

# Complete Genome of Uncultured Bacterial Endosymbionts of Cellulolytic Protists

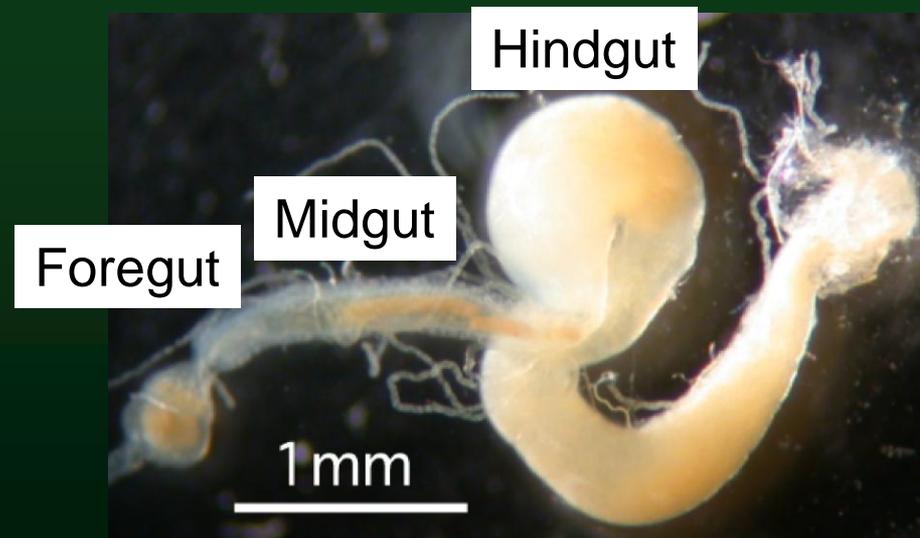
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Japan

Complex microbial community for efficient decomposition



A Japanese subterranean termite,  
*Reticulitermes speratus*

The dilated hindgut of termites harbors dense microbial population comprising both protists and prokaryotes.



50  $\mu\text{m}$

# Termite Gut Flagellated Protists

Symbiotic digestion  
of cellulose

Voluminous & high density

Difficult to cultivate

Unique for termites  
and the related *Cryptocercus roachei*

Inherited from their common  
ancestor and diversified within the  
gut

*Proc. R. Soc. B.* 2009

Belong to either  
**Parabasalia** or **Oxymonadida**

Comprise diverse species

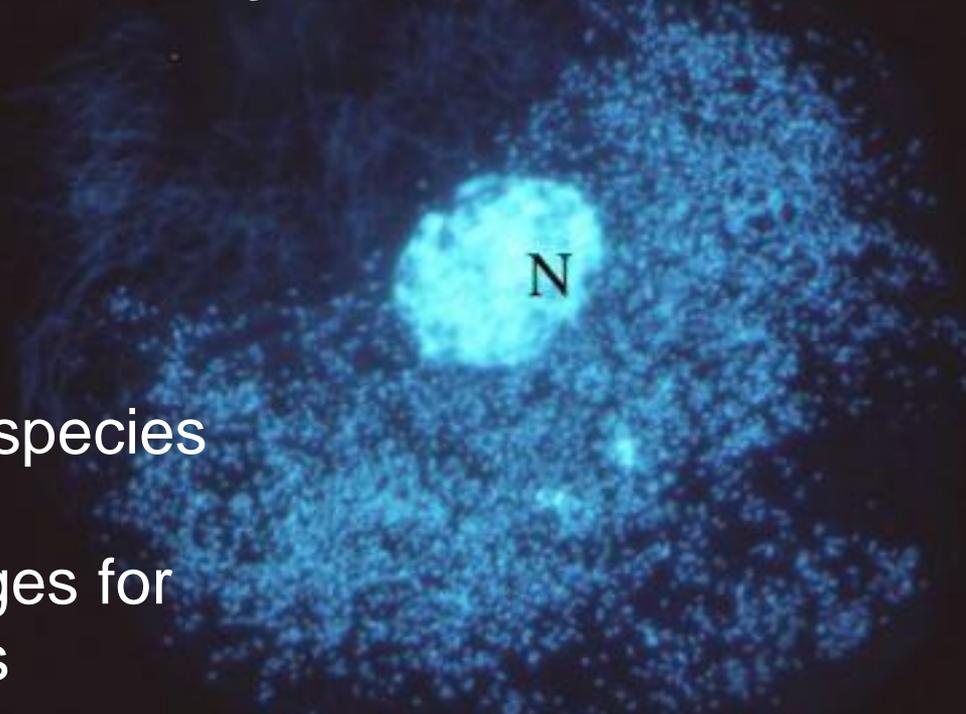
*Trichonympha* sp.

# Termite Gut Bacteria

(DAPI staining)

Many are associated with protists as their endo- or ecto-symbionts

A high density of endosymbiotic bacteria



Diverse and new species

Unique lineages for termites

Most are yet-uncultivated

**Culture-independent molecular approaches**

# Termite Gut Microbial Community

## Contents :

### **Diversity, novelty, and structure of the community**

- Importance of large protists and their associated bacteria

### **Functions and roles for efficient cellulose utilization**

#### **Bacterial endosymbionts**

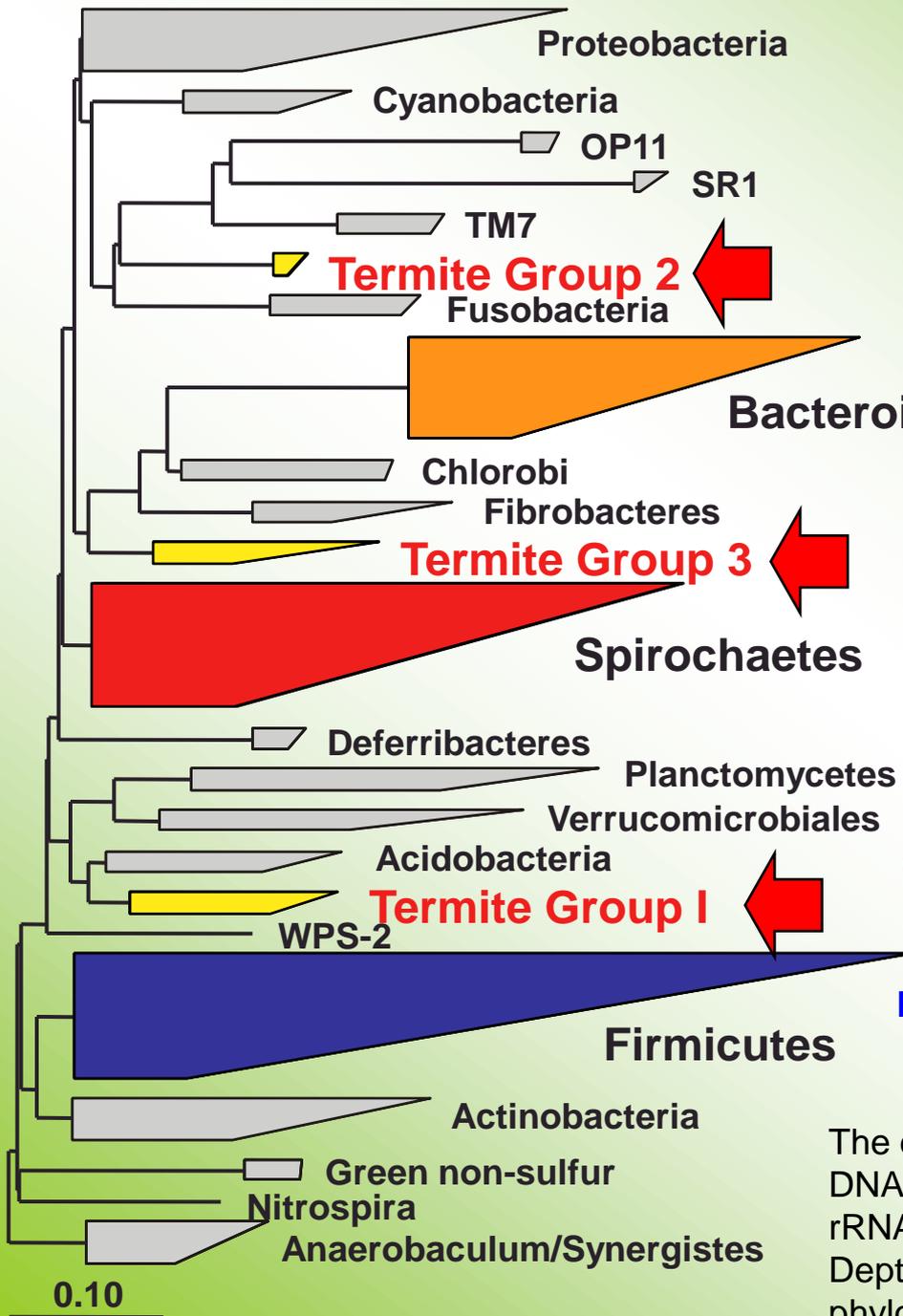
- complete genome of two endosymbiont species
- implications for the symbiotic interaction

#### **Host protists**

- meta-EST (meta-transcriptome) analysis
- primary metabolism & biochemistry of its key steps

# Diversity of bacteria in termite guts

(A phylotype represents clones showing >97% identity)



- 13 genera 16 species
  - 4000~ clones sequenced
  - 1000~ phylotypes
  - Estimated, 540-3600 per gut
  - >90% were new
  - 23 phyla
  - 3 new candidate phyla
- (Termite Groups 1, 2, 3)

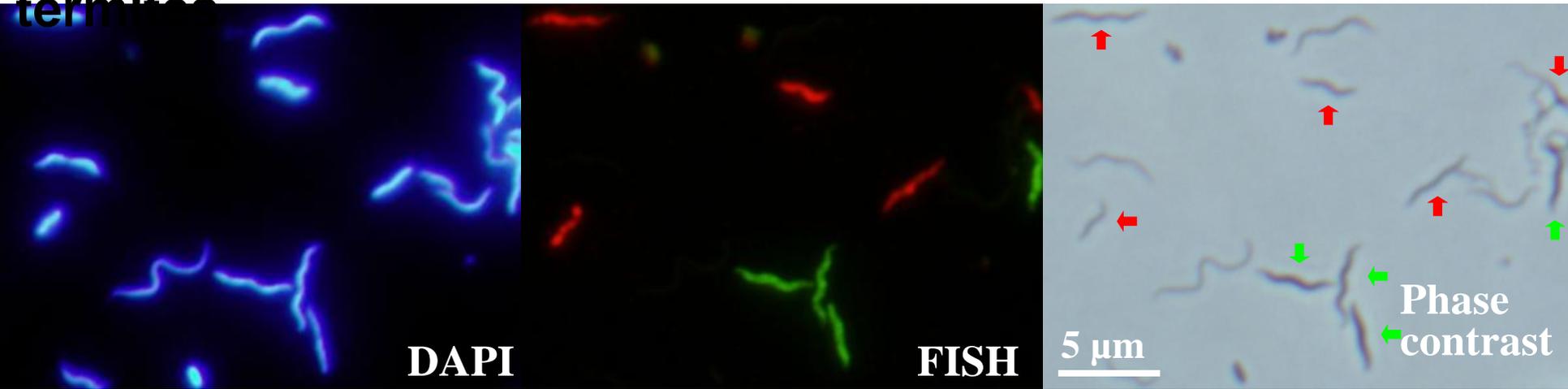
Phylum TG1 or TG3 shares ca.10% of gut bacteria depending on termite species

The diversity of bacteria was examined by extracting the DNA from the community, PCR-amplification of the 16 rRNA gene, and clonal analyses.

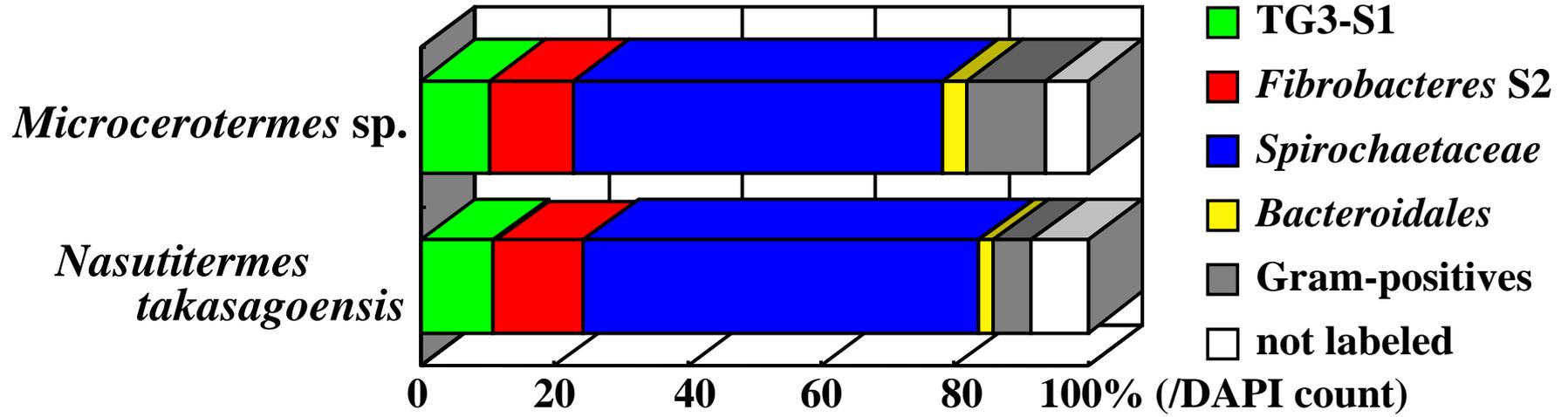
Depth and width of clusters roughly reflected phylogenetic diversity and abundance of the clones.

# Dominance and cell morphologies of the novel **phylum TG3** and a novel **subphylum in *Fibrobacteres*** in wood-feeding higher termites

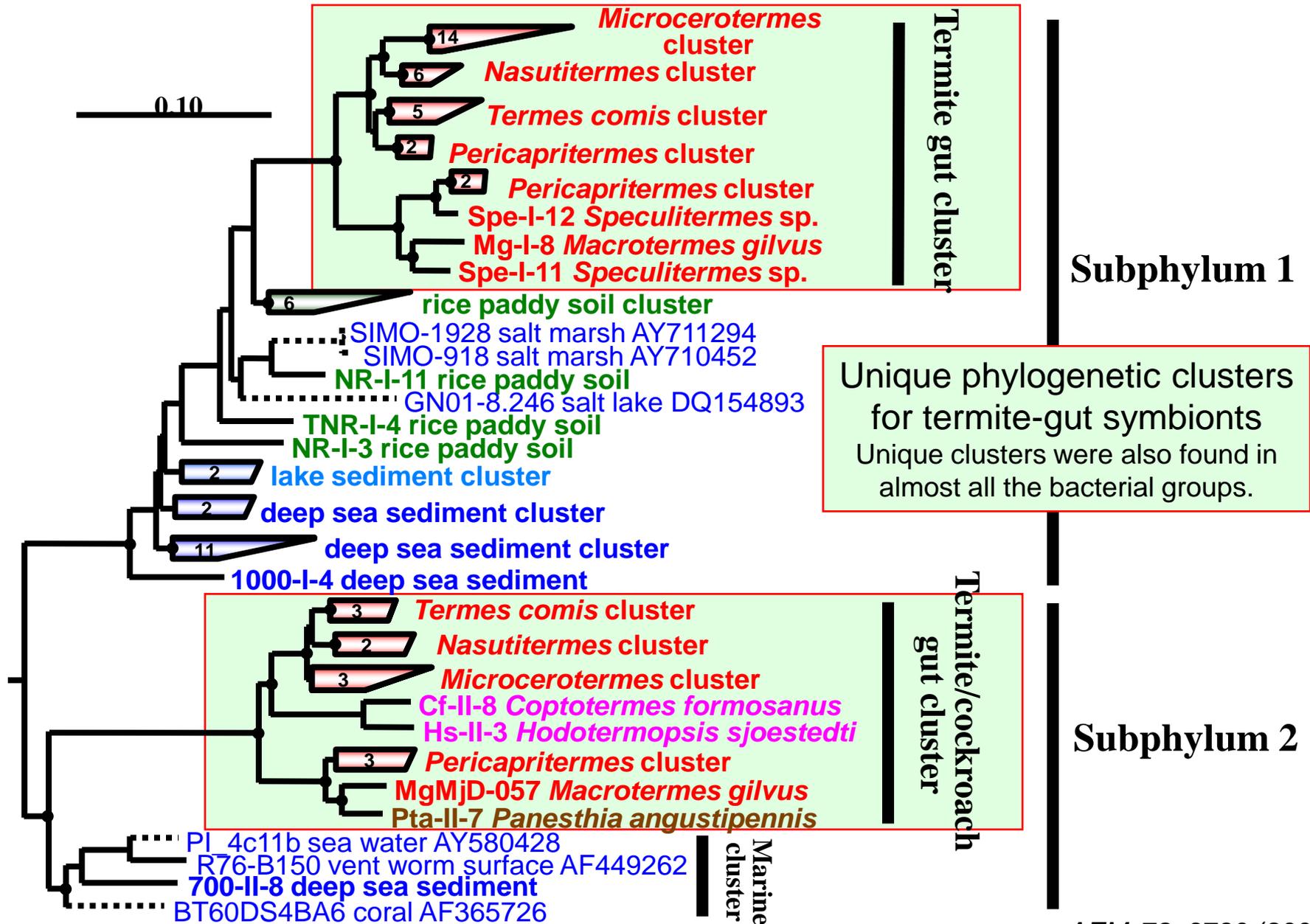
FISH identification of TG3-S1 (Green) and *Fibrobacteres* S2 (Red)



FISH counts of bacterial groups



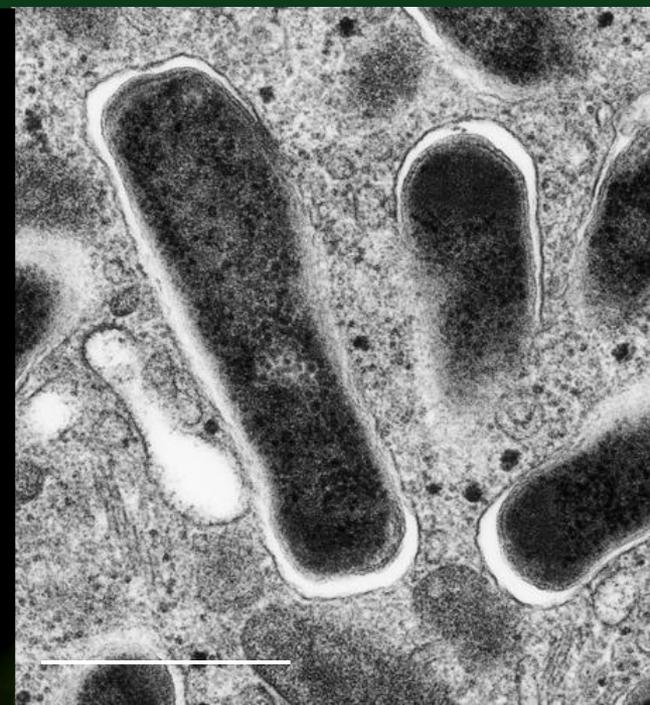
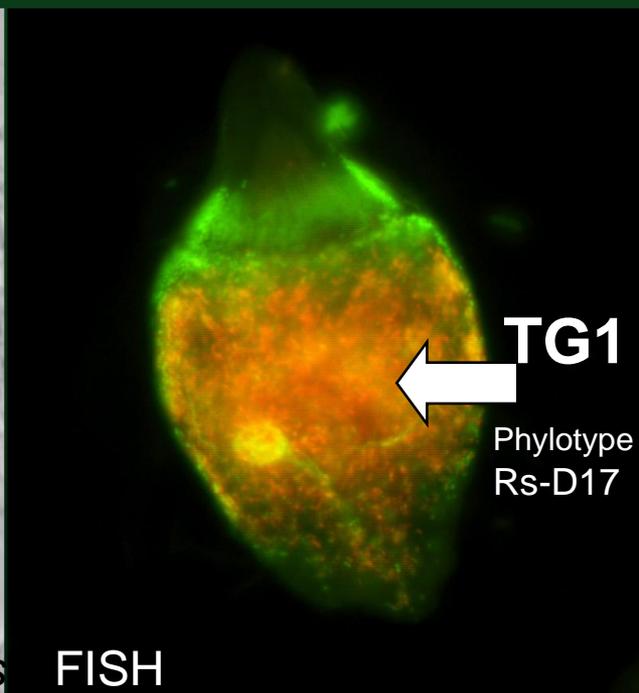
# Distribution and Phylogenetic diversity of the novel phylum TG3



# Uncultured endosymbiotic bacteria in the candidate phylum 'Termite Group 1 (TG1)'

- Candidate phylum with no cultured representative
- Specific endosymbionts of various protist species
- Several thousands in a single protist cell
- Up to 10% of total bacterial population in the gut

AEM 1996; FEMSEC 2003, 2007



# Phylogenetic identification of the associated bacteria of the protists as well as the protists themselves

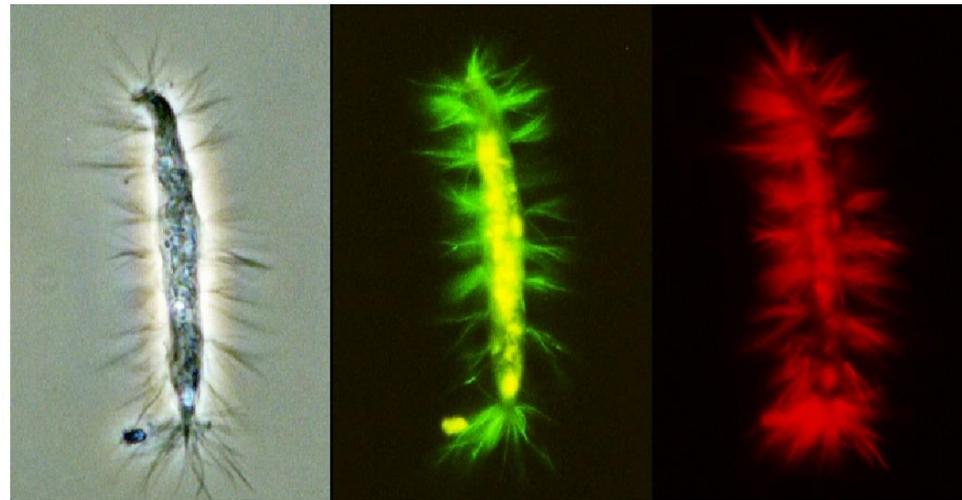
Manual isolation of specific protist cell(s) by aid of a micromanipulator



PCR, clone analyses, phylogeny  
rRNA gene & others

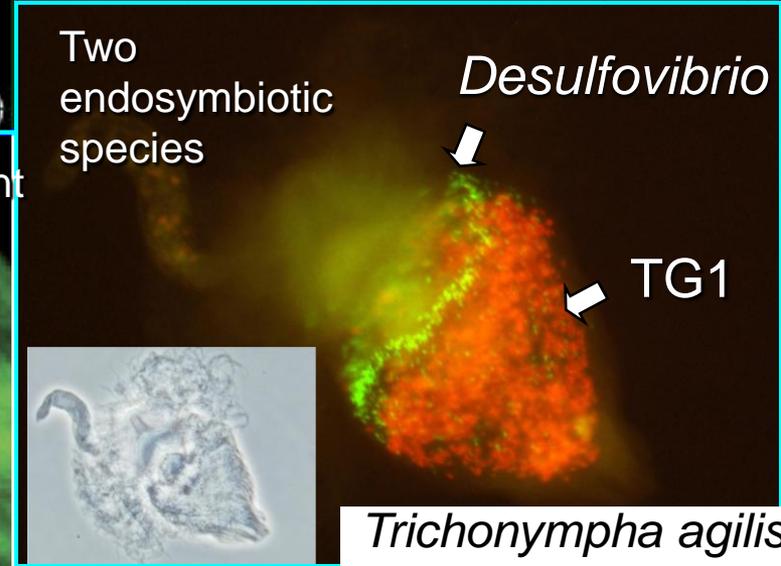
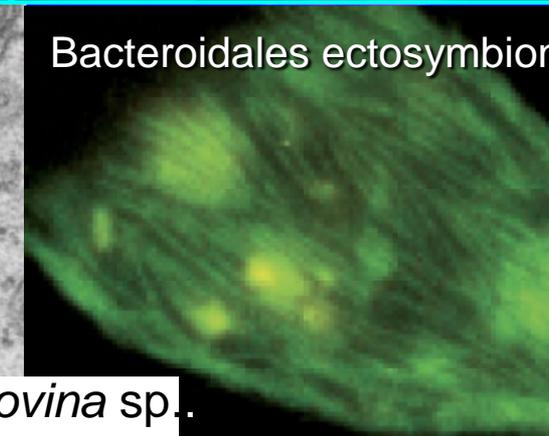
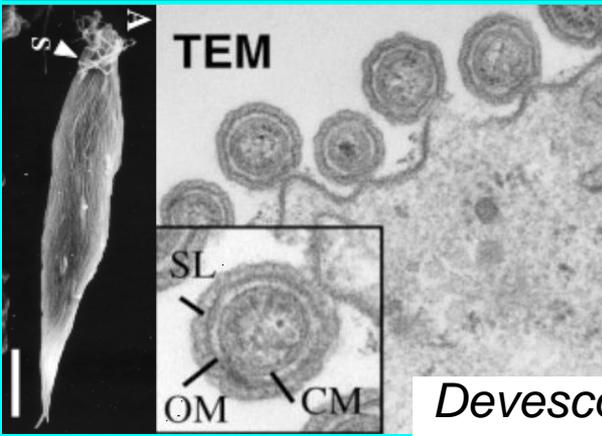
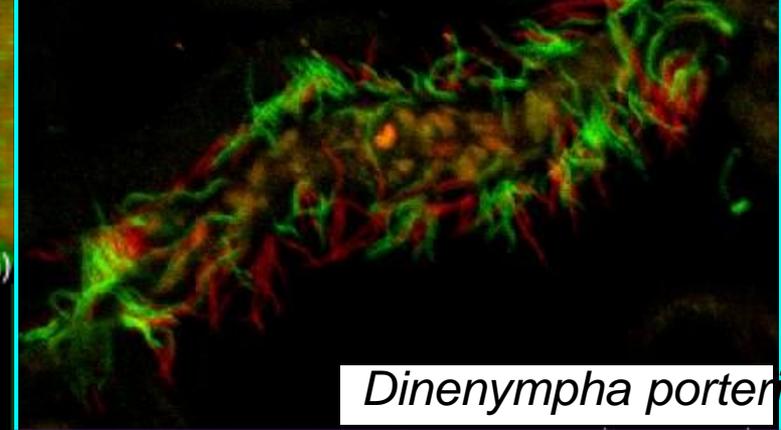
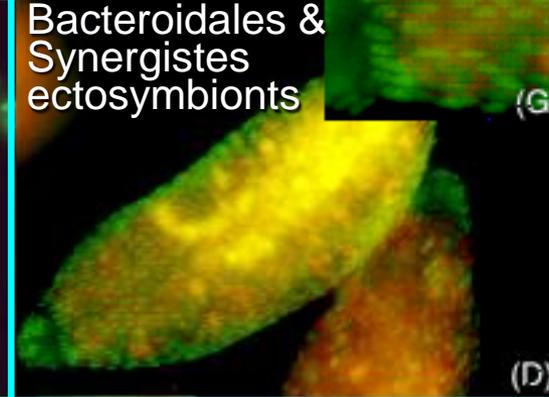
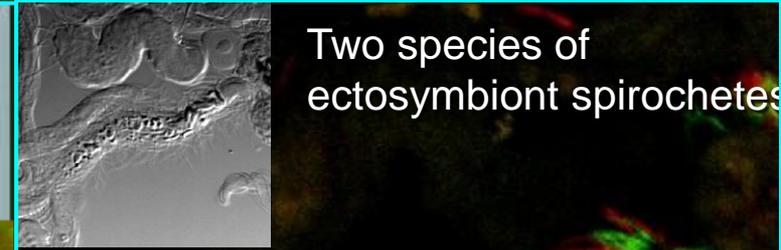
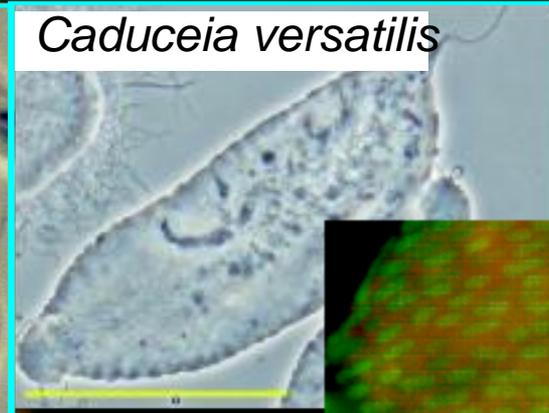


FISH (*in situ* identification)  
with specific probes



# Various Proist-Prokaryote Associations

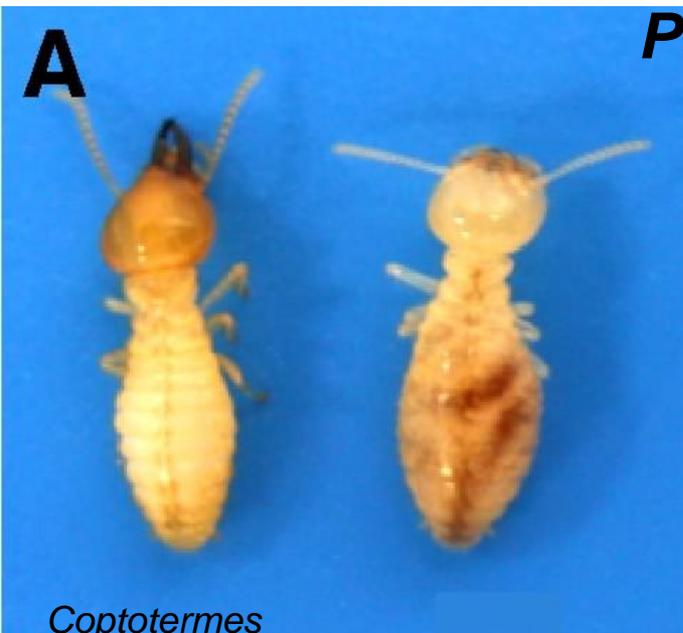
At least 7 phyla of bacteria involve in the associations with protists



# Endosymbiotic Bacteroidales bacteria of

## *Pseudotriconympha*

**A**

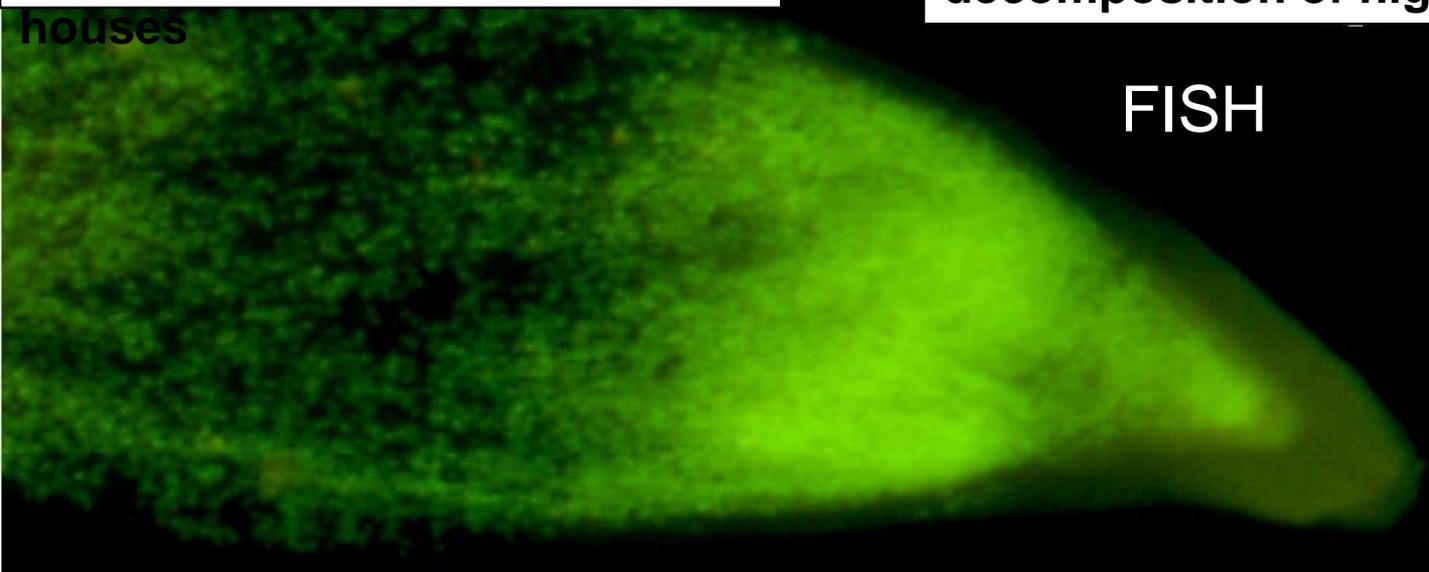


*Coptotermes*

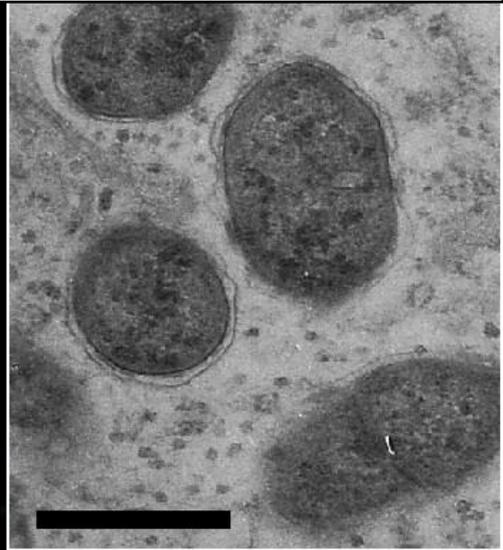


The *Coptotermes* termites are world-wide pests for woody houses

The protist species is responsible for decomposition of highly polymerized



FISH



# Abundance of the Bacteroidales endosymbiont of *Pseudotrichonympha* in the gut bacterial community of the termite *Coptotermes formosanus*

Quantitative method	% of the endosymbiont (mean $\pm$ SD)
rRNA cleavage	81.9 $\pm$ 9.0 %
FISH count	71.3 $\pm$ 6.0 %
Direct count (DAPI) *	66 %

* Endosymbiont	1.08 $\pm$ 0.04 x 10 <sup>5</sup> cells / protist
Protist	700-800 cells / gut
Total prokaryotes	1.30 $\pm$ 1.3 x 10 <sup>8</sup> cells / gut

A single endosymbiont species shares more than two-thirds of whole-gut bacteria

# Cospeciation in the triplex symbiosis of *Pseudotriconympha* protists, their endosymbionts, and their host termites

Host termite species

Tanglegram of three partners

**Protist**

SSU

**Termite**

mito. COII & 16S

- Coptotermes cf. testaceus* (C)
- Coptotermes formosanus* (C)
- Coptotermes formosanus* (C)
- Coptotermes* sp. (C)
- Coptotermes* sp. (C)
- Heterotermes longiceps* (H)
- Heterotermes tenuis* (H)
- Termitogeton planus* (T)
- Parrhinotermes* sp. (R)
- Schedorhinotermes* sp. (R)
- Schedorhinotermes* sp. (R)
- Rhinotermes hispidus* (R)
- Rhinotermes marginalis* (R)
- Psammotermes allocerus* (P)

**Endosymbiotic bacteria**

16S

TREEMAP analyses

$P < 0.05$

Cospeciation of the TG1 endosymbiotic bacteria and their host protists are also reported (Mol. Ecol. 2009)

# Importance of large protists and their associated bacteria

The protists occupy a large volume of the gut.

- The protists occupy > 90% of a dilated portion of the gut
- Due to their large size

The associated bacteria correspond to a significant portion of the population in the gut community.

- The associated bacteria represent the most abundant 16S rRNA gene phylotypes
- A single large protist cell harbors  $\sim 10^5$  cell of endosymbionts
- In a typical case, a single endosymbiont species accounts for 70-80% of gut bacteria

These observations suggest:

**The protists and their associated bacteria play major roles in the gut metabolisms**

The co-speciation of the associations implies their specific mutual relationship.

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# Prediction of functions of a bacterial symbiont through the genome analysis

## 1. Metagenome of the gut community



Fragmented sequences  
with ambiguous organismal assignments

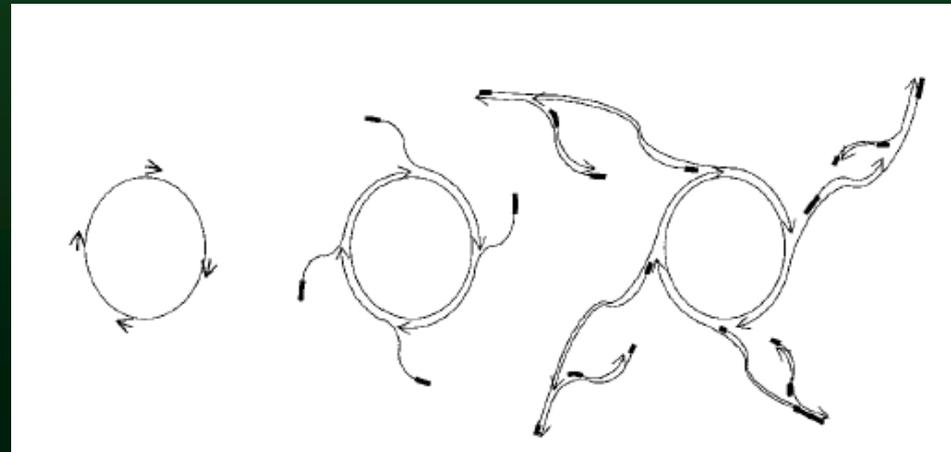
## 2. Complete genome of individual species



Starting from a small number of isolated cells

The genome of a single species is more informative than metagenome for understanding its precise functions and interactions with another..

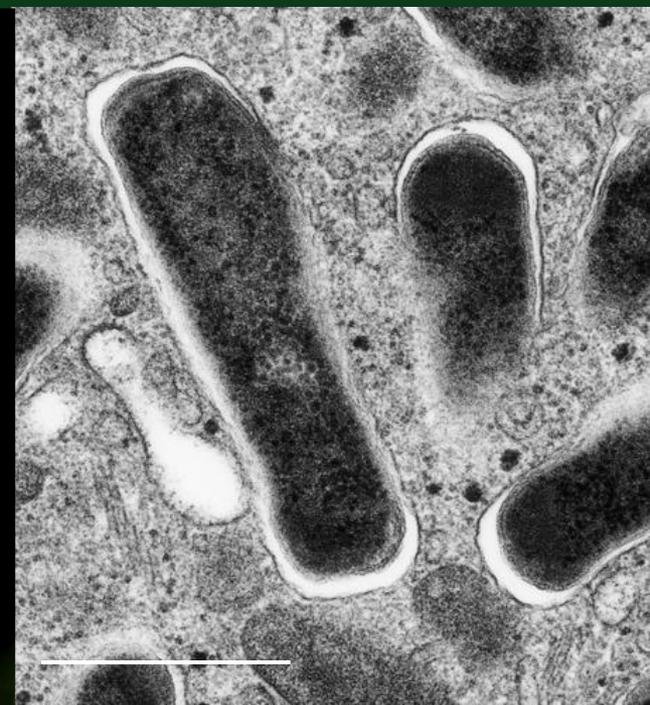
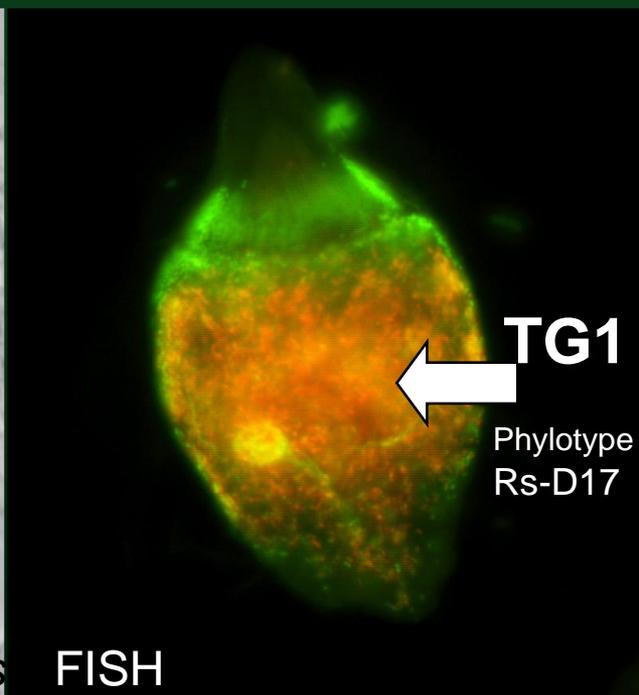
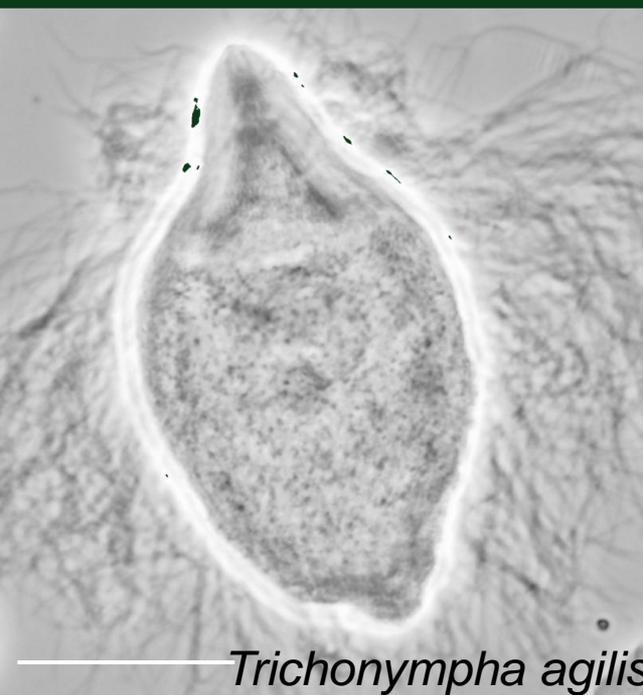
Whole genome amplification (WGA)  
with Phi29 DNA polymerase



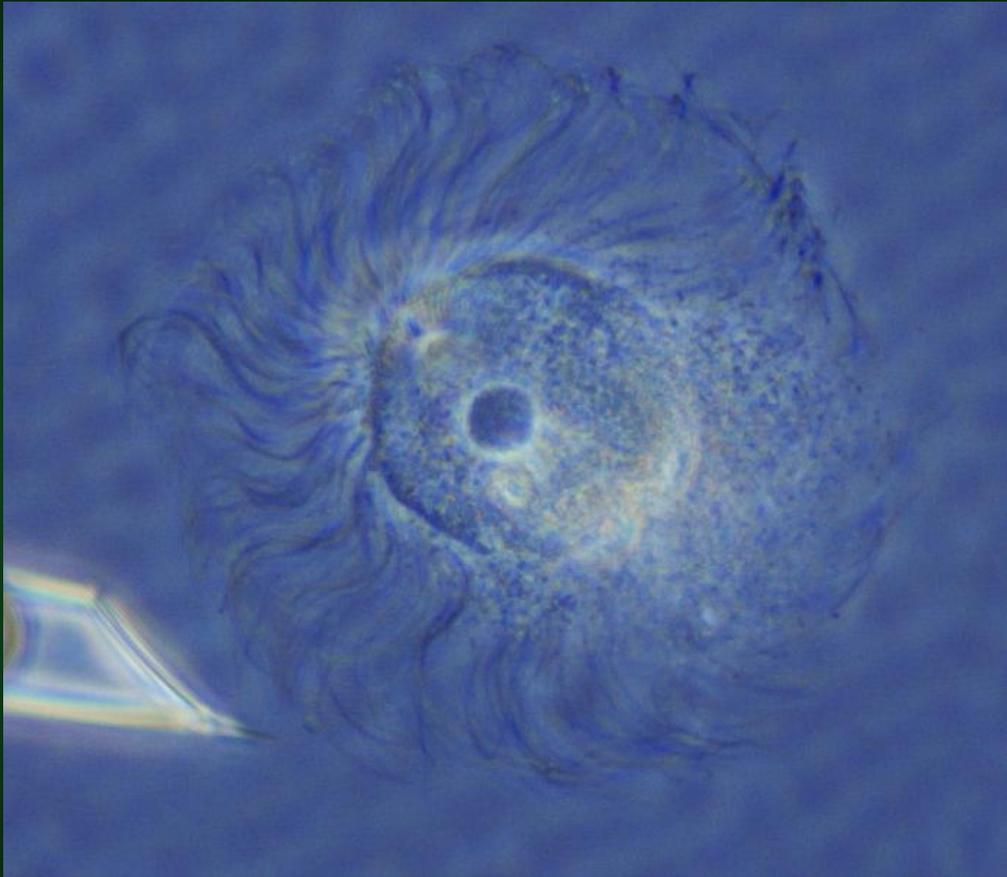
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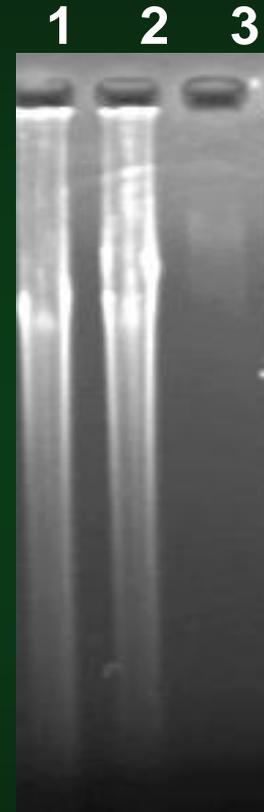
AEM 1996; FEMSEC 2003, 2007



# Whole genome amplification of TG1 endosymbiont from a single cell of host protist



To minimize the genome variation, a single protist cell was isolated and its endosymbionts were collected (several hundreds cells).



40kb

1 endosymbiont  
2 host nucleus  
3 buffer only

After whole genome amplification, the purity was checked by clonal analyses of 16S rRNA gene and other sequences.

# Complete genome of TG1 endosymbiont

The first complete genome of  
- a termite-gut symbiont

- a candidate phylum  
with no cultured  
representative

Small genome  
size

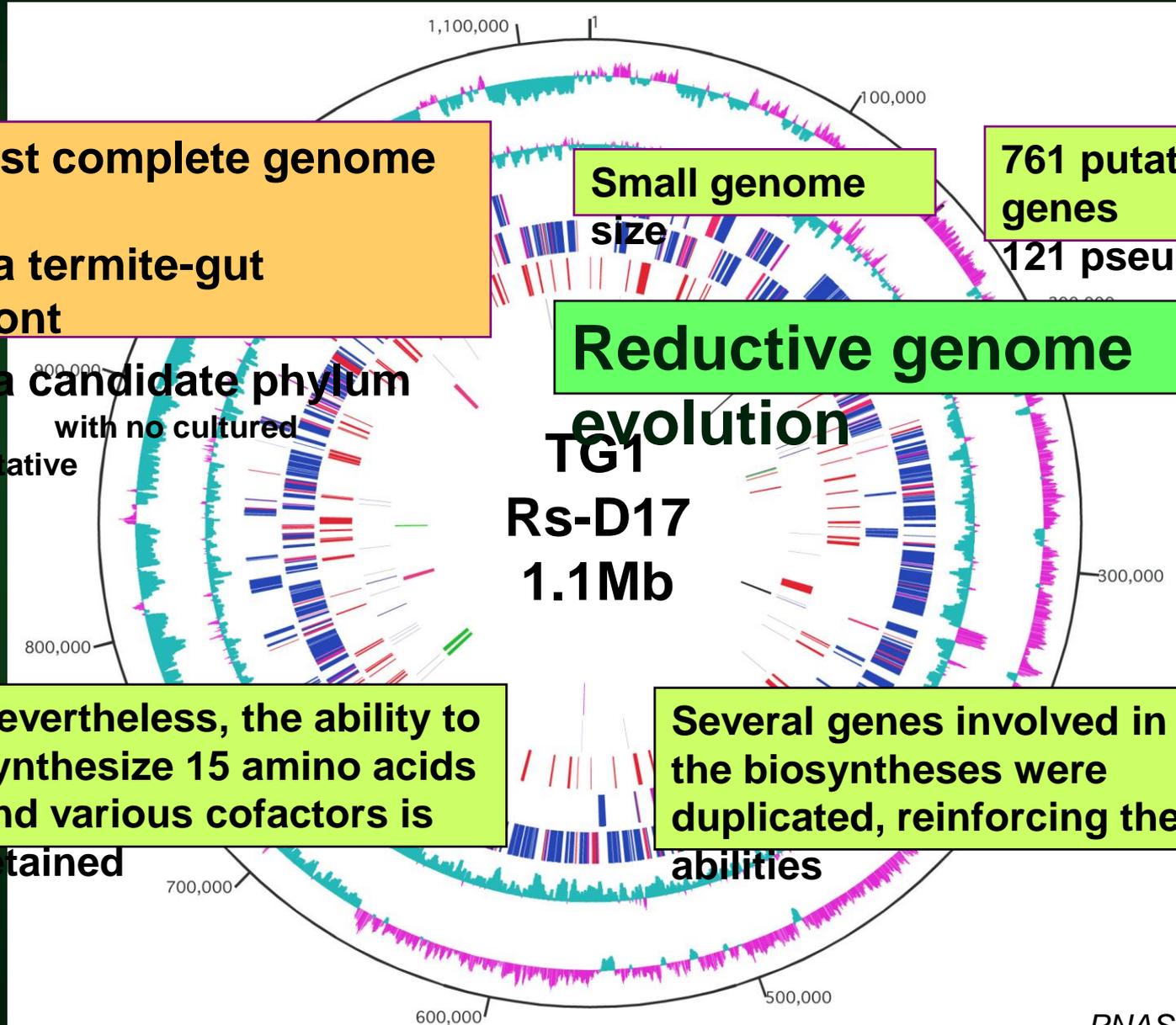
761 putative  
genes  
121 pseudogenes

Reductive genome  
evolution

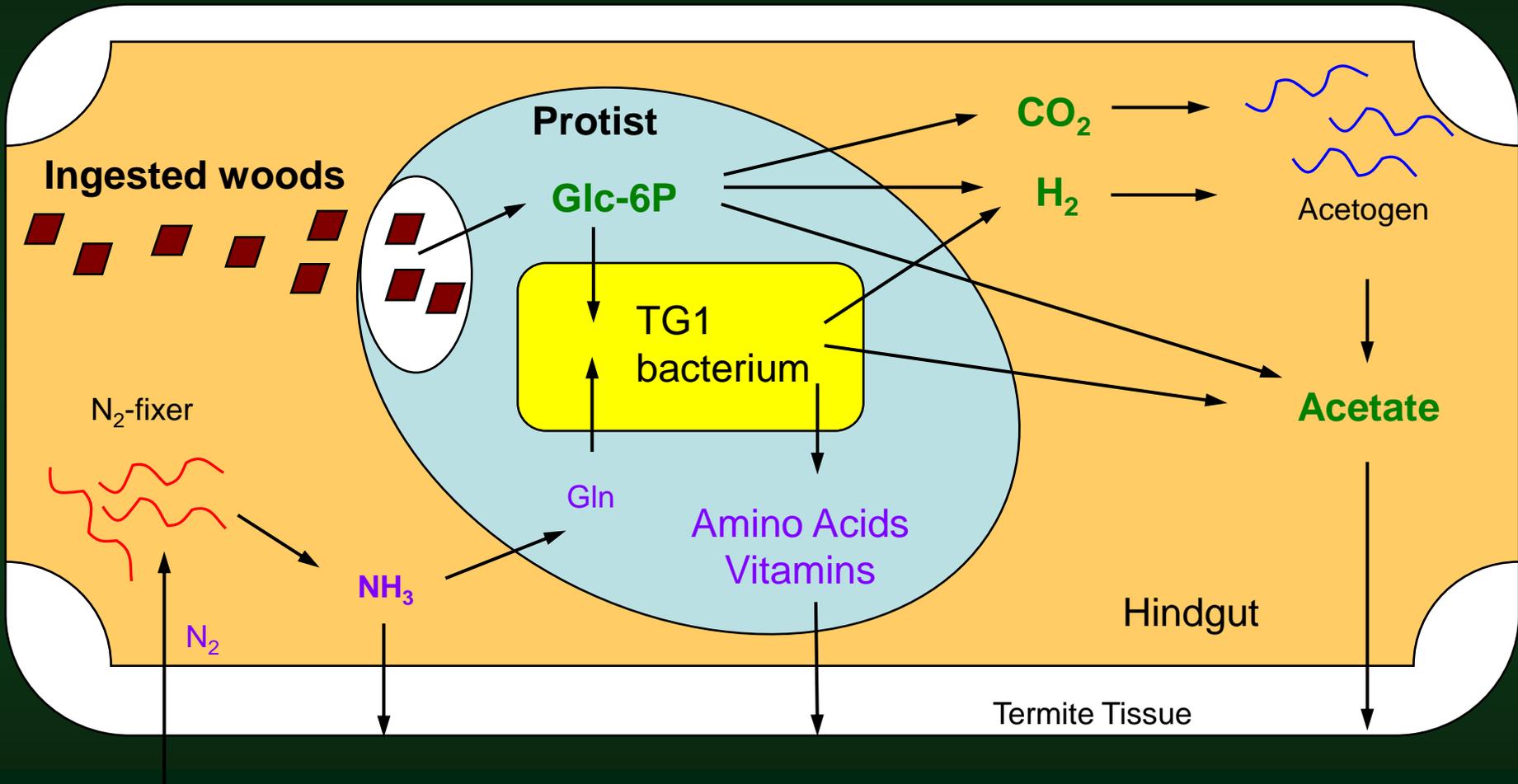
TG1  
Rs-D17  
1.1Mb

Nevertheless, the ability to  
synthesize 15 amino acids  
and various cofactors is  
retained

Several genes involved in  
the biosyntheses were  
duplicated, reinforcing the  
abilities

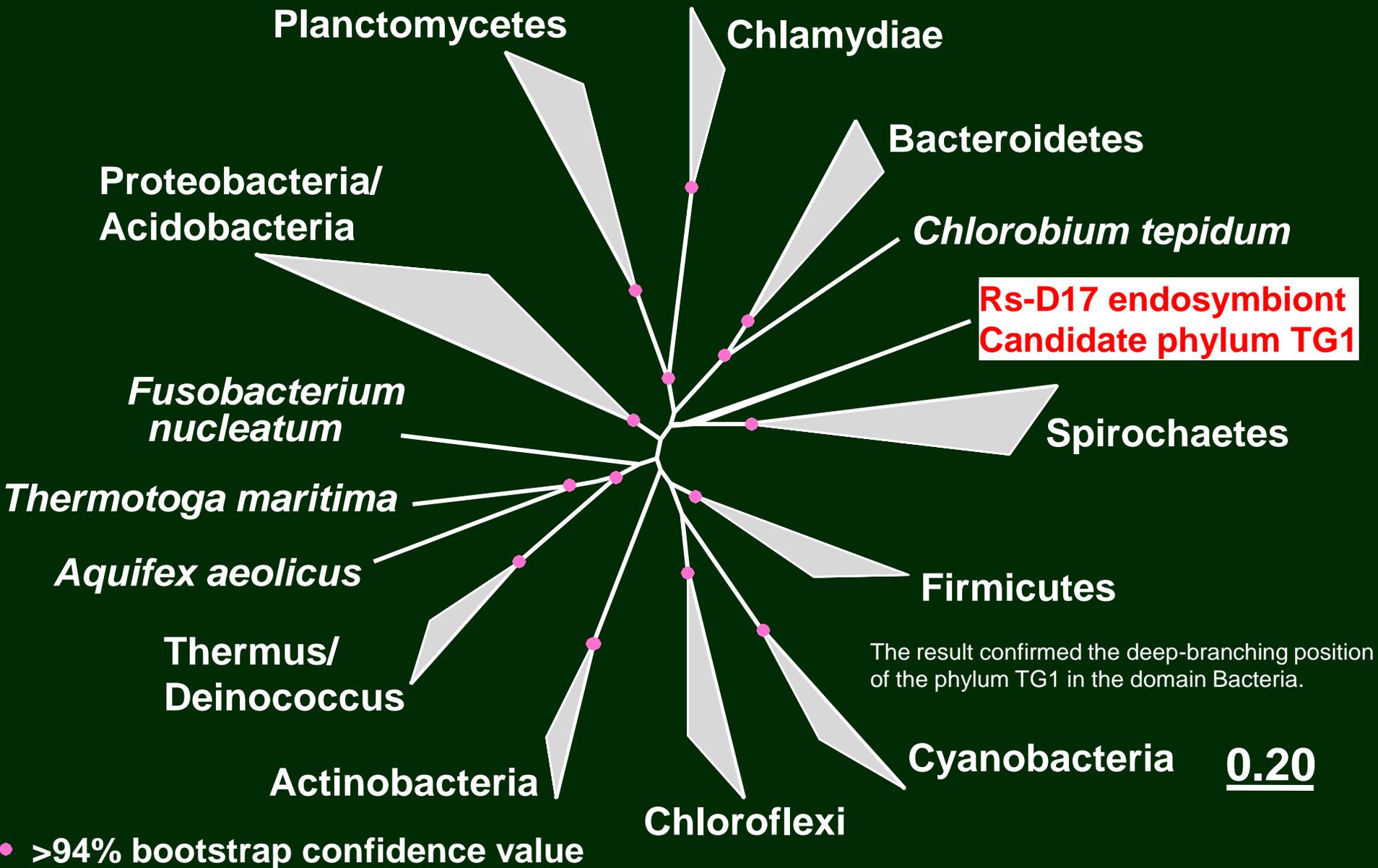


# Functional interaction between gut protist and its endosymbiont



The TG1 endosymbiont is a strict anaerobe depending solely on its energy production from substrate-level phosphorylation. The host protist provides sugar produced during cellulose decomposition, while the TG1 endosymbiont supplies essential nitrogenous nutrients deficient in ingested wood or cellulose to the host protists and termites.

# A ML tree of bacterial phyla based on a concatenated sequence of 38 ribosomal proteins



# Endosymbiotic Bacteroidales bacteria of

## *Pseudotriconympha*

AEM 2005; MEC 2007

**A**



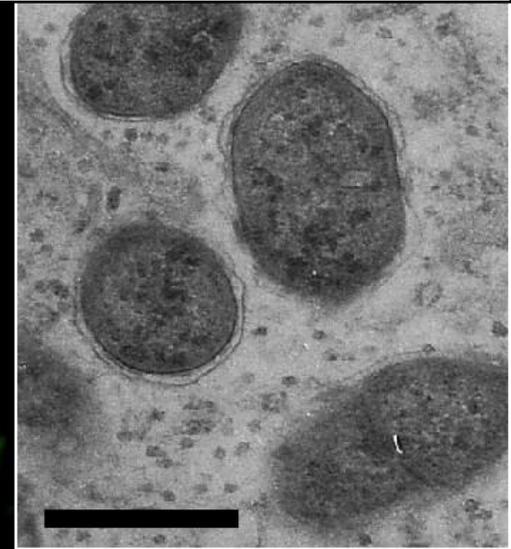
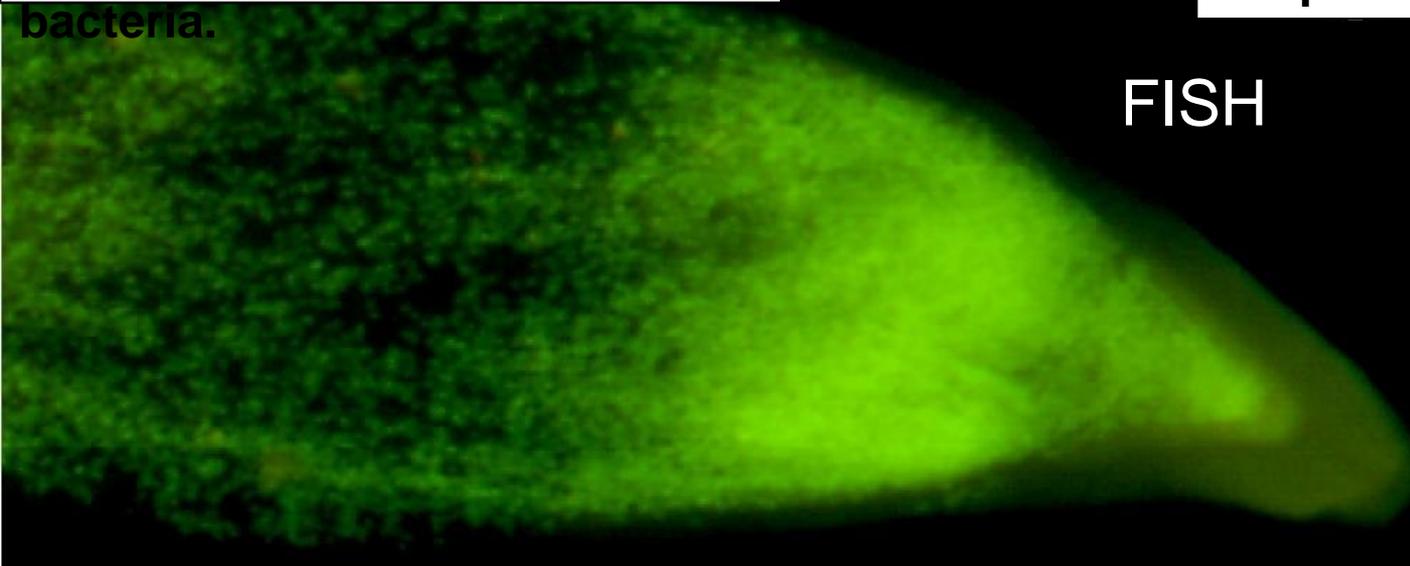
*Coptotermes*



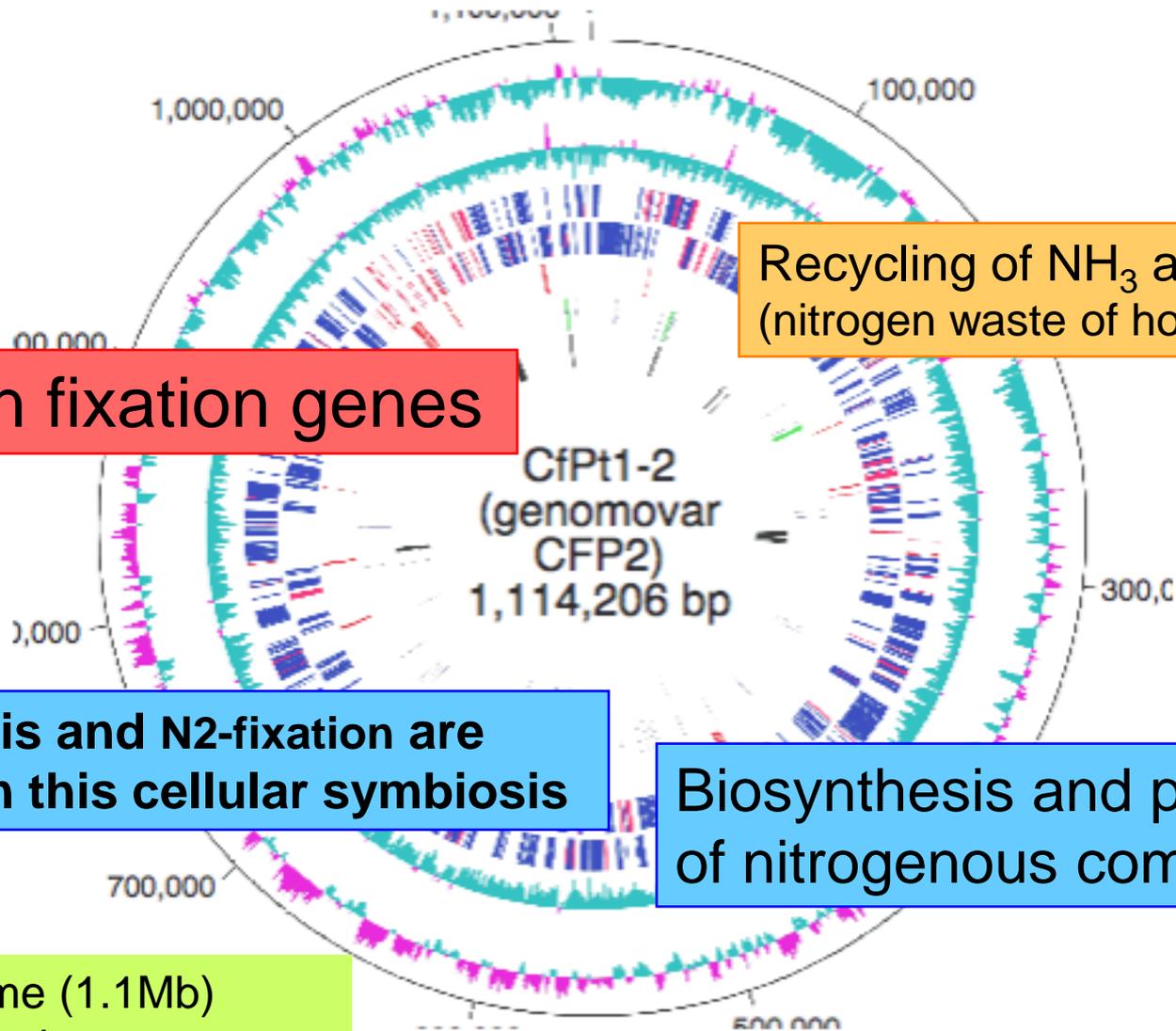
The endosymbiont species accounts for 70-80% of gut bacteria.

The endosymbionts have cospeciated with the host

FISH



# Complete genome of the endosymbiont of *Pseudotriconympha*



Nitrogen fixation genes

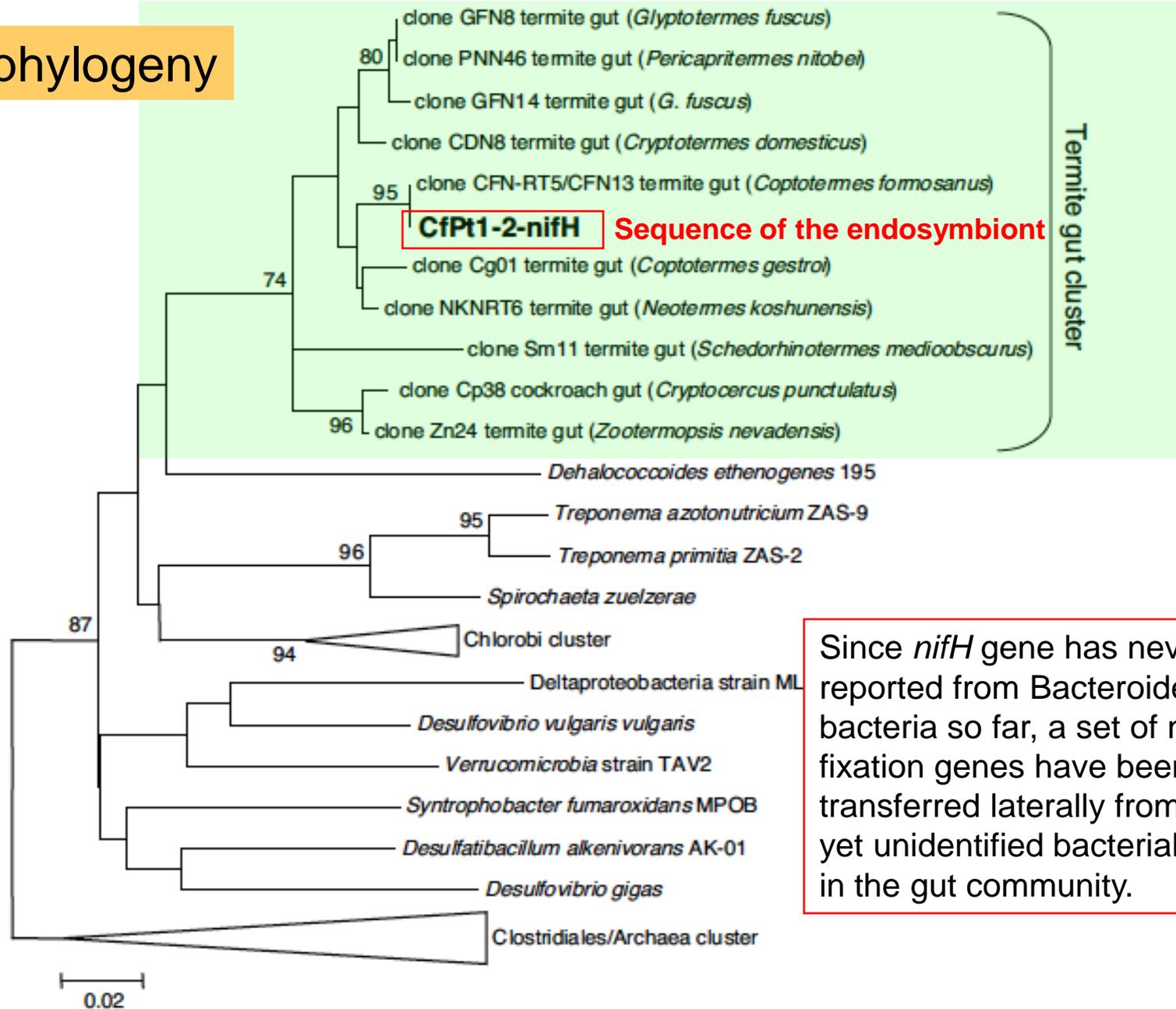
Recycling of  $\text{NH}_3$  and urea  
(nitrogen waste of host protist)

Cellulolysis and  $\text{N}_2$ -fixation are  
coupled in this cellular symbiosis

Biosynthesis and provision  
of nitrogenous compounds

Small genome (1.1Mb)  
but less pseudogenes  
758 CDSs, 22 pseudogenes

# NifH phylogeny



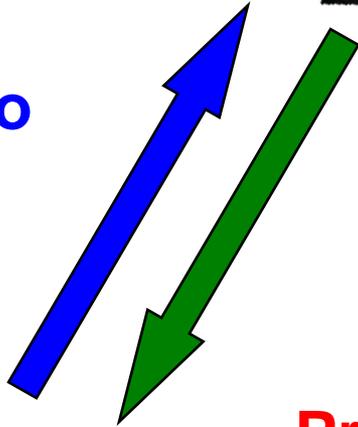
Since *nifH* gene has never been reported from Bacteroidetes bacteria so far, a set of nitrogen fixation genes have been probably transferred laterally from an as-yet unidentified bacterial lineage in the gut community.

A minimum evolution tree constructed with MEGA4.

# Multiplex symbiotic relationships among cellulolytic protists, their associated bacteria, and their host termite

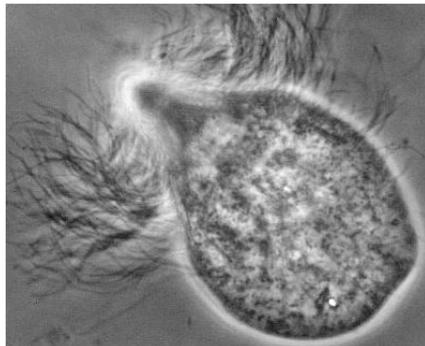
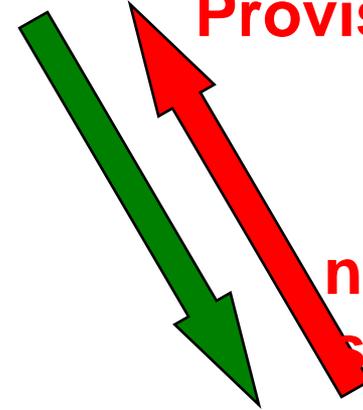


Cellulose fermentation,  
Provision of acetate



Provide stable habitat and cellulose

Provision of acetate  
Provision of nitrogenous nutrients



Provision of nitrogenous nutrients



Cellulose degradation,



# Complete genome of the endosymbionts

The complete genomes were successfully determined each from a single protist cell without cultivation by applying whole genome amplification.

The supply of essential nitrogenous nutrients for host protists is probably a common role of the associating bacteria.

The genomes are suggested to be still in a dynamic process of adaptation as endosymbionts of the gut protists, with their reductive genome evolution.

**The endosymbionts are becoming specialized organelles for supplying and upgrading N-nutrients !?**

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- cellulases

# Meta-EST (meta-transcriptome) of gut protistan community

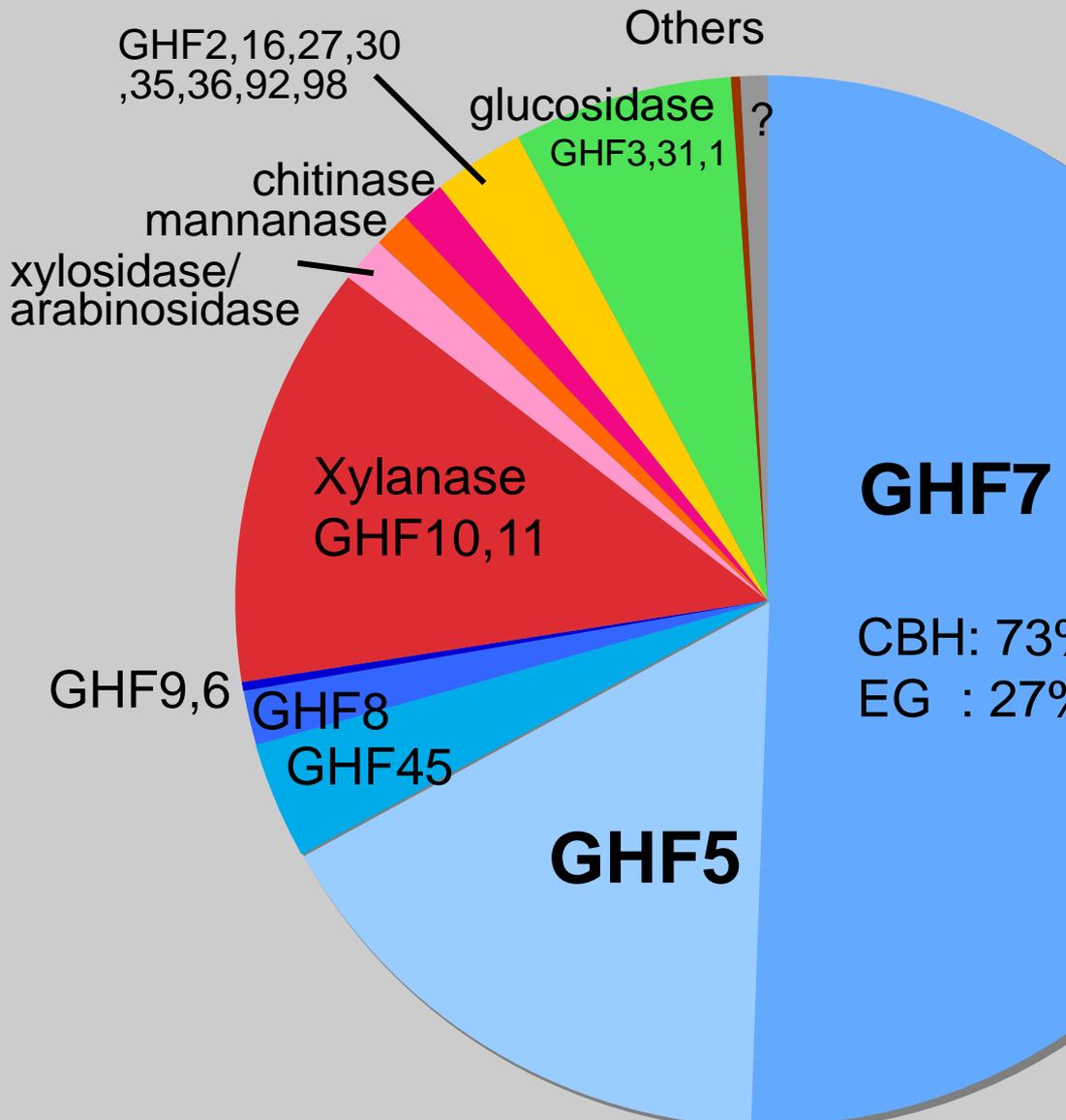
EST: Expressed Sequencing Tags

## Advantages of meta-EST:

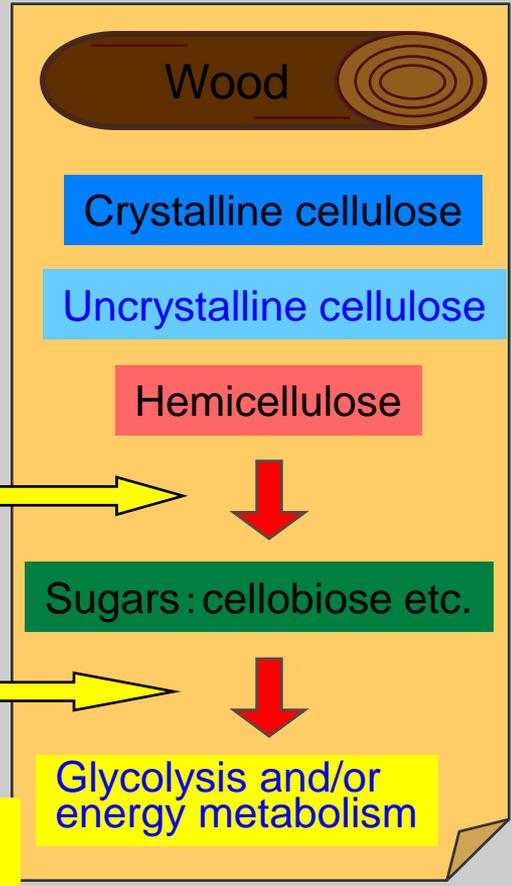
- An efficient screening method of functionally important genes of eukaryotes
- Concentration of only the protists' mRNA in a complex

Meta-EST and the following organismal identifications allow us

- to analyze molecular phylogeny with multiple proteins
- to investigate overall features of cellulose decomposition and primary metabolisms
- to use the gene sequences in the following studies



**Approximately 10% of ESTs corresponded to glycosyl hydrolases in 30 families**  
 (diverse genes even in a family)



**This diversity and the high expression level are probably keys for efficient decomposition**

# Termite Gut Microbial Community: Conclusions

The community is highly structured, coevolving symbiotic complex typified by various associations of protists and bacteria.

The complete genome of the associated bacteria disclosed their hitherto-unknown roles for the symbiotic interaction.

The meta-EST and biochemistry of host protists uncovered their metabolic characteristics for efficient cellulose utilization.

**These complementary approaches have gradually unveiled how this symbiotic complex functions to efficiently utilize recalcitrant lignocellulose.**

# Acknowledgements

## The RIKEN Team

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