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# Endosymbiont Phylogenesis in the Dryophthoridae Weevils: Evidence for Bacterial Replacement

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Intracellular symbiosis is widespread in the insect world where it plays an important role in evolution and adaptation. The weevil family Dryophthoridae (Curculionoidea) is of particular interest in intracellular symbiosis evolution with regard to the great economical and ecological features of these invasive insects, and the potential for comparative studies across a wide range of host plants and environments. Here, we have analyzed the intracellular symbiotic bacteria of 19 Dryophthoridae species collected worldwide, representing a wide range of plant species and tissues. All except one (*Sitophilus linearis*) harbor symbiotic bacteria within specialized cells (the bacteriocytes) assembled as an organ, the bacteriome. Phylogenetic analysis of the 16S rDNA gene sequence of the Dryophthoridae endosymbionts revealed three endosymbiotic clades belonging to  $\gamma$ 3-Proteobacteria and characterized by different GC contents and evolutionary rate. The genus name *Candidatus Nardonella* was proposed for the ancestral clade infesting Dryophthoridae 100 MYA and represented by five of nine bacterial genera studied. For this clade showing low GC content (40.5% GC) and high evolutionary rate (0.128 substitutions/site per 100 Myr), a single infection and subsequent cospeciation of the host and the endosymbionts was observed. In the two other insect lineage endosymbionts, with relatively high GC content (53.4% and 53.8% GC), competition with ancestral pathogenic bacteria might have occurred, leading to endosymbiont replacement in present-day last insects.

## Introduction

Symbiotic associations are widespread through the biotic world and occur at different levels of organismal complexity, ranging from bacterial associations (von Dohlen et al. 2001) to animal and plant symbioses (Nardon and Grenier 1991; Margulis 1993). The formed unit involves at least two separate species and may include several organisms belonging to distant phyla. These interspecific associations are believed to improve the partner's *fitness* through integrated endosymbiosis (Margulis 1993). Endosymbionts may also induce reproductive isolation and subsequent host speciation, such as in the *Wolbachia* endosymbioses (Werren 1997; Bordenstein, O'Hara, and Werren 2001). However, forces driving the establishment and evolution of symbioses are still poorly understood, and they probably vary from group to group. The early steps in symbiogenesis are often believed to be parasitic (environmental bacteria that possess infection capacities), during which time, the partners exchange virulence gene products, leading to selection at the population level (Yamamura 1996; Heddi 2003).

In insects subsisting on nutrient-deficient or unbalanced diets, such as blood (*Rhodnius*, *Glossina*), plant sap (aphids, psyllids, whiteflies, mealybugs), and cereal grains (*Sitophilus*), symbiosis involves intracellular bacteria (endosymbionts) living within specialized host cells termed *bacteriocytes*, which sometimes form an organ, the *bacteriome* (Buchner 1965). Intracellular bacteria, through their interaction with the bacteriocytes, are generally thought to supplement their host's diet with limiting nutrients (Wicker 1983; Douglas 1998; Heddi et al. 1999). Nevertheless, intracellular symbionts are not limited to

insects with unbalanced diets, but could also be involved in insect temperature resistance (Montllor, Maxmen, and Purcell 2002), host-plant detoxification (Dowd 1991), and parasite protection (Olivier et al. 2003).

Apparent symbiont-host coevolution is known or suspected in several systems where symbionts (living within bacteriocytes) are always transmitted vertically through female germ cells to the offspring (Baumann et al. 1995). Indeed, most of the insect associations studied so far involve monophyletic lineages of intracellular bacteria, whose phylogenesis follows host divergence (Munson et al. 1991; Bandi et al. 1995; Schröder et al. 1996; Chen, Li, and Aksoy 1999). Host-symbiont phylogeny concordance strongly implies a single bacterial infection of the host ancestor and subsequent cospeciation between the bacteria and the insects. Endosymbiont genome evolution is submitted to intracellular life-style constraints (i.e., maternal mode of transmission with population bottlenecks, bacteria insulation within bacteriocytes, and relaxed selection) and is characterized by evolutionary rates significantly higher and genomic GC content generally low when compared to closely related free-living bacteria (Brynnel et al. 1998; Heddi et al. 1998). This concordance is due to elevated mutation rate and biases, lack of recombination, and the fixation of deleterious mutations by genetic drift (Moran 1996) (for review see Wernegreen [2002]). Among-site rate variation and base composition heterogeneity challenge the reconstruction of phylogenetic trees, particularly when organisms are highly AT-biased and have evolved at relatively high rates (Loomis and Smith 1990; Hasegawa et al. 1993). Hence, the long AT-rich branches of insect endosymbionts have always been difficult to place in the phylogenetic tree with free-living relatives (Charles, Heddi, and Rahbé 2001).

The weevil family Dryophthoridae, which comprises about 140 genera, is of a particular interest with regard to intracellular symbiosis evolution. Most Dryophthoridae

Key words: intracellular symbiosis, insect, molecular phylogeny, GC content, evolutionary rate, evolution.

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**Table 1**  
**Symbiotic Status of Dryophthoridae Weevils**

Species	Origin DNA Template PCR	Plant Host (organ and tissue)	Sample Origin	First Endosymbiont Description
<i>Sitophilus granarius</i>	LB, OB	<i>Poaceae</i> (seed)	France	(Mansour 1930) <sup>c</sup>
<i>Sitophilus oryzae</i>	LB, OB	<i>Poaceae</i> (seed)	France	(Pierantoni 1927)
<i>Sitophilus zeamais</i>	LB, OB	<i>Poaceae</i> (seed)	France	(Musgrave and Homan 1962)
<i>Sitophilus vateriae</i>	MCB <sup>a</sup>	<i>Dipterocarpaceae</i> (seed)	India	(Nardon et al. 2002)
<i>Sitophilus linearis</i>	Asymbiotic	<i>Fabaceae</i> (seed)	Senegal	(Nardon et al. 2002)
<i>Sitophilus rugicollis</i>	LB, OB	<i>Myrtaceae</i> (seed)	India	This work <sup>c</sup>
<i>Yuccaborus frontalis</i>	LB	<i>Agavaceae</i> (stem)	Mexico	This work <sup>c</sup>
<i>Sphenophorus abbreviata</i>	OB	<i>Cyperaceae</i> (root)	France	This work <sup>c</sup>
<i>Cosmopolites sordidus</i>	LB, OB	<i>Musaceae</i> (root, stem)	Guadeloupe	(Nardon et al. 1985) <sup>c</sup>
<i>Trigonotarsus rugosus</i>	LB	<i>Xanthorrhoeaceae</i> (trunk)	Australia	(Nardon et al. 2002) <sup>c</sup>
<i>Rhynchophorus ferrugineus</i>	LB	<i>Arecaceae</i> (stem)	Cathar	(Buchner 1965)
<i>Rhynchophorus palmarum</i>	LB, OB	<i>Arecaceae</i> (stem)	Guadeloupe	(Nardon et al. 1985) <sup>c</sup>
<i>Metamasius hemipterus</i>	LB, OB	Polymorphic (stem)	Guadeloupe	(Nardon et al. 1985) <sup>c</sup>
<i>Metamasius callizona</i>	LB, OB	<i>Bromeliaceae</i> (stem)	USA	This work <sup>c</sup>
<i>Diocalandra frumenti</i>	LB	<i>Arecaceae</i> (stem)	Australia	(Nardon et al. 2002) <sup>c</sup>
<i>Sipalinus gigas</i>	LB <sup>b</sup>	Polymorphic (dead wood)	Australia	(Nardon et al. 2002)
<i>Scyphophorus yuccae</i>	OB	<i>Liliaceae</i> (stem)	USA	This work <sup>c</sup>
<i>Dynamis borassi</i>	LB <sup>b</sup>	<i>Arecaceae</i> (stem)	Brazil	(Nardon et al. 2002)

LB: larval bacteriome; OB: ovarian bacteriome; MCB: mesenteric caeca bacteriome.

<sup>a</sup> In *Sitophilus vateriae*, a bacteriome-like tissue was observed at the apex of the mesenteric caeca, but no positive PCR have been obtained on the tissue yet (work in progress).

<sup>b</sup> Bacteriome-tissue was unambiguously observed on larvae, but no positive PCR amplification was obtained probably because of DNA template damage.

<sup>c</sup> 16S rDNA sequencing in this work.

are restricted to feeding on monocotyledonous angiosperms and feed on leaves, stipes, and roots, while known exceptions include *Sipalinus* and *Trigonotarsus*, which feed on decaying wood, and *Sitophilus*, which feed on seeds, including some dicots (Delobel and Grenier 1993). To date, few Dryophthoridae species in four genera have documented endosymbiotic bacteria. These include *Sitophilus oryzae* (L.) (Pierantoni 1927; Nardon and Grenier 1988), *Sitophilus granarius* (L.) (Mansour 1930), *Sitophilus zeamais* (Mot.) (Musgrave and Homan 1962), *Cosmopolites sordidus* (Germar), *Metamasius hemipterus* (L.) (Nardon et al. 1985), and *Rhynchophorus ferrugineus* (Buchner 1965). In all these species, intracellular bacteria were shown to occur in the bacteriome organ and in the oocytes (Nardon et al. 2002).

The present work greatly broadens the known incidence of endosymbiosis occurrence to some 13 genera within the Dryophthoridae through concerted molecular and histological surveys of species collected worldwide, and it provides initial evidence of multiple origins and replacement of their endosymbionts.

## Material and Methods

### Insect Collections

Experiments were conducted with species reared in the laboratory (i.e., *S. oryzae*, *S. zeamais*, *S. granarius*) and with species collected from the field worldwide (table 1). Collected species were sent either alive or immersed in 70% alcohol or fixative fluid BMN (20 ml of 1.5% picric acid solution in 80% ethanol, 5 ml of 40% neutral formalin solution, 2 ml of glacial acetic acid, and 23 ml H<sub>2</sub>O).

### Total DNA Extraction

For living insects, ovaries or bacteriomes were dissected in buffer A (25 mM KCl, 10 mM MgCl<sub>2</sub>, 250 mM sucrose, 35 mM Tris-HCl, pH 7.5) and DNA extractions were performed on the symbiont-bearing organs. For fixed insects, bacteriome tissues were manually collected from toluidine blue-stained sections with a thin needle under the microscope. DNA was then extracted using the Nucleon HT Kit (Amersham, Life Science).

### 16S rDNA Polymerase Chain Reaction (PCR) Amplification and Sequencing

The primers used for 16S rDNA amplification were the eubacterial universal primers: 008for 5'-AGAGTTT-GATCMTGGCTCAG-3' (nucleotides 8–27, *Escherichia coli* numbering GenBank accession no. J01859) and 1389rev 5'-GACGGGCGGTGTGTACAA-3' (nucleotides 1,389–1,406). Reaction mixtures consisted of 2.6 units of Taq DNA polymerase and proofreading polymerase (Expand Long Template PCR System, Roche), 1.75 mM MgCl<sub>2</sub>, 0.35 mM deoxynucleotide triphosphate, 0.3 μM primers, and 10 ng of DNA template in a final volume of 50 μl. The PCR parameters (Biometra T-gradient, Biolabo) were 94°C for 2 min followed by three series of cycles: (1) four cycles of 94°C for 10 s, 51°C to 54°C (0.5°C increment each cycle) 30 s, and 68°C for 30 s; (2) six cycles of 94°C for 10 s, 54°C for 30 s, and 68°C for 30 s; (3) 20 cycles of 94°C for 10 s, 54°C for 30 s, and 20 s more each cycle, and 68°C for 30 s. 16S rDNA sequencing was performed on PCR products by Genome Express (Grenoble, France) according to the Applied Biosystems protocol on an automatic sequencer.

Ten new sequences of endosymbionts were obtained from 10 Dryophthoridae species: *Diocalandra frumenti*,

*Trigonotarsus rugosus*, *Rhynchophorus palmarum*, *Cosmopolites sordidus*, *Metamasius hemipterus*, *M. callizona*, *Sphenophorus abbreviata*, *Scyphophorus yuccae*, *Sitophilus rugicollis*, and *S. granarius*. Sequences were deposited in the GenBank database and their accession numbers are listed later in figure 2.

#### Fluorescence In Situ Hybridization (FISH) Procedure

Bacteriomes were dissected in buffer A (25 mM KCl, 10 mM MgCl<sub>2</sub>, 250 mM sucrose, 35 mM Tris-HCl, pH 7.5). These tissues were homogenized in a potter and fixed in 4% paraformaldehyde solution in PBS for 30 min at 20°C. After elimination of the fixative solution, the tissues were washed by centrifugation in PBS solution (7,000 × g for 5 min). Specific oligonucleotide probes were designed by sequence alignment of 16S rDNA Dryophthoridae endosymbionts. Two probes, 5' end labeled with rhodamine were used: SOPE probe (S) 5'-TACCCCCCTCTACGAGACTC-3' and *Nardonella* probe (N) 5'-ATCTAATGGCATAAGGTT-3'. Hybridization was performed at 45°C, for 4 h in a dark chamber in solution H (0.9 M NaCl, 20 mM Tris-HCl, 5 mM EDTA, 0.1% SDS, 10× Denhardt's solution) to which was added 50 ng of probe (S or N). Tissues were washed twice in the same buffer without probes at 48°C for 2 min, suspended, and mounted in Vectashield medium (Vector Laboratories) containing 4',6-diamidino-2-phenylindole (DAPI).

#### Phylogenetic Analyses

The alignments were performed using ClustalW followed by manual refinement (see *Supplementary Material*). Gaps and undetermined nucleotide sites were removed from the data sets for all analyses. Regions where the alignments were ambiguous were also excluded, resulting in a data set of 1,094 nucleotide sites. Because initial similarity analyses of the 16S rDNA sequences performed with additional sequences from free-living bacteria, phytopathogenic bacteria, and bacteria associated with insect digestive tracts (accession numbers are listed later in fig. 2) indicated that all Dryophthoridae endosymbionts belong to the  $\gamma$ -Proteobacteria group, these sequences were used in subsequent analyses.

Phylogenetic trees were constructed using the heterogeneous model of DNA sequence evolution developed by Galtier and Gouy (Galtier and Gouy 1998; Galtier, Tourasse, and Gouy 1999), allowing for both variations of substitution rates among sites and unequal base compositions. They were then implemented in the nhml software (URL: [ftp://pbil.univ-lyon1.fr/pub/mol\\_phylogeny/nhml](ftp://pbil.univ-lyon1.fr/pub/mol_phylogeny/nhml)). The most likely phylogenetic tree was obtained according to the "pruning-regrafting" method (Felsenstein 1993) by using the shake procedure of nhml. The eval procedure computes the log likelihood that a given tree will correspond to the sequence data. In addition, it computes the value of the evolutionary bias for the substitutions between GC and AT bases for each tree branch. Such biases quantify the tendency of an evolutionary lineage to change the GC content of the sequence, allowing for the estimation of (1) the equilibrium GC content of each

sequence (i.e., the GC content that a given sequence would have if it were to evolve indefinitely with the evolutionary bias of its terminal node) and (2) the GC contents of ancestral sequences (i.e., sequences represented by internal tree nodes). Such estimations and their accuracy are detailed in Galtier and Gouy (1998).

As calculation time greatly increased with sample size (10 to 20 days to estimate a tree with 20 sequences of 1,000 bp), methods of tree topology comparisons (Kishino and Hasegawa 1989) as well as bootstrapping cannot be employed with this model. A local exploration method was therefore developed to test specific phylogenetic hypotheses. This method (briefly described in the *Results*) is based on an a priori fixed topology where endosymbionts were successively added. The most likely topology is retained according to the log-likelihood score obtained.

#### Phylogenetic Topology Test

The phylogenetic topology test was performed to compare different topologies containing the same sequences. The alignment of 16S rDNA is bootstrapped (500 replicates) with Seqboot software. The procedure eval computes the log likelihood of the different topologies, to be compared, corresponding to the bootstrapping sequence data obtained before. A *t*-test was used on log likelihood scores obtained (500 values for each topology tested).

#### Relative Rate Tests

The rates of substitution of the 16S rDNA gene were compared between the Dryophthoridae clades and several representatives of free-living bacteria and other insect endosymbionts, by using the Kimura's 2-parameter distance and covariance estimations of Li and Bousquet (1992). Tests were performed using the RRTree software developed by Robinson-Rechavik and Huchon (Robinson and Huchon 2000). In each case, the *Aeromonas haywardensis* sequence (AF015258) was taken as the out-group. The null hypothesis is that the rate of substitution of the tested clade is the same as that of the reference group ( $K_{1-0} - K_{2-0} = 0$ ).

## Results

### Endosymbiosis Occurrence in the Dryophthoridae Family

Among the 13 species analyzed histologically, 12 were shown to bear intracellular bacteria in bacteriome structures: *Diocalandra frumenti*, *Trigonotarsus rugosus*, *Yuccaborus frontalis*, *Rhynchophorus palmarum*, *Sitophilus oryzae*, *S. zeamais*, *S. rugicollis*, *S. granarius*, *Sphenophorus abbreviata*, *Metamasius callizona*, *Scyphophorus yuccae*, and *Dynamis borassi*. For *S. linearis*, no bacteriome structures were seen. For *S. vateriae* and *Dynamis borassi*, symbiotic status needs to be confirmed; a bacteriome-like structure was observed in one individual only, but no 16S sequence have been obtained successfully (table 1).

Endosymbiotic bacteria appear pleiomorphic in all samples examined. They are rod-shaped, U-shaped, or

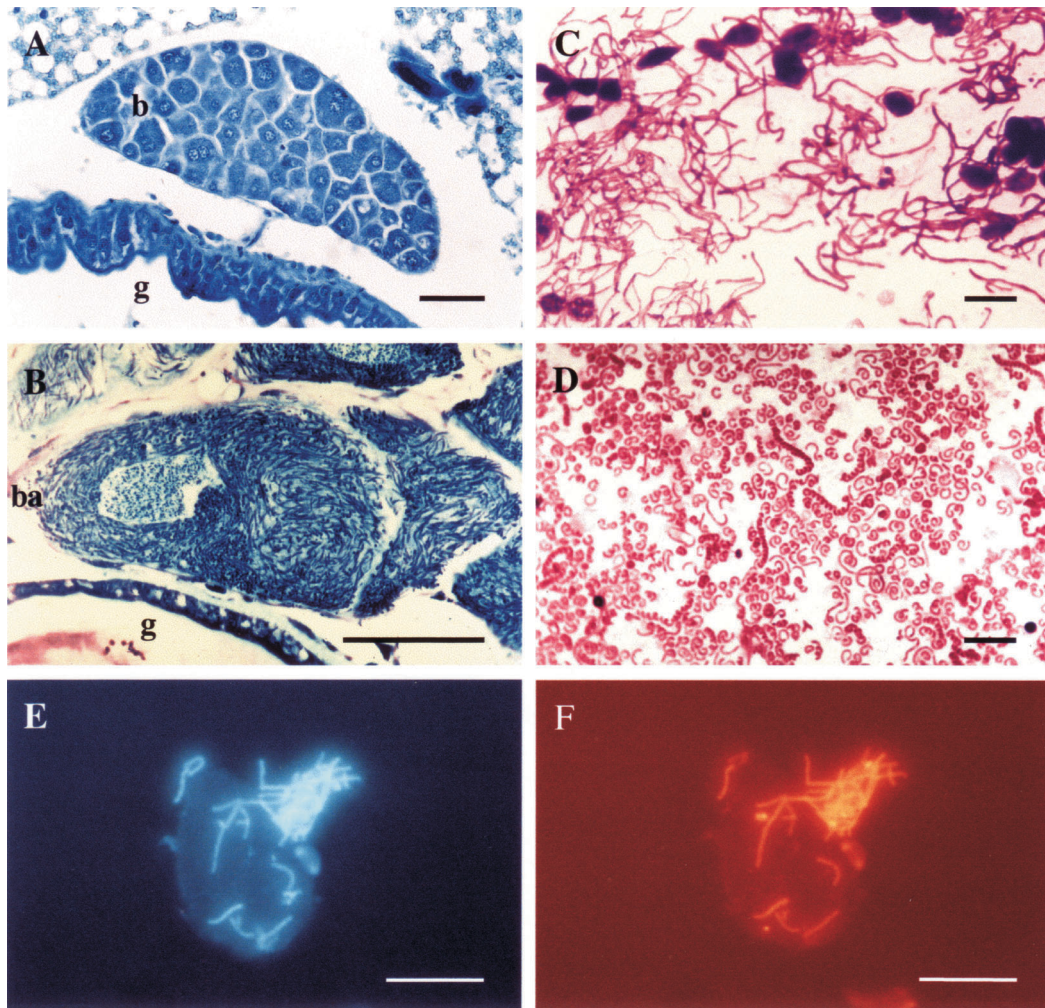


FIG. 1.—Histology of *Dryophthoridae* symbiosis. *A*. Larval bacteriome of *Trigonotarsus rugosus*. *B*. Endosymbiont within the cytoplasm of a bacteriocyte in *Metamasius hemipterus*. *C*. Spiral-shaped endosymbionts in *Sitophilus zeamais*. *D*. Long-shaped endosymbionts in *Sitophilus rugicollis*. FISH of *Metamasius hemipterus* endosymbiont: *E*. Control with DAPI fluorescence, *F*. Specific hybridization of the *Nardonella* probe (N). b: bacteriome; ba: bacteriocyte; g: gut. Scale bar for *A*, *B*, *E*, and *F*, 50  $\mu\text{m}$ ; for *C* and *D*, 10  $\mu\text{m}$ .

spiral-shaped, and their size vary from 2 to 200  $\mu\text{m}$  (fig. 1). Moreover, all symbiotic species are closely associated with bacteriocytes that form bacteriomes of similar shape and location within the larvae and at the apex of the adult ovaries. The larval bacteriome is always located at the junction foregut/midgut in intimate contact with but without any communication with the intestine (Nardon et al. 2002).

To confirm that the 16S rDNA sequences generated by PCR from insect bacteriome are derived from the endosymbionts we observed, we used the FISH technique. Specific probes were designed for the R-clade (probe N) and the S-clade (Heddi et al. 1999). However, no specific probe with enough sensitivity has been obtained for the D-clade. The probe N designed from sequence alignments of R-clade endosymbionts were shown to be highly specific to bacteria contained in the bacteriome of *M. hemipterus* (fig. 1), whereas no signal could be obtained when the probe was applied to the bacteriome of *S. oryzae*. Conversely, the probe S was found to be highly specific to the S-clade endosymbionts, and no signal could be

obtained when it was applied to the bacteriome of *M. hemipterus* (Heddi et al. 1999 and data not shown).

#### Phylogenetic Analyses

##### *Dryophthoridae* Endosymbiont Analysis

A first phylogenetic analysis was performed on the data set comprising the 13 *Dryophthoridae* endosymbiont 16S rDNA sequences. Three clades of endosymbionts were unambiguously separated regardless the phylogenetic method employed (distance, maximum likelihood, maximum parsimony) (data not shown). The D-clade includes endosymbionts of *Diocalandra frumenti* and *Trigonotarsus rugosus*; the S-clade comprises endosymbionts of *Sitophilus rugicollis*, *S. granarius*, *S. zeamais*, and *S. oryzae*; and the R-clade is represented by the endosymbionts of *Yuccaborus frontalis*, *Rhynchophorus palmarum*, *Cosmopolites sordidus*, *Sphenophorus abbreviata*, *Scyphophorus yuccae*, *Metamasius hemipterus*, and *Metamasius callizona*. These bacterial clades exhibit 53.4%, 53.8%, and 40.5% GC content, respectively, in their 16S

rDNA gene sequence. Sequences of the R-clade were highly AT biased and show many AT-rich insertions in positions 200 and 800, as described previously by Lambert and Moran (1998) in *Buchnera* endosymbionts (data not shown).

#### *Free-Living Bacteria Reference Topology and Local Exploration Method*

Phylogenetic analyses using standard methods (i.e., parsimony, distance, and maximum likelihood) were problematic, apparently because of extreme AT-richness and long branch attraction (Charles, Heddi, and Rahbé 2001). Therefore, a heterogeneous model of DNA evolution was used (Galtier and Gouy 1998; Galtier, Tourasse, and Gouy 1999). To reduce the space of the tree topology explored, a free-living bacteria topology was fixed a priori. The Enterobacteriaceae topology, published by Ahmad, Weisburg, and Jensen (1990) and based on fusion of genes implicated in aromatic amino acid biosynthesis, was taken as a reference. Three enteroclusters were fixed; enterocluster E1 includes the genera *Citrobacter*, *Klebsiella*, *Enterobacter*, *Escherichia*, and *Salmonella*; E2 includes the genera *Serratia* and *Erwinia*; and E3 comprises the genera *Kluyvera*, *Edwardsiella*, *Yersinia*, *Hafnia*, *Providencia*, and *Proteus*. The internal structures of the three enteroclusters were refined by “pruning regrafting” (Felsenstein 1993). To test the phylogenetic hypotheses concerning endosymbiont origin, a given endosymbiont sequence (or group of sequences) was fixed on each of branch of the reference topology; then for each new topology, including the new sequence, the corresponding log likelihood was estimated. The maximum log likelihood position was retained. Sensitivity tests entailed replacement of most of the pathogenic bacteria by other commensal free-living bacteria sequences, but no significantly different topologies were observed.

#### *Are the Dryophthoridae Endosymbionts Monophyletic or Polyphyletic?*

To test the hypothesis of endosymbiont monophyly, the three permutations of the three endosymbiont clades (i.e., [D, S],R; [D, R],S; and [S, R], D) were considered, and likelihoods were calculated for their placements on each of the 32 positions on the reference tree. A maximal log likelihood value of  $-7,389$  was obtained for the (D, R), S grouping, placed at position 8 (branch numbers are reported in fig. 2). The (S, R), D grouping obtained a log likelihood value of  $-7,391$  at the same position.

To test the hypothesis of polyphyly, the three clades were independently moved on the reference topology tree. The D-clade was unambiguously positioned on internode 5 (on *Haemophilus/Pasteurella*; fig. 2). It is noteworthy that the R-clade and the S-clade were always sister groups. These two clades were located on internode 8, with a maximal log likelihood value of  $-7,342$ . The ratio of log likelihood scores obtained ( $-7,342$  versus  $-7,389$ ) and the significant difference observed between these two topologies (phylogenetic topology test, monophyly versus

polyphyly:  $P < 10^{-4}$ ) supports the hypothesis of polyphyletic endosymbiont origin.

#### *Relative Position of Insect Endosymbionts*

Secondary endosymbionts of *Glossina pallidipes* (*Sodalis*), and endosymbionts of *Candidatus camponotii* and *Schizaphis graminum* (*Buchnera*) were added to the phylogenetic analysis on the tree topology including the three Dryophthoridae endosymbiont clades fixed as determined above. Then, local “pruning regrafting” procedures were performed to obtain the final topology (fig. 2).

#### *Relative and Absolute Rates of Evolution*

16S rDNA gene sequences of the R-clade exhibit much higher rates of substitution when compared to both Enterobacteriaceae and the S- and D-clades (table 2). *Buchnera* and Psyllids endosymbiont 16S rDNA sequences have previously been shown to evolve relatively faster when compared to the sequences of the free-living bacteria (Moran 1996; Fukatsu and Nikoh 1998). Here we show that endosymbiont sequences of the R-clade of the Dryophthoridae evolve even more rapidly. The relative substitution rates from the D-clade and the S-clade sequences were not found to be significantly different when compared to the Enterobacteriaceae sequences.

Using the Dryophthoridae family age estimation established by O'Meara and Farrell (personal communication), we have calculated the absolute rates of substitution (for the 16S rDNA gene) for two of the three Dryophthoridae clades. For the R-clade, which is about 100 Myr old, the estimate based on the Galtier and Gouy (1998) model of DNA evolution is 0.128 substitutions/site per 100 Myr, when considering the greatest divergences observed within the clade. Likewise, a value of 0.119 substitutions/site per 100 Myr was obtained for the S-clade (25 Myr). Therefore, the substitution rates of both the R-clade and the S-clade are much higher than those calculated for *Buchnera* (0.058 substitutions/site per 100 Myr), as well as for the enteric bacteria (0.007 to 0.018 substitutions/site per 100 Myr), (Clark, Moran, and Baumann 1999).

#### **Discussion**

Histological and molecular analyses reveal widespread symbiosis within the Dryophthoridae weevils. All but one Dryophthoridae species (*S. linearis*) analyzed so far were found to harbor pleiomorphic intracellular bacteria in the adult and the larval bacteriomes. Bacteriomes across a wide array of weevil species are very similar in shape and location. They are always connected to the foregut/mid-gut junction in the larvae, and at the apex of the ovaries. Such uniformity of structure of the widespread symbiosis in the Dryophthoridae is consistent with long association.

Three clades of Dryophthoridae endosymbionts (R, S, and D) were defined by phylogenetic analysis, indicating that all extant Dryophthoridae endosymbionts are derived from distinct bacterial species established in three ancestral

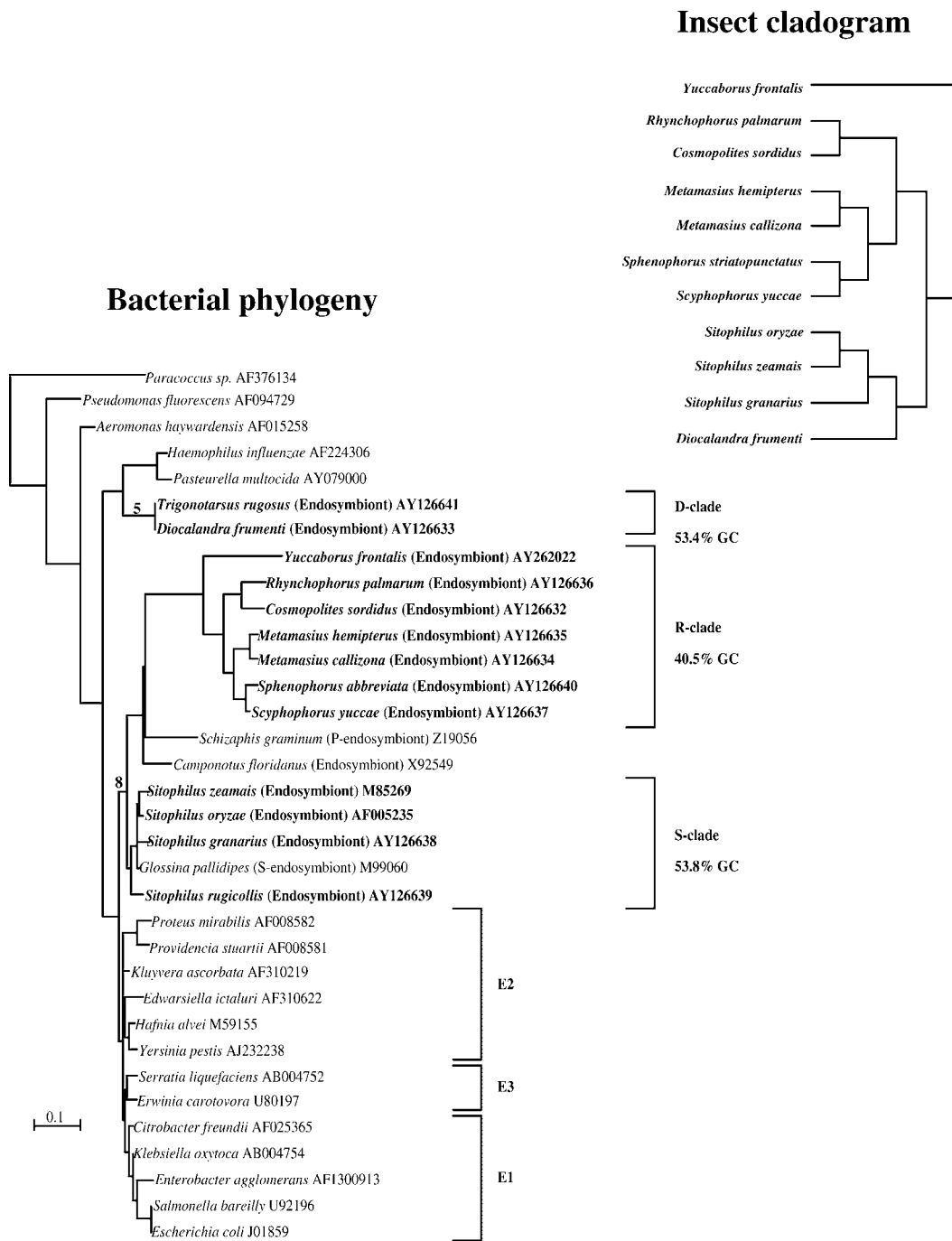


FIG. 2.—Phylogenetic tree of *Dryophthoridae* endosymbionts. Bacterial phylogeny: maximum likelihood phylogeny based on the 16S rDNA gene sequence of *Dryophthoridae* and others insect endosymbionts within the  $\gamma$ -Proteobacteria. Numbers on the tree (mentioned in *Results*) represent the most likely positions for endosymbiont clades branching on the reference tree topology. E1, E2, and E3: Enterocluster 1, 2, and 3 defined by Ahmad, Weisburg, and Jensen (1990). P-endosymbiont: principal endosymbiont (majority and vertically transmitted), S-endosymbiont: secondary endosymbiont (minority and can be horizontally transmitted). Insect cladogram: simplify cladogram from *Dryophthoridae* phylogenetic study from O'Meara and Farrell (personal communication).

*Dryophthoridae* species. However, a close phylogenetic proximity was observed between these three endosymbiont clades, particularly between R and S, which are also closely related to the other insect endosymbiotic bacteria. This finding is consistent with the “stem clade” hypothesis that suggests the existence within the  $\gamma$ 3-Proteobacteria of a limited group of bacteria that have generated intracellular

symbiosis in many insect species and at different evolutionary times (Charles, Heddi, and Rahbé 2001).

The R-clade endosymbionts were found in six of nine *Dryophthoridae* genera, and especially within *Yuccaborus frontalis*, the most ancestral insect of our data set (O'Meara and Farrell, personal communication). This clade shows much higher relative rates of substitution

when compared to both the Enterobacteriaceae and the other insect endosymbionts. A high AT bias and AT-rich insertions were also observed in the R-clade endosymbionts (40.5% GC on the 16S rDNA sequences). Estimation of the ancestral GC content of the R-clade (55.3% GC, node 8 in fig. 2) highlights this process of AT base accumulation during symbiotic evolution. Conversely, S-clade and D-clade endosymbionts are restricted to one and two genera, respectively. Moreover, they do not show a very great AT bias or a significant rates of evolution. These findings, along with dating estimations, the congruence between the host phylogeny (O'Meara and Farrell, personal communication), and the endosymbiont R-clade repartition within Dryophthoridae species, suggest that the R-clade endosymbiont represents the oldest and the ancestral bacterial infection.

The endosymbionts of the S-clade and the D-clade have been established more recently, probably by endosymbiont displacement. A hypothesis of endosymbiont replacement was postulated in aphids by Moran and Baumann (1994). More recently, a potential interplay between old-primary and recent-secondary endosymbionts was suggested by several workers who have noted a beneficial role of secondary endosymbionts for the host fitness in some aphid species (Sabater et al. 2001; Chen et al. 2000) or glossine species (Montllor, Maxmen, and Purcell 2002).

In the Dryophthoridae, the recent S-clade association is concomitant with the shift from stem-feeding, the ancestral habit in these weevils, to seed-feeding in *Sitophilus*. Likewise, the absence of intracellular bacteria in the unique asymbiotic species *S. linearis* could be interpreted as an endosymbiont loss associated with shift from monocots, the ancestral association in *Sitophilus*, to dicots. *S. linearis* feed on tamarind seeds (Fabaceae) that are much more nutritionally balanced than the cereal seeds used by the other symbiotic *Sitophilus* species (Delobel and Grenier 1993).

The D-clade is closely related to free-living parasitic bacteria such as *Haemophilus* and *Pasteurella*, which suggests that D-clade endosymbionts may also have evolved from an ancestor with a parasitic intracellular life-style. Moreover, recent studies on *Sodalis glossinidius*, the secondary endosymbiont of the tsetse flies (Dale et al. 2001), and *Sitophilus zeamais* endosymbionts (Dale et al. 2002) demonstrate that attachment and invasion of the host by the endosymbiont rely on functional genes of the type III secretion system, which are closely related to those of the pathogenic bacteria *Salmonella* and *Shigella*. Secretion systems are required for protein pathogen export and toxin delivery to the host cells. Hence, these results suggest that *Sodalis* spp. and the S-clade endosymbionts share functional infection faculties.

The position of *Sodalis glossinidius* is on the internode between *S. rugicollis* and the other *Sitophilus* species, suggesting that a horizontal transfer has occurred between *Sitophilus* weevils and tsetse fly. However, the widely separated areas of origin inferred from fossil studies for tsetse flies (Africa) and grain weevils (India) suggest, rather, an indirect transfer via some bacteria vectors.

In the early stages of symbiosis, horizontal transfers

**Table 2**  
**Evolutionary Rates of the 16S rDNA Gene Sequence in the Three Dryophthoridae Clades Relative to Other Clades Belonging to the  $\gamma$ -Proteobacteria and with *Aeromonas* (0) as Outgroup**

Tested Clade (1)	Reference Group (2)	K <sub>1-0</sub>	K <sub>2-0</sub>	P Value <sup>a</sup>
D-clade	<i>Pasteurella</i> / <i>Haemophilus</i>	0.117	0.140	0.01
D-clade	Enterobacteriaceae	0.116	0.100	0.20
D-clade	S-clade	0.116	0.108	0.45
S-clade	Enterobacteriaceae	0.112	0.106	0.29
S-clade	<i>Glossina pallidipes</i> (S-endosymbiont)	0.109	0.101	0.06
R-clade	Enterobacteriaceae	0.233	0.102	10 <sup>-7</sup>
R-clade	S-clade	0.233	0.106	10 <sup>-7</sup>
R-clade	D-clade	0.234	0.117	10 <sup>-7</sup>
R-clade	<i>Schizaphis graminum</i> (P-endosymbiont)	0.234	0.142	10 <sup>-7</sup>
R-clade	<i>Camponotus floridanus</i> (Endosymbiont)	0.227	0.135	10 <sup>-7</sup>

<sup>a</sup> Corresponds to the probability that Ho: K<sub>1-0</sub> = K<sub>2-0</sub>.

of endosymbionts with pathogenic capacities could have occurred much more frequently by taking advantage of similar diets, or prey-predator and even mutualistic relationships between their respective hosts. The high homology of 16S rDNA sequences found by Aksoy, Chen, and Hypsa (1997) within the group of *Sodalis* (about 0.6% divergence) suggests recent transfers between different *Glossina* hosts. Conversely, a high divergence is observed between S-clade endosymbionts (about 6% divergence), suggesting vertical endosymbiont transmission in this group, a finding which is testable with further sampling of *Sitophilus* species.

A molecular clock calculated for the weevil hosts (O'Meara and Farrell, personal communication) allows us to observe that the S-clade endosymbionts evolve even faster than *Buchnera* (Moran 1996). Values of 0.119 and 0.058 substitutions/site per 100 Myr were measured on the S-clade endosymbionts and *Buchnera*, respectively. The difference between the relative rate test (not significant for the S-clade; table 2) and the absolute rate of substitution indicates a pronounced rate change occurring during the evolution that led from free-living bacteria to endosymbiosis establishment.

The Dryophthoridae symbiosis is unique in that an apparently single origin of the bacterial endosymbiosis in these insects is associated with three independent origins of their primary endosymbionts. Symbiosis was apparently established 100 MYA in the R-clade, followed by at least two additional symbiont substitutions, resulting from endosymbiont competition, that generated the S-clade and the D-clade, possibly in conjunction with ecological shifts in these insects.

Modeling studies indicate that parasitism by the bacteria is strongly favored when bacterial transmission occurs horizontally, with many symbionts competing among themselves and when the host defense is weak (Ewald 1995; Frank 1996). Selection pressure may eliminate host insects that are neither able to tolerate nor able to circumvent bacterial virulence. Selected hosts may possess specific strategies to control the bacteria space by inducing specific cell differentiation, the bacteriocyte



for example. The high bacterial evolution rates may help to silence bacterial virulent genes through either gene mutations or deletions, thereby relaxing the selection pressure on the host and leading to insect diversification.

#### Proposition of “Candidatus” *Nardonella* for the R-Clade Endosymbionts

Symbiotic bacteria of the R-clade are unique to the weevil family Dryophthoridae. Following Murray and Stackebrandt (1995), microorganisms partially characterized and not cultivated on laboratory media might be given the designation “Candidatus.” Consequently, we propose to name the lineage corresponding to the R-clade of Dryophthoridae endosymbionts as *Candidatus* *Nardonella*, in honor of Professor Paul Nardon, who has first characterized endosymbionts in *Metamasius* and *Cosmopolites* (Nardon et al. 1985).

The description of “*Candidatus* *Nardonella*” is as follows: phylogenetic position,  $\gamma$ 3-subclass of Proteobacteria; cultivation, not cultivated on cell-free media; Gram reaction, negative; morphology, pleiomorphic (rod shape, U-shape, spiral shape), from 3–4 to 200  $\mu$ m in length, 1–2  $\mu$ m in diameter, surrounded by a mucopolysaccharide-like substance; basis of assignment, 16S rDNA sequences; association and host, intracellular symbionts of the Dryophthoridae (described in *Yuccaborus frontalis*, *Rhynchophorus palmarum*, *Cosmopolites sordidus*, *Metamasius hemipterus*, *Metamasius callizona*, *Sphenophorus abbreviata*, *Scyphophorus yuccae*); mesophilic, authors, Lefevre et al. (this study).

#### Supplementary Material

Sequence Alignment of the Dryophthoridae endosymbionts is provided as Supplementary Material online (fasta format) at the journal’s Web site.

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