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Chemoecology 12:133–138 (2002)
0937–7409/02/030133–06 $1.50 + 0.20
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CHEMOECOLOGY

Interspecific transmission of *Paederus* endosymbionts: relationship to the genetic divergence among the bacteria associated with pederin biosynthesis

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Summary. Endosymbiotic bacteria implicated in pederin production of *Paederus* (+)-females (Coleoptera: Staphylinidae) can be transmitted horizontally within and less frequently among the three species analyzed (P. *melanurus, P. riparius, P. sabaeus).* The 16S rDNA isolated from (+)-females reveals closely related bacterial sequences in the three species as well as in *Paederus fuscipes* and *Paederidus ruficollis.* This confirms the association of the undescribed endosymbiont and pederin biosynthesis in 5 of the 13 species that have been shown to contain the substance. In spite of the high sequence identities (> 99.5%), which suggest one species of endosymbiont, some of the heterospecific hosts were incompatible. This indicates adaptation and specific preferences of the endosymbiont for their natural host.

Key words. Endosymbiont – host – 16S rDNA – *Paederus* – Coleoptera – Staphylinidae – *Pseudomonas*

Introduction

Unlike other staphylinid beetles, the genus *Paederus is* known to many people as exemplified by various colloquial names in use especially in tropical regions of the world (e.g. creechy in Mamfé, Republic of Cameroon, for *P. sabaeus*). This is due to their vesicant hemolymph, which causes itching sensation followed by blisters if it comes into contact with human skin, a disease known as dermatitis linearis. Supposedly, the variegated color pattern of *Paederus* is aposematic (Frank & Kanamitsu 1987) although considerable variation of coloration can be observed in different species: The most common pattern (head and tip of abdomen black, pronotum and base of abdomen orange or red, dark elytra shining blue or green) is darkened in some species like the European *Paederidus* (all black abdomen) or lightened in others like *Paederus melanurus* (entirely orange with only a black apex of the abdomen) and other more complicated patterns have been described (Willers 1998). Indeed, there is no experimental proof of the combined effects of coloration and toxin on predators that might learn to avoid *Paederus* (Frank & Kanamitsu 1987). A defensive value of pederin, the toxic amide peculiar to *Paederus* hemolymph, could only be shown for the sombre larvae that are unpalatable to wolf spiders (Kellner and Dettner 1996).

Of the 621 recent species of *Paederus* (s. 1.) listed by Frank (1988) 35 have been found to definitively (Table 1) or possibly (according to indirect evidence) contain pederin. Although the exact classification of several species (particularly the tropical ones) remains to be clarified it is obvious that pederin is widely distributed within the Paederina & actually no *Paederus* (s. 1.) species has been shown to be devoid of the substance. In particular, it is known from the genus *Paederidus,* which is regarded as the most primitive genus within Paederina (Scheerpeltz 1957), and from four subgenera of *Paederus* (Table 1), which have been described with reference to their aedeagal structures. Furthermore, its distribution is not related to certain color patterns (e.g. the most typical coloration) of *Paederus* (s. l.).

Recent analysis of laboratory-reared specimens (Kellner 1999; Kellner 2001a; Kellner 2001b) has shown that this most complex nonproteinaceous insect toxin (Frank & Kanamitsu 1987) in fact is not a product of the biosynthetic capabilities of the *Paederus* species studied but can be attributed to a specific endosymbiont. Moreover, a highly dominant bacterium could be identified that is associated with pederin biosynthesis in (+)-females of *P. sabaeus* (Kellner 2002). As they also contain pederin, other *Paederus* species are expected to harbor the same or very similar endosymbionts. The endosymbiont is transmitted vertically from (+)-mothers to their daughters and is also able to colonize the daughters of conspecific (−)-females after ingestion of (+)-eggs during larval development (Kellner 1999). Similarly, horizontal transmission could enable the endosymbiont to spread among different *Paederus* species. Depending on the occurrence of such interspecific transmission, acquisition of the endosymbiont could be a comparatively recent event that happened after *Paederus* speciation. Identical endosymbionts in various unrelated species would testify horizontal transmission in the past whereas divergence of the endosymbionts of different species could be attributed to their isolation, which results in accumulation of differences. This study reports on the frequency of interspecific transmission among three species in the laboratory and compares the results with molecular

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Table 1 Current classification of the species (all belonging to the staphylinid subtribe Paederina, Frank, 1988) that proved to contain the hemolymph toxin pederin. 22 additional members of the Paederina (11 of them only classified as *Paederus* sensu lato, which applies to all Paederina species) might also contain the substance as they have been reported to cause skin blistering in mammals (Frank & Kanamitsu 1987; Whelan & Weir 1987; Morsy *et al*. 1996; Nikbakhtzadeh & Sadeghiani 1999)

Species	First pederin record
Paederidus rubrothoracicus (Goeze 1777)	Cardani *et al*. 1965
Paederidus ruficollis (Fabricius 1776)	Kellner, unpubl.
Paederus (Dioncopaederus) littoralis Gravenhorst 1802	Cardani *et al*. 1965
Paederus (Harpopaederus) baudii Fairmaire 1859	Kellner 1997
Paederus (Harpopaederus) brevipennis Lacordaire 1835	Kellner, unpubl.
Paederus (Harpopaederus) schoenherri Czwalina 1889	Kellner 1997
Paederus (Heteropaederus) fuscipes Curtis 1826	Pavan & Bo 1953
Paederus (Heteropaederus) sabaeus Erichson 1840	Kellner 2001a
Paederus (Paederus) balcanicus Koch 1938	Kellner 1997
Paederus (Paederus) melanurus Aragona 1830	Cardani *et al*. 1965
Paederus (Paederus) riparius (Linné 1758)	Kellner and Dettner 1995
Paederus[1] *rufocyaneus* Bernhauer 1912	Cardani *et al*. 1965
Paederus (s. 1.)[2] *columbinus* Laporte 1835	Pavan 1975

[1] subgeneric classification unknown
[2] unassigned to the finer generic division

data on the naturally occurring endosymbionts of these and other species.

Material and methods

Laboratory breeding of beetles

Imagines of *Paederus* were collected from their natural sites and bred in the laboratory to obtain (−)-larvae that could be fed with (+)-eggs. This procedure was applicable to the following three species: *Paederus melanurus* Aragona from Lombardia, Italy (Kellner 2001b), *Paederus riparius* (Linné) from northeastern Bavaria, Germany (Kellner 1998), and *Paederus sabaeus* Erichson from Littoral, Republic of Cameroon (Kellner 2001a). Reproduction was successful with pairs at constant conditions as previously described in the citations given above. 20°C and a photoperiod of 15L : 9D were used for the two European species (*P. melanurus* and *P. riparius*) while 27°C and 13L : 11D were chosen for the African *P. sabaeus*.

Individual females were classified as (+)- or (−)-females depending on the pederin content of their eggs (Kellner & Dettner 1995; Kellner 2001a; Kellner 2001b). As all daughters of (−)-females proved to be (−)-females (*P. melanurus*: n = 74, *P. riparius*: n = 61, *P. sabaeus*: n = 286) (−)-larvae descended from such (−)-females were used for analysis of interspecific transmission of endosymbionts. *P. riparius* could not be maintained for more than two filial generations in the laboratory and therefore only a few experiments were performed with offspring of (−)-females collected outdoors and of their first filial generation. (−)-Larvae of the other two species were selected from (−)-matrilines established during the laboratory breeding over several generations (*P. melanurus*: F_1 to F_5, *P. sabaeus*: F_2 to F_{12}).

Experimental treatment of (−)-larvae

During the laboratory rearing, when they were fed routinely with frozen *Drosophila melanogaster* and pieces of *Calliphora* pupae, experimental (−)-larvae received (+)-eggs of either their own species (Kellner 1999; Kellner 2001a; Kellner 2001b) or of another one. Generally 2 fresh (+)-eggs were fed to first stage (−)-larvae (L1) which are more susceptible to the endosymbionts than L2 (Kellner 2001b). Subsequently, the larvae were reared without access to other individuals. Males were preserved (−25°C) soon after imaginal eclosion while the resulting females were kept singly for two months (*P. melanurus* and *P. riparius*) or 14 days (*P. sabaeus*) at the same temperatures as before where they could biosynthesize pederin. Females of the two European species who died during that period were included in qualitative analysis (percentage of (+)-females) when they had survived for more than one month but they were excluded from quantitative comparisons.

Quantitative analysis

Several eggs of the (+)-females, whose eggs were needed in the experiments, were measured soon after deposition (as shown by their soft white chorion) using an ocular micrometer at a dissecting microscope (Olympus VMT-4): From their length and width the volume could be calculated according to the formula for ellipsoids. Weighing a sample of eggs from various *Paederus* species (Sartorius Supermicro 4504 MP 8) showed that their volumetric weight did not differ significantly from 1, thus enabling the easy conversion of volumes into weights. Length and width of eggs can be measured more accurately than weights because any adherent water on the surfaces, which must be kept moist, does not affect the measurement.

Pederin was extracted from single specimens and quantitatively analyzed as described previously (Kellner & Dettner 1995; Kellner 2001b). Individual pederin content was determined for at least 4 eggs of every (+)-female providing an estimation of her mean pederin transfer. Females containing more pederin than they could have ingested during experimental feeding in the larval stage were regarded (+)-females capable of own biosynthesis.

Cloning and sequencing of the endosymbionts' 16S rDNA

Template DNA was prepared separately from 1-2 females of the species chosen. Freeze-killed females were rinsed with ethanol and

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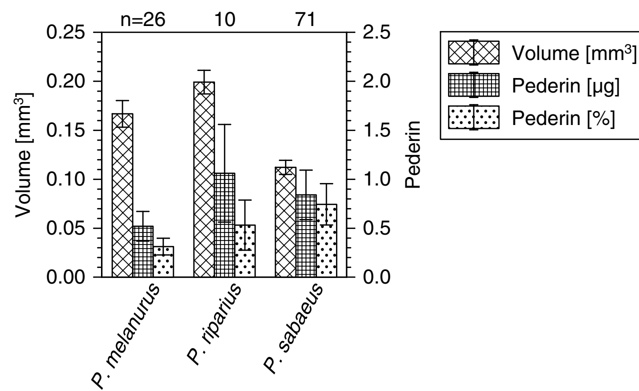

Fig. 1 Egg volume and transferred pederin (amount and percentage of fresh weight) in the (+)-females of three *Paederus* species whose eggs were used for the feeding experiments (means ± S.D.), n = number of females analyzed

extracted with QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR was used to amplify eubacterial 16S rDNA from total DNA with conserved primers 27f (5'-AGAGTTTGATCMTGGCTCAG, M = C:A) and 1492r (5'-TACGGYTACCTTGTTACGACTT, Y = C:T, Lane 1991). In the *Escherichia coli* standard, they correspond to positions 8 to 27 forward and 1513 to 1492 reverse and include > 97% of the 16S rRNA sequence (1542 bases). Individual reactions were performed in 50-µl volumes containing 5 µ1 10 × PCR-Buffer (15 mM $MgCI_2$, Promega, Madison, WI, USA), 120 µM of each dNTP, 50 pmol of each primer (MWG-Biotech, Ebersberg, Germany), about 100 ng of template DNA, and 2.5 U of *Taq* polymerase (Promega). Amplification was carried out with a PTC-200 thermal cycler (MJ Research, Waterton, MA, USA). Reaction conditions consisted of an initial 3-min denaturation step (94°C) followed by 35 cycles of denaturation (94°C, 1 min), annealing (50°C, 1 min), and extension (72°C, 2 min). A final 7-min chain elongation step (72°C) was included at the end of the PCR program (Polz and Cavanaugh 1997). After electrophoresis of 8-10 µl PCR mixture in ethidium-stained 1.2% agarose gels (NuSieve GTG, BioWhittaker Molecular Applications, Rockland, ME, USA), the bands of the appropriate size (1.5 kbp) containing the PCR product were excised, melted and directly cloned into pCR-4 plasmid DNA vector (TOPO TA Cloning Kit for Sequencing, Invitrogen, Groningen, The Netherlands), according to the manufacturer's instructions.

Plasmids were extracted from bacteria using QIAprep Spin Miniprep Kit (Qiagen), according to its specifications. They were digested with *Eco* RI (Promega) to show insert size and additionally with *Apa* I for an initial identification of sequences derived from the endosymbionts according to their characteristic restriction fragment length polymorphism pattern (Kellner 2002). Digestion products were analyzed by electrophoresis on 1.2% agarose gels and visualized with ethidium bromide. Plasmid inserts from at least 3 different transformed bacterial clones were sequenced completely on an ABI automated sequencer by GATC Biotech AG (Konstanz, Germany). Minor differences among the sequences were resolved after alignment resulting in a consensus sequence for the endosymbiont of every host species.

Phylogenetic analysis of endosymbionts' 16S rDNA

In addition to the three *Paederus* species bred in the laboratory and used for the feeding experiments two species were analyzed with regard to their expected endosymbionts for a more profound comparison among host species: *Paederus fuscipes* Curtis collected from northeastern Bavaria, Germany, and *Paederidus ruficollis* (Fabricius) from Languedoc, southern France. From their endosymbionts 16S rDNA sequences (1500 bases) were obtained and have been added to the EMBL database with accession numbers AJ316016-AJ316019 (*Paederus fuscipes, P. melanurus, P. riparius,* and *Paederidus ruficollis*). These new sequences were compared with the known sequence of the *P. sabaeus* endosymbiont (accession number AJ295331). The sequences were aligned with the Clustal W program (version 1.81, Higgins *et al*. 1996) including *Pseudomonas aeruginosa,* the most closely related bacterium (Kellner 2002), as an outgroup. Phylogenetic analysis was performed with Clustal W by excluding nucleotide sites with alignment gap(s) and applying the neighbor-joining method with Kimura's correction for multiple substitutions. The resulting tree was evaluated with the bootstrap method (l000 resamplings).

Results

The eggs of the three *Paederus* species chosen differed in volume ($F_{2,104} = 564.91$, $P < 0.001$) as well as pederin content ($F_{2,104} = 20.08$, $P < 0.001$). While the eggs of *P. riparius* were almost twice as great as *P. sabaeus* eggs and also contained more pederin, the eggs of the latter species achieved the highest concentration of the substance (Fig. 1). On the other hand, *P. melanurus* eggs contained comparatively small amounts of pederin and also had its lowest concentrations. Egg size could be related to the number of viable endosymbionts present as these are probably transmitted via the egg-shell. During the feeding experiments with (−)-larvae of the other two species, the smaller *P. sabaeus* eggs were thus offered in slightly higher numbers to compensate for such a possible effect of size: In most cases *P. riparius* (−)-larvae received 3 (+)-eggs and about half of the *P. melanurus* larvae were also fed with 3 (+)-eggs instead of only 2 in the remaining experiments. This means that they concomitantly could sequester more pederin from the (+)-eggs eaten.

Female (−)-larvae acquired biosynthetic capabilities through ingestion of (+)-eggs especially if conspecific (+)-eggs had been offered to them whereas the (+)-eggs of other species were less effective or did not produce any (+)-female (Fig. 2, *P. melanurus:* $\chi^2 = 21.58$, *P. riparius:* $\chi^2 = 20.81$, P. *sabaeus:* $\chi^2 = 35.15$, all species: $df = 2$, $P < 0.001$). There is an obvious pattern regarding the interspecific transmission of endosymbionts: They could be observed to colonize the other species in a few experiments performed with *P. melanurus* Ll and *P. riparius* (+)-eggs and vice versa. Although higher numbers of (−)-larvae were tested in combinations with *P. sabaeus* (Fig. 2), however, only a small number of (+)-females were obtained: 3 *P. melanurus* (+)-females after ingestion of *P. sabaeus* (+)-eggs and 2 *P. sabaeus* (+)-females after ingestion of *P. riparius* (+)-eggs and none at all in the other combinations.

The amount of pederin accumulated by (+)-females during a certain time-span varied among the three species ($F_{2,141} = 136.74$, $P < 0.001$) with *P. sabaeus* biosynthesizing most of the substance although the females of that species lived for only a quarter of the time of the other two. Remarkably, the order of median pederin content of (+)-females (Fig. 3) corresponds to the pederin concentration of (+)-eggs from the same species (Fig. 1). The (+)-females with heterospecific endosymbionts generally accumulated pederin amounts comparable to the control and not only minor quantities. Because of the small number of such (+)-females, they could not be analyzed statistically. However, their comparison with the medians and

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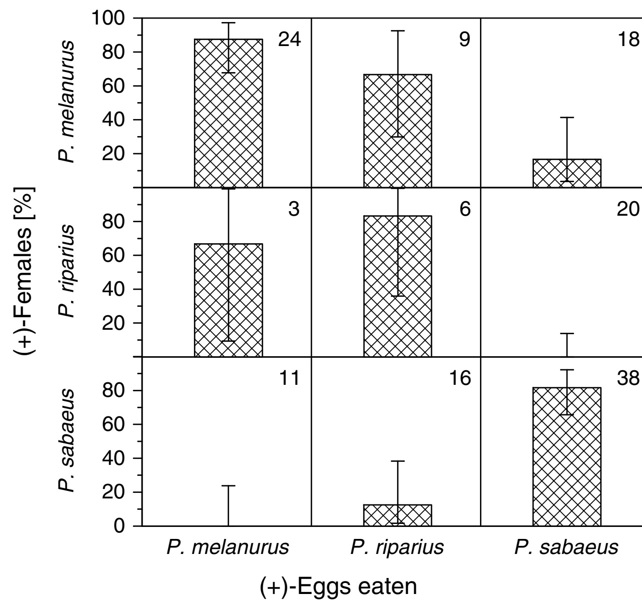

Fig. 2 Percentage of (+)-females (with 95% confidence intervals) in laboratory-reared descendants of (–)-females belonging to three species of *Paederus* that during larval development were fed with (+)-eggs of the same or another species. Numerals in the upper right corners indicate the number of females obtained in the respective experiment. The percentages of (+)-females from intraspecific feeding experiments are included for comparison: *P. melanurus* (+)-females' percentage as in Kellner (2001b), *P. riparius* (Kellner 1999) and *P. sabaeus* (Kellner 2001a) percentages recalculated based on experiments with the same refined methodology

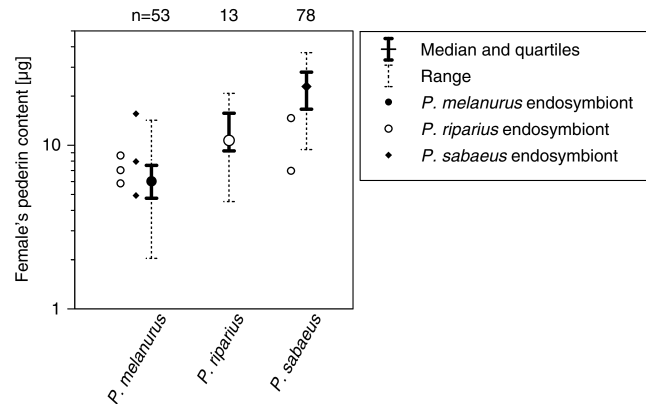

Fig. 3 Pederin content of individual (+)-females with endosymbionts acquired from other *Paederus* species as compared to the statistics (n = number of females analyzed) of the pederin content of (+)-females harboring their own endosymbiont. Within a given species only (+)-females of the same age were considered, but *P. sabaeus* females were younger than both *P. melanurus* and *P. riparius* females

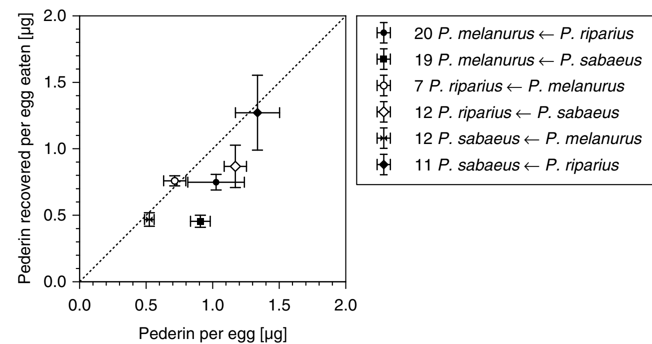

Fig. 4 Pederin recovered from the males of three species of *Paederus* (in front of the legend's arrow) that were descended from (–)-females and fed with (+)-eggs of another species (behind the arrow) during larval development (means ± SEM). The dashed line indicates the pederin amount that could be expected from the respective (+)-females' pederin transfer. The numerals in the legend show the numbers of males analyzed

non-overlapping quartiles of (+)-females with conspecific endosymbionts indicates that the type of endosymbiont influenced pederin accumulation (Fig. 3): *P. melanurus* (+)-females with endosymbionts derived from *P. riparius* or *P. sabaeus* tended to accumulate more pederin than those with their own endosymbiont (control). Half of the specimens had more pederin than three quarters of the control and one (+)-female harboring the *P. sabaeus* endosymbiont even exceeded the range shown by the control (+)-females. On the other hand, the *P. sabaeus* (+)-females with the endosymbiont of *P. riparius* accumulated less pederin than 75% of the other (+)-females and again one of these (+)-females was outside of the control's range.

Recovery of pederin ingested with the (+)-eggs of another species was comparatively high as shown by the males analyzed soon after completion of development (Fig. 4). As in the former experiment with conspecific (+)-eggs (Kellner 2001b), *P. melanurus* males contained clearly less than they could have received but nearly all of the pederin fed to *P. sabaeus* males (both tests) and *P. riparius* males given *P. melanurus* (+)-eggs could be recovered. The differences among the various tests could be related to the fact that (+)-eggs of *P. sabaeus* with their high concentration of pederin were often fed two at once resulting in lower incorporation rates whereas only one (+)-egg of the other two species was presented at a time.

16S rDNA sequences very similar to the sequence that has been found to be associated with pederin biosynthesis in *P. sabaeus* could be isolated from three additional *Paederus* species as well as from *Paederidus ruficollis.* There were only 1-7 nucleotide differences between any two of the endosymbionts derived from different host species resulting in high identities among the complete sequences (Table 2).

Phylogenetic analysis (Fig. 5) shows that the endosymbionts' sequences form a tight cluster well separated from *Pseudomonas aeruginosa,* which is the most closely related bacterium. Within the endosymbionts' cluster there is a well supported grouping of the endosymbionts of *P. melanurus* and *P. riparius,* two closely related species from the same subgenus. The endosymbionts of the other two species from a common subgenus, *P. sabaeus* and *P. fuscipes,* are, however, clearly separated and the endosymbiont of *Paederidus ruficollis* is actually affiliated with that of *P. sabaeus.*

Discussion

The finding that all species analyzed harbored very closely related bacteria which are affiliated to the bacteria associated

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Table 2 Percent identity (lower left) and number of nucleotide differences (upper right) in the 16S rDNA sequences of the endosymbionts of four species of *Paederus* and *Paederidus ruficollis*. 1458 aligned nucleotides (primer sequences used in PCR amplification truncated) were evaluated for the data matrix

Host species	1	2	3	4	5
1 *Paederus melanurus*		1	5	6	4
2 *Paederus riparius*	99.9		6	7	5
3 *Paederus fuscipes*	99.7	99.6		7	5
4 *Paederus sabaeus*	99.6	99.5	99.5		4
5 *Paederidus ruficollis*	99.7	99.7	99.7	99.7	

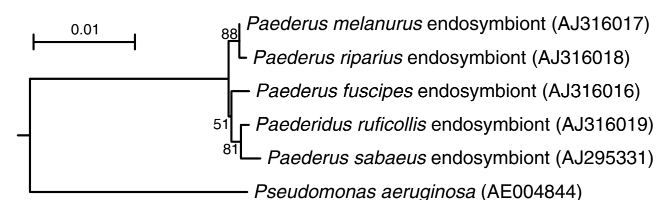

Fig. 5 Phylogenetic tree based on the 16S rDNA sequences of the endosymbionts of four species of *Paederus* and *Paederidus ruficollis* with their nearest relative, *Pseudomonas aeruginosa*, as an outgroup (EMBL accession numbers in parentheses). The neighbor-joining method with Kimura's correction for multiple substitutions was used for construction of the tree. Gaps were excluded from the analysis and there were 69 informative sites. The level of support of the individual nodes is shown according to percent bootstrap values (1000 resamplings). The scale bar represents 0.01 substitutions per nucleotide position

with pederin biosynthesis in *P. sabaeus* females (Kellner 2002) emphasizes the probable role of these hitherto undescribed bacteria in pederin biosynthesis. Representatives of this new type of endosymbiont could be detected in various species of the Paederina chosen from different lineages within that subtribe which were expected to contain the endosymbionts due to their possession of pederin. The consistency between capability of pederin biosynthesis and presence of these bacteria thus is evident from all species tested.

The endosymbiont has also been detected in samples of *P. sabaeus* (+)-eggs (Kellner 2002) and presumably is the agent hypothesized for horizontal transmission of biosynthetic capabilities for pederin. This type of transmission among unrelated specimens has been reported intraspecifically (Kellner 1999; Kellner 2001a; Kellner 2001b) but is now observed also interspecifically. While *P. melanurus* and *P. riparius* belong to the same subgenus *(Paederus* s. str.) and easily acquired the endosymbiont of the other species, *P. sabaeus* is placed in another subgenus *(Heteropaederus)* and transmission frequency of the endosymbionts to and from both other species was much reduced. Colonization of a heterospecific host may thus be facilitated in closely related *Paederus* species. This could be based on the degree of genetic similarity among the endosymbionts, which was analyzed according to their 16S rDNA sequences. *P. melanurus* and *P. riparius* contained endosymbionts that differed only in one nucleotide position whereas the difference to the *P. sabaeus* endosymbiont was much greater indicating divergence that might be responsible for incompatibility.

Alternatively, the observed differences in interspecific transmission of endosymbionts could be explained by the different temperatures used in rearing: 20°C for *P. melanurus* and *P. riparius* and 27°C for *P. sabaeus.* High temperatures are a well-known means of obtaining aposymbiotic insects, whose endosymbionts have been killed, but the temperatures used for that purpose are generally much higher (e.g. 36-39°C, Houk and Griffiths, 1980). Furthermore, (+)-eggs of *P. melanurus,* a species adapted to reproduce under comparatively high temperatures during the italian summer (Focarile 1964), did not produce any (+)-females when given to *P. sabaeus* larvae whereas those of *P. riparius* did, although that species has a more northern distribution. Likewise, a reduction of temperature as in the (+)-eggs of *P. sabaeus* presented to (−)-larvae of the other species cannot be expected to have the same effect as temperature increase in the reciprocal experiments but the outcome was quite similar.

The endosymbionts might thus have developed specialized preferences for their natural host and genetic distance could be used as an indicator of differences that accumulated since isolation. As expected from the subgeneric classification of *P. melanurus* and *P. riparius* their endosymbionts are most similar. Indeed, it has been suggested recently that irrespective of its distinct coloration *P. melanurus* could be regarded a subspecies of *P. riparius* according to its similar aedeagus (Adorno & Zanetti 1999). The separation between the two species might thus be imperfect allowing the continued exchange of the endosymbiont which could prevent them from being confined to one host.

Although characterized by a clearly lower frequency, transmission of endosymbionts between more distantly related *Paederus* species might be important on an evolutionary time scale. In the *Wolbachia* endosymbionts of various arthropods horizontal transmission has been suggested regarding their molecular phylogenies and the experimental transfer could be achieved by microinjection techniques (Boyle *et al*. 1993; Grenier *et al*. 1998). In the case of the *Paederus* endosymbiont, however, ingestion of heterospecific (+)-eggs inoculated with the endosymbiont would suffice for transmission. This could prevail in rare species occurring together with a common one where the larvae of the uncommon species have higher chances of finding eggs of the other one. *P. fuscipes* and *P. riparius* can be found side by side at the same sites in northeastern Bavaria, Germany, but they prefer clearly distinct microhabitats (Kellner, unpubl.). The differentiated endosymbionts of the two species show that interspecific transmission probably does not occur regularly.

Overall, the phylogenetic tree of the endosymbionts does not reflect the presumed phylogeny of their hosts. On the contrary, geographic distribution of the host species seems to be more important. Accordingly, the endosymbiont of *P. sabaeus* shows the greatest differences to the other species' endosymbionts. Whether one or more species of endosymbiont are involved cannot be decided from the 16S rDNA data alone. Sequence identities like that found among the endosymbionts (at least 99.5%) are generally regarded as representing one species (Fox *et al*. 1992) but partial

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incompatibility of the hosts would indicate a clear differentiation of at least certain endosymbiotic lineages. As in the *Wolbachia* endosymbionts (Zhou *et al*. 1998), the slowly evolving 16S rDNA precludes a fine-scale resolution of the endosymbionts' phylogeny by using these sequences. Therefore, other genes with fast evolutionary rate and higher variation like the *wsp* gene in *Wolbachia* (van Meer *et al*. 1999) need to be identified to adequately address this issue. Such an analysis could even show that strains with very similar or the same 16S rDNA sequence might belong to different species (Fox *et al*. 1992).

Acknowledgements

Thanks are due to Professors K. Dettner and K. H. Hoffmann (Bayreuth) for research facilities, and D. Ghiringhelli (Milan) and P. Kocieńzski (Southampton) for samples of authentic pederin. Marc Ehnert, Sandra Kellner, Kerstin Klemm, Markus Klier, Edda Roddewig, and Sonja Trenz kindly helped me to maintain the beetles over the years. The molecular analysis was supported by the Deutsche Forschungsgemeinschaft (KE 744/2-1) and technical assistance by Sonja Trenz is gratefully acknowledged.

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Received 3 September 2001; accepted 6 February 2002.