pringle et al. 2007). For example, the examination of synteny at the genomic scale can elucidate chromosome homology and provide a framework for predicting the locations of genes in other species (*C. elegans* Sequencing Consortium 1998).

The term synteny (σΰν - sun: together, ταινία – tainia: ribbon) is used in genetics to indicate the presence of two or more loci on the same chromosome (Renwick 1971). Conserved synteny can, in the strictest sense, be viewed as sequence conservation between chromosomes of two related species, irrespective of whether coding or non-coding sequence is examined. In addition, knowledge of gene order

or homologous chromosomes allows investigations of types and prevalence of chromosomes rearrangements (O'Brien et al. 1999). All genomes encode conserved genes and the arrangements of these genes on chromosomal elements is determined by a balance between stochastic rearrangements and functional constraints (Guiliano et al., 2002). Several gene clusters have been identified in the Metazoan. These include the Hox gene clusters (Ferrier & Holland, 2001), the histone genes (Hentschel & Birnstiel 1981) and the major histocompatibility complex (MHC) (Ohta et al., 2000), but most genes are believed to be free to move within the genome (Guiliano 2002). Vertebrate chromosomes are mosaic structures containing large conserved segments that can reside in different linkage groups in different species.

To facilitate the molecular genetics study in *P. uniformis* and enhance its utility for further comparative analysis, we sought to convert the transcriptome sequence assembly into a genome map in which the genome sequence and the genetic maps are linked to each other through common markers across the chromosomes. Here, I present the reconstruction of *P. uniformis* chromosomes by generating a genetic linkage map based on nuclear markers tested on a meiotic mapping panel of inbred lines.

2.4.2 Methods

2.4.2.1 Recombinant inbred lines

Two phylogenetically and ecologically divergent *P. uniformis* strains were used to generate recombinant inbred lines, RS5244 isolated from *Leptinotarsa decemlineata* (Chrysomelidae) in North America and RS5070 isolated from *Melolontha melolonhta* (Scarabaeidae) in Germany. Typically, dioceous species carry a high amount of heterozygosity. In *P. uniformis*, I tried to reduce heterozygosity by inbreeding the two selected strains for at least ten generations.

- [5 males RS5244 X 1 female RS5244] inbred for 10 generations
- [5 males RS5070 X 1 female RS5070] inbred for 10 generations

Inbred RS5244 and inbred RS5070 were then reciprocally crossed:

- C lines, [5 males RS5244 X 1 female RS5070] inbred for 10 or 11 generations
- M lines, [5 males RS5070 X 1 female RS5244] inbred for 10 or 11 generations

I set up 210 reciprocal crosses and observed only 64 lines surviving (38 M lines and 26 C lines). All these lines were then used to continue sibling crosses and the 42 that reached the 10th or 11th generation were used for the construction of the meiotic panel.

2.4.2.2 Polymorphic markers

The two distantly related *P. uniformis* strains were also used to detect polymorphic genetic markers. Known sequences of *P. pacificus* loci (from all six chromosomes) were used to search orthologous in the *P. uniformis* transcriptome (obtained with *454 pyrosequencing* technique of the reference inbred strain RS5244 in collaboration with C. Dieterich at the MDC in Berlin). Markers were chosen according to the *P. pacificus* genetic linkage map in order to cover all six chromosomes with an average distance of 10 cM between them. Using the Basic Logic Alignement Search Tool (Blast-n), the selected *P. pacificus* loci were compared with the *P. uniformis* transcriptome. *P. uniformis* contigs (sets of overlapping DNA segments) that show high similarity were used to design primers pairs with the on-line tool “Primer3” (http://frodo.wi.mit.edu/primer3/).

P. uniformis candidates were then tested in the two inbred strains RS5244 and RS5070 to discover polymorphic sites (table 4). I amplified *P. uniformis* loci via PCR (polymerase chain reaction) using the following protocol: genomic DNA was prepared from three overgrown 6-cm NGM plates. Plates were washed three times in distilled water. DNA was isolated with a Genomic DNA extraction kit (MasterPure^{TM} DNA Purification from EPICENTRE). The DNA was diluted to approximately 25 ng/µl for the polymerase chain reactions (PCR). PCR was performed in 25 µl 1x PCR buffer (Amersham) containing 1 U Taq DNA Polymerase (Amersham), 0.5 µM of each primer, 0.2 mM of each deoxynucleotide triphosphate and 4 µl of DNA lysate. PCR experiments were performed as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 50°C for 30 s and extension at 72°C for 60 s. A final incubation step at 72°C was performed for 7 min. Polymorphism detection was done via sequence analyses. PCR products were diluted 1:20 with ddH20, sequenced without further purification from both ends (Big Dye terminator protocol, Applied Biosystems, ABI373*xl* capillary platform) and sequence trace files were visualized with the software SeqMan (DNAStar Inc.). For this study, both single nucleotide polymorphisms (SNP) and insertion/deletion (INDEL) were used.

2.4.2.3 Linkage analyses

In total, 42 recombinant inbred lines were scored by genotyping 48 polymorphic markers (table 5). Each recombinant inbred line could show the RS5244, RS5070 or heterozygous genotype. Genotypes of each recombinant inbred line were entered into the meiotic panel scheme and then analysed in MapDisto software (available at http://mapdisto.free.fr/MapDisto/, Lorieux 2008) to elaborate the linkage groups.

Only markers and lines with clear genotypes have been included in the final analyses. This resulted in 44 markers studied in 38 recombinant inbred lines. Linkage groups assembly and marker order were determined using MapDisto software version 1.7.0 for MS Excel, employing the Haldane mapping unction, with a minimum LOD score of 5.0 and maximum recombination fraction of 0.30. Haldane

function applies to situations, in which interference of crossing over is absent. Considering that this is the case for *P.pacificus,* we assumed that *P. uniformis* genome behaves like *P. pacificus* (Srinivasan et al. 2002).

2.4.2.4 Mutagenesis and karyotype analyses

To test the possibility of obtaining mutant lines in a gonochoristic species in forward genetic approaches, I did preliminary mutagenesis tests on the reference strain of *P. uniformis* RS5244 with EMS (Ethyl methane sulfonate) mutagenesis. I followed the mutagenesis protocol described in Pires da Silva (2006).

In order to verify the agreement between the linkage groups detected with the meiotic panel analyses and the number of chromosomes, I analysed *P. uniformis* RS5244 karyotype using the following staining protocol. I fixed worms in Carnoy solution and stained DNA in the germ line with 1 µg/ml of 4,6-diamidino-2-phenylindole (DAPI). In detail, six plates of worms were washed with 1 ml of PBS and pelleted by centrifugation for 20 seconds at 6,000 rpm. Worms were fixed for 2 hrs in Carnoy solution at room temperature. Worms were then rinsed two times with PBS and after that were dried off as much as possible. To stain the DNA, 10 µl of VectaShield mounting medium (H-1000, Vector Laboratories, Inc.) containing 4′,6-diamidino-2-phenolindole (DAPI, D-1307, Molecular Probes) at a final concentration of 1 µg/ml was added and incubated over night at RT. VectaShield containing worms were transferred onto a slide and the cover slip was added and sealed with nail polish. Samples were viewed on a Axioplan 2 microscope (Zeiss) using a Polychromator Illuminator System (Visitron System,GmbH). Pictures were taken using the Meta View program (Meta Series 4.5, Visitron System, GmbH) and a digital camera (MicroMax 5MHz System, Princeton Instruments, Inc.).

2.4.3 Results

2.4.3.1 Inbred lines

After 10 generations of sibling crosses of RS5244 and RS5070, no inbreeding depression was observed. However, during the reciprocal crosses of RS5244 with RS5070, the speed of growth decreased dramatically. After the sixth generation, the crosses needed to be repeated several times until healthy offspring for the following generation crosses could be produced.

2.4.3.2 Polymorphic markers

Numerous loci were shown to be polymorphic (both with single nucleotide polymorphism and insertion-deletion) between the *P. pacificus* (PS312) and the *P. uniformis* (RS5244) sequence (table 4).

Table 4. List of markers that show sequence polymorphisms between *P. pacificus* (PS312) and *P. uniformis* (RS5244)

primer n°	primer name	primer n°	primer name	primer n°	primer name	primer n°	primer name	primer n°	primer name	primer n°	primer name
22876_77	G 1	22902_03	G8*	22624_25	G 16*	22938_39	G 25	22958_59	G 29*	22982_83	G 40
22878_79	G 2*	22440_41	G9	22386_87	G 17	22446_47	G 26	22644_45	G 30*	22984_85	G 41*
22880_81	G 3*	22378_79	G10	22926_27	G 18	22396_97	G 27	22454_55	G 31*	22988_89	G 42
22600_01	G 4	22616_17	G11	22928_29	G 19*	22952_53	G 28	22960_61	G 32	22990_91	G 43
22598_99	G 5	22438_39	G12*	22930_31	G 20	25035_36	G 57*	22408_09	G 33	22994_95	G 44
22594_95	G 6	22908_09	G13	22932_33	G 21	25044	G 58	22636_37	G 34	22456_57	G 45*
22894_95	G 7*	22910_11	G14	22444_45	G 22			22642_43	G 35	23006_07	G 46
22432_33	G 53*	22916_17	G15	22384_85	G 23			22634_35	G 36*	23000_01	G 47
22434_35	G 54			22934_35	G 24			22972_73	G 37	23004_05	G 48
24991_92	G 55							22974_75	G 38	23008_09	G 49
25015_16	G 56							22976_77	G 39		
25081_82	G 59										
25091_92	G 60										
25095_96	G 61										
22372_73 (rpl)	G 50*										
22374_75 (rpl)	G 51*										
22376_77 (rpl)	G 52*										

The chromosome number refers to *P. pacificus* chromosomal organization. * indicates that the marker was not used in the genetic linkage map because amplification was not possible for at least 42 recombinant inbred lines.

2.4.3.3 Linkage groups

Chromosomal rearrangement comparison between species belonging to the same genus with sequence-based approach showed evidence for conservation of synteny between *P. pacificus* and *P. uniformis* (table 5 and figure 12). The presence of six linkage groups matched with the number of chromosomes that was detected with DAPI staining (figure 13).

2.4.3.4 *P. uniformis* karyotype and morphological mutants

Typical *Pristionchus* karyotype was conserved in *P. uniformis*. Figure 13 shows that six condensed chromosomes are present. Preliminary ethyl methane sulphonate (EMS) mutagenesis of the *P. uniformis* reference strains (RS5244) was performed to obtain mutant animals. I obtained different morphological mutants (figure 14).

Table 5: Meiotic mapping panel

P.pacificus chromosome	markers	M 33	M 32	C 44	C 102	C 53	C 83	M 100	M 106	C 89	C 24	M 47	C 73	C 12	C 48	M 7	M 86	M 53	M 45	C 85	M 18	M 1	C 42	C 3	M 60	C 20	M 79	M 94	C 15	M 43	C 45	C 6	M 77	M 52	M 17	C 59	M 49	M 23	M 46
		lines																																					
I	G1	C	C	C	C	C	C	C	C	C	C	C	C	C	M	M	M	M	M	M	M	M	M	M	M	n.d.	M	M	M	M	M	M	M	M	M	M	M	M	M
	G4	C	C	C	-	C	M	M	M	-	M	-	M	M	M	M	M	M	M	M	M	M	M	M	M	-	M	M	M	M	M	M	-	M	M	M	M	M	M
	G5	C	C	C	C	M	C	C	C	C	C	-	C	C	C	C	C	C	C	C	C	C	C	C	C	C	-	C	C	-	C	-	C	C	C	M	M	-	M
	G6	C	C	C	C	M	C	C	C	C	C	-	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	-	C	C	M	C	M	M	M
	G54	C	C	C	C	M	M	M	M	n.d.	M	n.d.	M	M	M	n.d.	M	M	M	M	M	M	M	M	n.d.	M	M	M	n.d.	C	n.d.	M	M	n.d.	M	M	M	M	M
	G55	-	M	-	M	M	M	M	M	n.d.	n.d.	M	M	-	C	-	M	M	M	M	-	M	M	M	M	C	M	M	M	M	M	-	C	n.d.	-	M	-	M	M
	G56	C	C	C	n.d.	M	C	C	C	n.d.	C	-	C	C	C	n.d.	-	C	C	C	C	C	C	C	C	C	C	C	C	-	C	C	C	n.d.	C	C	M	-	C
	G59	C	C	C	C	C	C	M	M	n.d.	C	M	M	M	M	M	M	M	C	C	C	M	M	M	M	M	C	M	n.d.	M	M	M	C	M	M	M	M	M	C
	G60	C	C	C	M	C	n.d.	C	C	n.d.	n.d.	n.d.	M	n.d.	M	n.d.	M	M	C	M	C	M	M	n.d.	n.d.	C	M	n.d.	n.d.	M	n.d.	M	C	n.d.	M	M	n.d.	n.d.	n.d.
	G61	C	C	C	C	M	C	C	C	n.d.	C	n.d.	C	C	C	C	n.d.	C	C	C	C	C	C	C	C	C	C	C	n.d.	n.d.	n.d.	C	C	M	C	C	M	M	C
II	G9	n.d.	n.d.	C	n.d.	C	C	C	C	M	C	M	CMd -	n.d.	-	M	C	M	n.d.	C	C	M	M	n.d.	M	C	C	C	C	M	C	C	C	C	n.d.	CMd -	n.d.	M	C
	G10	-	C	C	C	M	-	C	C	M	M	M	C	C	-	M	C	M	C	-	C	M	M	-	M	C	C	-	C	M	C	C	M	C	M	-	C	M	C
	G11	C	C	C	C	C	C	C	C	M	C	M	-	C	-	M	C	M	C	C	C	M	M	C	M	C	C	C	C	M	C	C	C	C	M	-	C	M	C
	G13	C	C	C	C	M	M	C	n.d.	M	M	n.d.	C	C	C	n.d.	C	C	M	n.d.	C	n.d.	n.d.	n.d.	M	C	C	C	C	C	C	C	n.d.	C	C	C	C	n.d.	M
	G14	C	C	C	M	M	-	n.d.	C	M	M	M	C	n.d.	-	M	C	M	C	M	C	M	n.d.	-	M	C	C	-	C	n.d.	C	C	M	C	n.d.	C	C	M	C
	G15	C	C	C	M	M	C	M	C	C	C	C	M	C	M	C	M	C	M	C	C	M	M	M	M	M	M	C	M	C	C	M	M	M	M	C	M	M	M
III	G17	C	C	C	C	-	C	C	C	-	C	M	-	M	C	M	-	M	C	M	M	M	M	M	M	-	M	M	M	M	-	M	M	n.d.	M	C	-	C	M
	G18	C	C	M	C	-	-	C	C	C	C	M	-	M	C	M	C	C	C	M	M	M	M	M	-	-	M	M	M	M	-	M	M	C	C	C	-	C	-
	G20	C	C	C	C	M	-	C	C	C	C	M	C	M	C	M	C	C	C	M	M	M	M	n.d.	C	M	-	M	C	M	-	M	C	n.d.	M	C	M	C	-
	G21	n.d.	C	C	n.d.	C	C	n.d.	n.d.	C	C	M	C	n.d.	C	n.d.	n.d.	C	C	n.d.	M	n.d.	n.d.	n.d.	n.d.	n.d.	C	n.d.	C	n.d.	C	n.d.	C	C	C	n.d.	n.d.	C	n.d.
	G22	C	C	C	C	-	-	-	C	C	C	M	-	M	C	M	-	C	C	M	M	M	M	M	C	-	-	M	C	M	-	M	M	C	-	C	-	C	-
	G23	C	C	C	C	-	-	C	C	-	M	M	-	M	C	M	M	M	-	M	M	M	C	M	-	-	M	M	M	M	C	M	M	n.d.	-	C	-	C	-
	G24	C	C	C	C	-	n.d.	-	n.d.	C	C	M	n.d.	n.d.	C	n.d.	n.d.	C	C	M	M	M	n.d.	n.d.	-	-	M	M	C	M	-	M	M	n.d.	-	C	M	C	-
IV	G25	M	C	M	M	C	C	M	M	-	C	C	M	M	M	M	C	C	M	M	C	M	C	M	M	C	M	M	M	C	M	-	M	M	M	M	M	C	M
	G26	C	n.d.	n.d.	M	M	M	C	C	M	C	C	n.d.	n.d.	n.d.	C	M	M	n.d.	C	C	M	n.d.	n.d.	C	C	M	M	C	C	C	n.d.	n.d.	n.d.	n.d.	n.d.	C	M	C
	G27	M	M	M	M	M	M	M	M	M	M	M	M	M	C	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	C	M	M	M	M
	G28	M	M	M	M	C	M	C	C	M	-	M	C	M	M	C	C	M	C	-	-	C	M	C	M	C	M	C	M	M	M	M	C	M	C	M	M	C	-
	G58	C	C	M	M	M	n.d.	C	C	n.d.	M	C	C	C	C	M	C	C	M	M	M	M	C	M	M	C	M	M	M	C	n.d.	M	M	n.d.	M	M	M	M	M
V	G32	C	C	C	C	M	M	M	M	-	C	C	-	C	C	C	M	C	C	C	C	C	C	-	C	C	M	M	C	C	C	-	C	C	C	C	C	-	C
	G33	C	C	C	C	M	C	C	M	C	C	C	C	C	C	C	M	C	C	C	C	C	C	-	M	C	M	M	C	C	C	M	C	C	C	C	C	C	C
	G34	C	C	C	n.d.	M	C	C	n.d.	C	C	C	C	C	C	C	n.d.	C	C	C	n.d.	n.d.	C	n.d.	C	C	C	M	C	C	C	C	C	n.d.	C	C	C	C	C
	G35	C	C	C	C	M	M	-	M	-	C	C	M	C	-	C	M	C	C	C	C	C	C	-	M	M	M	M	C	C	C	-	C	C	C	C	C	-	C
	G37	C	C	C	C	M	n.d.	n.d.	M	n.d.	C	C	n.d.	M	C	C	M	n.d.	n.d.	C	C	C	n.d.	n.d.	C	C	M	M	C	C	C	M	C	C	C	C	C	n.d.	n.d.
	G38	C	C	C	C	C	M	C	n.d.	C	n.d.	M	C	M	C	C	M	M	C	M	M	M	C	M	C	C	n.d.	M	n.d.	M	M	M	C	M	C	C	C	C	C
	G39	C	C	M	M	M	M	M	M	C	C	C	M	n.d.	M	C	C	C	M	C	M	M	C	n.d.	C	M	M	M	M	C	M	M	M	n.d.	n.d.	M	M	C	M
X	G40	n.d.	n.d.	C	M	M	M	M	M	C	M	n.d.	n.d.	M	M	M	M	n.d.	n.d.	M	M	M	n.d.	M	M	n.d.	M	M	n.d.	M	M	M	n.d.	n.d.	n.d.	M	M	M	n.d.
	G42	-	C	M	M	M	C	C	M	M	M	C	C	C	C	C	C	C	M	C	M	M	C	M	M	C	M	M	M	C	C	M	M	C	M	M	M	M	M
	G43	-	-	C	M	M	M	-	M	-	M	-	n.d.	-	-	M	M	-	M	M	M	M	-	M	M	-	M	M	M	-	M	M	-	M	M	-	M	-	M
	G44	-	-	C	n.d.	M	n.d.	M	n.d.	n.d.	M	n.d.	M	-	-	n.d.	M	M	M	M	M	M	-	M	M	M	M	M	M	n.d.	M	n.d.	n.d.	M	n.d.	-	M	M	M
	G46	C	C	C	C	M	M	M	M	M	C	C	M	C	-	C	M	C	C	C	C	C	C	C	C	C	M	M	C	-	C	-	C	C	C	C	C	-	C
	G47	n.d.	n.d.	C	M	M	M	M	n.d.	n.d.	M	M	M	M	C	M	n.d.	M	n.d.	M	M	n.d.	n.d.	M	M	n.d.	M	M	n.d.	M	n.d.	M	M	n.d.	M	M	n.d.	n.d.	M
	G48	-	M	C	M	M	M	C	M	M	M	M	M	n.d.	n.d.	n.d.	n.d.	M	M	M	M	M	n.d.	n.d.	M	M	n.d.	M	M	M	M	M	M	n.d.	M	M	M	M	M
	G49	C	C	C	C	-	n.d.	-	-	n.d.	n.d.	M	C	M	C	M	n.d.	C	C	M	M	M	M	M	-	-	M	-	C	M	C	C	n.d.	n.d.	n.d.	C	M	C	n.d.

In the first column from the left, *P. pacificus* chromosome numbering. In the second column the polymorphic markers between RS5244 and RS5070 are listed. They are grouped according to the *P. pacificus* chromosome grouping (e.g. G1 marker amplifies a *P. uniformis* locus sequence, orthologous to a locus in *P. pacificus* chromosome I). In the subsequent columns the recombinant inbred lines genotypes are displayed. Orange (C) indicates that the recombinant inbred line resulted in C lines-like genotype, blue (M) corresponded to the M lines-like genotype and green indicated an heterozygous genotype.

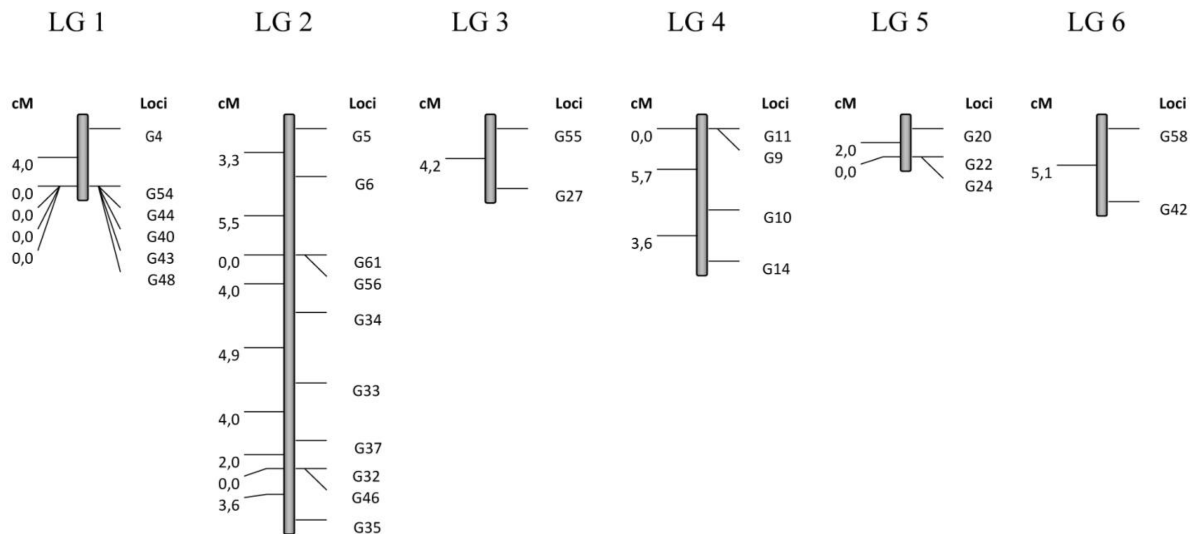

Figure 12. Preliminary genetic linkage groups of the *P. uniformis* genome. Numbers to the right on each bar are the markers and the numbers on the left indicate the genetic distance in centimorgan (cM). The numbering of LGs (linkage groups) is based on homology with the *P. pacificus* chromosomes.

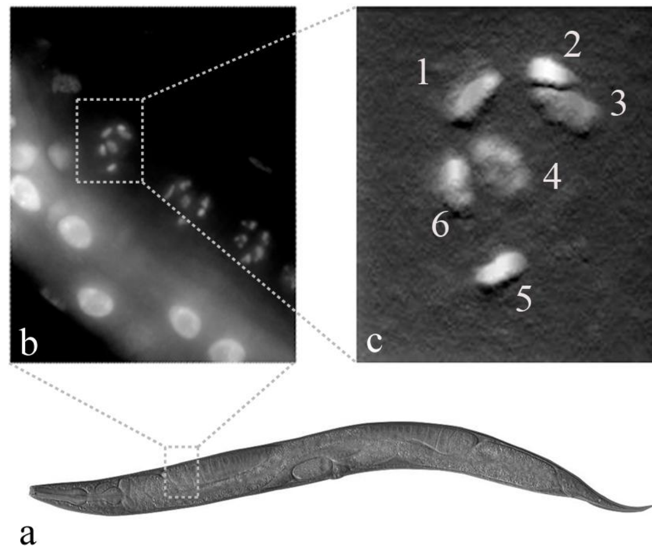

Figure 13. *P. uniformis* karyotype. In an adult nematode the mature oocytes is marked with the dashed square (a) (differential interference contrast (DIC) microscopy illumination). (b) DAPI fluorescence highlighted the condensed chromosomes. In (c) a deconvoluted and magnified image of the marked oocytes, 6 chromosomes are clearly visible.

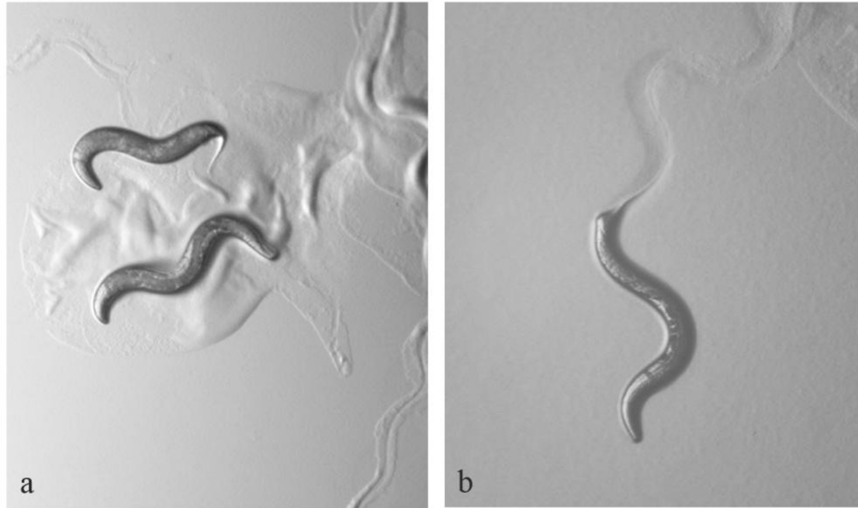

Figure 14. *P. uniformis* putative mutants. In (a) a comparison between an adult *Dumpy* like mutant (worm above) and a wild type *P. uniformis* female is presented. Typically adult *Dumpy* mutants show a shorter and fatter phenotype than the wild type. Figure (b) shows a putative defective defecation-like mutant. Pictures were taken with a Zeiss V12.

2.4.4 Conclusion

The limited interchromosomal rearrangements between *P. uniformis* and *P. pacificus* indicate the existence of synteny between this *Pristionchus* species. This was predictable considering that synteny conservation had already been detected between the more distantly related *P. pacificus* and *C.elegans* (Lee et al. 2003).

If synteny and gene order are strongly conserved across Diplogastrids, the utilization of the genomic resources developed in *P. pacificus*, such as a fully assembled genome, will become a powerful resource for predictive gene finding, and comparative linkage mapping in other *Pristionchus* species.

2.5 Unique association between *P. uniformis* and the unwelcome Colorado potato beetle. Toxicity tests with *Leptinotarsa decemlineata* haemolymph

2.5.1 Background

One of the *P. uniformis* hosts, the aposematic *L. decemlineata* (Colorado potato beetle) is well protected against predators by the secretions of defensive glands (Daloze & Pasteels 1986; Pasteels & Rowell-Rahier, 1990) and by toxic substances present in the haemolymph (Hsiao & Fraenkel 1969). The major haemolymph compound is the protein β-leptinotarsin d or h ("d": when extracted from *L. decemlineata* and "h" when derived from *L. haldemani*). The molecular weight of the protein was determined as being between 45 and 57 kDa (Armer & Berry 2004; Miljanich et al. 1988). The polypeptide leptinotarsin seems to be present in all developmental stages and does not derive from the host plant; in fact the Colorado potato beetle does not sequester the glycoalkaloids present in their food plant (Solanaceae) (Rothschild 1972; Armer 2004).

Haemolymph extracts were seen to be toxic for both, insects and vertebrates (Hsiao and Fraenkel 1969). The toxic effect of potato beetle haemolymph on different animals was determined by the injection of purified haemolymph. For example, the minimum lethal dose for the house fly was 500 mg per kg (Hsiao and Fraenkel 1969). In contrast, the minimum dose required to kill white mice within 40 hours is about 25 mg per kg (Hsiao and Fraenkel 1969). These authors reported that the haemolymph from one potato beetle larva would be sufficient to kill five mice.

Leptinotarsin has been characterized as a neurotoxin. It promotes the influx of Ca^{2+} and the release of neurotransmitters from the presynaptic nerve terminal (McClure et al. 1980; Crosland et al. 1984; Madeddu et al. 1985; Miljanich et al. 1988). In detail, leptinotarsin protein interacts with the presynaptic terminal to modify the process of release and induces release of acetylcholine. This property means that leptinotarsin is also a useful tool for studying neurotransmitter activity; leptinotarsin could indeed act by activating specifically the voltage dependent Ca^{2+} channels (for instance in rat, mouse, cow or guinea pig) (Crosland et al. 2005).

The entomoparasitic nematode *Heterorhabditis marelatus* can kill the Colorado potato beetle, but cannot reproduce on it (Blackburn et al. 2007). This could be explained by the observation that the Colorado potato beetle haemolymph can negatively affect the symbiotic bacteria of *H. Marelata*, *Photorhabditis luminescens* (Armer et al. 2004). Dalzoe et al. (1986) isolated a toxic dipeptide containing a nonprotein amino acid from potato beetle defensive secretions and identified it as γ–L–glutamyl-L–2–amino–3(*Z*),5–hexadienoic acid. This dipeptide exhibited toxic properties when ingested by ants (*Myrmica rubra*). However, they did not show any correspondence with the compound defined as leptinotarsin.

We hypothesized that the absence of other nematodes in the Colorado potato beetle could be due to the toxicity in the beetle haemolymph and that *P. uniformis* may have evolved

mechanisms to overcome the toxicity associated with the *L. decemlineata* habitat. To test whether *P. uniformis* is characterized by a unique toxicity resistance, I compared the effects of Colorado potato beetle haemolymph on diverse nematode species.

2.5.2 Methods

2.5.2.1 Nematode culture and development comparison

The nematode strains used in this study were *Caenorhabditis elegans* reference strain N2, *Pristionchus pacificus* reference strain PS312 and the *P. uniformis* isolated from Colorado potato beetle, RS5244. Breeding and maintenance of *Pristionchus* spp. follows *C. elegans* standard culture methods that have been described previously (Brenner 1974). To generate dauer larvae, I followed the "wet plate protocol" described in Brown et al. 2011.

The timing of worms to adult stage development was investigated in *P. pacificus* PS312 and *P. uniformis* RS5244. I scored the total hours necessary to reach the adult stage from egg laying. I placed gravid hermaphrodites or females on agar plates and transferred the freshly layed eggs onto single plates. Plates were kept at a constant temperature of 20° C.

2.5.2.2 Haemolymph extraction

Haemolymph was extracted from fourth instar larvae or pupae of *L. decemlineata* reared on potato (*Solanum tuberosum*) in the laboratory. The group of beetles used to establish the laboratory culture was originally collected in Tübingen (Germany). One experiment was done by extracting haemolymph from freshly field collected *L. decemlineata* from Valdobbiadene (Italy).

Leptinotarsin is heat labile, ammonium precipitable and non-dialyzable. Therefore, after pricking the fourth instar larvae abdomen with a sterile syringe, the freshly collected haemolymph was kept in an ice bath to prevent coagulation and darkening. Extracted haemolymph was centrifuged at 10,000 rpm for 10 minutes in a refrigerated centrifuge. The supernatant was frozen until use (freezing does not denature the protein, Hsiao and Fraenkel 1969). Toxicity tests were carried out with both centrifuged and pure haemolymph but no differences were noticed.

2.5.2.3 Haemolymph bath toxicity tests

In the first kind of "haemolymph bath" test, nematodes (adults or dauer) were placed in sterile plastic well plates covered by lids and kept in the dark during the trial. Each well contained variable amounts of the dense extracted haemolymph. To facilitate nematode observation, the haemolymph was diluted in S-medium (a salt buffer commonly used for nematode liquid cultures, consisting of: NaCl, K_2HPO_4, cholesterol (5mg/ml in ethanol), dd-H20, 1M Potassium citrate pH 6, 1M $CaCl_2$, 1M $MgSO_4$). In the control wells only S-medium was applied. A comparison between

heated (10 minutes at 60 °C) and unheated haemolymph was performed. Different haemolymph concentrations were also applied.

During the second kind of "bath" experiment, the nematodes were exposed to direct contact with the beetles. If disturbed, from the potato beetle individuals can ooze a secretion derived from the haemolymph.

2.5.2.4 Haemolymph ingestion toxicity tests

Taking into account the big protein size that characterizes the toxic leptinotarsin compound, I tried to test whether bacteria food enriched with haemolymph extract would have a harmful effect on the different nematode species tested. The common bacterial food, *Escherichia coli* (OP50 strain), was grown in a liquid culture and concentrated by long centrifugation, until dry pellets were formed. Approximately 100 µl of concentrated *E. coli* (OP50) mixed with 40 µl of haemolymph were applied on agar plates prepared without peptone and cholesterol addition. Parallel controls were done by pouring the dry bacteria without the haemolymph. Third larval stage nematodes were applied in the test plate and observed for several days.

2.5.3 Results

2.5.3.1 *P. uniformis* develops slower than *P. pacificus*

P. pacificus and *P. uniformis* species reach L4 stage in about three days. To develop until the reproductive stage, the gonochoristic *P. uniformis* species requires about 10 hours more than the hermaphroditic *P. pacificus*.

Table 6. *P. pacificus* and *P. uniformis* development duration comparison

stage	*P. pacificus* *(RS2333)*	*P. uniformis* *(RS5244)*	hours difference
Egg laid	0 hr	0 hr	
L1	15.9 hr	21.7 hr	5.8 hr
L2	24.3 hr	43.2 hr	18.9 hr
L3	44.6 hr	54.8 hr	10.2 hr
L4	63 hr	72 hr	9 hr

Total time (hours) required to develop the different larval stages are indicated in the second and third column. In the fourth column, the time discrepancy between and *P. uniformis* and *P. pacificus* is indicated per each stage.

2.5.3.2 *P. uniformis* does not show peculiar haemolymph resistance

To study a particular effect of Colorado potato beetle haemolymph on the survival of *P. uniformis, P. pacificus* and *C. elegans*, the response of L4 and dauer larvae was tested. Heat treated or untreated haemolymph in different concentrations did not significantly affect the survival of nematodes in the "bath test". Results are shown for *C. elegans* (N2), *P. pacificus* (PS312) and *P. uniformis* (RS5244) in figure 15.

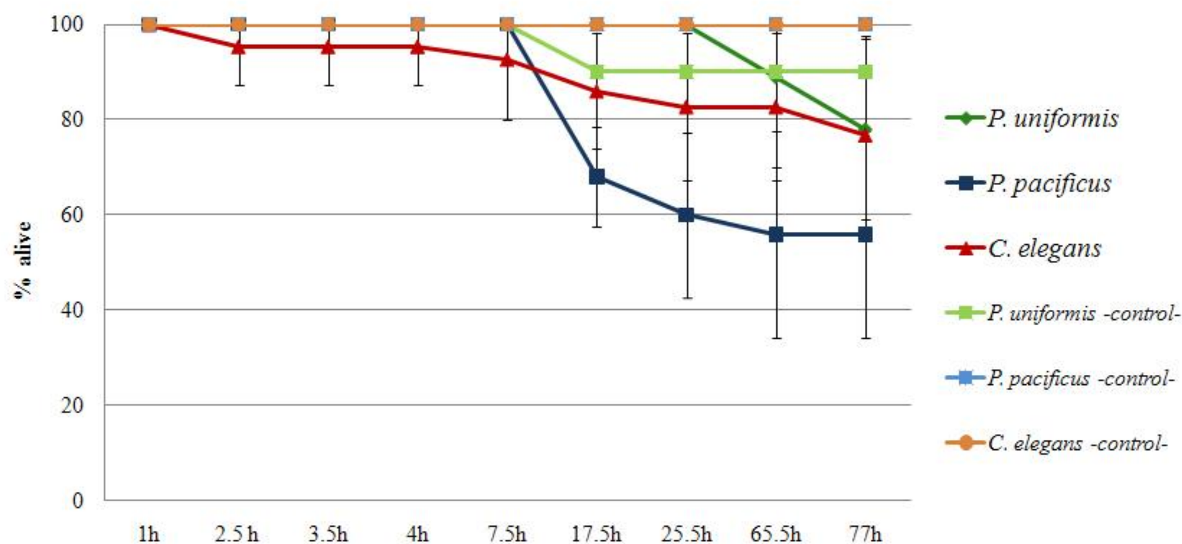

Figure 15. L4 larvae *C. elegans* (N2), *P. pacificus* (PS312) and *P. uniformis* (RS5244) were immersed in wells containing untreated haemolymph (5% in the total volume of 50 µl) extracted from fourth instar *L. decemlineata* larvae.

To test whether a long laboratory culture may have affected Colorado potato beetle physiology, I repeated the "bath tests" extracting haemolymph from beetles freshly collected from the wild. Haemolymph was extracted from pupae. In this experiment, presented in figure 16, I also used nematode in the arrested dauer stage instead of adults or L4, and increased the haemolymph concentration. Considering that laboratory dauer induction does not allow enough *P. uniformis* to develop and that this test aimed to check if the two non-potato beetle-associated nematode species would be affected by a stronger toxin concentration, *P. uniformis* was not included.

Given that the toxic haemolymph has a defensive role, the toxicity should have a rapid effect on the invading nematodes once they come in contact. Therefore, in the experiments shown in figure 16**,** nematodes were carefully checked for only three hours. In some cases, nematodes in contact with haemolymph died faster than the control samples; therefore, haemolymph may have a negative influence on nematode survival. In any case, the goal of these experiments was to investigate whether *P. uniformis* species shown a peculiar difference in surviving haemolymph toxins when compared with other nematode species.

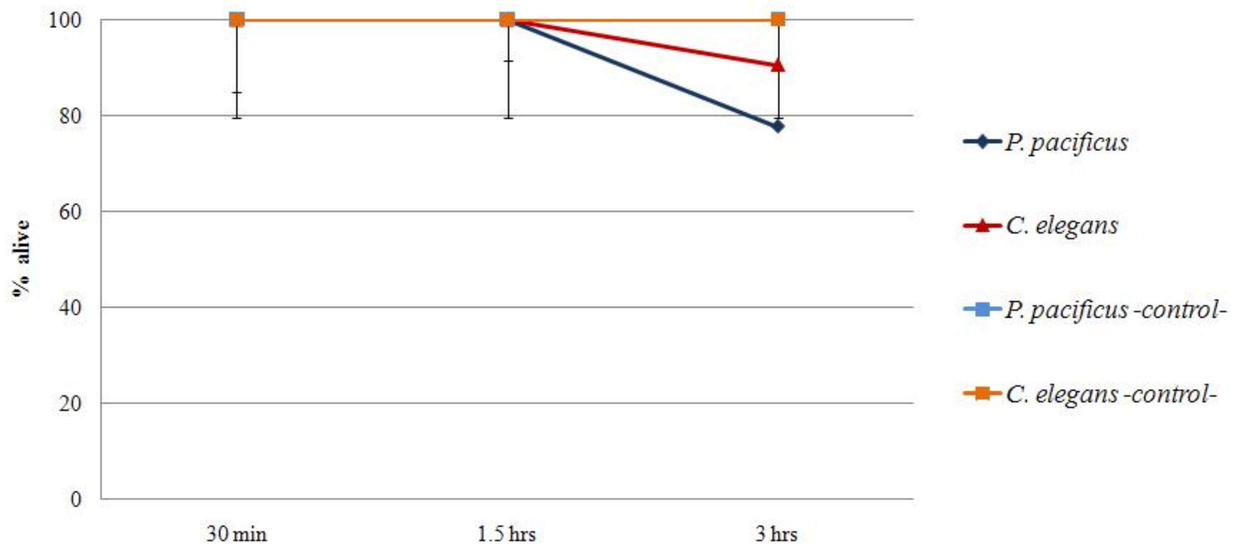

Figure 16. Dauer larvae *C. elegans* (N2), *P. pacificus* (PS312) were immersed in wells containing untreated haemolymph (67% in the total volume of 75 µl) extracted from pupae *L. decemlineata* larvae.

In the second kind of "bath test", the nematodes were exposed directly to the whole beetles, fourth instars or pupae. The beetle was place in the well, covered by S-medium and then the nematodes were applied. Figures 17 and 18 show that there is no significant difference in the nematode survival between treated and untreated samples.

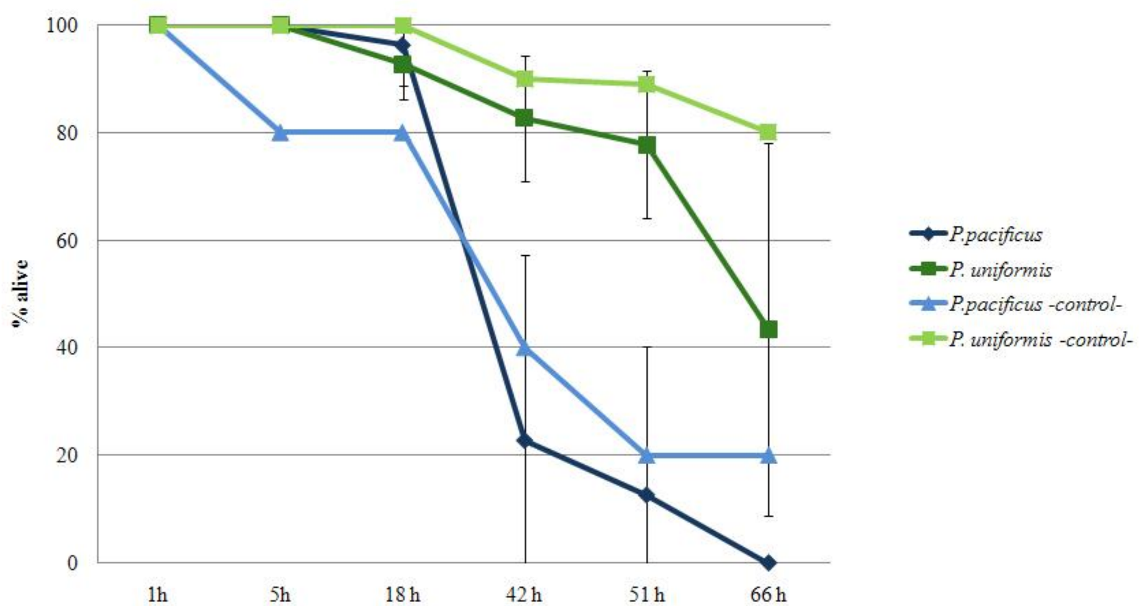

Figure 17. L4 larvae of *P. pacificus* (PS312) and *P. uniformis* (RS5244) were immersed in wells containing one fourth instar of *L. decemlineata.*

The results of figure 18 indicate that beetle that freshly moulted from pupae to adult stage had a weaker effect on nematode survival since all nematodes survived during the complete time of observation.

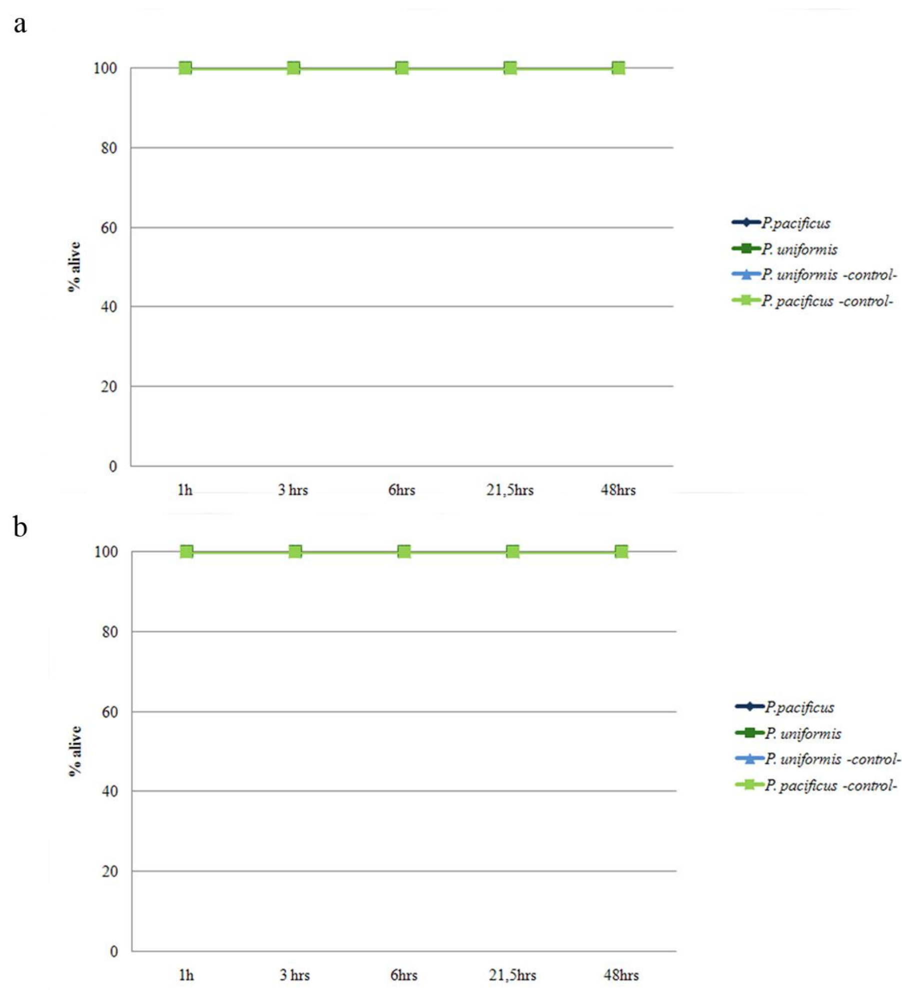

Figure 18. In (a) synchronized L3 larvae of *P. pacificus* (PS312) and *P. uniformis* (RS5244) were immersed in wells containing one freshly moulted adult of *L. decemlineata*. In (b) synchronized L3 larvae of *P. pacificus* (PS312) and *P. uniformis* (RS5244) were immersed in wells containing haemolymph extracted from freshly molted adult of *L. decemlineata.* No nematode survival differences were recorded, therefore all wells showed 100% of nematodes alive during the 48 hour observation period.

In general, *P. uniformis* survives longer in the assay than *P. pacificus*. This is possibly due to its slower development (table 6) and the divergence from *P. pacificus* survival is not sufficient to support a higher toxicity resistance explanation.

2.5.3.3 Haemolymph ingestion does not affect *Pristionchus* spp. and *C. elegans* nematodes

Considering that the leptinotarsin protein size might prevent the toxin from passing through the nematode cuticle, I tested whether bacteria food enriched with haemolymph extract would have any harmful effect on the different nematode species. Forty µl of potato beetle haemolymph were added to approximately 100 µl of dried OP50 and then spotted on six centimeter plates (without peptone and cholesterol). Afterwards, 10 L3 larvae of *C. elegans* (N2), *P. pacificus* (PS312) and *P. uniformis* (RS5244) were added to the test plate. On the first day the plates were checked every hour. The test was then examined for one week but no harmful effects were recorded. All three species started to reproduce in the test plates and no effect on nematodes survival was observed.

2.5.4 Conclusion

The initial hypothesis that *P. uniformis* nematodes might have evolved a mechanism to overcome the toxicity associated with the *L. decemlineata* habitat could not be verified. I was unable to show a strong lethal haemolymph effect in all three nematode species tested. Differently from my tests, all previous reports of leptinotarsin high toxicity were obtained through direct injection of haemolymph. I choose to avoid injection considering that in the natural situation nematodes might encounter potato beetle haemolymph only through external contact.

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PUBLICATIONS

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Host-finding behaviour in the nematode *Pristionchus pacificus*

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Costs and benefits of foraging have been studied in predatory animals. In nematodes, ambushing or cruising behaviours represent adaptations that optimize foraging strategies for survival and host finding. A behaviour associated with host finding of ambushing nematode dauer juveniles is a sit-and-wait behaviour, otherwise known as nictation. Here, we test the function of nictation by relating occurrence of nictation in *Pristionchus pacificus* dauer juveniles to the ability to attach to laboratory host *Galleria mellonella*. We used populations of recently isolated and mutagenized laboratory strains. We found that nictation can be disrupted using a classical forward genetic approach and characterized two novel nictation-defective mutant strains. We identified two recently isolated strains from la Réunion island, one with a higher proportion of nictating individuals than the laboratory strain *P. pacificus* PS312. We found a positive correlation between nictation frequencies and host attachment in these strains. Taken together, our combination of genetic analyses with natural variation studies presents a new approach to the investigation of behavioural and ecological functionality. We show that nictation behaviour in *P. pacificus* nematodes serves as a host-finding behaviour. Our results suggest that nictation plays a role in the evolution of new life-history strategies, such as the evolution of parasitism.

Keywords: ambush; behavioural genetics; body-waving; host attachment; nictation; *Pristionchus pacificus*

1. INTRODUCTION

Behavioural adaptations have been important in enabling nematodes to successfully exploit diverse habitats [1,2]. But little is known of the extent to which changes in behaviour influence the evolution of new life-history strategies in organisms.

One model used in foraging theory separates predatory and parasitic animals into two categories: cruisers and ambushers [3–5]. Cruisers are in constant movement and actively search for food (high energy cost), whereas ambushers tend to stand still and wait for their prey/host to approach (low energy cost) [3–5]. Infective juveniles of some ambusher nematode species show a specific search behaviour in which the animals stand on their tail and wave. This standing behaviour has been termed 'winken' [6], 'nictation' [7–9], 'standing' [10–12] and most recently 'body waving' [13]. For a complete revision about the terminology of this behaviour, see Kruitbos & Wilson [14]; for the sake of simplicity, we refer to this behaviour as nictation.

Nictation consists of raising the anterior and middle body regions of the juvenile off the ground, supported only by the tip of its tail [8,15]. In different species of nictating nematodes, juveniles can either stay in an erect pose or wave their bodies in three-dimensional spirals and loops [7,8,15]. In the laboratory, nictation behaviour is only observed when the nematodes are exposed to irregular substrates [13]. Foraging strategies of host finding in nematodes from the order Rhabditida have been extensively studied, e.g. in the parasitic *Steinernema* spp., *Heterorhabditis* spp. and *Phasmarhabditis hermaphrodita* because of their importance to pest management. The slug-parasitic nematode *P. hermaphrodita* attaches to hosts by crawling and is devoid of nictation, whereas insect parasitic *Steinernema* spp. attach by nictating [13,16]. Nictation behaviour has been also described in the animal-parasitic nematodes *Heligmosomoides polygyros* [17] and *Strongyloides ratti* [18]. By contrast, foraging strategies in the Diplogastridae, which are often associated with insects but do not necessarily parasitize them, have been poorly investigated. In this context, multiple questions can be addressed: do non-parasitic insect-associated nematodes show cruiser and/or ambusher behaviour? Is there a nictation-like behaviour? Such questions could best be addressed using laboratory model species in which behavioural assays could be complemented with genetic analyses.

Nematodes of the genus *Pristionchus* have a necromenic association with scarab beetles, in which arrested dauer-stage nematodes invade the insect, wait for the host to die and then resume development by feeding on growing micro-organisms on the carcass [19,20]. *Pristionchus pacificus*, a satellite model nematode for evolutionary and developmental studies, is known to live in association with scarab beetles in nature [21,22].

The *P. pacificus* community has up-to-date genetic and genomic tools available as well as transgenic techniques and the nematode is also amenable to studies of behaviour and neurobiology owing to the relative simplicity and detailed description of its nervous system (D. Bumbarger & R. J. Sommer 2011, personal communication). Therefore, the

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features of *P. pacificus* allow us to address the question of whether nictation behaviour provides a selective advantage for nematode–host associations.

Molecular and morphological similarities support the hypothesis that the infective juvenile stage of parasitic nematodes is homologous to the dauer stage of non-parasitic nematodes, such as *Pristionchus* [23–26]. Harsh environmental conditions, such as high temperature, low food availability and high population density, induce many non-parasitic nematodes to develop into an alternative developmental juvenile stage referred to as 'dauer'. The dauer stage of *Pristionchus* species is responsible for host finding and attachment to host [20], and it is the only stage of development in which nictation occurs.

Nictation is proposed to provide a selective advantage that allows dauer juveniles to attach to passing hosts. On this basis, nictation could serve as an adaptation for the dauer juveniles to make contact with a host, in most cases an insect, for transportation, i.e. a phoretic interaction [27]. A consequence of a phoretic interaction is the permanent adherence of the dauer to the cuticle of its host, and in some cases cuticle penetration into body cavities of the host [28]. Therefore, phoresis has been suggested to serve as a pre-adaptation for the evolution of parasitism [9,23,24,26].

Herein by the use of an experimental set-up with *P. pacificus* strains, we tested the hypothesis that nictation behaviour favours the ability to attach to its beetle host with recent isolated and laboratory-generated mutant strains. Using forward genetics, we screened for mutants lacking nictation behaviour in *P. pacificus*. We isolated two mutants that can develop into proper dauers but are nictation-defective. We compared these two mutant strains with recently isolated strains from la Réunion island, with variable nictation rates, to test their ability to attach to artificial hosts under laboratory conditions.

2. MATERIAL AND METHODS

(a) *Nematode culture*

Breeding and maintenance of *P. pacificus* follow *Caenorhabditis elegans* standard culture methods that have been described previously [29,30]. Nematode strains used in this study were: (i) *P. pacificus* reference PS312 strain; (ii) *P. pacificus* RS5401 and RS5386, which are recent isolates from isogenic lines of *Oryctes borbonicus* scarab beetles or from soil samples from la Réunion island as described by Herrmann *et al.* [31]; and (iii) mutagenized *P. pacificus tu426* and *tu427*, which are nictation-defective strains isolated using a classic forward genetics approach [32]. In this study, we adopt the definition of strain described in Herrmann *et al.* [19]. Strain stocks were maintained on NGM plates with *Escherichia coli* OP50 lawns [29,30]. All *P. pacificus* strains used in this study are available upon request.

To generate dauers, we performed dauer inductions using the 'wet-plate method'. We resuspended three 6 cm NGM plates with fully grown mixed-stage worms into 1 ml of OP50 liquid medium and added it onto 10 cm NGM plates. Worms were grown at 20–25°C for approximately 14 days or until a sufficient number of dauers were found on the plate (A. Weller, 2009, personal communication). Timing of worms to dauer formation was investigated in *P. pacificus* PS312 and the two nictation-defective mutants *tu426* and *tu427*. We scored total hours necessary to reach the highest amount of dauers in the dauer induction plates.

Steinernema feltiae, *Steinernema carpocapsae* and *P. hermaphrodita* were supplied by Becker Underwood, UK. Dauer juveniles of *S. feltiae* and *S. carpocapsae* were cultured following Kaya & Stock [33]. Briefly, approximately 1000 *S. feltiae* or *S. carpocapsae* dauer juveniles were added to moistened Whatman filter paper in a 10 cm Petri dish and 5 to 10 *Galleria mellonella* were added. Plates were sealed with Parafilm and incubated at room temperature for 4 days. Once dead, then *G. mellonella* were transferred to White traps and dauers collected in the surrounding water. Nematodes were washed three times in M9 buffer before use and were stored at 4°C in 200 ml tissue culture flasks. *Phasmarhabditis hermaphrodita* (Nemaslug) were mixed with tap water (1 g in 100 ml) and stored similarly to *Steinernema* spp.

(b) *Nictation and attachment tests*

Nictation test arenas consisted of 6 cm NGM plates sparsely covered with sterile sand grains evenly spread with a shaker. Either 1000 or 5000 dauers, previously washed and resuspended in distilled water, were applied to the centre of the Petri dish and left to dry. The dishes were left covered (to preserve environmental conditions within the plate) at room temperature and nictation was recorded after the first hour and every 12 h thereafter for a period of 5 days. We used the first time point (1 h) to test for nictation differences between *P. pacificus* strains. To obtain the proportion of nictating dauers and dauers attached to host, we scored nictation activity for individual dauers that lifted their body off the substrate. For the host attachment studies, a single host was applied onto the plate already containing dauers and sand, prepared as described above. In two independent assays, we used 1000 and 5000 dauers, respectively, per plate and placed one *G. mellonella* moth larva (commercially available from HW Terra, Germany) on each plate; i.e. each replicate consisted of either 1000 or 5000 dauers/host/plate for each assay. All replicates contained the same stage, and the same size hosts. Nematodes were exposed to the host for a 2 hour period in covered plates. The hosts were removed, dissected and resuspended in water to allow release of dauers from the carcass. We counted the number of resuspended dauers.

(c) *Mutant screen*

We screened for nictation-defective mutant strains using EMS mutagenesis [32] in *P. pacificus* PS312. We screened approximately 350 gametes (1300 homozygous F_2 lines) in two mutagenic screens over a six-month period. We isolated homozygous F_2 single worm clones in 96-well plates each containing 40 µl OP50 solution per well and allowed the generation of enough dauers (modified wet-plate method for large-scale screenings). We screened for nictation depletion after approximately 14 days in 30 µl containing more than 300 dauers per well; the remaining 5–10 µl was used to recover the homozygous lines. Candidate strains were confirmed by multiple searches for the defective phenotype in at least three independent dauer inductions (with 6000–60 000 dauers per induction) using the wet-plate method described above.

(d) *Nictation and attachment calculations*

We counted the total number of dauers in nictation from $n =$ 1000 dauers, and calculated means and standard errors from three to 12 independent replicates. Standard errors of the mean were corrected for small sample size ($n < 20$) [34]. We tested the normality of our data using the Shapiro–Wilk test.

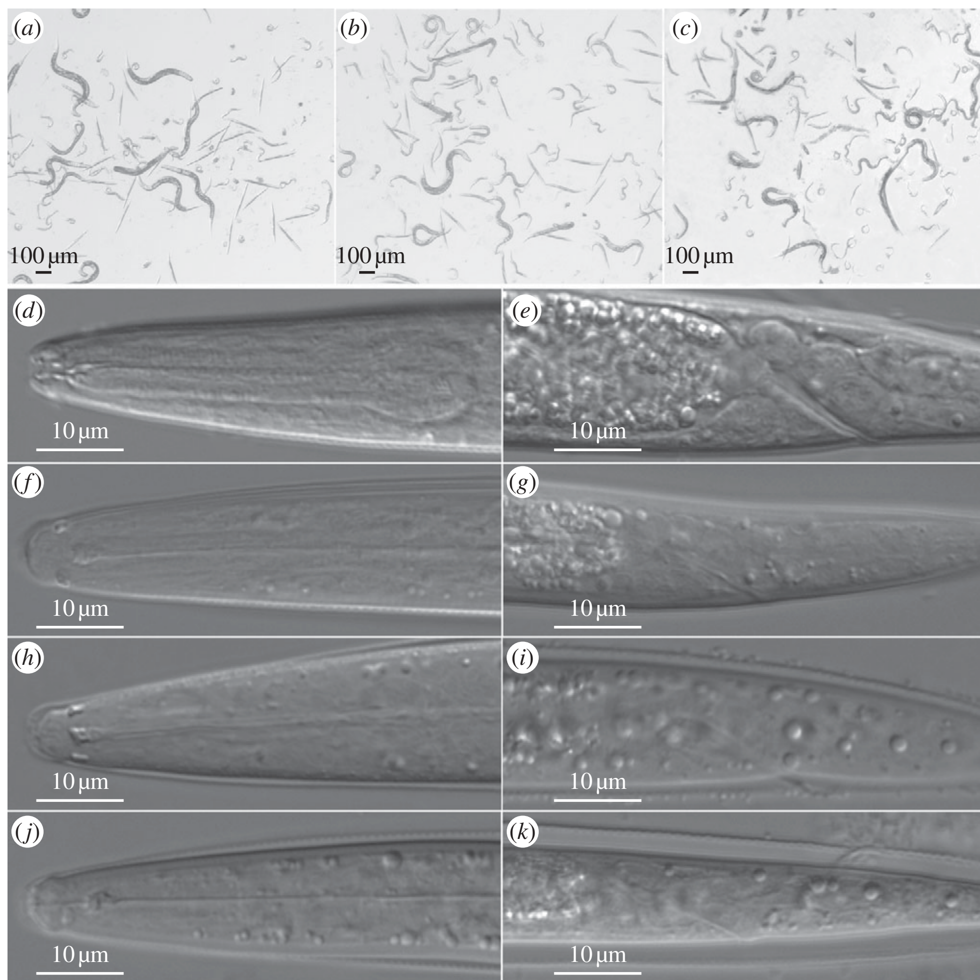

Figure 1. *Pristionchus pacificus* dauer juveniles after 15 days of induction in liquid culture: (*a*) PS312 strain, (*b*) *tu426* strain, and (*c*) *tu427* strain. Partial dauers are observed only in the nictation-defective mutants: (*b*) *tu426* and (*c*) *tu427*. A dorsal view of the head region shows closed oral orifices and constricted pharynges of (*f*) PS312, (*h*) *tu426*, and (*j*) *tu427* dauer juveniles when compared with reference PS312 third juvenile stage (*d*). Lateral view of PS312 third juvenile stage with an open anus is shown (*e*). By contrast, lateral views of strains PS312 (*g*), *tu426* (*i*), *tu427* (*k*) display a closed anus of dauer juveniles. The dauer juvenile stage (*f–k*) shows higher amounts of lipid droplets throughout the body than the third juvenile stage (*d,e*).

We performed an ANOVA between the groups, and a Tukey's honestly significant difference (HSD) test to assess significant differences in nictation between the strains. To assess for differences in the time to dauer formation between *P. pacificus* PS312 and the mutant strains, we used a non-parametric Mann–Whitney *U*-test because some of the data did not follow a normal distribution.

To study the relationship between nictation and attachment, we used multiple replicates of *single* assays. We calculated the number of dauers in nictation on each plate immediately before host exposure and the number of dauers on the host 2 h later. We tested nictation and attachment to host relationship in two independent assays, with two replicates of 1000 dauers and three replicates of 5000 dauers, respectively. The correlation coefficient (r) and coefficient of determination (r^2) were calculated. ANOVAs and Tukey's HSD tests were also performed on these data.

3. RESULTS

(a) *Morphological characterization of* Pristionchus pacificus *dauer juveniles*

The dauer stage is an alternative developmental stage to the J3 stage of directly developing nematodes (figure 1). Under stressful conditions or high densities, *P. pacificus* juveniles enter the dauer stage (figure 1*a*). Specialized morphological and physiological characteristics, such as high lipid storage, strong and impermeable cuticle, no food intake and extended lifespan, among others, develop in the dauers for endurance (table 1). Several characteristics distinguish arrested dauer juveniles from the active J3 juveniles (table 1 and figure 1*d–k*). Specifically, *P. pacificus* (PS312) dauers possess thin bodies and a dark intestine, which indicates their non-functional gut (figure 1*f*,*g* and table 1). Dauers contain a constricted pharynx and a closed oral orifice with an internal plug (figure 1*f*,*h*,*j* and table 1) and closed anus (figure 1*g*). Dauers also show a specialized cuticle and a stereotypical gonadal arrest with fewer germ cells than J3 juveniles (not shown) as has been previously described for *C. elegans* (table 1) [47–49]. The mid-body region of dauers is characterized by a high density of lipid droplets, which is absent in the J3 stage.

(b) *Nictation behaviour in* Pristionchus pacificus *dauers and other nematode species*

We induced nictation behaviour in dauers by adding sand grains to the substrate of the worms. As previously described for other nematode species [8], *P. pacificus*

Table 1. Dauer characteristics in *P. pacificus*. Most of these characteristics have been described previously (see references); differences found in *C. elegans* are shown in bold.

category	dauer characteristic	references
(*a*) morphology	thin and dense body; axial ratio for *P. pacificus* is 16:1 (length:width), **for *C. elegans* it is 30:1**.	[35–37]
	remodelled foregut pharynx	[38]
	dark sealed intestine, generally darker than corresponding J3 stage, **or L2 for *C. elegans***	[35–37]
	closed mouth and constricted pharynx	[35–37,39]
	gonadal arrest	[35–37]
	strengthened, specialized cuticle with lateral ridges: peripheral ridges become more pronounced in *P. pacificus*, **whereas conspicuous lateral alae become visible for *C. elegans***	[35–37,39]
	fat bodies in intestinal and hypodermal cells	[40]
	remodelled neurons in *C. elegans*; has not been characterized for *P. pacificus*	[41]
(*b*) physiology	developmental arrest	[42]
	increase in lifespan reduced metabolic activity and dependence on internal energy storage; work in progress for *P. pacificus* (M. Mayer, A. Ogawa & R. Sommer, 2009, personal communication)	[37,43,44]
	resistance to environmental stress heat, cold, desiccation, oxidative stress and detergents such as SDS in *C. elegans*; has not been tested for *P. pacificus*	[35–37,43–46]
(*c*) behaviour	lethargic needle-like pose with reduced activity	[35–37]
	nictation	[35]

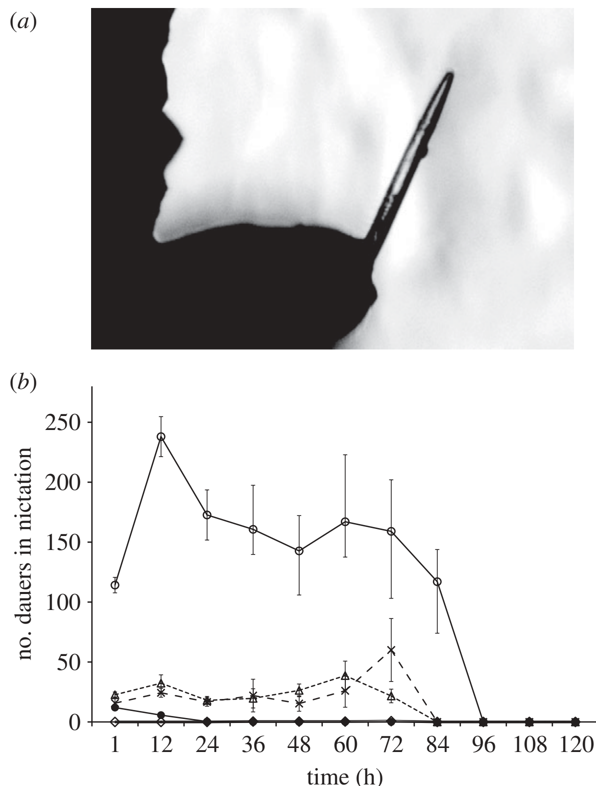

Figure 2. Nictation in *P. pacificus*. (*a*) A representative dauer in nictation. Its posterior end remains attached to the substrate, whereas its anterior stands erect with occasional waving. Note a lipid droplet often observed on the side of the pharynx region of dauers in nictation. (*b*) Number of dauers in nictation in populations ($n = 1000$) of two entomopathogenic *Steinernema* species (*S. feltiae* (dashed lines with triangles) and *S. carpocapsae* (dashed lines with crosses)), the slug parasitic *P. hermaphrodita* (double line with rhombi), and two *P. pacificus* strains (PS312 (solid lines with filled circles) and RS5386 (solid lines with open circles)). Error bars represent standard errors of the mean (s.e.m.) corrected for small sample size $n < 20$.

dauers lift all of the body off the substrate except the posterior tip. The nematodes keep a primarily straight posture (figure 2*a*) and occasionally wave their bodies in three-dimensional spirals and loops (electronic supplementary material, movie S1). Thus, *P. pacificus* dauer juveniles show a nictation behaviour typical of other nematodes.

We scored nictation and compared *P. pacificus* with other nematodes species (figure 2*b*). Initially (after 1 h), the free-living *P. pacificus* (PS312) exhibited nictation behaviours similar to that of the entomopathogenic nematodes *S. carpocapsae* and *S. feltiae*. Species began to exhibit differences after 12 h. *Pristionchus pacificus* PS312 started at around 15 dauers in nictation, and dropped to nearly zero after 24 h (figure 2*b*). The entomopathogenic *S. carpocapsae* and *S. feltiae* maintained 20–30 dauers in nictation until 36 h. Interestingly, *S. carpocapsae* reached a peak of nictation of around 60 dauers at 72 h, whereas *S. feltiae* reached a peak of around 40 dauers at 60 h (figure 2*b*). The slug parasitic *P. hermaphrodita* did not show any nictation throughout the whole period. By contrast, only the recently isolated *P. pacificus* strain RS5386 showed a higher proportion of nictation from the beginning and up to 96 h after-sand addition. Over 100 *P. pacificus* RS5386 dauers displayed nictation after 1 h, followed by a sharp increase at 12 h (over 200 dauers in nictation), followed by a drop to *ca* 150 dauers after 24 h, which persisted up to 4 days post-sand addition.

(c) *Nictation-defective* Pristionchus pacificus *mutant strains*

To test the hypothesis that nictation behaviour affects attachment abilities for host association, we used forward genetics to generate strains lacking this behaviour in the *P. pacificus* PS312 strain. In total, we isolated 11 nictation-defective mutants. Most of these mutant strains showed dauer entry defects, referred to as 'partial dauers' (table 2) [42,49,50]. To reduce the probability

Table 2. Resemblance of dauer characteristics in nictation-depleted *P. pacificus* mutants to reference PS312 dauers: 1, low; 2, medium; 3, high. Most dauer-like mutants (highest similarity index) are shown in bold.

dauer characteristics	***tu427***	*tu428*	*tu429*	*tu430*	*tu431*	*tu432*	*tu433*	***tu426***	*tu434*	*tu435*	*tu436*
dark body	**3**	1	3	3	2	2	3	**3**	1	3	2
thin body	**3**	1	3	2	2	2	2	**3**	2	2	2
constricted pharynx (does not pump)	**3**	1	3	3	3	3	3	**3**	2	3	3
thin dark intestine	**3**	1	3	2	3	3	2	**3**	2	2	2
lipid droplet accumulation	**3**	1	2	2	3	3	3	**3**	2	3	3
gonad arrest	**3**	1	2	2	3	3	2	**3**	2	3	2
absence of intermediate or partial dauers	**2**	1	2	1	1	1	1	**3**	1	1	1
similarity index (total)	**20**	7	18	15	17	17	16	**21**	12	17	15

that severe anatomical defects are the cause of nictation-defective phenotypes, we compared the morphological characteristics of dauer juveniles between mutants and reference animals (table 2 and figure 1). We focused on conspicuous morphological characteristics (table 1) that define the dauer stage by assigning a similarity score to reference dauers, ranging from low to high (1–3) (table 2). We used the nictation-defective mutants with the highest similarity index to reference dauers for the rest of the study, i.e. *tu427* (score of 20) and *tu426* (score of 21). The nictation-defective mutants *tu426* (figure 1*b*,*h*,*i*) and *tu427* (figure 1*c*,*j*,*k*) showed all dauer characteristics described for *P. pacificus* PS312 (figure 1*a*,*f*,*g* and electronic supplementary material, tables S1 and S2). Specifically, the oral orifices are closed and contain an internal plug [48] and their pharynges are constricted (figure 1*f*,*h*,*j*) [49]. In all three strains, the intestine is reduced and the mid-body region is characterized by a high density of lipid droplets. In figure 1*g*,*i*,*k*, the closed anus of the dauers is shown.

We observed an asynchrony in dauer formation between the reference and nictation-defective mutant strains (electronic supplementary material, figure S1). *Pristionchus pacificus* PS312 developed dauer juveniles approximately 120 h after eggs laying begins, whereas *tu427* mutant animals developed dauer juveniles after 125 h. The *tu426* mutant animals were significantly slower (Mann–Whitney test, $U = 2967.5$, *tu426* $n = 53$, PS312 $n = 56$, $p < 0.001$ two-tailed) in developing dauers; they spent more than 200 h in liquid culture before dauers appeared (electronic supplementary material, figure S1). Age differences observed in dauer formation between the different strains have not been shown to affect nictation behaviour. Previous observations in our laboratory comparing same dauer strains 12 or 25 days after induction showed no relevant differences in nictation numbers (data not shown).

(d) *Relationship between nictation in* Pristionchus pacificus *strains and attachment to the laboratory host* Galleria mellonella

To resolve the role of nictation in *P. pacificus*, we measured nictation rates of different strains and studied their attachment abilities to the larva of the moth *G. mellonella* (figures 3 and 4). The two mutant strains, *tu427* and *tu426*, show a complete absence of nictation during the nictation induction assays (figures 3 and 4). By contrast, one recent isolate from la Réunion island, which was

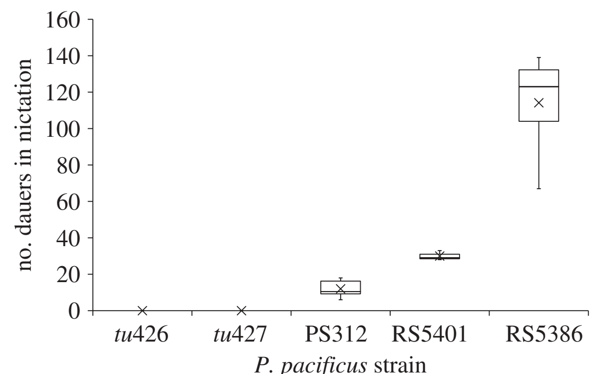

Figure 3. Number of dauers in nictation in populations ($n = 1000$) of *P. pacificus* nictation-defective mutant strains *tu426* and *tu427*, the reference laboratory strain PS312 and two strains from la Réunion island: RS5401 and RS5386. Maximum and minimum values are shown by whiskers, upper (75%) and lower (25%) quartiles are shown by the upper and lower limits of each box, medians and means are shown by the straight line and cross within each box, respectively.

kept in the laboratory for less than six months, showed a significantly higher nictation rate than the reference strain PS312 (ANOVA: $F = 142.17$, d.f. $= 4$, $p < 0.0001$; Tukey's HSD test: $p < 0.01$; electronic supplementary material, tables S1 and S3). RS5386 exhibits a two- to 10-fold higher nictation rate than the reference strain PS312 (figures 3 and 4). RS5401 showed a slightly higher nictation rate than PS312 (Tukey's HSD test: $p < 0.05$; table 3).

We found a proportional increase in the attachment to the host, which is directly related to the increase in nictation behaviour previously recorded for the different strains. The ability to attach to hosts increases dramatically when *P. pacificus* strains are able to nictate, as shown for reference and la Réunion strains (figure 4). In a sample containing 1000 dauers, we found absence of dauers attached to the hosts after 2 h of exposure for *tu427* and *tu426* (figure 4*a*). By contrast, we found approximately 20 *P. pacificus* PS312 dauer juveniles on host grubs after a similar exposure (figure 4*a*). The strains from la Réunion, RS5401 and RS5386, showed a relative increase in average attachment. However, despite the generally higher attachment of RS5401 to host grubs, this increase is not significantly higher than that of PS312 (ANOVA: $F = 106.25$, d.f. $= 4$, $p < 0.0001$; Tukey's HSD test: $p > 0.05$ (non-significant); figure 4*a* and

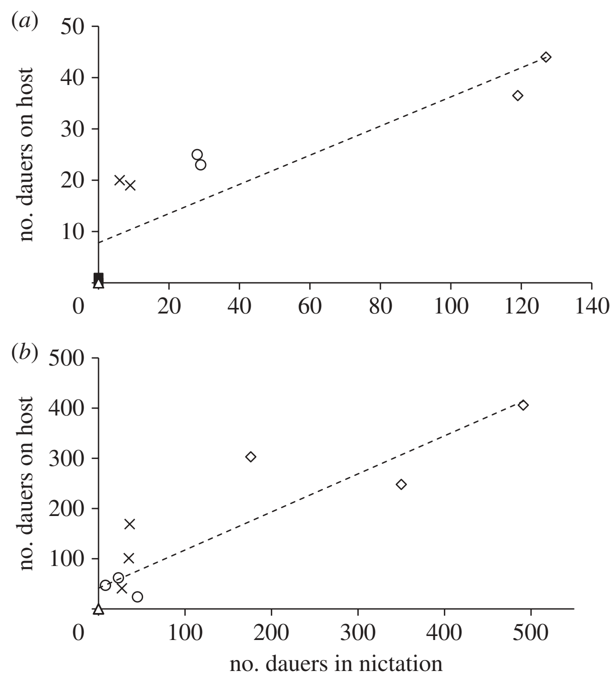

Figure 4. Relationship between nictation and attachment to the laboratory host *G. mellonella* for two nictation-defective mutant strains (*tu426* in solid squares, and *tu427* in open triangles), the reference laboratory strain (PS312 shown in crosses) and two recently isolated strains of la Réunion island (RS5401 in open circles, and RS5386 in open rhombi) in two different dauer concentrations assays: (*a*) total dauers on plate $n = 1000$. Correlation coefficient $r = 0.87$, and coefficient of determination $r^2 = 0.76$. (*b*) Total dauers on plate $n = 5000$. Correlation coefficient $r = 0.90$, and coefficient of determination $r^2 = 0.80$.

Table 3. Pairwise comparisons of nictation in *P. pacificus* strains using Tukey's honestly significant difference (HSD) test (cf. electronic supplementary material, table S1 and figure S3). n.s., non-significant differences.

strain A	strain B	p
tu426	*tu427*	n.s.
tu426	PS312	n.s.
tu426	RS5401	<0.01
tu426	RS5386	<0.01
tu427	PS312	n.s.
tu427	RS5401	<0.01
tu427	RS5386	<0.01
PS312	RS5401	<0.05
PS312	RS5386	<0.01
RS5401	RS5386	<0.01

electronic supplementary material, tables S2 and S4). By contrast, RS5386 showed nearly 40 worms attached to host and showed significantly higher numbers than the reference strain PS312, which only showed about 20 worms attached to the host (Tukey's HSD test: $p < 0.01$; figure 4*a* and electronic supplementary material, tables S2 and S4). The two nictation-defective mutant strains showed no significant difference in attachment ability when compared with each other (Tukey's HSD test: $p > 0.05$ (non-significant); figure 4*a* and table 4). In summary, a positive correlation ($r = 0.87$, $r^2 = 0.76$, $p \leq 0.001$) was found between nictation and attachment to hosts in *P. pacificus* strains.

To test whether population density had any effect on nictation or attachment to insect hosts, we repeated these experiments with a fivefold higher initial number of dauers ($n = 5000$ dauers) (figure 4*b* and electronic supplementary material, tables S2 and S4). We found that the relationship was maintained with relationship values that were almost identical ($r = 0.9$, $r^2 = 0.8$, $p < 0.001$). We conclude that density does not play a role in the positive relationship observed between nictation and host attachment. Taken together, we found a high positive correlation between the number of worms in nictation and the number of worms attached to host moth larvae. Coefficient of determination (r^2) and correlation coefficient (r) of nictation and attachment approach a value of 0.8 and 0.9, respectively, suggesting a relationship between both variables (figure 4). Thus, populations of nematodes with higher proportions of nictation are found to be more effective at attaching to mobile insects.

4. DISCUSSION

(a) *Use of mutant strains in combination with wild strains to study behavioural phenotypes in laboratory settings*

We took advantage of laboratory tools to combine, for the first time, mutagenesis-generated strains defective in a specific behaviour with recently isolated wild strains showing variable degrees of the specific behaviour. Classical forward genetic screens in *C. elegans* have proved to be a powerful tool for studying behaviours relevant to the species survival, such as mechanosensation mutants [51], chemosensation mutants [52], thermotaxis [53] and egg laying [54]. Populations of *P. pacificus* strains used in our study show a gradient in the proportions of nictation of each strain, which range from complete disruption of nictation in *tu427* and *tu426* mutants to high nictation in RS5386. Such a gradient of mutants, together with recently isolated strains, is required to correlate this behaviour to a particular function, such as host attachment. The low gamete number required to generate the nictation-defective strains *tu427* and *tu426* in our mutant screen shows that this behaviour may be complex and regulated by multiple genetic loci.

Nictation is absent in all stages of development, except in dauers; therefore programmes that act upstream of dauer formation, such as dauer induction or entry, may also affect behavioural phenotypes of the dauer. Presence of partial dauers in our nictation-defective mutants (table 2) suggests that nictation in some of our mutants may be affected owing to dauer formation defects. Previous studies in *C. elegans* of dauer formation mutants (daf), such as *daf-2*, have shown that these genes act independently on different aspects of development, such as entry and exit to dauer, intestinal pigmentation and reproduction [42]. Furthermore, previous studies have reported partial or intermediate dauer formation by mutations in *daf-9, daf-15, daf-16, daf-18, daf-20*, *daf-12* and unmapped *sy5315* X-linked mutation [49,55–58]. It remains to be tested whether any of these mutants also show defects in nictation behaviour. *Pristionchus pacificus* nictation-defective mutant strains that form partial dauers might contain mutations in the

Table 4. Pairwise comparisons of **nictation** (bold) and *attachment* (italics) results of *P. pacificus* strains at different densities (1000 and 5000 worms in single assays) using Tukey's HSD test (cf. electronic supplementary material, table S2 and figure S4). n.s., non-significant differences.

1000 dauers	*tu426*	*tu427*	PS312	RS5401	RS5386
tu426	—	*n.s.*	$p < 0.01$	$p < 0.01$	$p < 0.01$
tu427	**n.s.**	—	$p < 0.01$	$p < 0.01$	$p < 0.01$
PS312	**n.s.**	**n.s.**	—	n.s.	$p < 0.01$
RS5401	**$p < 0.01$**	**$p < 0.01$**	**$p < 0.01$**	—	$p < 0.01$
RS5386	**$p < 0.01$**	**$p < 0.01$**	**$p < 0.01$**	**$p < 0.01$**	—
5000 dauers	*tu426*	*tu427*	PS312	RS5401	RS5386
tu426	—	—	—	—	—
tu427	—	—	n.s.	n.s.	$p < 0.01$
PS312	—	**n.s.**	—	n.s.	$p < 0.01$
RS5401	—	**n.s.**	**n.s.**	—	$p < 0.01$
RS5386	—	**$p < 0.01$**	**$p < 0.01$**	**$p < 0.01$**	—

orthologues of any of the formerly mentioned genes. Further experiments are necessary to determine how genes that regulate dauer entry also affect behaviour.

In our study, we isolated and characterized mutants with a complete absence of nictation behaviour, but we did not screen for mutants with defects in specific individual steps and events of nictation. Nictation most probably consists of a number of individual events that are controlled by independent regulatory genetic units. Such regulatory units might in turn have different effects on host finding and/or attachment. Other behaviours in *C. elegans* have been observed to have different sensitivities. Mutant strains such as *unc-97* (uncoordinated) and *mec-3* (mechanosensation defective) showed different degrees of sensitivities in behavioural mechanosensory responses [58]; whereas male mating behaviour also contained multiple independent sub-behaviours controlled by different neuronal and genetic inputs [59,60]. For example, it is known that vulva location by the males is mediated by the neurons HOA and HOB, and that the genes *lov-1* and possibly *klp-6* and *pkd-2* mediate these responses [59,60]. Therefore, nictation may also be divided into sub-behaviours regulated independently as described for previous behaviours.

(b) *Nictation behaviour is relevant for host finding*

The evolution of parasitism involves a series of events, including an initial association with a host. Previous comparisons of nictating species and insect associations of different entomopathogenic nematodes suggested that nictation may provide a higher chance of contact with a host owing to a higher body surface area exposure to the transitory insect [9,13,61]. Comparative studies in *Caenorhabditis* species also suggested that nictation may be associated with the attachment of transitory animals [47,62]. However, few studies so far have investigated host finding or nictation behaviour in the diplogastrids [6,63], although these nematodes often show specific insect associations. *Pristionchus pacificus* is a necromenic nematode, i.e. uses its dead host as a source of food, and shows a life history tightly associated with beetles [19,20]. Previous comparisons of nictation in different nematode species suggested a relationship to host attachment, but the present study is the first to show a relationship of the evolutionary history of foraging behaviour in nematodes at the population level by the use of different *P. pacificus* strains.

Many aspects of nematode host finding are still unclear. Nematodes perceive their environment primarily by chemosensation, thermosensation and mechanosensation. Rhabditid nematodes are commonly described as 'cruisers' if they spend most of their time crawling and searching for resource-associated cues, such as insect host chemicals. The slug parasitic nematode *P. hermaphrodita* shows minimal nictation in our study, as has been previously reported [13], and may therefore apply a cruiser strategy. In *P. pacificus*, interception of the chemical communication system of the insect is likely to be involved in host preferences [64]. 'Ambushers' are instead more sedentary. It was initially assumed that ambush foragers were not as responsive to chemical cues as cruise foragers. However, it has since become apparent that they do respond to chemical cues, although their response is fundamentally different from cruise foragers [65]. *Steinernema* species, both cruiser and ambusher, respond strongly to volatile cues [66]. Our experiments show that *Pristionchus* species first have the ability to recognize and move towards host-associated volatiles by chemotaxis, which typically applies to a cruiser strategy [22,64]. Second, *P. pacificus* show nictation behaviour that applies to a typical ambusher behaviour as well. For other ambush foragers, stimuli from the environment have been demonstrated to be important for host finding [67], and environmental cues are used to assess patch quality [68,69] and select ambush sites [70,71]. Therefore, we propose that *P. pacificus* dauers may also have the ability to scan the surrounding environment, as shown for some *Steinernema* species [11]. We speculate that the differences in the variability observed within each strain may be a consequence of environmental differences across replicates and unidentified strain-specific traits related to host attachment, e.g. host sensing. It should be noted, however, that other differences between the genotypes/strains also affect these traits. Furthermore, we propose that nictation behaviour may also facilitate scanning and detecting host-associated cues by the dauer, such as volatile chemicals [12].

In conclusion, we provide evidence at the intraspecific level that nictation is associated with attachment. It is

tempting to speculate that nictation or nictation-like host finding behaviours are crucial during the initial steps of the evolution of parasitism. The specificity of this behaviour to the host-finding stage of nematodes, both in parasitic and non-parasitic species, reveals the relevance of nictation to understanding the origins of parasitism. Future studies should aim to understand the genetic and sensory regulation of this behaviour.

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Ecology and Evolution

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Pristionchus uniformis, should I stay or should I go? Recent host range expansion in a European nematode

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Abstract

Pristionchus pacificus has been developed as a model system in evolutionary developmental biology, evolutionary ecology, and population genetics. This species has a well-known ecological association with scarab beetles. Generally, *Pristionchus* nematodes have a necromenic association with their beetle hosts. Arrested dauer larvae invade the insect and wait for the host's death to resume development. Only one *Pristionchus* species is known to frequently associate with a non-scarab beetle. *Pristionchus uniformis* has been isolated from the chrysomelid *Leptinotarsa decemlineata*, also known as the Colorado potato beetle, in Europe and North America, but is also found on scarab beetles. This unusual pattern of association with two unrelated groups of beetles on two continents requires the involvement of geographical and host range expansion events. Here, we characterized a collection of 81 *P. uniformis* isolates from North America and Europe and from both scarab beetles and *L. decemlineata*. We used population genetic and phylogenetic analyses of the mitochondrial gene *nd2* to reconstruct the genetic history of *P. uniformis* and its beetle association. Olfactory tests on beetles chemical extracts showed that *P. uniformis* has a unique chemoattractive profile toward its beetle hosts. Our results provide evidence for host range expansion through host-switching events in Europe where *P. uniformis* was originally associated with scarab beetles and the nematode's subsequent invasion of North America.

Introduction

The expansion of the host or the geographic range of an organism can be favored by host-switching events (Secord and Kareiva 1996). Host-switching is defined as the horizontal transfer from one host to another and represents a process that has attracted increasing consideration in ecology and evolutionary biology in recent times (Ricklefs and Fallon 2002; Page 2003; Zarlenga et al. 2006). Host-switching by parasites refers to colonization of "foreign" host species in which it did not occur previously (Clayton et al. 2003), although host-switching as a concept is not restricted to parasites. One process that often involves host-switching events is species invasions (Sax et al. 2005). The invasion of a host can favor host-switching for two reasons. First, an invasive host can carry microorganisms that can infest new hosts. Second, an invasive host species can be infected by new microorganisms in the invaded area. There is a growing awareness on species invasion that turned a major threat to native biological diversity, such as that associated with trading and tourism. Species invasion and host-switching often result in the extinction of native organisms, particularly on islands, making these processes tremendously important for the sustainability of biodiversity (Sax et al. 2005).

Nematodes are ubiquitous, mostly small animals that have successfully invaded marine, freshwater, soil, and parasitic habitats. Several nematode species have been developed as important model systems in biology, including *Caenorhabditis elegans*, which is one of the best-studied model organisms in modern biology (The *C. elegans* Research Community 2011) and *Pristionchus pacificus*, a model organism in evolutionary biology and ecology (Hong and Sommer 2006a; Herrmann et al. 2007, 2010; Zauner et al. 2007; Brown et al. 2011). Field studies revealed that *Pristionchus* nematodes have a necromenic association with their beetle hosts, in such a case, arrested dauer larvae invade the insect, wait for the host to die naturally, and resume development by feeding on growing microorganisms on the carcass (Herrmann et al.

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Figure 1. Worms resume development on beetle carcass. *Pristionchus uniformis* adult nematodes feeding on a *Leptinotarsa decemlineata* carcass. Image courtesy of Andreas M. Weller.

2006a; Weller et al. 2010; Bento et al. 2010) (Fig. 1). More than 20 *Pristionchus* species have been identified in worldwide samplings in association with scarab beetles, whereas other beetle and insect groups (with two exceptions, see below) do not harbor *Pristionchus* nematodes on a regular basis (Herrmann et al. 2006a, b, 2007, 2010; Weller et al. 2010). The phylogeny of *Pristionchus* nematodes has been studied by detailed molecular investigations indicating the existence of three clades, a European, a North American, and a basal Asian clade (Mayer et al. 2007, 2009). Several *Pristionchus*–beetle associations have been characterized, such as the oriental beetle (*Exomala orientalis*) with *P. pacificus*, the cockchafer (*Melolontha melolontha*) with *P. maupasi*, and dung beetles (*Geotrupes* spp.) associated with *P. entomophagus* (Herrmann et al. 2006a).

So far, only one *Pristionchus* species could also be frequently recovered from a non-scarab beetle. *Pristionchus uniformis* has been isolated from the chrysomelid *Leptinotarsa decemlineata*, also known as the Colorado potato beetle, in Europe and North America (Herrmann et al. 2006a, b). Interestingly, *P. uniformis* also shows an association with scarab beetles on both continents. The gonochoristic species *P. uniformis* is unique in the genus *Pristionchus* for two reasons. First, it has stable associations on two continents with members of two ecologically disparate families of beetles. Second, while scarab beetles are commonly associated with numerous nematode species, *L. decemlineata* does not harbor other nematodes suggesting that *P. uniformis* has evolved specific traits that allow the identification, infestation, and survival on this chrysomelid beetle.

A potential correlation between host-switching and species invasion also exists in *P. uniformis*. While most *Pristionchus* species are restricted to single continents, *P. uniformis* is one of three species in this genus that was found on several continents (Herrmann et al. 2006a, b). *Pristionchus pacificus* is a true cosmopolitan species that associated with different scarabs on different continents (Herrmann et al. 2010), *P. entomophagus* is a species that is found in association with multiple insects (Herrmann et al. 2010; Herrmann and Sommer, pers. comm.), whereas *P. uniformis* is the only species of that genus that is frequently found on two groups of beetles. Interestingly, one of the beetle hosts of *P. uniformis*, *L. decemlineata*, is an example of a biological invasion from the United States to Europe (Balachowsky 1963). Given the *P. uniformis*–*L. decemlineata* association, it is therefore tempting to speculate that their invasions into Europe might be correlated. However, phylogenetic analysis of *Pristionchus* nematodes clearly indicates that *P. uniformis* is part of the European clade of the genus and not the North American clade (Mayer et al. 2007). Therefore, a simple coinvasion of nematode and beetle seems unlikely.

Despite the growing awareness of species invasion and host-switching, little is known about the genetic conditions and the population genetic structures associated with these processes. The major economic pest of potato crops, *L. decemlineata*, is an interesting exception, as recent molecular studies indicate that European *L. decemlineata* populations contain only a small fraction of the genetic variability known from North America (Grapputo et al. 2005).

Here, we describe a population genetic study on an invasive nematode. The aim of this study was to analyze the directionality of both biological invasion and host-switch during geographic and host range expansion events. Based on a collection of 81 *P. uniformis* isolates from North America and Europe, both from scarab beetles and *L. decemlineata*, we used population genetic and phylogenetic analyses of the mitochondrial gene *nd2* to reconstruct the genetic history of the obligate outbreeding species *P. uniformis* and its beetle association. Our results provide evidence for host-switching events in Europe where *P. uniformis* was first associated with scarab beetles. Population structure of European *P. uniformis* strains show a much higher genetic diversity than American strains arguing for an invasion from Europe to North America.

Materials and Methods

Nematode sampling and breeding conditions

Strains used in this study are the result of various collections from diverse localities (see Table S1). Nematodes were isolated from insects collected during field trips or sent to our Institute from other laboratories. Some strains were isolated from soil samples. The nematode lines used in the study were obtained using the standard procedure to isolate *Pristionchus* nematode from the field (Herrmann et al. 2006a). The insect or the soil samples were transferred to the laboratory and placed on nematode growth medium (NGM) agar plate. The insects were sacrificed by cutting them in

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half. Using a dissecting scope, the plates were checked daily over a period of 1–3 weeks for emerging and reproducing nematodes. From the emerging nematodes, we established isofemale lines: gravid females were transferred to new plates to establish laboratory lines. For taxonomic determination, morphological and molecular methods were used (see next paragraph). For breeding and maintenance of *P. uniformis*, we followed standard culture methods described previously for *C. elegans* (Brenner 1974). Stock maintenance, fecundity, and generation time tests were done on NGM agar plates with *Escherichia coli* OP50 lawns and kept at 20°C. Mating experiments for species identification and fecundity tests between *P. uniformis* strains were performed on plates with one virgin female together with five males.

Biological properties of *P. uniformis*

To study the population genetics of *P. uniformis* and the potential patterns of species invasion and host-switching, we sampled a total of 81 isolates of *P. uniformis* in different locations in Europe and North America. Twenty-one of these strains have been collected from different scarab beetles, 32 from *L. decemlineata*, whereas the remaining strains were obtained from various sources including soil and rotten plants (Table S1). To study the biology of *P. uniformis*, we have first observed if major difference in the life-history traits was detectable among the different 81 strains. We checked brood size, generation time, and sex ratio between strains from scarab beetles, from *L. decemlineata*, isolated in Europe and North America, performing crossing in plates with one virgin female together with five males.

Hypotheses to be tested

In *P. uniformis*, we can test hypotheses about geographic and host range expansion. Different hypotheses are possible. Concerning how species invasion might have occurred, *P. uniformis* could have been primarily present in North America and subsequently invaded Europe (Fig. 2A1) or vice-versa (Fig. 2A2). A European origin of *P. uniformis* is supported by the observation that this species is part of a European clade of *Pristionchus* species (Mayer et al. 2007). In the context of *P. uniformis* beetle association origin, we could also test the direction of the host-switching between the two major host, scarabs and *L. decemlineata* (Fig. 2B). Under both of these circumstances (Fig. 2A and B), invasion and host-switching, the genetic variability in *P. uniformis* isolates obtained from the receiver location and host might have resulted in a reduction, similar to what has been described for the *L. decemlineata* (Grapputo et al. 2005). A final, alternative scenario would be that no genetic structure exists between material from different beetle hosts and geographic origins due to multiple independent switches and invasions (Fig. 2C).

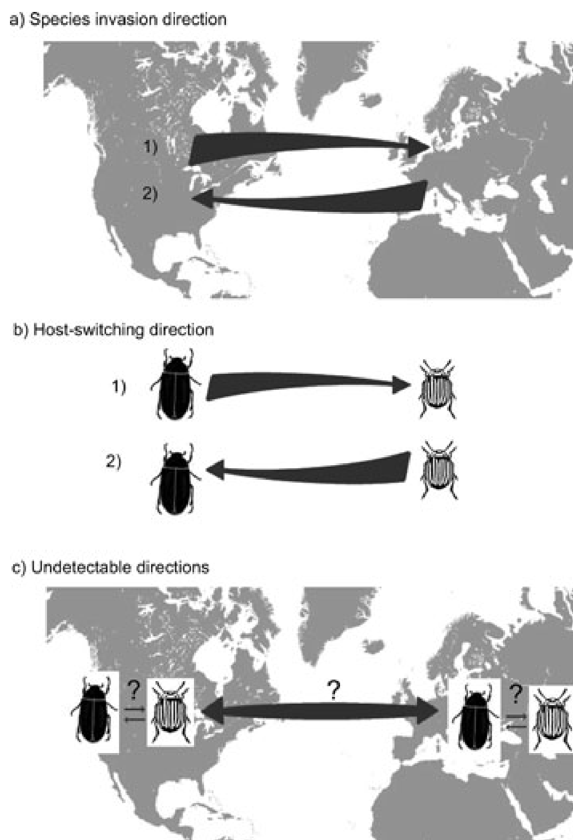

Figure 2. *Pristionchus uniformis* geographical and host origin hypotheses. *Pristionchus uniformis* possible migration direction, from North America toward Western Europe (A1) or vice-versa (A2). (B) Primary *P. uniformis* host association and following host-switch hypotheses: from scarab spp. to *Leptinotarsa decemlineata* (B1) or vice-versa (B2). (C) When no clear *P. uniformis* genetic structure is found, neither species invasion nor host-switch direction can be detected. Big arrows size is proportional to the *P. uniformis* population genetic diversity. Darker cartoon represents a scarab spp. host and the striped cartoon represents the *L. decemlineata*. World map modified after Graphic Factory CC.

Sequencing

For species identification, DNA was prepared from single individual nematodes per strain, and species identity was assessed by their having identical small subunit ribosomal RNA (SSU) sequences as described in Herrmann et al. (2006a). To evaluate the genetic variability between *P. uniformis* isolates, by conducting BLAST searches of the *P. pacificus* mitochondrial sequence to an early version of the *P. entomophagus* genome, a species closely related to *P. uniformis* (Mayer et al. 2009), we then obtained the mitochondrial genes for *nd2* and *cyt b*. The following polymerase chain reaction (PCR) primers were designed: IS12109 CGCAAAAGATATACGC-CAAT and IS12120 TTCTCCCAAAGGAACTTTACC. The *nd2* mitochondrial gene fragment of 789 bp was cloned and sequenced from both ends in all 81 *P. uniformis* strains used

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in this study. Genomic DNA was prepared from three overgrown 6-cm NGM plates. Plates were washed three times in distilled water. DNA was isolated with a genomic DNA extraction kit (MasterPure™ DNA Purification from Epicentre Biotechnologies, Madison, WI, USA). The DNA was diluted to approximate 25 ng/μl for the PCRs. PCR was performed in 25-μl 1× PCR buffer (Amersham Biosciences [currently GE Healthcare Europe Gmbh], Munich, Germany) containing 1 U *Taq* DNA Polymerase (Amersham Biosciences [currently GE Healthcare Europe Gmbh], Munich, Germany), 0.5 μM of each primer, 0.2 mM of each deoxynucleotide triphosphate, and 4 μl of DNA lysate. PCR experiments were performed as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, primer annealing at 50°C for 30 sec, and extension at 72°C for 60 sec. A final incubation step at 72°C was performed for 7 min. PCR products were diluted 1:20 with ddH_2O and sequenced without further purification from both ends (Big Dye terminator protocol, Applied Biosystems, CA, USA, ABI373*xl* capillary platform).

Data analyses

Sequence trace files were visualized with the software SeqMan (DNAStar Inc.) and aligned with Bioedit version 7.0. 5. 3; (Hall 1999). The sequences have been deposited in GenBank and can be retrieved by their accession code JN555657-JN555737 (*P. uniformis*) and JN562471 (*P. maupasi*). For phylogenetic analyses, the best-fit substitution model and the parameter settings were determined in Find Best-Fit Substitution Model (ML) in MEGA version 5 (Tamura et al. 2011) using the Akaike information criterion (Akaike 1974; Posada and Crandall 1998). The parameters for the selected model HKY+I+Γ were as follows: estimated base frequencies: $A = 0.331$, $C = 0.081$, $G = 0.109$, $T = 0.479$; proportion of invariable sites $(I) = 0.40$. Phylogenetic relationship among the *P. uniformis* strains was assessed based on 789 bp of the *nd2* mitochondrial gene with in MEGA 5 (Tamura et al. 2011), and heuristic search using maximum likelihood (ML) as optimality criterion. The bootstrap consensus tree was inferred from 10.000 replicates. The tree was edited using Dendroscope (Huson et al. 2007). Divergence parameters, values for neutrality tests, and haplotype data were obtained with the program DNASP version 5.10.01, (Librado and Rozas 2009). The population parameter θ was calculated from the number of segregating sites. Haplotypes variation output file, with parsim informative sites highlighted, was generated with MEGA 5 (Tamura et al. 2011). Haplotypes distribution among host and F_{ST} statistics were calculated with Arlequin version 3.5. 1. 2; (Excoffier et al. 2005). Network analyses of the mitochondrial sequences were performed with Network version 4.6.0.0 available at fluxus-engineering.com (Bandelt et al. 1999).

Beetle extracts and chemotaxis assays

The attraction of *Pristionchus* nematodes toward specific compounds in chemotaxis assays might recapitulate their behavioral response in nature toward their preferred host (Hong and Sommer 2006b). Chemical extracts from the insect host of *P. uniformis* were used to investigate attraction profiles of two *P. uniformis* strains. Feeding adult males of the scarab *Phyllophaga anxia* were collected with pheromone traps in Geneva (NY), USA in May 2007. Pupae of *L. decemlineata* were derived from our laboratory culture that was initiated in 2006 from a group of beetles collected in Tübingen (Germany). For the cuticular hydrocarbon extract, three *P. anxia* adults and 25 *L. decemlineata* pupae were placed in glass sample tubes. The beetles were then soaked in dichloromethane (CH_2Cl_2) for 24 h at 23°C. The washes were then vacuum dried at 30°C in small glass sample tubes, then resuspended in 150 μl of pure ethanol. Dichloromethane without a beetle specimen but processed the same way served as a counter-attractant control. Chemotaxis assays were performed on 8.5-cm Ø NGM agar plates, as previously described for *Pristionchus* species (Hong and Sommer 2006b). The host odorants have been tested on two *P. uniformis* strains (RS5167 and RS5303) and for comparison on *P. pacificus* (PS312). Mixed stage nematodes containing mostly adults were washed three times in M9 buffer and then loaded onto the agar plates, which had been prepared with two point sources of odors. As attractant, the host extract and control a solvent and sodium azide to anesthetize the nematodes on opposite sides of each plate. The chemotaxis index is defined as [the number of worms at the attractant site – worms at control site]/total number of worms scored. At least two separate experiments were conducted for each strain, and each experiment consisted of 6–10 replicates. On average, each replicate represented the outcome for 30–100 worms. Only the highest chemotaxis index was recorded, which peaked between 15 and 16 h at 23°C. Two-tailed two-sample Student's t-test was done in Microsoft Excel.

Results

Life-history traits variation in *P. uniformis*

First, we analyzed the biological properties of 11 representative strains of *P. uniformis* (the reference strain and isolates from different host association and with different geographic origin) to evaluate if different host associations were correlated with differences in life-history traits. No obvious differences among *P. uniformis* isolates were found. For example, the sex ratio was close to 50% males in all strains. Similarly, the generation time was between 3 and 4 days and fecundity was between 91 and 170 (Table S2), which is typical for the genus *Pristionchus*. These findings suggest that the different host association of *P. uniformis* did not result in adaptive differences in life-history traits.

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Phylogenetic and network analyses for *P. uniformis*

To determine the genetic variance of *P. uniformis*, we analyzed all strains by comparing the rapidly evolving mitochondrial marker *nd2*. A total of 789 bp were compared among the 81 strains isolated from various localities, sources, and hosts. The *nd2* sequences were used to construct a phylogeny based on the genotypes of these 81 strains. As outgroup, the sequence of *P. maupasi* RS0143 was included. Figure 3 shows a rooted, ML tree of the phylogenetic relationship between the mitochondrial *nd2* gene genotypes of *P. uniformis* isolates. The majority of the strains fall into a derived monophyletic group of strains that are genetically very similar to each other. In this clade, there is no clear separation between strains associated with scarab or chrysomelid beetles (Fig. 3A). Similarly, strains from North America and Europe are interspersed, and several *P. uniformis* strains with identical haplotypes, such as RS5303 and RS5255, were found on scarab beetles and *L. decemlineata* in the United States and Germany, respectively (Fig. 3A). Similarly, multiple *P. uniformis* strains, that is, RS5287, RS5312, RS5323, and RS5308, as well as RS5048, RS5256, RS5245, and RS5240 were collected from the same site, but are genetically unrelated (Fig. 3B). The major findings of the phylogenetic analysis can be summarized as follows. First, North American strains always share a clade with European strains. Second, there are three clades formed by European strains only. Third, there are no deeper clades and there is no support for grouping the clades together. Taken together, these data suggest a European origin of *P. uniformis* and multiple colonization events from Europe to North America. Also, no population genetic structure that would completely dissociate hosts and localities can be detected.

In Figure 4, the *nd2* gene network defines 32 haplotypes in a selection of 53 strains isolated from scarab spp. and *L. decemlineata*. Each haplotype is restricted to one of the four predefined groupings (*L. decemlineata* from Europe; *L. decemlineata* from North America; scarab from Europe; scarab from North America) except for eight haplotypes that are shared between the two beetle groups and three cases also between continents (Fig. 4A; Table S3). Genetic diversity of scarab derived *P. uniformis* ($\pi = 0.102$) was nearly twice that of the *L. decemlineata* derived strains ($\pi = 0.056$) (Table 2). Additionally, the estimation of the genetic differentiation between these two host association subgroups of *P. uniformis* resulted to be significant ($F_{ST} = 0.091 \pm 0.000$, P-value $= 0.000$).

Population structure for *P. uniformis*

The deep sampling of *P. uniformis* and the availability of type material from Europe and North America make it possible to study the ancestral origin of this species. The *nd2* gene analysis suggests that the *P. uniformis* strains collected in Europe are more genetically diverse than the strains from North America. As mentioned in the previous paragraph, North American *P. uniformis* are always found in clades together with European strains. In contrast, European strains are found in all major clades covering a higher sequence divergence and occupying more basal positions in the rooted phylogeny with three clades formed by European sequences only.

Only one group of American isolates, RS5505, RS5500, and RS5506, has a more basal position in the phylogeny (Fig. 3B). This group is clearly distinct from the other American strains, supporting the hypothesis of multiple expansions from Europe to North America.

The European origin of *P. uniformis* is confirmed by considering the nucleotide diversity found between *P. uniformis* strains from North American and Europe. Specifically, North American strains show a lower nucleotide diversity (π) of 0.04, whereas the European strains show a π of 0.10 (Table 2). The nucleotide diversity of *P. uniformis* is substantially higher than the diversity of related hermaphroditic species (Tables 2 and S4). Specifically, the comparison of *P. pacificus* strains from North America, Europe, and La Réunion in the Indian Ocean revealed diversity values that were a factor of two to three times lower than those observed for *P. uniformis* (Table 2). Furthermore, the estimated genetic differentiation (F_{ST}) was significant between the two geographic subgroups of *P. uniformis* (Europe vs. North America) ($F_{ST} = 0.103 \pm 0.000$; P-value $= 0.000$).

P. *uniformis* host recognition

Previous studies found that *Pristionchus* species associated with different beetles have distinct chemotaxis profiles toward insect compounds (Hong and Sommer 2006b). To test whether *P. uniformis* strains show specificity in their host recognition, we performed chemotaxis experiments on two American *P. uniformis* strains, one isolated from *L. decemlineata* (RS5167) and one from *P. anxia* (RS5303). Both of these strains fall genetically in the derived clade A (Fig. 3A). Chemotaxis profiles obtained from exposing nematodes to cuticular extracts of the two beetles provide three important observations. First, the *P. anxia* derived strain RS5303 shows a stronger attraction to *P. anxia* beetle extract than *L. decemlineata* derived RS5167 (t-test, P-value $= 0.018$) (Fig. 5A). Second, RS5303 and RS5167 show similar attraction to washes of the "novel" beetle host *L. decemlineata* and both are significantly more attracted than *P. pacificus*, which has never been found associated with any of the test-hosts (Fig. 5B) (t-test, P-value $= 0.000$). These results suggest that, first, chemoattraction mechanisms evolve rapidly, and second, that some *P. uniformis* strains have lost the ability to recognize certain scarab beetles as potential hosts.

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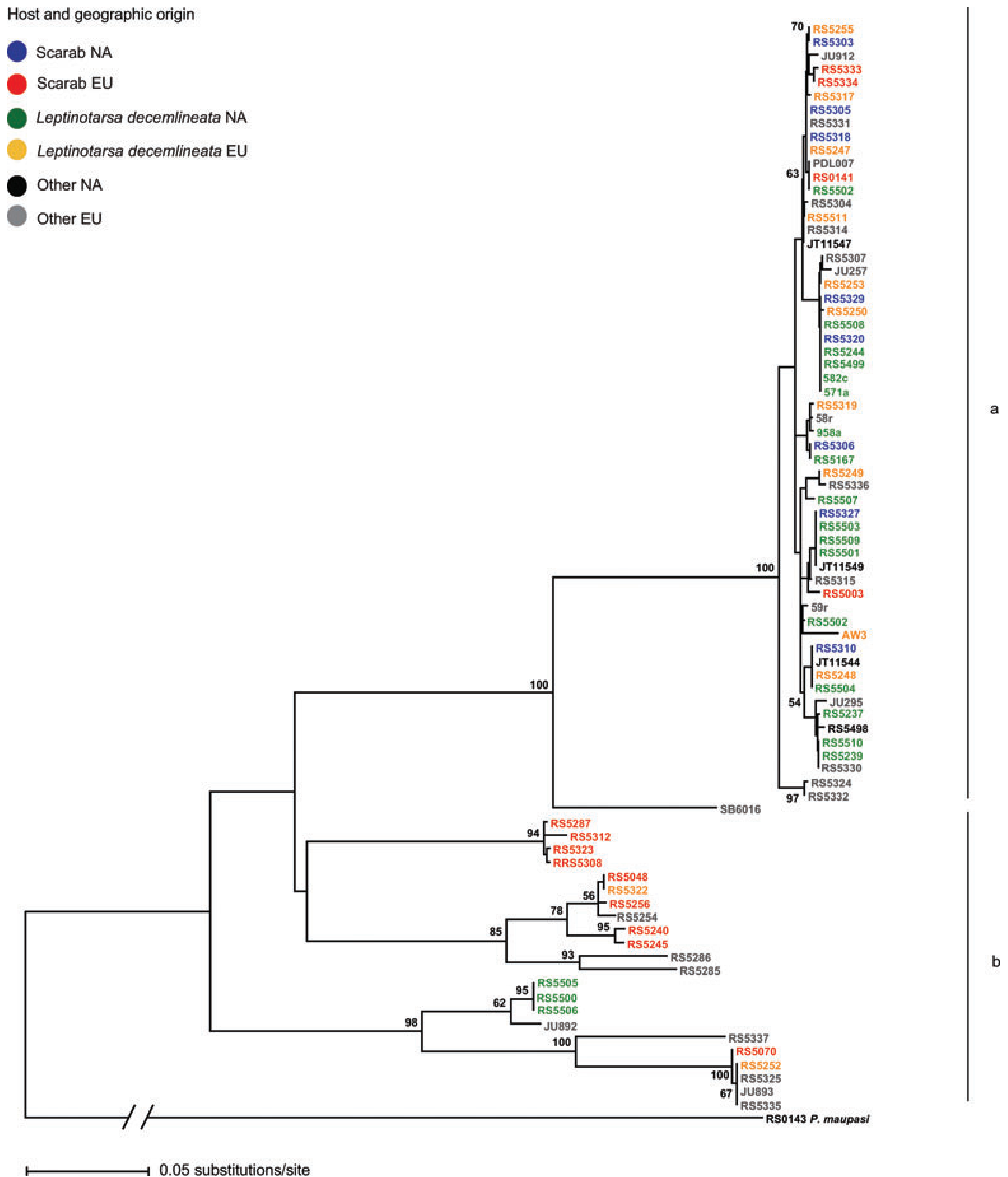

Figure 3. Phylogenetic relationship of 81 *Pristionchus uniformis* strains. The maximum likelihood (ML) tree was reconstructed from aligned mitochondrial gene *nd2* sequences. Robustness of the tree topology was evaluated by 10.000 ML replications. The support values are shown at the nodes. Branch lengths are proportional to genetic divergence. Geographical origin (EU = European; NA = North America) and host association are color coded at the taxon label. Strains with "other" origin were isolated from soil or non-scarab or non-*Leptinotarsa decemlineata* beetles. Letters indicate geographical subgrouping.

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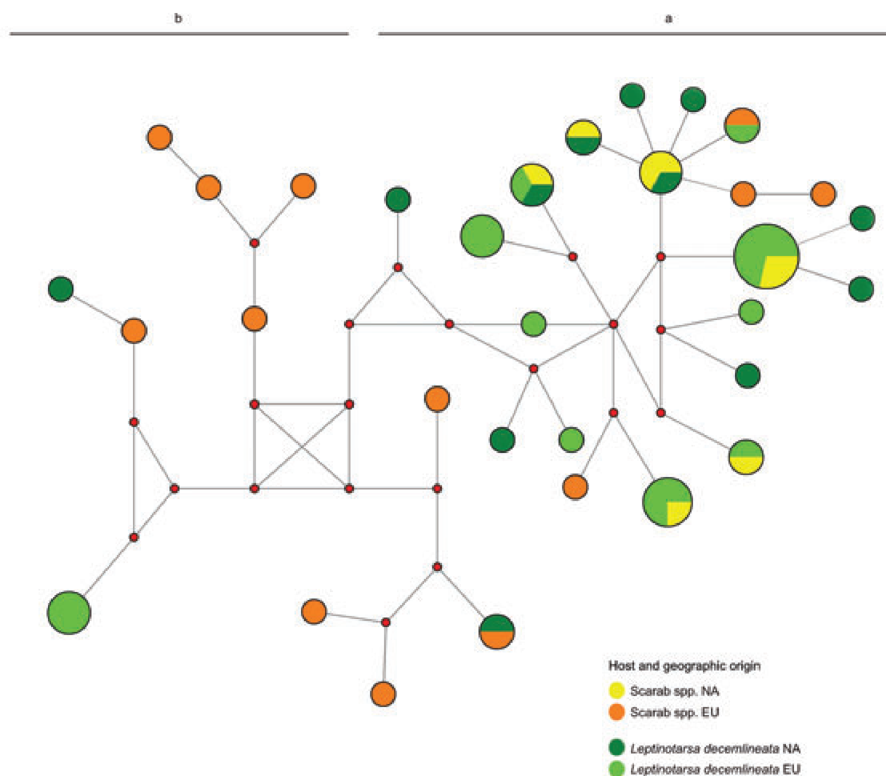

Figure 4. Median joining network for a selection of 53 mitochondrial haplotypes of the *nd2* gene amplified in *Pristionchus uniformis* strains collected in scarab spp. or *Leptinotarsa decemlineata*. Circles represent unique haplotypes. The areas of the circles are proportional to the number of samples sharing each haplotype. Different colors represent different geographic origin (EU = European; NA = North American) and host associations as indicated on the figure. Letters indicate subgroups detected also in Figure 3.

Discussion

Growing interest in invasion biology has mirrored the escalation of species invasion. (Elton 1958; Richardson and Pyšek 2007). While species invasions are often a problem in agriculture, recent studies also focus on the basic biogeography of species invasion to gain insight into the factors and processes that control diversity and distribution at different scales (Richardson and Pyšek 2007). Here, we investigated the biogeography of the nematode *P. uniformis* that has been found tightly linked to one of the most famous insect invaders of Europe, the Colorado potato beetle (*L. decemlineata*).

Nematode associations with other organisms are common and numerous entomophilic nematodes have associations with their hosts, ranging from loose phoresy to strict species-specific parasitism (Sudhaus 2008). Many insect hosts spend most parts of their life cycle in habitats that facilitate nematode attachment. For example, scarab beetle females usually deposit eggs in the soil and grubs feed on roots often for several years, for example, the American May beetle *P. anxia* and the European cockchafer *M. melolontha*. The beetle *L. decemlineata* has a different and much shorter life cycle approximately 30 days long. After hatching from eggs on potato leaves, larval instars feed on leaves, only entering the soil during the last instar to pupate. Thus, scarab beetles and *L. decemlineata* are in the soil where they can get into contact with nematodes, during overlapping but distinct parts of their life cycles. However, a quantitative assessment of the soil–beetle exchange of *Pristionchus* nematodes awaits further study, which may be considerably advanced by involving transgenic technology, available for *Pristionchus* nematodes, for monitoring the movement of nematodes.

Numerous studies on parasite–host associations have described a horizontal transfer from one host to another, defined as host-switching (Page 2003). The switch to a new host is considered one of six types of events that are commonly found in host–parasite evolution (Page 2003). A host-switch involves an initial "expansion" of the parasite's host range and often, the parasite persists on the original host. Successful "colonization" of foreign hosts requires that the parasite "disperse" to that host and is able to "establish" a viable breeding population on it.

Our results revealed that Europe is the likely native area of *P. uniformis* because genetic diversity is greater in Europe than in North America. Thus, *P. uniformis* most likely *expanded* to a new continent, namely from Europe to North

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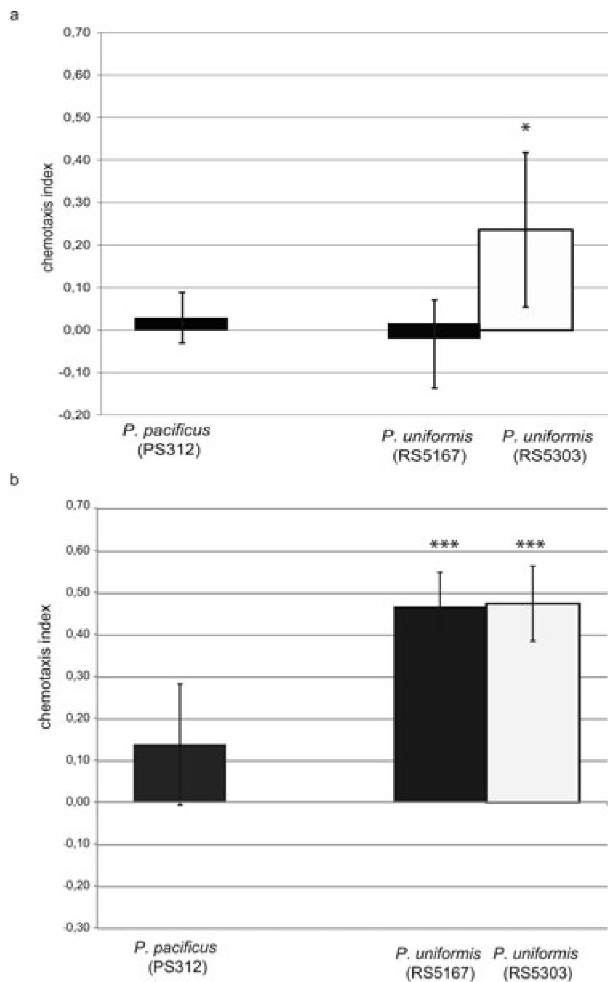

Figure 5. Chemoattraction assay of *Pristionchus uniformis* strains compared to *P. pacificus*. (A) Nematode attraction toward dichloromethane extraction of *Phyllophaga anxia* adults. *Significant difference between *P. uniformis* (RS5167), *Leptinotarsa decemlineata* derived, and (RS5303) *P. anxia* derived, $P < 0.05$ by two-sampled *t*-test. (B) Nematode attraction toward dichloromethane extraction of *Leptinotarsa decemlineata* pupae When compared to *P. pacificus* (PS312), both *P. uniformis* strains (RS5167 and RS5303) are significantly more attracted towards the *L. decemlineata* extract, $^{***}P < 0.001$ by two-samples *t*-test. Error bars denote 95% confidence intervals and each bar represents 10–15 replicates.

America and succeeded in *colonizing* a "new" insect host, the Colorado potato beetle (Table 1). We found eight cases, in which a mitochondrial haplotype is shared between the two hosts, supporting the hypothesis that *P. uniformis* can successfully associate and reproduce on both beetle groups (Fig. 4; Table S3). Assuming that identical mitochondrial sequences did not originate independently in different hosts or locations by parallel (or convergent) mutations, these findings provide evidence for host-switching during recent *P. uniformis* evolution.

Table 1. Divergence estimates in host-associated subgroups based on the mitochondrial gene *nd2*.

P. uniformis (length = 789 bp)	*n*	*S*	*H*	*H*d	π	θ
Total	53	278	32	0.969	0.078	0.097
Scarab spp.	21	250	19	0.990	0.102	0.109
Leptinotarsa decemlineata	32	241	21	0.960	0.056	0.093

S = number of segregating sites, H = number of haplotypes, Hd = haplotype diversity, π = nucleotide diversity, θ = level of polymorphisms from S.

Our analysis of a collection of 81 *P. uniformis* genotypes from North America and Europe favors scenario 2 of the potential biological invasions offered in Figure 2A. We provide clear evidences for a European origin of *P. uniformis* based on the basal positions of European clades and much higher genetic diversity of strains found in Europe (Table 2). Colonization has probably happened at least twice, apparent from a clade of North American nematodes (RS5505, RS5500, RS5506) distinct from that of the highly related clade (Fig. 3B). This finding is supported by the phylogenetic position of *P. uniformis* in the European group of species within the genus *Pristionchus* (Herrmann et al. 2007; Mayer et al. 2007). Given the recent invasion of *L. decemlineata* to Europe, host-switching event might have occurred in Europe. Under these circumstances, *P. uniformis* most likely has invaded North America from Europe. However, it remains unknown if *P. uniformis* invaded North America in association with a host beetle or by another way of transportation. Also, our results do not allow to exclude the potential scenario that *P. uniformis* has been introduced to North America prior to the invasion of Europe by *L. decemlineata*. Data presented herein, provide the first molecular support to characterize the complexity of species invasion and host-switching events of a *Pristionchus*

Table 2. Divergence estimates in geographic subgroups based on the mitochondrial gene *nd2*.

	n	*S*	*H*	*H*d	π	θ	Tajima's D
P. uniformis							
Total	81	319	49	0.979	0.083	0.105	–0.515
Europe	49	316	41	0.991	0.105	0.117	–0.515
North America	32	156	14	0.915	0.038	0.055	–0.923
P. pacificus							
Total	22	126	19	0.983	0.037	0.049	–0.605
North America	7	28	5	0.857	0.013	0.015	–0.474
Europe, Asia, La Réunion	12	133	12	1.000	0.046	0.053	–0.207

S = number of segregating sites, H = number of haplotypes, Hd = haplotype diversity, π = nucleotide diversity, θ = level of polymorphisms from S, Tajima's D test for the assumption of neutral sequence selection evolution. *Pristionchus uniformis* sequence length = 789 bp and *P. pacificus* length = 777 bp. Data for *P. pacificus* are from Zauner et al. 2007 and Molnar et al. 2011.

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nematode. The ease with which these nematodes can be isolated and characterized might make them a useful system to further investigate the biogeography of insect associated nematodes.

So far *P. uniformis* is the only *Pristionchus* species that is found consistently on disparate families of hosts among more than 20 species collected worldwide (Mayer et al. 2009). These observations suggest that host-switching is exceptional in *Pristionchus* evolution and might require special genetic features. The adaptation to the new ecological niche requires a new host-seeking behavior, special chemoattractive properties, and novel survival attributes. Among these adaptations, chemoattraction can be studied under laboratory conditions by using chemoattraction assays. We were able to show that *P. uniformis* has a species-specific preference for its beetle hosts' odor profile. *Pristionchus uniformis* significantly diverged from the model species *P. pacificus* in its chemoattraction profiles toward the extract of the scarab *P. anxia* and the chrysomelid *L. decemlineata*. Interestingly, we observed also an intraspecific discrepancy in chemotaxis when testing the two *P. uniformis* strains on *P. anxia* extracts. The *P. anxia* odor was attractive only for the *P. anxia* derived *P. uniformis* strain. These findings suggest that chemoattraction mechanisms can evolve rapidly, and that some *P. uniformis* strains have lost the ability to recognize certain scarab beetles as potential hosts. It is important to note that this finding might be influenced by the high diversity of scarab beetles. In particular in North America, the group of scarab beetles is very diverse (Arnett et al. 2002) and investigations about the species specificity of *Pristionchus* with these North American scarab beetles await future analysis.

One other, crucial factor for successful host-switching is the ability to overcome competitive exclusion by other residents of the new host (Barker 1994). Interestingly, the "new" *P. uniformis* host *L. decemlineata* is well protected against predators by the secretions of defensive glands (Dalzoe et al. 1986) and by the toxic substances present in the haemolymph (Hsiao and Fraenkel 1969). Indeed, *L. decemlineata* is poor in associated nematodes, most likely because toxic substances prevent a successful invasion by nematodes. Therefore, the switch of *P. uniformis* toward *L. decemlineata* was not influenced by competition from resident nematodes and might have been favored by low predation rates. At the same time, *P. uniformis* must have evolved a mechanism to overcome the toxicity associated with the *L. decemlineata* habitat. However, we have so far been unable to identify such mechanisms; our investigations of the *L. decemlineata* haemolymph did not show any evidence for resistant mechanisms of *P. uniformis*, when compared to other nematode species.

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Supporting Information

Additional Supporting Information may be found online on Wiley Online Library.

Table S1. *Pristionchus uniformis* strains used in the study.
Table S2. Comparison of life-history traits among representative strains.

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Table S3. *nd2* mitochondrial gene haplotype variation and distribution among hosts.
Table S4. Divergence estimates of mitochondrial genes in different *Pristionchus* species in *cyt b*.

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