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Parthenogenesis-inducing microorganisms in *Aphytis* (Hymenoptera: Aphelinidae)

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Abstract

Production of males in uniparental lines of two species in the parasitic wasp genus *Aphytis* was induced by rifampicin, and male sexual functioning was determined. *Wolbachia*-specific 16S rDNA primers were used in a PCR in order to: (1) assess correlation between thelytokous reproduction and the presence of *Wolbachia*; (2) detect the loss of *Wolbachia* DNA in uniparental *A. lingnanensis* following antibiotic treatments, with or without the presence of a host; and (3) clone and sequence part of the *Wolbachia* 16S rDNA from the uniparental *Aphytis* species for phylogenetic studies. Males produced viable sperm that was transferred to the female spermatheca following mating. However, sperm failure to effect egg fertilization resulted in all-male progeny. *Wolbachia* were found in the two uniparental (*A. lingnanensis* and *A. diaspidis*) but not in the two biparental (*A. lingnanensis* and *A. melinus*) *Aphytis* lines tested. They can be detected in wasps up to 7 days following antibiotic treatments, regardless of the presence of host. The 16S rDNA for the symbionts in the two *Aphytis* species is virtually identical, and is most closely related to the *Wolbachia* found in *Muscidifurax uniraptor* (Pteromalidae).

Keywords: antibiotic, endosymbionts, PCR, thelytoky, *Wolbachia*.

Introduction

In parasitic Hymenoptera, severe sex-ratio distortions are sometimes associated with the presence of symbiotic microorganisms. Such symbionts include the son-killer, a bacterium that causes differential mortality of male embryos (Skinner, 1985; Werren *et al.*, 1986) and *Wolbachia*, a rickettsia-like microorganism that induces non-reciprocal cross incompatibility (Richardson *et al.*, 1987; Breeuwer & Werren, 1990). Microorganisms are also involved in thelytoky, the most extreme case of sex-ratio bias. Symbiont-induced uniparental reproduction was first discovered in *Trichogramma* spp. (Trichogrammatidae) (Stouthamer *et al.*, 1990, 1993; Stouthamer & Werren, 1993), and has since been reported from *Muscidifurax uniraptor* (Pteromalidae) (Stouthamer *et al.*, 1993), *Encarsia formosa* (Aphelinidae) (Zchori-Fein *et al.*, 1992; Stouthamer *et al.*, 1994) and *Aphytis lingnanensis* (Aphelinidae) (Zchori-Fein *et al.*, 1994). In each of these species, production of males was successfully induced by antibiotic treatments.

Repeated attempts to culture these symbionts have failed, hampering progress in identification and inference of phylogenetic interrelationships. Recent development of the polymerase chain reaction (PCR) has facilitated the use of different gene sequences for phylogenetic analysis. For various reasons, the DNA sequence encoding the 16S rDNA gene is the most widely used (Woese, 1987). Other genes such as 23S rDNA are also used in phylogenetic studies (Rousset *et al.*, 1992). Using prokaryote-specific 16S rDNA primers, Stouthamer *et al.* (1993) were able to amplify and sequence that gene from six strains (three species) of uniparental *Trichogramma* and one strain of uniparental *Muscidifurax uniraptor*. Phylogenetic analysis of these sequences showed that parthenogenesis microorganisms are most closely related to the *Wolbachia* that induce cytoplasmic incompatibility in parasitic wasps and insects in other orders. Stouthamer *et al.* (1993) also showed that the parthenogenesis/incompatibility group consists of two subgroups that can be distinguished by diagnostic base positions. In 1992, a pair of *Wolbachia*-specific primers capable of amplifying about 1000 bp of the 16S rDNA was designed (O'Neill *et al.*, 1992).

About a quarter of all species of the aphelinid genus *Aphytis* (Hymenoptera: Aphelinidae) whose sexuality is known, exhibit thelytokous parthenogenesis in which unfertilized females produce female offspring. In these species, males are produced regularly, usually at a low rate of 1–5% (Rosen & DeBach, 1979). Electron microscopy, as well as antibiotic treatments, have suggested that parthenogenesis-inducing microorganisms are associated with thelytokous reproduction in one of these species, *Aphytis lingnanensis* (Zchori-Fein *et al.*, 1994). Similar

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antibiotic treatments described below have indicated that thelytokous *A. yanonensis* also carries such symbionts (R.T. Roush, pers. comm.).

In the present study we have investigated the sexual functioning of antibiotic-induced males, and the possibility of establishing a biparental line from the uniparental one. In order to determine the systematic status of the *A. lingnanensis* parthenogenesis-inducing microorganisms, specific primers were used in the amplification of the 16S rDNA, and the amplified products were cloned, sequenced and used for the construction of a phylogenetic tree. *Wolbachia*-specific primers were used as a diagnostic tool to determine whether the presence of *Wolbachia* is correlated with uniparental reproduction in other *Aphytis* species. They were also used for detection of *Wolbachia* in antibiotic-treated uniparental *A. lingnanensis.*

Results and Discussion

Male sexual functioning

Uniparental *A. lingnanensis* females fed with 50 mg/ml rifampicin did not produce a significantly higher proportion of males in the F1 (11%, $n = 99$), compared to the control (7% males, $n = 88$). However, virgin F1 of antibiotic-treated females produced 93% males ($n = 135$), as opposed to 10% males in the control ($n = 96$). In *A. diaspidis*, this trend was even clearer, F1 of antibiotic-fed wasps producing all-female broods, which in turn produced 100% male offspring. Thus, the influence of antibiotic treatments is evident only in the F2. Antibiotic-induced males carry sperm (Fig. 1), and when the spermathecae of females confined with such males were dissected, about 54% (15/28 found) contained viable sperm. However, although the males were capable of inseminating the females, mated females of the F1 of antibiotic treatments produced exclusively male progeny. The functionality of heat-induced males in thelytokous species has been reviewed by Stouthamer *et al.* (1990). Such males were generally considered non-functional, but in several species they were able to produce offspring when mated with their conspecific females (e.g. Legner, 1985). The sexual functioning of antibiotic-induced males ranges from possessing sperm although lacking the ability to transmit it in *Encarsia formosa* (Zchori-Fein *et al.*, 1992) to the successful production of daughters in *Trichogramma* (Stouthamer *et al.*, 1994; Stouthamer & Kazmer, 1994). *A. lingnanensis* was found to be somewhere within that range: the males are capable of transmitting viable sperm, but some reproductive barrier prevents the successful completion of fertilization.

Presence of Wolbachia*-like symbionts in* Aphytis *lines*

Using *Wolbachia*-specific 16S rDNA primers in a polymerase chain reaction, DNA fragments of about 1 kb were detected in two uniparental *Aphytis* lines (*A. lingnanensis* and *A. diaspidis*), but were not recovered from biparental lines (*A. lingnanensis* and *A. melinus*) (Fig. 2). The presence of *Wolbachia*-like symbionts in the uniparental line of *A. lingnanensis* had previously been confirmed by electron microscopy studies and antibiotic treatments (Zchori-Fein *et al.*, 1994). *Wolbachia* was also detected, using specific primers, in the uniparental species *Aphytis yanonensis* (R.T. Roush, pers. comm.). These data show good corre-

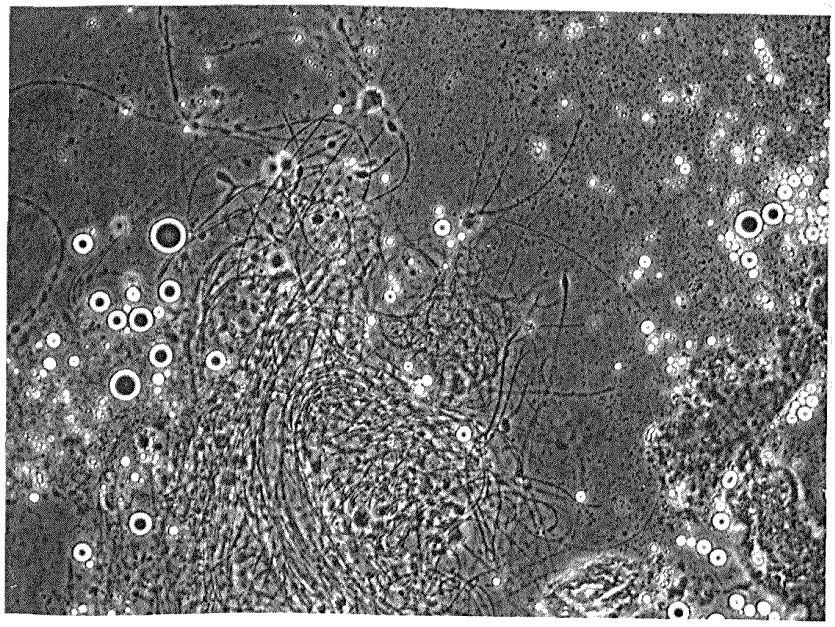

Figure 1. Squash of reproductive tract of an antibiotic-induced *Aphytis lingnanensis* male, showing sperm (×1000).

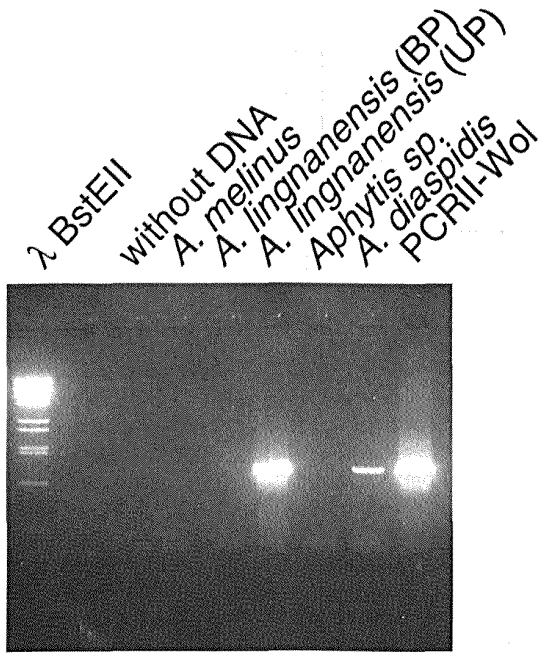

Figure 2. PCR analyses of *Wolbachia* infection in uniparental and biparental *Aphytis* lines, using specific primers. PCR products were electrophoresed on 1% agarose gel and stained with ethidium bromide. (BP = biparental; PCRII-WOL = plasmid containing the *Wolbachia* 16S rDNA; UP = uniparental.) *Aphytis* lines are described in the experimental procedure.

lation between uniparental reproduction and the presence of symbionts in *Aphytis*.

It has been suggested that thelytoky is an advantage in a natural enemy, as production of all-female brood would ensure a higher rate of population increase (Speyer, 1927) and establishment in new habitats. However, it has been shown that, on average, the hatching success of uniparental insect species is only two-thirds that of biparental ones (Lamb & Willey, 1978). Species of *Aphytis* are usually the most abundant, and by far the most effective, natural enemies of armored scale insects (Homoptera: Diaspididae) (Rosen & DeBach, 1979, 1990). Whether the presence of thelytoky-inducing symbionts in these economically important parasitoids is an advantage or a disadvantage is still to be determined.

Table 1. Diagnostic nucleotide positions in the 16S rDNA of subgroups of the parthenogenesis microorganisms. Type 1 are from *Trichogramma* (six strains, three species); type 2 are from *Muscidifurax raptor*, *Aphytis lingnanensis* and *A. diaspidis*.

Position	Type 1	Type 2
189	T	C
587	T	G
589	G	A
593	A	G
614	G	A
645	A	G
646	T	C
649	G	A
650	C	T
682	G	A
683	A	G
693	A	G
760	A	G
842	–	T
989	T	C

Antibiotic treatments

In the absence of hosts for oviposition, *Wolbachia* 16S rDNA was detected in *A. lingnanensis* females even 7 days after antibiotic treatment (Fig. 3). A much weaker PCR product was obtained up to 3 days post-treatment when the wasps were permitted to oviposit. This difference in signals can be explained either by the presence of non-degraded DNA of dead *Wolbachia* in the ovaries, or by the inability of antibiotics to penetrate the mature oocyte. The fact that the F1 offspring of antibiotic-treated females yielded no product in PCR (Fig. 3) supports the former explanation, since

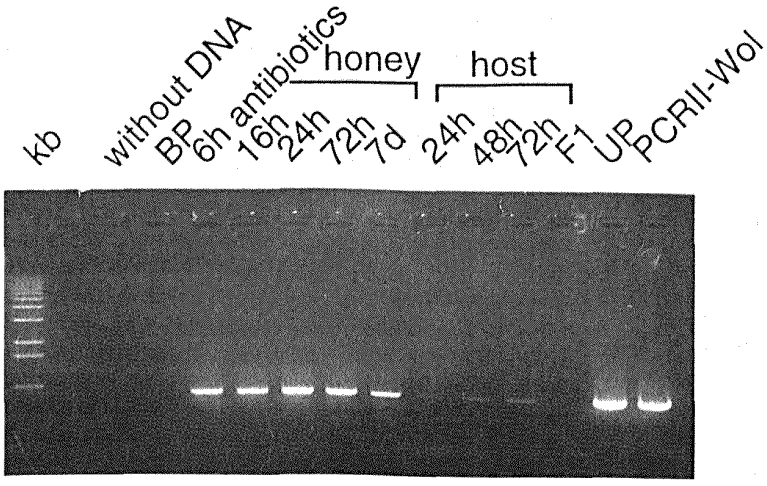

Figure 3. PCR analyses of *Wolbachia*-like 16S rDNA in antibiotic-treated *Aphytis lingnanensis*, using specific primers. Female wasps were fed for 6 h on 50 mg/ml rifampicin, kept either with or without hosts, and collected at different times. (BP = biparental; F1 = the female offspring of antibiotic-treated mothers; PCRII-Wol = plasmid containing the 16S rDNA; UP = uniparental.)

Wolbachia should have been detected in at least some of the F1 females if the antibiotic were incapable of penetrating the oocyte. A more detailed PCR analysis of sequential eggs produced by antibiotic-treated females is necessary in order to resolve this issue. As discussed above, the F1 offspring of antibiotic-treated females were mainly females. These females are rickettsia-free (Zchori-Fein *et al.*, 1994), and in turn produce all-male broods. This suggests that the development of a female from an unfertilized egg does not depend on the actual presence of the *Wolbachia*, but rather that thelytokous reproduction is induced by an unknown factor released by the symbiont.

Sequencing and phylogenetic analysis

For the symbionts of the two uniparental *Aphytis* species studied, the sequences of 16S rDNA obtained from three different transformed bacterial colonies had only minor differences between them, and these differences were not in the positions differentiating between 'type 1' and 'type 2' of the known *Wolbachia*. It was generally possible to determine the actual base unambiguously, with the exception of positions 335 and 467 in *A. lingnanensis*, which were assigned the letter N. The consensus sequence, generated by sequencing plasmids of *A. lingnanensis* and *A. diaspidis*, was 898 and 897 bases long, respectively. The sequences generated from *A. lingnanensis* and *A. diaspidis* (deposited in the GeneBank under access codes X87406 and X87407, respectively), differed only in two positions, and both of them were used in the phylogenetic analysis. The most parsimonious tree constructed by the PAUP procedure shows that parthogenesis microorganisms from *Aphytis* most closely resemble the ones found in *Muscidifurax uniraptor*, and are clearly separate from those sequenced from *Trichogramma* wasps (Fig. 4). The *Wolbachia* from *Aphytis*, therefore, falls into the 'type 2' subgroup of Stouthamer *et al.* (1993), resembling cytoplasmic incompatibility-inducing *Wolbachia* in *Drosophila simulans*, for example, more than symbionts associated with parthenogenetic reproduction in *Trichogramma* (analysis not shown). There was a total of twenty-three variable positions between the different sequences. Of these, fifteen were fixed in all six strains of *Trichogramma* (type 1) and *Aphytis* and *M. uniraptor* (type 2) (Table 1) and show a consistent difference between the two.

A. diaspidis and *A. lingnanensis* are representatives of the *proclia* and *lingnanensis* species-groups of *Aphytis*, respectively, which are not closely related phylogenetically, the former being more primitive than the latter (Rosen & DeBach, 1979). The fact that both species harbour what appear to be almost identical *Wolbachia* symbionts seems to support the hypothesis of horizontal transfer of these symbionts (O'Neill *et al.*, 1992). It is less likely for ancestral symbionts to have remained unchanged through the long evolutionary process that has separated their *Aphytis* hosts.

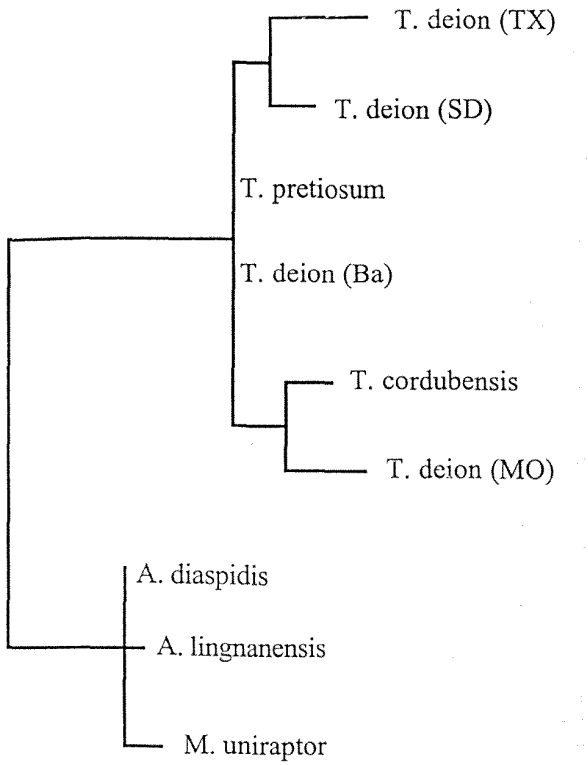

Figure 4. A most parsimonious phylogenetic tree of parthenogenesis microorganisms based on 16S rDNA sequences. The tree was constructed using the branch-and-bound method of PAUP, with *Ehrlichia platys* as an outgroup (100 bootstrap, gap treated as 'fifth base'). A = *Aphytis*; M = *Muscidifurax*; T = *Trichogramma*; abbreviations in parentheses are after Stouthamer *et al.* (1993).

Experimental procedures

Male sexual functioning

In order to induce the production of males in the uniparental line of *A. lingnanensis*, 0–24-h-old females were fed 50 mg/ml rifampicin for 6 h and then pure honey for an additional 18 h. About twenty fed females were caged for oviposition on suitable stages of the oleander scale, *Aspidiotus nerii*. After 13 days, pupae were collected individually in gelatin capsules, and their sex was determined upon emergence. Female progeny were again caged with hosts, and their offspring were collected and sexed in the same way. This procedure was also performed with *A. diaspidis*, using the cactus scale, *Diaspis echinocacti*, as host. Honey-fed wasps were used as a control. Sexual functioning of antibiotic-induced males was tested on three levels: sperm production, sperm transfer, and viable female offspring. (1) Sperm production. The reproductive tracts of ten males were dissected and squashed in saline (9% NaCl), and examined under a stereoscopic microscope. (2) Sperm transfer. The presence of sperm in the spermathecae was observed in females confined in a glass tube with males for 24 h and provided with honey. This was repeated four times with an average of twelve females and eight males each time. The females

were then dissected in a drop of saline on a microscope slide. The spermatheca was carefully removed, covered with a coverslip and immediately examined under a compound microscope for the presence of active sperm. (3) Viable female offspring. Pupae of antibiotic-treated F1 were collected individually in gelatin capsules and sexed upon emergence. One male and one female were confined in a capsule and were carefully watched for 30 min. Pairs that were seen copulating were left for 2 more hours, and then were caged with suitable hosts. Their offspring were collected as pupae, and their sex was determined upon emergence. This procedure was repeated with twenty pairs.

Aphytis *lines*

Total DNA used for the detection of *Wolbachia* was extracted from the following lines:

A. lingnanensis (uniparental): originally collected in 1987 from *Aonidiella* sp. in the Philippines, and subsequently maintained in the laboratory in Israel on *Aspidiotus nerii*.

A. lingnanensis (biparental): a parasite of the California red scale, *Aonidiella aurantii*, imported to Riverside, California, from China in 1947, and from there to Israel in 1988, where it has been subsequently maintained in the laboratory on *A. nerii*.

A. melinus (biparental): a pesticide-resistant strain (Spollen & Hoy, 1992), imported from California in 1992. In Israel it has been maintained in the laboratory on *A. nerii*. Only biparental lines are known in this species.

A. diaspidis (uniparental): collected in 1992 in Rehovot, Israel, from the cactus scale, *Diaspis echinocacti*, and reared in the laboratory on that host. Both uniparental and biparental lines are known (Rosen & DeBach, 1979; Rössler & DeBach, 1973).

Aphytis sp. (suspected uniparental): collected in 1993 in the Negev desert, Israel, from *Duplachionaspis* sp. About thirty individuals emerged, all females, and these were used for the survey.

Antibiotic treatments

The influence of antibiotics on the presence of *Wolbachia* was tested by allowing 0–24-h-old females from the uniparental line of *A. lingnanensis* to feed on 50 mg/ml rifampicin (Sigma) in honey. After 6 h, the antibiotic was removed and the wasps were either provided with honey or placed without honey on suitable hosts and allowed to oviposit. Wasps were collected at different times after the antibiotic treatment and frozen. Wasps that had fed on pure honey for 6 h served as a control, and F1 female offspring of antibiotic-treated females were also included in the analysis. Wasp DNA from each treatment was extracted and processed as described below.

Nucleic acid extraction

About ten adult female wasps were frozen and then suspended in a 100 μl solution of 100 μg/ml proteinase-K and 0.4% SDS. The suspension was homogenized, incubated for 1 h at 55°C, and the DNA was extracted with phenol chloroform isomyl alcohol (25:24:1, phenol saturated with tris buffer at pH 8). Total nucleic acids were concentrated and cleaned by the GeneClean kit (Bio 101, La Jolla, Calif.).

DNA amplification

Bacterial 16S rDNA was amplified using PCR in a volume of 50 μl (1 μl of 100 ng DNA sample, 5 μl buffer × 10, 5 μl of 250 μM nucleotide mix, 1 μl of 200 μM primer I, 1.6 μl of 200 μM primer II, 2 u Taq polymerase (Invitrogen), and ddH_2O added to a final volume of 50 μl). Specific primers for *Wolbachia pipientis* were designed according to O'Neill *et al.* (1992), with minor modifications (primer I: T instead of C in position 8, primer II: C instead of T in position 17), based on the sequence published by Breeuwer *et al.* (1992) from *Nasonia*. Primer I: 5′-TTGTAGCTTGCTATGGTATAACT, which is in the variable V1 region and corresponds to *Escherichia coli* positions 76–99 forward, and primer II: 5′-GAATAGGTATGATTTCCATGT, which is the reverse complement of the variable V6 region and corresponds to *Escherichia coli* positions 1012–994 reverse. PCR cycling conditions were one cycle (10 min at 96°C, 10 s at 55°C, 55 s at 72°C), 30 cycles (45 s at 95°C, 50 s at 60°C, 55 s at 72°C), and one cycle (7 min at 72°C) (Techne PHC-2). The enzyme was added during the 10 s at 55°C of the first cycle. After PCR, 5 μl of amplified product were run on an ethidium-stained 1% agarose gel. The remainder of the PCR product of the uniparental *A. lingnanensis* was used for cloning. The cloned PCR fragment was used as a positive control in the screenings of *Aphytis* and in the antibiotic treatments.

Cloning and sequencing

PCR products from *A. diaspidis* and the uniparental *A. lingnanensis* were directly cloned into a TA cloning vector kit (Invitrogen), according to the manufacturer's directions. Plasmids were extracted from bacteria using the Wizard minipreps DNA purification systems kit (Promega), according to their specifications. In order to verify the presence of an insert, the plasmids were digested using Eco-RI and the products were run on 1% agarose gel. Plasmids from three different colonies that were found to contain the expected insert of about 1000 bp were sequenced by the 373A automatic sequencer (Applied Biosystems), using SP6, T7 and internal primers. The plasmids containing the 16S rDNA were designated PCRII-Wol.

Construction of a phylogenetic tree

The sequences were introduced into the GCG and aligned against other 16S rDNA of parthenogenesis microorganisms. The numbering of nucleotide positions is based on the sequence published for *Escherichia coli* (Brosius *et al.*, 1978). In order to determine the systematic position of the endosymbionts of *Aphytis* in comparison with other parthenogenesis-inducing microorganisms, evolutionary tree analyses were performed. The tree was constructed by the branch-and-bound method of the PAUP computer program (phylogenetic analysis using parsimony) 3.1 for Macintosh, with *Ehrlichia platys* as an outgroup (100 bootstrap, consensus tree constructed from all most parsimonious trees by the strict method, gaps treated as a 'fifth base') (Swofford, 1990).

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