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Nematodes of the genus *Pristionchus* are closely associated with scarab beetles and the Colorado potato beetle in Western Europe

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Abstract

Evolutionary developmental biology examines how changes in developmental programmes give rise to developmental and, ultimately, morphological novelty. To this end, comparisons of related but distinct organisms have to be performed. The diplogastrid nematode *Pristionchus pacificus* has been developed as a satellite system for a detailed comparison of various developmental processes to the model organism *Caenorhabditis elegans*, a rhabditid nematode. In addition to developmental and genetic studies, a genomic platform has been established to analyse the biology of this organism. However, only little is known about where and how *Pristionchus pacificus* and its relatives live in the wild. Here we show that nematodes of the genus *Pristionchus* live in close association with scarabaeoid beetles and the Colorado potato beetle. In total, we generated 371 isogenic female lines from 4242 beetles collected at 25 sampling sites all over Europe. Isogenic female lines were subjected to sequence analysis and mating experiments for species determination. The 371 isolates fell into six species. Two hermaphroditic species account for about 60% of the collected nematodes. We found *Pristionchus maupasi* almost exclusively on cockchafers and *Pristionchus entomophagus* predominantly on dung beetles. Colorado potato beetles carried the gonochoristic species *Pristionchus uniformis*, which was only rarely observed on scarabaeoid beetles. We describe the initial evidence for the association of *Pristionchus* nematodes with beetles and provide a phylogeny based on sequence analysis of the small subunit ribosomal RNA gene.

Keywords: *Pristionchus*; Nematoda; Scarabaeoid beetles; Colorado potato beetle; *Caenorhabditis elegans*

Introduction

The nematode *Caenorhabditis elegans* is one of the most important model organisms in modern biology (Wood, 1988; Riddle et al., 1997; WormBook, 2005). As a consequence of a comparative approach in evolutionary developmental biology, the diplogastrid *Pristionchus pacificus* has been developed as a satellite organism. *Pristionchus pacificus* is a hermaphroditic species that can feed on *Escherichia coli* and has a 3–4 day generation time at 20 °C (Sommer et al., 1996). Over the last few years, developmental, molecular and genetic studies in *Pristionchus pacificus* have been complemented with a genomic initiative including the generation of a genetic linkage map and a physical map (Srinivasan et al., 2002, 2003). A whole genome sequencing project has been announced in 2004 and should result in a draft of the complete genome sequence in 2006. *Caenorhabditis*

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elegans and *Pristionchus pacificus* shared a last common ancestor about 200–300 million years ago, indicating that the comparison of the two species is a macroevolutionary approach (Pires-daSilva and Sommer, 2004).

Despite the generation of a technical toolkit and the increasing knowledge about developmental processes in *Pristionchus pacificus*, little is known about the evolution and ecology of this species. In particular, it is unknown where *Pristionchus pacificus* lives and what it feeds on under natural conditions. This is not untypical of nematodes. For example, the environmental niche of the model organism *Caenorhabditis elegans* has not been known either. Only recently did several studies indicate that *Caenorhabditis elegans* occurs in compost heaps and is sometimes observed on the snail *Helix aspersa* (Barrière and Félix, 2005; Caswell-Chen et al., 2005; Kiontke and Sudhaus, 2005).

For *Pristionchus pacificus* and all available species of the genus *Pristionchus*, isolates were obtained from various scientists around the world, often isolated from soil samples, or in other cases from fungal or insect samples. However, all of the available strains resulted from haphazard samplings, and none of them was isolated as part of a detailed systematic analysis. Here we show that nematodes of the genus *Pristionchus* live in close association with scarabaeoid beetles and the Colorado potato beetle. We report the results of our sampling in Western Europe in 2004 and 2005. In total, 371 isogenic female lines fall into six species, five of which are morphologically indistinguishable from each other. We provide a species designation regime based on sequence analysis and mating experiments and suggest a collection code involving the deposition of material in museums, a frozen stock centre at our institute and a molecular barcode system.

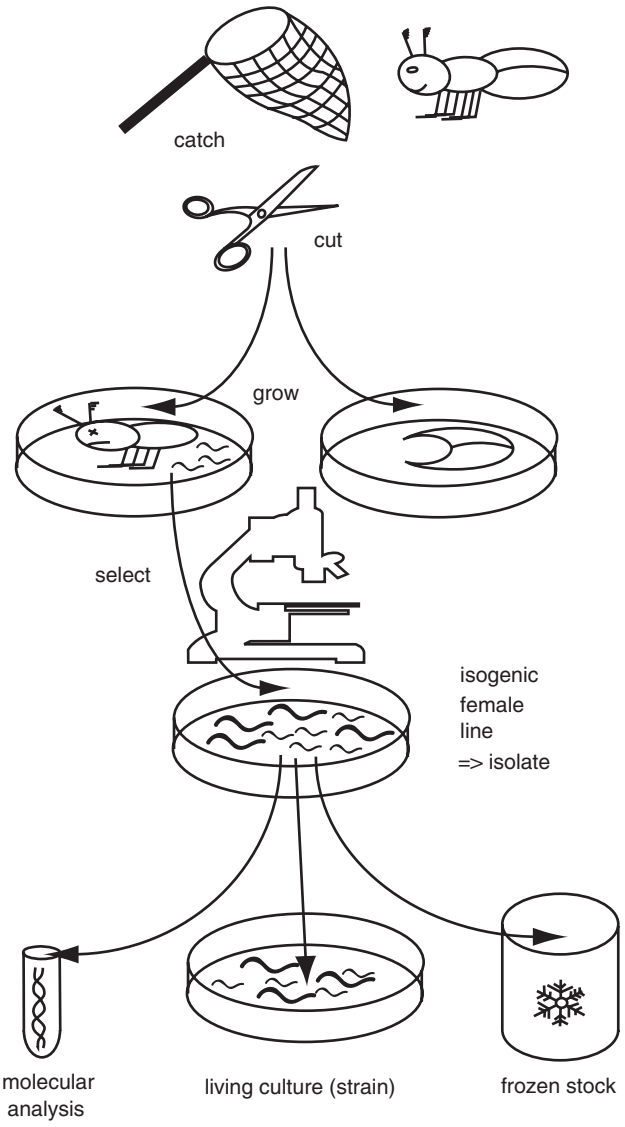

Fig. 1. Scheme depicting the isolation and establishment of nematode lab strains. See text for details.

Materials and methods

Isolation of nematodes

We collected different insects at the adult stage using sweeping nets, blacklight traps and pitfall traps baited with dung (Fig. 1). Insect larvae were isolated from soil. The insects were mainly beetles belonging to the superfamily Scarabaeoidea and the family Chrysomelidae but we also sampled other groups, such as the Elateridae (click beetles) and the Cantharidae (soldier beetles). The insects were transferred to the lab alive, sacrificed by cutting them in half transversely, and put on NGM agar plates (6 cm diameter) seeded with 300 µl of the slowly growing *E. coli* strain OP50 (Fig. 1). Using a Zeiss Stemi 2000 dissecting scope, the plates were checked daily over a period of 1–3 weeks for emerging and reproducing nematodes. From the emerging nematodes we produced isogenic lines by transferring single gravid females or hermaphrodites to new plates. To check whether the isolated nematodes belonged to gonochoristic or hermaphroditic/parthenogenetic species, virgin larvae were singled out onto plates. The presence of offspring indicated that they were hermaphroditic or parthenogenetic species, which could be distinguished by checking the *spermatheca* for the presence of sperm, which is present in hermaphroditic but usually absent in parthenogenetic nematodes.

Taxonomic determination

Emerging nematodes were determined to family level with a Zeiss Stemi 2000 dissecting scope and to genus

level with a Zeiss Axioplan 2 microscope using the key proposed by Sudhaus and Fürst von Lieven (2003). For determination, several worms were transferred onto microscope slides covered with a 0.5 mm layer of 5% agar and either immobilised by heating the slide over an open flame to about 60 °C for a few seconds or anesthesised with sodium azide. Permanent slides were produced as described by Cobb (1918). Within the genus *Pristionchus* many species cannot be identified by morphological methods. We therefore chose to use molecular tools and mating experiments with reference strains to distinguish the different species.

Molecular species identification

Genomic DNA from single nematodes was prepared using the NaOH digestion procedure described by Floyd et al. (2002). A single worm was transferred to 20 µl of 0.25 M NaOH, incubated overnight at 25 °C and heated to 99 °C for 3 min before 4 µl of 1 M HCl, 10 µl of 0.5 M Tris–HCl (pH 8.0) and 5 µl of 2% Triton X-100 were added. The mixture was heated to 99 °C for 3 min, frozen at −20 °C and reheated at 99 °C for further 3 min. Two microlitres of this extract were used for subsequent polymerase chain reaction (PCR). The remaining lysate was kept frozen at −20 °C.

A 1 kb fragment of the small subunit ribosomal RNA gene (*SSU*) was amplified by PCR using the primers SSU18A (5′-AAAGATTAAGCCATGCATG-3′) and SSU26R (5′-CATTCTTGGCAAATGCTTTCG-3′) (Blaxter et al., 1998; Floyd et al., 2002). The reactions were performed in 25 µl of 1 × PCR buffer (Amersham Biosciences, Freiburg, Germany) containing 2.5 mM $MgCl_2$, 0.16 mM of each deoxynucleoside triphosphate, 0.5 µM of each primer, 2 µl of the lysate, and 2 U of *Taq* DNA polymerase (Amersham). The reactions were started by initial denaturation at 95 °C for 2 min in a PTC-200 (MJ Research, Biozym, Hess. Oldendorf, Germany) or T gradient (Biometra, Göttingen, Germany) thermocycler, followed by 35–40 cycles of denaturation at 95 °C for 15 s, primer annealing at 50 °C for 15 s, and extension at 72 °C for 2 min. A final incubation step at 72 °C for 7 min concluded the reaction. PCR products were purified by the Qiagen PCR product gel extraction kit (Qiagen, Hilden, Germany). Approximately 500 bp of the 5′-terminal end were sequenced using the primer SSU9R (5′-AGCTGGAATTACCGCGGCTG-3′) and the Big Dye terminator protocol (Applied Biosystems, Darmstadt, Germany).

Sequences were aligned manually using the Seqpup 0.6f software for Macintosh. The substitution model for the reconstruction of phylogenetic relationships was selected by the hierarchical likelihood ratio test as implemented in the Modeltest 3.7 software (Posada and Crandall, 1998).

Sequence comparison and analysis confirmed that all strains previously assigned to the same species based on mating experiments (Srinivasan et al., 2001) had identical sequences, while 7–19 nucleotide substitutions exist between species, corresponding to 1.5–4.0% sequence divergence (Fig. 2). The sequence divergence of *Pristionchus* to the closely related diplogasterid *Koerneria* ranged from 83 to 95 substitutions (17.7–20.2%). Thus, the *SSU* gene proved to be a suitable marker to classify nematodes into molecular operational taxonomic units (MOTU) and to distinguish species within the genus *Pristionchus*.

Phylogenetic trees were determined using the heuristic search algorithm under the maximum likelihood (ML) criterion using the PAUP*4.0b10 program (Swofford, 2002). Trees were rooted at midpoint. Neighbour joining (NJ; Saitou and Nei, 1987) and maximum parsimony (MP) trees were drawn by the same program. Alignment gaps were eliminated from the analysis. The topological stability of the trees was assessed by 1000 bootstrap replications (Felsenstein, 1985).

Mating experiments

To confirm the species identification by molecular sequencing of a novel isolate, we performed mating experiments with the reference strain of the respective species (see below for definitions). Five virgin females of each species were put on a plate with a small spot of OP50 (so-called ‘‘mating plate’’) together with five males of the reference strain. On a second plate we used the opposite sexes of the two strains to test for reciprocity. If there was no offspring after 1 week, the experiments were repeated two more times. If fertile offspring occurred we considered the two strains to belong to the same species.

In all cases in which isolates had identical *SSU* sequences, mating experiments with the reference strain were successful. Correspondingly, when we tested for mating between strains with different *SSU* sequences no fertile offspring was observed.

Isolate and strain definition

Given the collection procedure introduced above (Fig. 1), we use the following definitions to distinguish ‘‘isolates’’ and ‘‘strains’’. An isolate is an isogenic female line that derives from a beetle (or soil) sample and is subjected to molecular and experimental analysis. After species identification (see above) we established one isolate per species and location as a strain. A strain is permanently cultured in the lab, has a strain number and is also kept as a frozen stock. From the 371 isolates originally obtained we generated 83 strains. For each species designated by molecular sequence analysis and

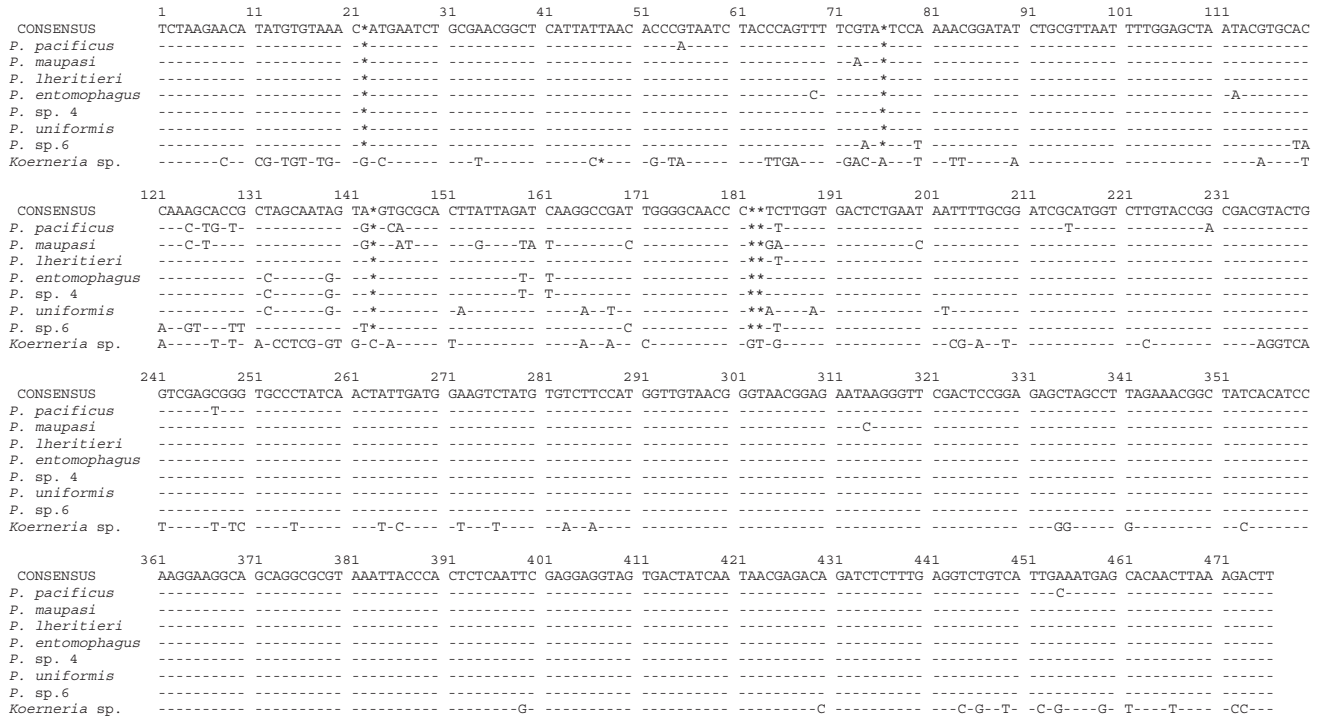

Fig. 2. Alignment of SSU sequences. The sequences of seven *Pristionchus* and one *Koerneria* species were aligned. Numbering refers to the SSU segment obtained in this study. Dashes (–) indicate conformity to the majority consensus sequence at the top; asterisks (*) indicate alignment gaps. The sequences have been submitted to the GenBank database and are available under the following accession codes: SSU_PS312 DQ270018, SSU_RS0143 DQ270019, SSU_SB245 DQ270020, SSU_RS0144 DQ270021, SSU_RS0141 DQ270022, SSU_RS5050 DQ270023, SSU_RS5101 DQ270024, SSU_RS1982 DQ270025.

mating experiments, one reference strain was established: RS0143, Berlin (Germany); SB245, Berlin (Germany); RS0144, Berlin-Dahlem (Germany); RS0141, Menz (Brandenburg, Germany); RS5050, Vailhauques (France); RS5101, Tübingen (Germany).

Assigning names to reference strains

According to the designation of reference strains (see above), the 371 isolates we obtained from beetle material fall into six *Pristionchus* species, three of which were already cultured in our lab (reference strain SB245 *Pristionchus lheritieri*, ref. strain RS0143 *Pristionchus maupasi* and RS0144, *P.* sp. 1). The three others could not be identified and were provisionally named *Pristionchus* sp. 2, 4 and 6, respectively. *Pristionchus* sp. 3 and sp. 5 are provisional names of species found outside of Europe. We tried to match unidentified species with the most suitable descriptions listed in the catalog provided by Sudhaus and Fürst von Lieven (2003) and found that *P.* sp. 1 is identical to *Pristionchus entomophagus* (Steiner, 1929) and *P.* sp. 2 corresponds to *Pristionchus uniformis* Fedorko and Stanuszek, 1971 (see Table 1 and discussion for justification). So far we were able to obtain the following seven *Pristionchus* species from Western Europe, including *Pristionchus pacificus*, which was not found in this study:

Pristionchus entomophagus (Steiner, 1929)	(ref. str. RS0144)
Pristionchus lheritieri (Maupas, 1919)	(ref. str. SB245)
Pristionchus maupasi (Potts, 1910)	(ref. str. RS0143)
Pristionchus pacificus Sommer et al., 1996	(ref. str. PS312)
Pristionchus uniformis Fedorko & Stanuszek, 1971	(ref. str. RS0141)
Pristionchus sp. 4	(ref. str. RS5050)
Pristionchus sp. 6	(ref. str. RS5101)

Freezing protocol for *Pristionchus*

Nematodes were cultivated on plates as described above. Plates with high numbers of J2 larvae were chosen for freezing. Nematodes were washed off with M9 buffer (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 85 mM NaCl, 1 mM $MgSO_4$) and washed several times on ice in the same buffer by gravity sedimentation. Finally, nematodes were resuspended in 2 ml of M9; 2 ml of

Table 1. Comparison of *P. entomophagus* and *P. uniformis* original descriptions with recently isolated strains

	Pristionchus entomophagus (Steiner 1929) $n = 8$	Strain RS0144 $n = 10$		
Reproductive mode	Hermaphroditic	Hermaphroditic		
Sex ratio (males/hermaphrodites)	0/hundreds	0/thousands		
Total length (μm)	668.0	691 (627–795)		
Width (μm)	41.4	43 (37–56)		
Depth of buccal cavity (μm)	5.3	6 (5.2–7.9)		
Pharynx length (μm)	142.9	138 (124–150)		
Distance anterior end–median bulb (μm)	95.5	84 (77–99)		
Distance anterior end–vulva (μm)	394.1	337 (328–356)		
Distance anterior end–anus (μm)	557.1	571 (521–674)		
Anal body width (μm)	22.7	25 (22–28)		
Tail length (μm)	111.0	120 (102–139)		
Finding circumstances	On dead insects	On *Geotrupes stercoarius*		
	P. uniformis Fedorko and Stanuszek 1971 Females ($n = 50$)	Strain RS0141 Females ($n = 10$)	*P. uniformis* Males ($n = 50$)	Strain RS0141 Males ($n = 10$)
Reproductive mode	Gonochoristic	Gonochoristic		
"Hosts"	*Leptinotarsa decemlineata* and *Melolontha* sp.		*L. decemlineata* and *Melolontha* sp.	
Total length (μm)	1460.0 (1100–1760)	1336.0 (1091–1622)	760.0 (630–1350)	830.0 (744–1049)
Width (μm)	125.4 (87–147)	107.0 (88–132)	63.5 (48.1–75.4)	72.0 (64–80)
Depth of buccal cavity (μm)	10.3 (9.2–12.2)	7.1 (6.7–7.6)	7.9 (6–9.8)	6.4 (5.1–7.1)
Vulva (%)	49.0 (48–51) μm	56.0 (45–65) μm		
Spicula length (μm)			46.0 (31.2–123.6)	40 (36–46)
Gubernaculum length (μm)			17.1 (13.4–37.3)	17 (15–17)
Anal body width (μm)	42.1(35–63)	41(35–47)	38.8 (32.8–86.8)	41 (37–45)
Tail length (μm)	250.7 (229.1–345.8)	198.0 (133–235)	131.3 (111.3–305)	122.0 (95–137)

sterile freezing solution (15 mM NaCl, 15 mM KH_2PO_4, 0.5 mM $MgSO_4$, 0.4% Bacto agar, 24% glycerol) prepared with local tap water warmed to 50 °C was added and mixed carefully. The suspension was aliquoted into four cryotubes and frozen immediately in styrofoam racks in a −70 °C freezer. The following day the tubes were transferred to liquid nitrogen for permanent storage. To assess viability of the frozen stocks, an aliquot was thawed and spread on NGM agar plates seeded with *E. coli* OP50.

Museum material

Permanent slides will be sent to the following museums and institutions: The Natural History Museum, London, England; Le Muséum National d'Histoire Naturelle, Paris, France; the University of California Riverside, USA; Zoologische Staatssammlung, München, Germany.

Results

Background: life history of nematodes of the genus *Pristionchus*

At the beginning of this study, our collection contained 13 strains of the genus *Pristionchus* from soil samples from all over the world (Srinivasan et al., 2001). These strains belonged to four different species, i.e. *Pristionchus pacificus*, *Pristionchus maupasi*, *Pristionchus lheritieri* and *Pristionchus entomophagus* (Srinivasan et al., 2001). Detailed analysis revealed that the four species are morphologically indistinguishable from one another. They can only be distinguished by their mode of reproduction and by use of mating experiments. *Pristionchus pacificus*, *Pristionchus maupasi*, and *Pristionchus entomophagus* are hermaphroditic, whereas *Pristionchus lheritieri* is gonochoristic.

Despite this morphological uniformity within *Pristionchus*, 27 species of the genus have been described in

the literature, including the four species named above. Many of the descriptions are rather short and lack illustrations. Therefore, it is most likely that the 27 species descriptions contain a number of synonyms. In addition, type material does not exist in most cases – either because it never existed or because it was lost.

Eighteen of the 28 diplogastrid genera are known to include insect-associated species and six of them are completely insect associated, as, for example, the genus *Micoletzkya* with bark beetles (Sudhaus and Fürst von Lieven, 2003). In the genus *Pristionchus*, several of the strains that were given to us over the years by nematologists from all over the world were also insect associated, in particular with beetles (Srinivasan et al., 2001). Two of the *Pristionchus maupasi* strains, for example, had been isolated from cockchafers (*Melolontha* spp.) and some of the available *Pristionchus* species descriptions mentioned an association with beetles, such as Fedorko and Stanuszek (1971) for *Pristionchus uniformis*, Kotlàn (1928) for *Pristionchus brevicauda* or Lespès (1856) for *Pristionchus migrans*. We therefore set about to test if nematodes of the genus *Pristionchus* indeed show a close association with beetles, and with scarabaeoid beetles in particular.

Overview of the nematode–beetle association

In the years 2004 and 2005 we collected and analysed a total of 4242 beetles from Germany, France, Spain, Switzerland and the Netherlands (Fig. 3). Spanish, Swiss and Dutch samples did not contain *Pristionchus* and were not depicted in the map. Additional samples were sent to us from Italy, Belgium and Poland. Three hundred and seventy-one beetle-derived isolates were analysed molecularly and by mating experiments, resulting in six *Pristionchus* species; three of them, *Pristionchus maupasi*, *Pristionchus lheritieri* and *Pristionchus entomophagus*, were rediscovered species already present in the laboratory (Srinivasan et al., 2001). The remaining three species could not be classified to any of the previously molecularly characterised species. These are *P. uniformis*, *P.* sp. 4, and *P.* sp. 6. *Pristionchus uniformis* and *P.* sp. 4 are gonochoristic, *P.* sp. 6 is hermaphroditic, representing the fourth hermaphroditic species in the genus *Pristionchus*. All species differ in their *SSU* sequences (Fig. 2). The two most closely related species are the hermaphrodite *Pristionchus entomophagus* and the gonochorist *P.* sp. 4, which differ by only two substitutions and form a sister group with different reproductive modes (Fig. 2).

Justification for identifying *P.* sp.1 as *Pristionchus entomophagus* and *P.* sp. 2 as *Pristionchus uniformis* is provided in Table 1. In the following, we describe in detail the results of our analysis.

Cockchafers (*Melolontha*)

We examined about 1100 *Melolontha* individuals of the two most common European species *Melolontha melolontha* and *Melolontha hippocastani* (Fig. 4, Table 2). The dominating nematodes on cockchafers were two diplogastrids of the genus *Diplogasteroides* (Manegold and Kiontke, 2002). *Diplogasteroides magnus* or *Diplogasteroides nasuensis* were observed on more than 60% of the analysed cockchafers and were always the first nematodes to appear, usually 2–3 days after the beetles were killed. Interestingly, if *Pristionchus* occurred on these plates they only occurred with a delay of 7–10 days. As populations of the *Diplogasteroides* species were diminishing, the *Pristionchus* populations were growing. In *Melolontha melolontha* we found up to 10% of the beetles of a population to be infested with *Pristionchus* species. The dominating *Pristionchus* species on this beetle was *Pristionchus maupasi* and we established more than 60 isolates from 1095 beetles (Table 2). In addition, we found *Pristionchus uniformis* on 10 beetles and *Pristionchus entomophagus* on two beetles.

June beetles and garden chafers (*Amphimallon* and *Phyllopertha*)

We also examined *Amphimallon solstitiale* L. and *Phyllopertha horticola* L., close relatives of *Melolontha*. In *Amphimallon solstitiale*, no *Pristionchus* species were observed ($n = 237$). In *Phyllopertha horticola* we found a high infection with a *Koerneria* species. At one collecting site nearly 90% of the individuals were infested. Only eight beetles were infested with *Pristionchus*, six with *Pristionchus entomophagus* and two with *Pristionchus uniformis* (Table 2).

Rose beetles (*Cetonia*)

We investigated 220 rose beetles of the species *Cetonia aurata* and established only one isolate of *Pristionchus maupasi* and two of *Pristionchus entomophagus* (Table 2). All three isolates came from the same location in Schwäbisch Hall (Germany). These were the only records of *Pristionchus* from *Cetonia* spp. At other locations, e.g. in the Mediterranean region, we never found any *Pristionchus* on rose beetles.

Dung beetles (*Geotrupes*)

Geotrupes (*Anoplotrupes*) *stercorosus* L. was the most reliable source for *Pristionchus* nematodes (Fig. 4; Table 2). In a forest in Tübingen (Germany), we found *Pristionchus* in up to 70% of a beetle population. At other locations, *G. stercorosus* was also regularly

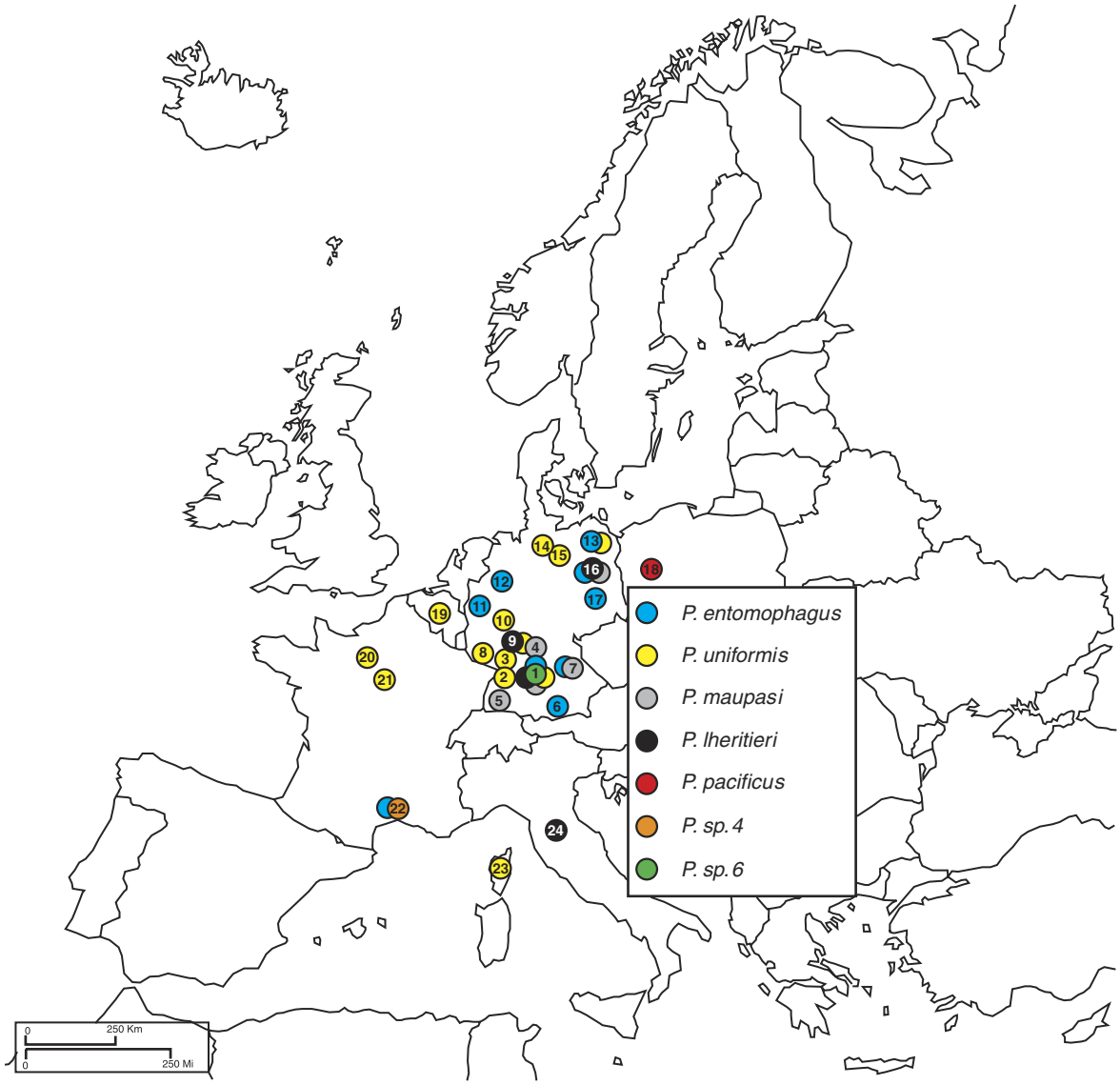

Fig. 3. Map of Western Europe showing the distribution of sampled *Pristionchus* species. The species are differentiated by colour as indicated. Sampling localities: 1, Tübingen; 2, Dielheim; 3, Obergrombach; 4, Wiesloch; 5, Freiburg; 6, Bad Buchau; 7, Schwäbisch Hall; 8, Saarbrücken; 9, Neunkirchen; 10, Limburg; 11, Bonn; 12, Münster; 13, Usedom, 14, Wittenburg; 15, Menz; 16, Berlin; 17, Königswusterhausen; 18, Poland; 19, Belgium; 20, Vallee de la Visnoise; 21, Etrechy; 22, Vailhauques; 23, Corsica; 24, Arrezzo.

infested with *Pristionchus* but the numbers of infested beetles did not reach values as high as the ones described for the Tübingen area. *Pristionchus entomophagus* is the predominant species on dung beetles, making up 94% of all obtained isolates (Fig. 4). In addition, we established five isolates of *Pristionchus lheritieri* and five isolates of *P.* sp. 6, a novel hermaphroditic species that has so far only been observed at the Tübingen location.

If dung beetles were caught directly at dung piles, other non-diplogastrid nematodes such as the rhabditid *Pelodera icosiensis* (a nematode that is known to occur frequently in dung) were also commonly found. However, dung beetles caught at sapping trees did not carry *Pelodera icosiensis* but were highly infested with *Pristionchus*. These results suggest that *Pelodera icosiensis* uses dung beetles for short-term transport (phoresy) to new feeding sites, while *Pristionchus* stay on the beetles for longer time periods.

In the genital chamber of the beetles we frequently observed a non-*Pristionchus* diplogastrid nematode, which there only occurred in the dauer stage and never became adult. Similar results were reported earlier by Théodoridès (1955) and Kühne (1995). We were able to determine (by sequencing) that this nematode is closely related to the members of the genus *Koerneria* which we isolated from *Phyllopertha* or received from Walter

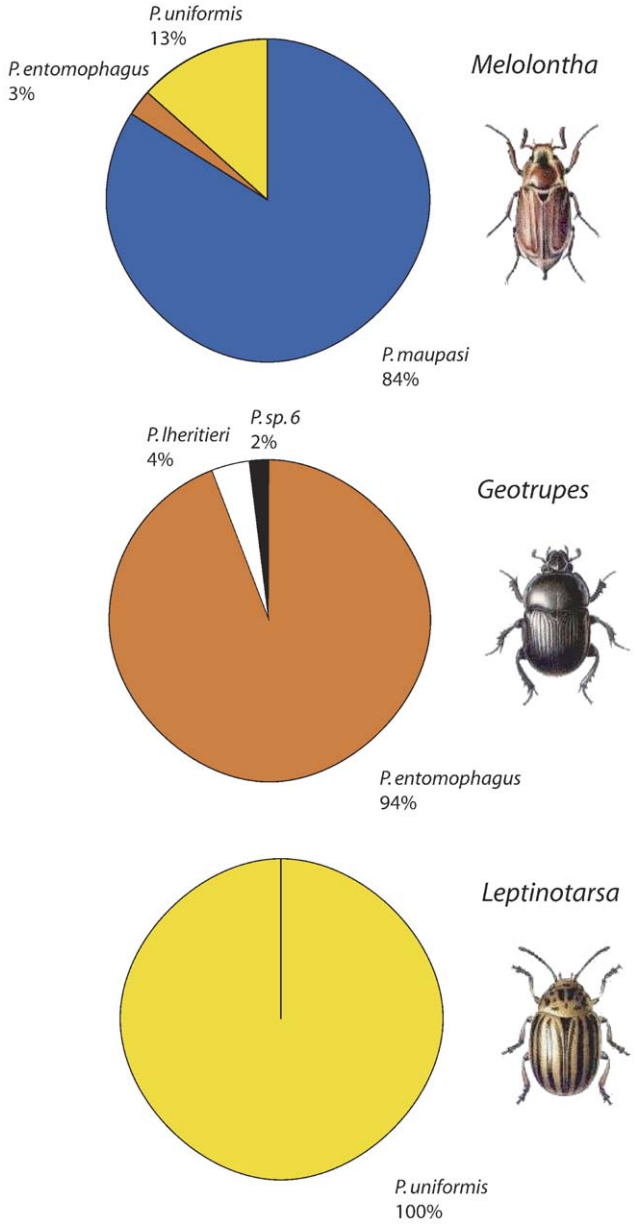

Fig. 4. Pie charts of the occurrence of different *Pristionchus* species on *Melolontha*, *Geotrupes*, and *Leptinotarsa*.

Sudhaus (FU Berlin, Germany) (data not shown). These results are consistent with the fact that Kühne (1995) described three adult nematode species occurring in the brood balls of *Geotrupes*. One of them was named *Diplogaster labiomorpha* (Kühne, 1995), the others were *Diplogaster henrichiae* Sachs, 1950 and *Diplogaster hirschmannae* Sachs, 1950. Sudhaus and Fürst von Lieven (2003) placed all three of them into the genus *Koerneria*.

To check if *Pristionchus* nematodes are restricted to a particular body part of the beetle, we analysed *Pristionchus entomophagus* on dung beetles as they had the highest infestation rate in our study. Surprisingly, *Pristionchus entomophagus* is found on all three body parts of the beetle. Forty-one percent of the nematodes were observed on the head, 33% on the thorax and 26% on the abdomen (Fig. 5). Considering the relative size of the body parts, the preference for the head is obvious. However, we have been unable to find a specific location of the nematode on or in the beetle.

Other dung beetles (Onthophagus *and* Aphodius)

Most of the *Geotrupes* beetles we analysed were from horse dung. At most collection sites, *Geotrupes* coexisted with other dung beetle species, mainly of the genera *Onthophagus* and *Aphodius*. We analysed a total of 453 dung beetles of these genera and never observed any *Pristionchus* (Table 2). This observation supports the notion that the association of *Pristionchus* and *Geotrupes* is highly specific and goes much further than a loose phoretic association. It should be noted that in contrast to *Geotrupes*, other dung beetles such as members of the genus *Aphodius* do not form brood chambers in the soil.

Colorado potato beetles (*Leptinotarsa decemlineata*)

The Colorado potato beetle belongs to the family of the leaf beetles (Chrysomelidae). Fedorko and Stanuszek (1971) reported that during a screening for potential entomopathogenic species, *Pristionchus uniformis* was found on the Colorado potato beetle (*Leptinotarsa decemlineata*) as well as on cockchafers. As the Colorado potato beetle is a fast reproducing pest species, it was quite easy to collect a large number of beetles from different infested potato fields. In our analysis, the incidence of *Pristionchus* on *L. decemlineata* varied between 0% at some sites near Tübingen and up to 20% at one site near Usedom in North-eastern Germany (Fig. 4; Table 2). Sequence analysis revealed that all *Pristionchus* isolates from the Colorado potato beetle are identical to *P. uniformis*, a species that was also observed on cockchafers. This observation is similar to that of Fedorko and Stanuszek (1971). Interestingly, *Pristionchus uniformis* is the only *Pristionchus* species on the Colorado potato beetle. This is in contrast to scarabaeoid beetles, which often show a strong preference for, but never an exclusive infestation with one particular *Pristionchus* species.

Soldier beetles and click beetles (*Rhagonycha* and *Ampedus*)

We also analysed beetles of other families than the Scarabaeidae and Chrysomelidae to address the specificity of the associations. Neither soldier beetles (Cantharidae) of the genus *Rhagonycha* ($n = 50$) nor

Table 2. Frequencies of *Pristionchus* isolates on beetles

Beetle species	No. of individuals	*Pristionchus* species					
		P. maupasi	*P. lheritieri*	*P. entomophagus*	*P. uniformis*	P. sp. 4	*P.* sp. 6
Melolontha melolontha	477	48 (10%)	—	2 (0.4%)	8 (1.6%)	—	—
Melolontha hippocastani	618	15 (2.4%)	—	—	2 (0.3%)	—	—
Phyllopertha horticola	447	—	—	6 (1.3%)	2 (0.4%)	—	—
Cetonia aurata	220	1 (0.4%)	—	2 (0.8%)	—	—	—
Lucanus cervus	20	—	—	3 (15%)	—	1 (5%)	—
Geotrupes stercorosus	760	—	5 (0.7%)	143 (19%)	—	—	5 (0.7%)
Leptinotarsa decemlineata	832	—	—	—	130 (16%)	—	—
Phyllopertha horticola	330	—	—	6 (1.8%)	2(0.6%)	—	—
Amphimallon solstitiale	237	—	—	—	—	—	—
Aphodius spp.	220	—	—	—	—	—	—
Onthophagus spp.	233	—	—	—	—	—	—
Rhagonycha fulva	50	—	—	—	—	—	—
Ampedus sp.	50	—	—	—	—	—	—

Numbers in parentheses indicate percent of individual beetles infested.

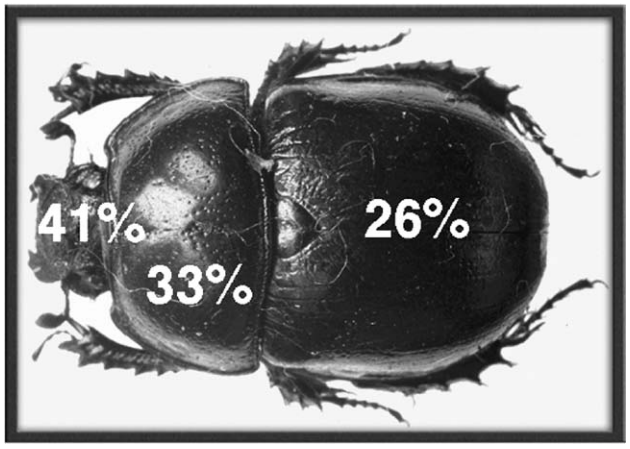

Fig. 5. *Pristionchus* distribution on various body parts of *Geotrupes* (*Anoplotrupes*) *stercorosus*. Values of occurrence are given in percent.

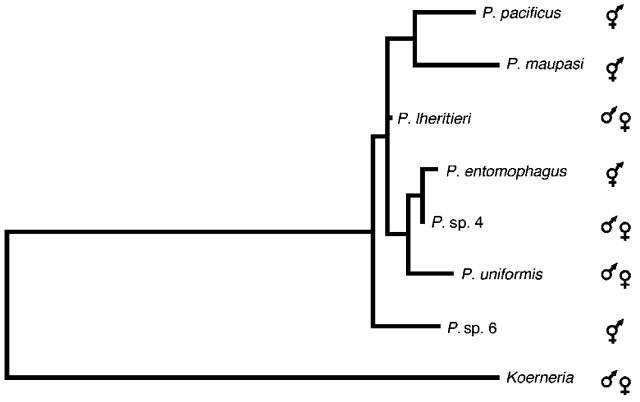

Fig. 6. Phylogenetic tree of *Pristionchus* species as inferred by maximum likelihood analysis of *SSU* sequences. *Koerneria* sp. was included as a closely related genus to *Pristionchus*. The tree was rooted at midpoint. The same tree topology was obtained when neighbour-joining or maximum parsimony methods were used. Hermaphroditic and gonochoristic species are indicated.

click beetles (Elateridae) of the genus *Ampedus* ($n = 50$) carried *Pristionchus*.

Molecular phylogenetic analysis

In order to obtain a provisional phylogenetic framework of the genus *Pristionchus*, we used the *SSU* sequences of the six *Pristionchus* species obtained in this study. In addition, we included the satellite model organism *Pristionchus pacificus* and a member of the closely related genus *Koerneria* (Sudhaus and Fürst von Lieven, 2003) as outgroup. The resulting phylogenetic tree is shown in Fig. 6. The tree illustrates the close relationship between all *Pristionchus* species, well separated from *Koerneria*. Two main branches are evident, one grouping the hermaphroditic species pair *Pristionchus pacificus* and *Pristionchus maupasi*, the other clustering the hermaphroditic *Pristionchus entomophagus* with its gonochoristic sister species *P.* sp. 4 and *Pristionchus uniformis*. The gonochoristic species *Pristionchus lheritieri* assumes an intermediate position, whereas the novel hermaphroditic species *P.* sp. 6 branches off at a basal position with regard to the other species of the genus. Although the bootstrap values were low, additional support for the tree is provided by NJ and MP trees that all show the same topology (data not shown). However, to provide better support for the phylogenetic relationship of the *Pristionchus* species, extensive sequencing efforts of more genes are necessary.

Discussion

We have shown that nematodes of the genus *Pristionchus*, a genus that includes the satellite organism *Pristionchus pacificus*, are closely associated with certain scarabaeoid beetles and the Colorado potato beetle. In total, we observed six *Pristionchus* species, three hermaphroditic and three gonochoristic ones, on these beetles. There is a clear preference of certain nematodes for a specific beetle. *Pristionchus maupasi* and *Pristionchus entomophagus* are the two dominating species on *Melolontha* and *Geotrupes* species, respectively. In this context it is interesting to note that both of these nematode species are hermaphroditic. We did not observe a single case in which a nematode species was restricted to one scarabaeoid beetle only, and similarly, most scarabaeoid beetles carry multiple *Pristionchus* species, sometimes concurrently. In contrast, the only *Pristionchus* species we observed on the Colorado potato beetle was *Pristionchus uniformis*. Thus, the *Pristionchus* community structure seems to be beetle-specific.

Pristionchus species and their presumed association with beetles

Pristionchus pacificus Sommer et al., 1996

Pristionchus pacificus is a hermaphroditic species that has been established as a satellite species in evolutionary developmental biology (Sommer, 2005). Several isolates from North America are available, the two most important strains for laboratory use being isolates from Pasadena (California, USA) and Port Angeles (Washington, USA). The name *Pristionchus pacificus* is based on the observation that both of the original isolates were from the Pacific area (Sommer et al., 1996).

Only one strain of *Pristionchus pacificus* has so far been isolated in Europe. This strain, RS106, was given to us by Einhard Schierenberg (Cologne, Germany), who had isolated it from Polish soil samples (Srinivasan et al., 2001). Our study of scarabaeoid beetles and the Colorado potato beetle indicates that in Western Europe *Pristionchus pacificus* does not occur, at least not very frequently, on this group of beetles. *Pristionchus pacificus* has never been observed during any of the many soil sampling efforts of various European nematologists. Future work on beetles in North America and Asia will reveal if the species is associated with certain beetles in these geographic regions.

The fact that *Pristionchus pacificus* has not been observed on beetles in our studies suggests two possibilities. First, the species lives in Europe but is not associated with beetles or not associated with the beetles investigated in this study. Second, *Pristionchus pacificus* does not naturally occur in Europe, but rather lives in North America or Asia. Only additional collection data can clarify these issues. One should note that the scarabaeoid beetle fauna in North America is completely different from the European fauna, making it likely that other *Pristionchus* species do exist in this part of the world. Also, in the random soil samples we have obtained over many years, *Pristionchus pacificus* was found six times in samples from North America and three times in samples from Asia (M.H., W.E.M. and R.J.S., unpublished data).

Pristionchus maupasi (Potts, 1910)

Pristionchus maupasi has been found at 11 different locations in Germany on the two most common cockchafer species. It should be noted that *Pristionchus maupasi* is the predominant *Pristionchus* species on cockchafers. *Pristionchus maupasi* is a hermaphroditic species with a high incidence of males. Many of the isolates that were directly obtained from beetles had up to 10% of males, an observation that supports the importance of males in nature. It should be noted that the existence and importance of males in natural populations of *Caenorhabditis elegans* has recently been strongly debated (Barrière and Félix, 2005; Chasnow and Chow, 2002; Cutter, 2005; Cutter and Ward, 2005; Stewart and Phillips, 2002). Our observation of *Pristionchus maupasi*, although in another nematode species, clearly demonstrates the presence of males. We could only establish one isolate of *Pristionchus maupasi* from another beetle genus than *Melolontha*. This came from a rose beetle from Schwäbisch Hall (Germany).

Pristionchus lheritieri (Maupas, 1919)

Pristionchus lheritieri is a gonochoristic species that was found several times in *Geotrupes* (*Anoplotrupes*) dung beetles in Tübingen and Neunkirchen. One strain was established from mushrooms near Berlin (kindly provided by Sudhaus). Taking into account that *Geotrupes* (*Anoplotrupes*) *stercorosus* is known to be a mycetophilic beetle (Benick, 1952), sometimes even burrowing mushroom parts into its tunnels, we think that *Pristionchus lheritieri* is mainly associated with geotrupid beetles. In comparison to *Pristionchus maupasi* and *Pristionchus entomophagus*, *Pristionchus lheritieri* occurred on beetles less often.

Pristionchus entomophagus (Steiner, 1929)

Pristionchus entomophagus was the predominant hermaphroditic species on dung beetles. It also occurred on other beetles, but in lower numbers (Fig. 4). *Pristionchus entomophagus* is morphologically very similar to the three species described above and can best be distinguished by its *SSU* sequence and by its hermaphroditic mode of reproduction. Unfortunately, males have never been observed in cultures generated from beetles or cultures that were exposed to stress

conditions. This is consistent with Steiner's observation (1929) of a hermaphroditic *Pristionchus* species that occurs on dead insects. Accordingly, he named it *Pristionchus entomophagus*. He also noted that males never occurred in *Pristionchus entomophagus*. Table 1 provides the morphometric data of *Pristionchus entomophagus* (Steiner, 1929). These measurements are identical to those obtained from the strain RS0144 from beetles (Table 1). Thus, we classified the provisional *P.* sp.1 as *Pristionchus entomophagus*. Since males of this species were not available for mating experiments, we used the reciprocal set-up of virgin hermaphrodites of *Pristionchus entomophagus* with males of other gonochoristic or hermaphroditic species. These mating experiments never resulted in males in the F1 generation, indicating that *Pristionchus entomophagus* is a distinct species.

Pristionchus uniformis Fedorko and Stanuszek, 1971

Pristionchus uniformis is a gonochoristic nematode that in our study was found on the Colorado potato beetles (*L. decemlineata*) at several locations. In 10 cases it also occurred on cockchafers. This observation conforms to the description of a *Pristionchus* species by Fedorko and Stanuszek (1971). In a 3-year study in Poland, the authors often found a gonochoristic *Pristionchus* species on Colorado potato beetles and cockchafers and named it *Pristionchus uniformis*. Table 1 provides the morphometric data of *Pristionchus uniformis* Fedorko and Stanuszek 1971, which are identical to those obtained from the strain RS0141. The thickening of the tip of the spicules as shown by Fedorko and Stanuszek (1971, Fig. 7d) was only observed in a few males and is not a general characteristic of RS0141. We therefore classified the provisional *P.* sp. 2 as *P. uniformis*.

In contrast to our observations, Fedorko and Stanuszek also describe *Pristionchus uniformis* as a facultative parasite of the beetles because in some cases the nematodes had invaded the insects' body cavity. We have been unable to repeat this finding and cannot provide any evidence for a parasitic relationship between any *Pristionchus* species and beetles. Thus, the genus *Pristionchus* is not actively involved in killing the beetles. Rather, the nematodes feed on the carcass of the dead beetle, a phenomenon that has been called "necromeny" (Sudhaus, 1976; Sudhaus and Schulte, 1989).

Pristionchus sp. 4

P. sp. 4 is a gonochoristic species, the males of which do not cross with females/hermaphrodites of any other species. We only established one isolate from a stag beetle from southern France. *P.* sp. 4 is morphologically similar to all other species but can be clearly distinguished at the sequence level and by mating experiments (Fig. 2).

Pristionchus sp. 6

P. sp. 6 is a hermaphroditic species that is unique among the *Pristionchus* species observed in our study in that it is the only one that is morphologically distinct. A more detailed morphological description is in progress. We established five isolates from different dung beetles at the Tübingen location. It should be noted that the molecular phylogenetic analysis places *P.* sp. 6 at the base of the genus, which makes this species very important for phylogenetic reconstruction.

Conclusion

Our observations of *Pristionchus* species in Western Europe are by no means complete. *P.* sp. 4 has only been obtained from one beetle at one location. For *P.* sp. 6 we established five isolates, all of which derive from the same dung beetle species at the same location. We plan long-term studies to obtain a more complete picture of the species distribution in Europe. In addition, many other observations await further analysis. How general are the findings we made in Europe? Does the scarabaeoid beetle fauna in North America harbour other *Pristionchus* species? Is *Pristionchus pacificus* an indigenous species in North America? All of these biogeographic questions can only be answered by collecting material in other parts of the world.

Another area of future research will be the exact determination of the life cycle of *Pristionchus* species on the beetles. As part of this study we have been able to show that *Pristionchus maupasi* infests larvae, pupae and adult stages of the cockchafers, indicating that there is no restriction to a particular stage of the insect life cycle.

In our dissections of dung beetles we did not obtain any evidence for *Pristionchus* being associated with the intestine or other internal body parts (data not shown). At the same time, our analysis indicated that *Pristionchus* occurs only in small numbers and lives on the beetle as a dauer stage. *Pristionchus* occurs very late on the carcass of the beetles. In the most extreme case of the cockchafers, *Pristionchus* emerged only from 10–14-day-old carcasses. The presumed succession of nematodes from *Pelodera* and *Diplogasteroides* to the final appearance of *Pristionchus* raises the interesting possibility that they form a food net, with *Pristionchus* at least partially feeding on the other nematode species. The specialised buccal cavity of *Pristionchus* and other highly derived diplogastrids makes it possible for these nematodes to feed on bacteria, fungi and other nematodes (Fürst von Lieven, 2001), all of which are present on carcasses of beetles. We plan a more detailed analysis of this

nematode succession, which will, however, require long-term studies given the slow generation time and the limited access to cockchafers.

We observed adult *Pristionchus* individuals more than a week after the killing of the beetle in the laboratory. In several cases we first observed dauer larvae indicating that *Pristionchus* remains on beetles in the dauer stage. We have not observed any evidence for reproduction of *Pristionchus* nematodes on living beetles. Most of the beetles we analysed in the lab were obtained as healthy animals from the wild. Also, we have been culturing dung beetles in the laboratory for many months without observing any health problems in the beetles or a reduction of their lifetime. However, when we killed some of these beetles and tested for nematode infestation, we found similar infestation rates as in beetles tested immediately after capture from the wild. Thus, we have no evidence for considering *Pristionchus* a parasite of insects.

On some insects we could never find *Pristionchus*. Neither elaterids nor cantharids, which do not belong to the superfamily Scarabaeidae, carried a diplogastrid nematode. But even within the scarabs there were certain groups without *Pristionchus* infestation, e.g. the genus *Aphodius* – dung beetles living in diverse kinds of manure. Interestingly, *Aphodius* species spend all their life cycle in the dung piles and never burrow down into the soil.

The *Pristionchus*–beetle association as described in this study provides the unique opportunity to combine research in evolutionary developmental biology with ecology. Scarabaeoid beetles and Colorado potato beetles can be cultured in the laboratory and we have successfully established such cultures. Thus, the nematode–beetle interaction can be studied under laboratory conditions. Such studies will be of importance for understanding many aspects of the biology of the nematode, e.g. the genetic regulation of dauer formation and olfaction. Ultimately, knowledge about the natural environment of *Pristionchus* and the possibility to culture nematodes under these "environmental" conditions will also be of importance for the analysis of the evolution of developmental processes. For example, genetic and molecular studies of *Pristionchus pacificus* revealed fundamental differences in the control of vulva development when compared to *Caenorhabditis elegans* (Zheng et al., 2005). The long-term goal of this two-species comparison – a comprehensive analysis of the genetic and molecular changes that occurred in the evolutionary lineages giving rise to *Pristionchus pacificus* and *Caenorhabditis elegans* – requires an appreciation of ecology, because any adaptation results from environmental conditions that organisms are exposed to.

The type of relationship between *Pristionchus* and its hosts has been called "necromeny" (Sudhaus, 1976; Sudhaus and Schulte, 1989). This association is much more specific than a phoretic relationship, which only serves for the transportation of nematodes to new habitats. It has been suggested that in evolutionary terms necromeny is an intermediate step preceding true parasitism. In this context it will be interesting to study the association of *Pristionchus* with beetles in more detail in order to learn more about the evolution of parasitism. In particular, the upcoming completion of the genome sequencing project of *Pristionchus pacificus* will provide a powerful tool to investigate the genomic and genetic features necessary for the evolution of parasitism.

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