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‘*Candidatus* Liberibacter europaeus’ sp. nov that is associated with and transmitted by the psyllid *Cacopsylla pyri* apparently behaves as an endophyte rather than a pathogen

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Running title: '*Ca*. Liberibacter europaeus' associated with *C. pyri*

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Summary

'*Candidatus* Liberibacter spp.' cause serious plant diseases. '*Ca.* L. asiaticus', '*Ca.* L. americanus' and '*Ca.* L. africanus' are the aetiological agents of citrus greening (Huanglongbing) in Asia, America and Africa. '*Ca.* L. solanacearum' causes diseases in Solanaceae in America and New Zealand. All four species are vectored by psyllid insects of different genera. Here, we show that the pear psyllid pest *Cacopsylla pyri* (L.) hosts a novel liberibacter species that we named '*Ca.* Liberibacter europaeus'. It can bloom to high titres in the psyllid host, with more than 10^9 16S rRNA gene copies per individual. Fluorescent *in situ* hybridization experiments showed that '*Ca.* L. europaeus' is present in the host midgut lumen, salivary glands and Malpighian tubules. '*Ca.* L. europaeus' has a relatively high prevalence (> 51%) in *C. pyri* from different areas in the Piedmont and Valle d'Aosta regions in Italy and can be transmitted to pear plants in experimental transmission trials. However, even though high titres of the bacterium (more than 10^8 16S rRNA gene copies g^{-1} of pear plant tissue) could be detected, in the pear tissues no specific disease symptoms could be observed in the infected plants over a six-month period. Despite liberibacters representing potential quarantine organisms, '*Ca.* L. europaeus', first described in Italy and Europe, apparently behaves as an endophyte rather than a pathogen.

Introduction

Huanglongbing (HLB, from the Chinese yellow dragon disease) is a destructive disease of citrus plants that is caused by Gram-negative, phloem-restricted nonculturable α-Proteobacteria belonging to the candidate genus "*Candidatus* Liberibacter" (Bové, 2006 and references therein). To date, three species from HLB liberibacters infecting citrus plants have been recognized: '*Candidatus* L. asiaticus', '*Ca.* L. africanus' and '*Ca.* L. americanus'. Although '*Ca.* L. africanus' is found only in Africa, '*Ca.* L. asiaticus', first described in Asia, has also been found in the Americas together with '*Ca.* Liberibacter americanus' (Lopes *et al.*, 2009; Teixeira *et al.*, 2005). In nature, the transmission of these quarantined (in Europe) pathogens from plant to plant is carried out by citrus psyllids: *Diaphorina citri* (Kuwayama) in Asia and America and *Trioza erytreae* (Del Guercio) in Africa. Recently, another psyllid species, the potato/tomato psyllid *Bactericera cockerelli* (Sulc), has been reported to host and transmit a fourth liberibacter species '*Ca.* L. solanacearum'. It has been reported to cause psyllid yellows and zebra chip diseases on solanaceous plants in New Zealand and America (Abad *et al.*, 2009; Hansen *et al.*, 2008; Liefting *et al.*, 2009; Secor *et al.*, 2009).

Duan *et al.* (2009) showed that '*Ca.* L. asiaticus' is the dominant bacterium associated with *D. citri*, three and half orders of magnitude more abundant than the primary endosymbiont '*Candidatus* Carsonella ruddii'. They suggested that the liberibacter behaves as a symbiont in the psyllid host and thereby is capable of a double lifestyle, pathogenic in the plant and symbiotic in the insect (Duan *et al.* 2009).

To date, the Mediterranean area is still free of the HLB, psyllid yellows and zebra chip diseases as well as the three corresponding vectors (Teixeira *et al.*, 2008). However, other psyllid species living on several temperate fruit trees and vegetables are present (Ben Khalifa *et al.*, 2007; Conci *et al.*, 1993; Micheletti *et al.*, 2005; Tedeschi *et al.*, 2002).

To the best of our knowledge, there have been no reports in the literature dealing with the association of liberibacters with insect hosts other than psyllids. This evidence suggests that psyllids are important insect hosts of these pathogenic bacteria. However, no investigations have yet checked if or how other psyllids can host liberibacters or studied the diversity of liberibacters in different psyllid species.

The objective of this study was to investigate if liberibacters are associated with the psyllid species *Cacopsylla pyri* that is known as an important vector of '*Ca.* Phytoplasma pyri', the cell wall-less Mollicutes that causes pear decline (PD) in pear trees (Seemüller and Schneider, 2004). We characterized the microbiota associated with the psyllid and showed for the first time that a new liberibacter species, here named '*Ca.* L. europaeus', is hosted by *C. pyri* and can be transmitted to pear plants. '*Ca.* L. europaeus' is particular because it can infect pear plants at high titre but without causing any apparent specific symptoms.

Results

Characterization of the bacterial community associated with adults of C. pyri

The diversity of symbiotic bacteria associated with *C. pyri* individuals collected from pear orchards in Valle d'Aosta and Piedmont regions, north-western Italy, was analysed by 16S rRNA gene PCR-DGGE, using as template DNA isolated from whole single individuals. We first used this technique to analyse 36 *C. pyri* individuals (19 males and 17 females). An example of the DGGE profiles is shown in Figure 1. Some variability in the community profiles was observed between different individuals, even though certain bands were conserved among almost all the individuals examined, such as bands A1 and A3 that were detected in 15 and 14 of the 18 individuals examined, respectively (Table 1). Moreover, the intensity of the different bands ranged from intense to very faint. However, PCR-DGGE cannot be used for the quantitative estimation of the target templates, and so a large difference in the intensity among bands suggests that different cell amounts per individual of the different symbionts were observed. The sequences obtained from the different bands excised from the DGGE gels are presented in Table 1 together with their closest relatives found by BLAST search. Band A1 showed 100% identity with '*Candidatus* Carsonella ruddii' the primary endosymbiont of psyllids (Clark *et al.*, 2001; Spaulding and von Dohlen, 1998; Thao *et al.*, 2000), whereas band A2 showed 100% identity with the PD phytoplasma '*Ca.* Phytoplasma pyri' (Seemüller and Schneider, 2004). Band A3 showed 98% identity with both the secondary endosymbiont of *Cacopsylla pyricola* (Förster) (Spaulding and von Dohlen, 2001) and the *Arsenophonus* endosymbiont of *Trichobius yunkeri* Wenzel (Trowbridge *et al.*, 2006). *Arsenophonus* has been reported as a secondary endosymbiont of psyllids such as *D. citri* (Subandiyah *et al.*, 2000a). Band A5 had 100% identity with *Ralstonia sp*. (AB503703). Bacteria of the genus *Ralstonia* have been reported from xylem tissues of the clove tree as well as from the insect *Hindola* spp. in Indonesia (Vaneechoutte *et al.*, 2004). Band A6 had 96% identity with both the *Sodalis*-allied secondary endosymbiont of *Curculio sikkimensis* (Heller) (AB517595) and the primary endosymbiont of *Sitophilus oryzae* (L.) (AF548139).

Interestingly, band A4 had 98% identity with '*Ca.* L. asiaticus' (AB480102). Bacteria in the genus '*Ca.* Liberibacter' are known as plant pathogens causing HLB in different citrus varieties, as well as zebra chip and psyllid yellows in solanaceous crops (Abad *et al.*, 2009; Adkar-Purushothama *et al.*, 2009; Bové, 2006; Hansen *et al.*, 2008; Liefting *et al.*, 2009; Secor *et al.*, 2009; Teixeira *et al.*, 2005; Tyler *et al.*, 2009). Since liberibacters have not yet been reported in pear psyllids and in Europe, efforts were concentrated on the characterization of the phylogenetic position and the evaluation of the prevalence of the newly discovered '*Ca.* Liberibacter' species together with '*Ca.* Phytoplasma pyri' in *C. pyri* individuals and pear plants.

*Phylogeny of the newly discovered '*Candidatus *Liberibacter' sp.*

The almost full length of the 16S rRNA gene (1410 bp) as well as the 16S–23S rRNA gene ITS sequences were obtained from different *C. pyri* individuals by combining the specific Lib223F and Lib451R primers with the universal eubacterial primers as described in the experimental procedures. When aligned by BLAST, the 16S rRNA sequence was shown to share 96% similarity with '*Ca.* L. americanus' (AY742824, EU754742) and 94% with '*Ca*. L. africanus' (L22533, EU754741) and '*Ca.* L. solanacearum' (EU834130). In the phylogenetic tree constructed on the basis of the almost full length of the 16S rRNA gene (Fig. 2), the newly discovered '*Ca.* Liberibacter' clustered in a separate branch from the other four liberibacter species, suggesting that it represents a new species in the genus '*Ca.* Liberibacter'. This conclusion was confirmed by the analysis of the 16S–23S rRNA ITS sequence. DNA amplified with primers Lib223F and ITSREub was directly sequenced in both directions, resulting in a 613 bp long sequence different from the 591, 620 and 1184 bp ITS of '*Ca.* L. americanus' (FJ263693), '*Ca.* L. asiaticus' isolate GuangXi-GL-1 (DQ778016) and '*Ca.* L. asiaticus' from Florida (FJ263704), respectively. The ITS sequence has the same length as the ITS region from the '*Ca*. L. africanus' strain Mpumalanga-UPCRI-06-0071 (EU754741). The ITS region was found to contain the sequences for two tRNA genes: alanine and isoleucine. The comparison of the ITS region from the newly discovered '*Ca.* Liberibacter' with that of the other '*Ca.* Liberibacter' species resulted in 80% identity with the partial sequence of '*Ca.* L. solanacearum' (EU834130) followed by 79% with '*Ca*. L. americanus' (FJ263693). Also, in the phylogenetic tree constructed based on the ITS sequences, the newly discovered '*Ca.* Liberibacter' clustered in a separate branch from the other species (Fig. 3).

These results indicate that the liberibacter identified in *C. pyri* represents a new species for which the name '*Ca.* Liberibacter europaeus' is proposed.

*Localization of '*Candidatus *Liberibacter europaeus' and '*Candidatus *Phytoplasma pyri' in adults of* C. pyri

Fluorescent *in situ* hybridisation (FISH) using specific probes for '*Ca*. L. europaeus' and '*Ca*. P. pyri' allowed their detection in different organs of the insect body. '*Ca.* L. europaeus' was detected in the midgut (Fig. 4A). Both bacteria were colocalized in Malpighian tubules (Fig. 4B) and salivary glands (Fig. 4C). No specific signals were observed in the male (Fig. 4D) and female (Fig. 4E) reproductive organs tested here with all four specific probes. FISH signals with liberibacter/phytoplasma-specific probes were (when present) analogous to those observed by using bacterial probe EUB338, whereas no signals were observed in tissues treated with RNase or in the absence of the probes.

*Prevalence of '*Candidatus *Liberibacter europaeus' and '*Candidatus *Phytoplasma pyri' in* C. pyri *and pear plants*

Specific nested PCR approaches were used to detect '*Ca.* L. europaeus' and apple proliferation (AP) group phytoplasmas in insects and plants. The restriction fragment length polymorphism (RFLP) analyses with SspI and RsaI on nested PCR amplicons confirmed in all cases the identity of the phytoplasma as '*Ca.* P. pyri'. We screened using nested PCRs 238 *C. pyri* individuals and 227 pear plant samples recovered from the field for the presence of the liberibacter and the phytoplasma.

'*Ca.* L. europaeus' and '*Ca.* P. pyri' were detected in 51.7% (123/238) and 26.5% (63/238) of the individuals, respectively. Simultaneous infection with both bacteria was detected in 14.3% (34/238) of the individuals. Out of 131 *C. pyri* male individuals, 54.2% (71/131), 27.5% (36/131) and 15.3% (20/131) were infected by '*Ca.* L. europaeus', '*Ca.* P. pyri' and both the bacteria, respectively. Among the 107 *C. pyri* females tested, 45.8% (49/107), 25.2% (27/107) and 13.1% (14/107) were infected by '*Ca.* L. europaeus', '*Ca.* P. pyri' and both the bacteria, respectively.

Out of the 227 field pear plant samples tested, '*Ca.* L. europaeus' and '*Ca.* P. pyri' were detected in 50.2% (114/227) and 45.3% (103/227) of the plants, respectively. Both the bacteria were simultaneously present in 22.9% (52/227) of the plants.

Nested PCR is a sensitive method for detecting low copy number templates. However, it does not provide the target copy number, as real-time RT-PCR does. To determine the 16S rRNA gene copy number of '*Ca.* L. europaeus' and '*Ca.* P. pyri', quantitative real-time RT-PCR was performed on 76 *C. pyri* individuals and 98 pear plants. Among the 76 *C. pyri* individuals tested, 72.4% (55/76) were positive for the presence of the liberibacter, 51.3% (39/76) for the phytoplasma and 35.5% (27/76) for both bacteria. The higher percentage of positive samples obtained with the quantitative real-time RT-PCR than with the nested PCR can be explained by a higher sensitivity achieved with the former, as shown for the detection of '*Ca.* L. americanus' (Teixeira *et al.*, 2008). The range of '*Ca.* L. europaeus' 16S rRNA gene copies varied from 8.31×10^0 to 1.13×10^9 copies per *C. pyri* individual. Taking into account that there are three copies of rRNA operons in the genome of '*Ca.* Liberibacter' (Duan *et al.*, 2009), there were between 2.77×10^0 and 3.77×10^8 cells of '*Ca.* L. europaeus' per *C. pyri* individual. The range of '*Ca.* P. pyri' 16S rRNA gene copies varied from 2.86×10^1 to 1.7×10^6 copies per *C. pyri* individual. Taking into account that there are two copies of rRNA operons in the genome of '*Ca.* P. pyri' (Schneider and Seemüller, 1994), there were between 1.43×10^1 and 8.50×10^5 cells of '*Ca.* P. pyri' per *C.* pyri individual. Based on the range of eubacterial 16S rRNA gene copies varying between 4.12×10^4 and 4.96×10^9, '*Ca.* L.

europaeus' was shown to represent between 0.001% and 29.8% of the total bacterial community of the insect, whereas '*Ca.* P. pyri' corresponded to 0.001% to 34.4% of the insect microbiome. Among the 98 pear plants tested, 91.8% (90/98) and 84.7% (83/98) were infected with '*Ca.* L. europaeus' and '*Ca.* P. pyri', respectively, whereas 78.6% (77/98) were positive for the presence of both bacteria. Between 6.03×10^{2} and 1.62×10^{8} 16S rRNA gene copies of '*Ca.* L. europaeus' and between 6.32×10^{3} and 1.05×10^{11} gene copies of '*Ca.* P. pyri' were present per gram of plant tissue. Based on the number of 16S rRNA gene operons in the genomes of '*Ca.* Liberibacter' and the PD phytoplasma, there were between 2.01×10^{2} and 5.40×10^{7} cells of '*Ca.* L. europaeus' and between 3.16×10^{3} and 5.26×10^{10} cells of '*Ca.* P. pyri' per gram of plant tissue.

*Transmission of '*Candidatus *Liberibacter europaeus' and '*Candidatus *Phytoplasma pyri' to young pear plants by* C. pyri

Among the 100 field psyllids employed for the transmission trials, DNA was extracted from 62 individuals that showed by nested PCR percentages of infection of 70.2%, 64.5% and 43.6% for '*Ca.* L. europaeus', '*Ca.* P. pyri' and both the bacteria, respectively. Among the 20 psyllids confined in each cage, the percentage of infection ranged from 50% to 100% for '*Ca.* L. europaeus', 50% to 83% for '*Ca.* P. pyri' and 30% to 80% for both bacteria. Two months after the inoculation access period, the first screening of the pear seedlings for the presence of both bacteria was performed and resulted in two out of the 10 plants exposed to *C. pyri* positive for the presence of only '*Ca.* L. europaeus'. All the plants were re-examined 15 days later and the same results were obtained. The presence of the phytoplasma was detected three months after the inoculation access period in one plant that was previously negative for '*Ca.* L. europaeus'. By contrast, none of the samples showing the liberibacter infection in the earlier tests were positive for this bacterium. PCR screenings performed during the following three months (fourth to sixth month after the inoculation access period) resulted in the same infection pattern and no leaves' co-infection events (of the same plant leaf by both bacteria) were shown to happen. The first reddening symptoms were observed on the plant infected with '*Ca.* P. pyri' four months after the acquisition access period, whereas none of the two liberibacter-infected plants showed any symptom of disease even after six months of the monitoring period.

Discussion

Relatively simple community patterns showing six major different bands were observed by PCR-DGGE analysis of the 16S rRNA gene among the different individuals of *C. pyri* analysed. We concentrated our attention on the bacterium that showed a nucleotide identity with '*Ca.* L. asiaticus' and on its association with the pathogen '*Ca.* P. pyri', because of the emerging importance of liberibacters as plant pathogens. Indeed, bacteria in this genus, quarantine agents in the Mediterranean area, have been known to have an economically important impact, such as pathogens of citrus trees in Africa, Asia and America and solanaceous crops in America and New Zealand. An almost full length (1410 bp) sequence of the 16S rRNA gene was obtained by the extension of the '*Ca.* L. asiaticus'-related band detected in the DGGE analysis. This sequence showed 96% similarity to '*Ca.* L. americanus' (AY742824, EU754742) and 94% to both '*Ca*. L. africanus' (L22533, EU754741) and '*Ca.* L. solanacearum' (EU834130, EU935004). Stackebrandt and Goebel (1994) considered 97.5% similarity in the 16S rRNA gene sequence as a minimum to cluster two bacteria in the same species. The percentage similarity in the case of the new liberibacter and '*Ca*. L. americanus' (with which the liberibacter found in *C. pyri* has the highest nucleotide identity) was 96%, indicating that the two liberibacters represent two different species. Indeed, the phylogenetic tree inferred from the 1410 bp 16S rRNA gene sequence (Fig. 2) clustered the bacterium in a separate branch from the four reported species. This phylogenetic position was also confirmed by the 16S–23S rRNA ITS region. The 16S–23S rRNA ITS sequence was 613 bp long and contained the $tRNA^{Ala}$ and $tRNA^{Ile}$-coding genes. The 16S–23S rRNA ITS of '*Ca.* L. asiaticus' (Jagoueix *et al.*, 1997), '*Ca.* L. americanus' (Teixeira *et al.*, 2008), '*Ca.* L. africanus subsp. capensis' (Garnier *et al.*, 2000) and '*Ca.* L. solanacearum' (Liefting *et al.*, 2009) also contained $tRNA^{Ile}$ and $tRNA^{Ala}$, whereas that of '*Ca.* L. africanus' was reported to contain only $tRNA^{Ala}$ (Jagoueix *et al.*, 1997). Compared with the 16S–23S rRNA ITS of the four other `*Candidatus* Liberibacter' species, a sequence identity of 80% and 79% with '*Ca.* L. solanacearum' and '*Ca.* L. americanus' was found, respectively. It has been reported that the ITS sequence does not vary much within a given liberibacter species with isolates of the same species having either identical or very similar intergenic sequences (Ding *et al.*, 2009; Jagoueix *et al.*, 1997; Subandiyah *et al.*, 2000b; Tomimura *et al.*, 2009; Wen *et al.*, 2009). However, a low similarity value was found when the 16S–23S rRNA ITS sequences of two different species were compared. For example, the recently described species '*Ca.* L. solanacearum' had 80%, 79% and 78% sequence similarity with the 16S–23S rRNA ITS sequences from '*Ca.* L. asiaticus', '*Ca.* L. americanus' and '*Ca.* L. africanus', respectively. '*Ca.* L. solanacearum' had 80% similarity with the newly found sequence from *C. pyri,* which confirms the results based on the 16S rRNA gene sequence comparisons and indicates that the new liberibacter is a novel species. Also, in the phylogenetic tree inferred based on the ITS sequence, the bacterium clusters in a branch separated from the other four species. Hence, based on the 16S rRNA gene, the 16S–23S rRNA ITS sequences and the novel host range, the newly discovered sequence should be considered a novel species in the candidate genus '*Candidatus* Liberibacter' to which we attributed the name '*Ca.* L. europaeus' because this is the first time that this genus is reported to occur in Europe. Altogether, both for the 16S rRNA gene and 16S–23S rRNA ITS sequences, the percentage nucleotide identity shows that ‘*Ca.* L. europaeus’ is more closely related to ‘*Ca.* L. solanacearum’ and ‘*Ca.* L. americanus’ than to ‘*Ca.* L. asiaticus’ and ‘*Ca.* L. africanus’. This phylogenetic position was also confirmed and reflected in the phylogenetic trees constructed based on the 16S rRNA gene sequences (Fig. 2) and 16S–23S rRNA ITS sequences.

The localization of ‘*Ca.* L. europaeus’ and ‘*Ca.* P. pyri’ in different organs of *C. pyri* was determined by FISH using specific probes. ‘*Ca.* L. europaeus’ was detected in the midgut and both bacteria were colocalized in Malpighian tubules and in the salivary glands, whereas no specific signals were observed from the testes and ovaries with the four specific probes used (Figs. 4, A, B and C). These results suggest that, similar to ‘*Ca.* P. pyri’ (Hogenhout *et al.*, 2008), once acquired by *C. pyri* from infected plants, ‘*Ca.* L. europaeus’ multiplies in different organs of the insect and mainly in the salivary glands, before being transmitted to other plants during insect feeding events. In addition to this horizontal transmission during feeding events, Hansen *et al.* (2008) reported that '*Candidatus* L. psyllaurous' is also vertically transmitted by *B. cockerelli* to the offspring at different rates depending on the host plant on which the psyllid was reared. By contrast, in the case of *D. citri*, laboratory experiments showed no transovarial transmission of ‘*Ca.* L. asiaticus’ (Hung *et al.*, 2004). Our FISH experiments did not detect liberibacter in the gonads, suggesting that it is not vertically transmitted by *C. pyri*. However, in light of the abovementioned studies, further experiments on larger numbers of *C. pyri* individuals and populations must be performed to definitely exclude a vertical transmission route.

The presence of ‘*Ca.* L. europaeus’ and ‘*Ca.* P. pyri’ was assessed in different adults of *C. pyri* and pear plant samples. Being present in about 52% of the insects tested, ‘*Ca.* L. europaeus’ was more prevalent than the phytoplasma detected in less than 27% of the 238 *C.*

pyri individuals analysed. An infection rate of 45.2% for '*Ca.* L. asiaticus' has been reported in *D. citri* individuals from Indonesia (Subandiyah *et al.*, 2000a). By contrast, Manjunath *et al.* (2008) reported that '*Ca.* L. asiaticus' was present in only 10.5% (105 out of 1200 nymphs and adults) of *D. citri* individuals tested in Florida. This divergence could be because of the inoculum sources (e.g. evergreen vs. deciduous plants) and/or variable efficiencies in the liberibacter uptake in relation to age effects. Among the 227 plant samples tested by conventional and nested PCR, similar infection rates of 50.2% and 45.3% for '*Ca.* L. europaeus' and '*Ca.* P. pyri' were observed, respectively, whereas 22.9% of the plants were positive for the simultaneous presence of both bacteria.

Quantitative real-time RT-PCR analysis allowed the detection of less than three cells (or genome copies) of liberibacter per individual of *C. pyri* and 200 cells per gram of plant tissue. In the case of the phytoplasma, a minimum of about 14 cells per *C. pyri* individual and 3.16×10^3 cells per gram of plant tissue were detected, respectively. The real-time RT-PCR data showed that '*Ca.* L. europaeus' was present in a high density in both insects and plants reaching up to 10^8 cells per individual and up to 10^7 cells per gram of plant tissue, respectively. Similar results were also found for '*Ca.* L. asiaticus' in previous studies performed on *D. citri* in Florida when 3% of the infected population was shown to carry up to 10^8 cells per insect (Duan *et al.*, 2009). In a recent study (Li *et al.*, 2009), an average of 10^{10} '*Ca*. L. asiaticus' genomes per gram of tissue were detected in both the root samples and the above-ground portions of naturally infected citrus trees. By contrast, an average of 10^7 genome copies of '*Ca*. L. americanus' was detected in symptomatic sweet orange plants (Teixeira *et al.*, 2008).

Since '*Ca.* Liberibacter europaeus' was detected both in *C. pyri* salivary glands (as shown by FISH experiments) and pear plant phloem tissue, transmission trials were set up, under outdoor conditions, to confirm the occurrence of the transmission of the bacterium from insects to healthy plants. The results obtained showed that '*Ca.* L. europaeus' could be transmitted to healthy plants during insect feeding events. Although the presence of the bacterium was confirmed in the test plant tissues, we observed no particular symptoms. The absence of symptoms in pear plants (both from the field and the psyllid-inoculated ones under laboratory conditions) could be because of the endophyte nature of '*Ca.* L. europaeus' in pear plants and/or pears might represent an intermediate reservoir of the bacterium that explicate virulence towards other plants when properly transmitted. One fact that supports a role as an endophyte is the presence of the liberibacter in about 92% of the pear plants tested by real-time RT-PCR (90/98) without any disease symptom. If the liberibacter was an endophyte, it could exert a protective effect on the plant because of the competitive displacement of the phytoplasma by competition for the same niche, the phloem cells. Further experimental trials are ongoing to better study such a hypothesis.

In conclusion, the analysis of the microbial community within *C. pyri* allowed the identification of a novel species of liberibacter that is transmitted to pear plants. The results highlight that the diversity of liberibacters is higher than previously reported, with regard to the insect and plant host range and virulence. This bacterium was found to infect pear plants at a high titre without disease symptoms in the field-collected samples or in the laboratory inoculated plants, suggesting an endophytic rather than a pathogenic nature. However, based on the history of liberibacters as plant pathogens, further investigations on these bacteria from plants and insects from other climatic areas and on their transmission and potential virulence should be performed to confirm a virulence-free nature.

Experimental procedures

Insect collection, plant material and DNA isolation

Adults of *C. pyri* (131 males and 107 females) were collected from pear trees between 2008 and 2009 from eight different pear orchards in the Valle d'Aosta (one orchard) and Piedmont (seven orchards) regions, north-western Italy, in areas affected by PD disease. The samples were kept in pure ethanol at –20°C until DNA extraction. Single *C. pyri* individuals were washed three times in distilled water before total DNA was isolated by sodium dodecyl sulfate-proteinase K-cethyltrimethyl ammonium bromide treatment (Sambrook *et al.*, 1989) with one modification: insects were ground in 500 μl of TE buffer, pH 8, using sterile pestles and fine quartz powder. The precipitated DNA was resuspended in 30 μl of TE buffer, pH 8 and kept at –20°C until use. Pear branches from 227 plants were collected from the same eight pear orchards as *C. pyri*. DNA was extracted from 100 mg (wet weight) of plant phloem tissue previously ground with liquid nitrogen in a sterile mortar, according to the DNeasy Plant Mini Kit protocol (Qiagen, Italy) instructions. DNA was eluted in 100 μl of elution buffer and kept at –20°C until used.

PCR amplification and DGGE analysis of the microbiota associated with adults of C. pyri

For PCR-DGGE analysis, primers GC357F, containing a 40 bp GC clamp, and 907R were used to amplify a 550 bp fragment of the 16S rRNA gene using as template total DNA isolated from single *C. pyri* individuals as previously described (Sass *et al.*, 2001). Seven percent (of a 37:1 acrylamide:bisacrylamide mixture in 1× Tris-acetate-EDTA [TAE] buffer) polyacrylamide gels with a denaturing gradient of 40% to 60% (100% denaturing polyacrylamide was defined as 7 M urea and 40% formamide) were made with a gradient maker (Bio-Rad, Milan, Italy) according to the manufacturer's guidelines. For polymerization 50 μl 10% APS-solution (ammonium-persulfate, w/v) and 7 μl TEMED were added. Gels were run for 16 h at 90 V in 1× TAE buffer at a constant temperature of 60°C in a D-Code electrophoresis system (Bio-Rad). The gels were stained for 30 min in 1× TAE buffer containing 1× SYBR Green (Invitrogen, Milan, Italy) and then washed in distilled water for 30 min. Visualization and digital image recording were performed with GelDoc 2000 apparatus (Bio-Rad, Milan, Italy) using the Quantity one software (Bio-Rad).

Sequencing of DGGE bands

DGGE bands were excised from the gel using a sterile blade, eluted at 37°C for 6 h in 50 μl of MilliQ water and kept at –20°C until reamplification. Primers 357F (without GC clamp) and 907R were used for the reamplification of 1 to 9 μl (based on the intensity of the DGGE band) of the eluted DNA fragments. PCR products were sent to Primm (Primm srl San Raffaele Biomedical Science Park, Milan, Italy) for purification and sequencing. Sequencing was performed using the 357F primer as in DGGE but without the GC clamp. The resulting sequences were compared with the sequence database at the National Center for Biotechnology Information using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al.*, 1990; Johnson *et al.*, 2008).

Based on the sequences of the DGGE bands, the sequence of the 16S rRNA of the new '*Ca*. Liberibacter' species was extended by two additional specific PCRs using two specific primers designed in this study. These two primers, named Lib223F, 5'-CCAGGGCTCAACCCTGGAACG-3' and Lib451R, 5'-CTACGCCACTGAATGGTAAAA-3', respectively, corresponding to positions 538–558 and 766–786 of the 16S rRNA gene of '*Ca.* L. solanacearum' (NZ082226) were used in combination with the universal reverse 1494R (5'-CTACGGCTACCTTGTTACGA-3') and the forward 27F (5'-AGAGTTTGATCCTGGCTCAG-3') primers to amplify the flanking regions at the 5' and 3' ends of the DGGE fragment. After amplification, the fragments were sequenced in both directions and aligned with the original sequence from the DGGE fragment. The final sequence obtained by assembling the two 16S rRNA gene contigs was then used to analyse the phylogenetic position of the bacterium. A partial 16S rRNA gene sequence was also obtained by combining the specific and universal primers from pear plant samples using template total DNA isolated from plant phloem.

Sequencing of the 16S-23S rRNA intergenic transcribed spacer

To confirm the taxonomic position of the bacterium characterized based on the 16S rRNA gene sequence, sequencing of the 16S–23S rRNA ITS was also performed. The specific primer Lib223F was used in combination with the universal primer ITSREub (5'-GCCAAGGCATCCACC-3') (Cardinale *et al.*, 2004) for the amplification of the 16S–23S ITS sequences. Based on previous works reporting only one ITS haplotype for the genus '*Ca.* Liberibacter', PCR products were directly sequenced in both directions with primers Lib223F and ITSREub. The obtained ITS sequence was then used to characterize the phylogenetic affiliation of the bacterium.

Phylogenetic analysis

Both partial 16S rRNA and full length 16S–23S rRNA ITS sequences of the new liberibacter species identified in *C. pyri* and pear-infected leaves were subjected to BLAST analysis and aligned with close relatives, as well as with other unrelated eubacterial sequences. Phylogenetic analyses were performed using the Jukes and Cantor distance estimation with the TREECON 1.3b package (Van de Peer and De Wachter, 1994). A 50% majority rule bootstrap consensus tree (1,000 replicates) was generated.

Fluorescent in situ *hybridization*

FISH analysis was carried out on tissues dissected from field-collected *C. pyri* adults in a sterile saline solution. The dissected organs were fixed for 2 min at 4℃ in 4% paraformaldehyde and washed in PBS. All hybridization experiment steps were performed as previously described (Crotti *et al.*, 2009), using the specific fluorescent probes Lib223 (5'-CGTTCCAGGGTTGAGCCCTGG-3') and Lib451 (5'-CTACGCCACTGAATGGTAAAA-3'), matching with the 16S rRNA of '*Ca.* Liberibacter europaeus' and PDF1 (5'-GACCATAGACTTATTAAACCG-3') and PDF2 (5'-CTCAGGCGGAGTACTTAATGC-3'), targeting the 16S rRNA of '*Ca.* P. pyri'. Probes Lib223 and Lib451 were labelled at the 5' end with the fluorochrome Cy5 (indodicarbocyanine, absorption/emission at 650/670nm), whereas probes PDF1 and PDF2 were marked with the fluorochrome Cy3 (indocarbocyanine, absorption/emission at 550/570 nm). As a positive control for the hybridization experiment, a universal bacterial probe EUB388 labelled with fluorescein isothiocyanate (FITC, absorption/emission at 494/520 nm) was also used (Fuchs *et al.*, 1998), by applying the same treatments described in Crotti *et al.* (2009). The probe EUB338 is routinely used as a universal bacterial probe even though several phyla are not completely covered (Daims *et al.*, 1999). Control experiments were performed by applying either the treatment of slides with RNase before the probe hybridization step or the absence of the probe. After hybridization, the samples were mounted in antifading medium and then observed in a laser scanning confocal microscope SP2-AOBS (Leica).

*Prevalence of '*Candidatus *Liberibacter europaeus' and '*Candidatus *Phytoplasma pyri'*

The Lib223F and Lib451R specific primers amplified a 228 bp fragment of the newly discovered sequence. This primer pair was used in heminested PCRs to search '*Ca.* L. europaeus' from insect individuals and plants that did not give an amplification in direct conventional PCR using the universal primer 27F and the specific one Lib451R. PCR amplifications were performed in a 25 μl reaction mixture containing 1× PCR buffer (Invitrogen), 0.12 mM of each dNTP, 0.3 μM of each primer, 1 U of Taq polymerase (Invitrogen) and 1 μl of DNA template. The cycling conditions were as follows (both for direct and nested PCRs): 94°C for 4 min; 10 cycles of 94°C for 1 min, 61°C for 30 s and 72°C for 1 min; 20 cycles of 94°C for 30 s, 56°C for 45 s and 72°C for 1 min; and 7 min at 72°C.

To search '*Ca.* P. pyri', conventional PCRs with phytoplasma universal primers P1/P7 and nested PCR with primers fO1/rO1, specific for AP group phytoplasmas, were performed using the previously described conditions (Schneider *et al.* 1995; Lorenz *et al.*, 1995). Afterwards, 7 µl of the nested PCR products were digested (37°C, 3 h) using 3 U of each of the endonucleases SspI and RsaI. The restriction products were then separated on a 1.5% agarose gel, and '*Ca.* P. pyri' was identified based on the RFLP profile. A total of 238 *C. pyri* individuals, including those examined by DGGE, and 227 pear plants were screened by PCR.

Quantitative real-time RT-PCR for 'Candidatus *Liberibacter europaeus' and* 'Candidatus *Phytoplasma pyri'*

Quantitative real-time RT-PCRs were performed on the Chromo4 real-time detector (Bio-Rad) using Lib223F/Lib451R, PDF for1 (5'-CGGTTTAATAAGTCTATGGTC-3')/PDF rev1 (5'-CTCAGGCGGAGTACTTAATGC-3'), primer sets designed in this study or bacterial universal primers 357F (5'-CTACGGGAGGCAGCA G-3') and 907R (5'-CCGTCAATTCCTTTGAGTTT-3'). To realise standard curves, a 781 bp fragment of the 16S rRNA gene of '*Ca.* L. europaeus' amplified by PCR with primers 27F/Lib451R and a fragment of about 1800 bp fragment obtained with primers P1/P7 (Lorenz *et al.*, 1995) were cloned using a pGEM T-easy Vector Cloning Kit (Promega). Following the calculation of the 16S rRNA gene copies of bacteria and '*Ca*. L. europaeus', the liberibacter-to-bacterial 16S rRNA gene copy ratio (LBR) was calculated and used as an estimate of the relative abundance of '*Ca*. L. europaeus' in the bacterial community associated with different individuals of *C. pyri*. Similarly, the phytoplasma-to-bacterial 16S rRNA gene copy ratio (PBR) was also calculated. For pear plants, the results were expressed as 16S rRNA gene copy numbers (of liberibacter and phytoplasma) per gram of plant phloem tissue, since LBR and PBR could not be estimated because chloroplast and mitochondrial DNA are preferentially amplified when the bacterial primer set used in this study was applied for the amplification of bacterial DNA from plants (Overbeek *et al*., 2006; Tyler *et al*., 2009). The detection limit of the real-time RT-PCR was 8.31×10^{0} 16S rRNA gene copies of '*Ca.* L. europaeus' (corresponds to about three cells of the bacterium) per *C. pyri* individual.

*Transmission of '*Candidatus *Liberibacter europaeus' to pear leaves by* C. pyri

Adults of *C. pyri* were collected from pear orchards where '*Ca.* L. europaeus' was found to be highly present both in plants and insects, to ensure the presence of naturally infected individuals among those used for the trial. *C. pyri* individuals were caged onto young pear plants (three to six months old) obtained from seeds and that had tested negative for the presence of '*Ca.* L. europaeus' by real-time RT-PCR. The insects were confined in five batches of 20 (10 females and 10 males), inside 200 × 200 × 300 mm Plexiglas cages with two sides and a top made of a fine gauze (30/10 net) and containing two test plants. The psyllids were left to feed on the young plants for one week after which they were removed and kept in pure ethanol at –20°C until used for PCR screening. The test plants were also treated with an insecticide (a. i. thiametoxan, trade name Actara®, 30 g hl^{-1}) to kill putative remaining psyllids. Afterwards, the treated seedlings were maintained inside a psyllid-proof screenhouse

(20/10 net) for all the trial period. Two other young pear plants obtained from seeds and that were tested negative for the presence of '*Ca.* L. europaeus' by real-time RT-PCR were caged and treated in the same way as the test plants but with no exposure to psyllids and used as controls. Two months after the insects' removal, one leaf sample from each plant was recovered every 15 days for a total period of four months. Leaf samples were used for DNA extraction and specific PCR screening for the presence of '*Ca.* L. europaeus' and '*Ca.* P. pyri'.

Sequence accession numbers

The sequences of the 16S rRNA gene and 16S–23S rRNA ITS of '*Ca*. L. europaeus' (named strain NR-01) from *C. pyri* (a male individual named ppl6 captured in Valle d'Aosta, Italy) have been deposited in the DDBJ-EMBL-GenBank databases under the accession numbers FN678792 and FN678796, respectively.

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Tables

Table 1. Identification of microorganisms associated with *C. pyri* adults according to DGGE profiles. Band ID is defined according to Fig. 1.

Figures

Fig. 1. Bacterial diversity associated with *C. pyri* adult individuals. Example of DGGE profiles, in 7% polyacrylamide gels with 40% to 60% denaturation gradients, of partial 16S rRNA bacterial genes amplified from DNA extracted from whole insects collected in 2008–2009 from pear orchards in Valle d'Aosta and Piedmont, Italy. Names over the lanes refer to the ID of the tested individuals. The identity of sequences of bands marked with arrows is given in Table 1 according to the band ID (A1–A6).

Fig. 2. Phylogenetic affiliation of the almost entire 16S rRNA gene of '*Ca.* Liberibacter europapeus' constructed using the TREECON 1.3b package. Numbers at each node represent percentages of bootstrap replications calculated from 1,000 replicate trees.

Fig. 3. Phylogenetic affiliation of the entire 16S–23S ITS of the '*Ca.* Liberibacter europaeus' constructed using the TREECON 1.3b package. Numbers at each node represent percentages of bootstrap replications calculated from 1,000 replicate trees.

Fig. 4. Localization of '*Ca*. L. europaeus' and '*Ca.* P. pyri' cells in different organs of *C. pyri*. (A) Midgut. a) Interferential contrast micrograph showing a gut portion of an adult individual. b) CLSM image of FISH of the insect gut after hybridization with the FITC-labelled Eub338 probe targeting all eubacteria. c) CLSM image showing same gut portion after hybridization with the Cy5-labeled probes Lib223 and Lib451 specific for '*Ca*. L. europaeus'. (B) Malpighian tubules. The image of a Malpighian tubule pictured by interferential contrast is shown in (a); the tubule lumen is visible. After hybridization with the eubacterial Eub338 probe, the tube lumen appears to be full of bacterial cells (b), belonging to '*Ca*. L. europaeus', as observed

after hybridization with the Cy5-labeled *Liberibacter* probes (c) and a much weaker signal was obtained after hybridization with the phytoplasma-specific Cy3-labeled probes (d). (C) Salivary glands of *C. pyri*. a) Interferential contrast micrograph of a salivary gland lobe. b–d) CLSM image of FISH of the same salivary gland portion after hybridization with the FITC-labelled universal Eub338 probe (b), with the Cy5-labelled probes Lib223 and Lib451 specific for '*Ca*. L. europaeus' (c) and with the Cy3-labeled probes PDF1 and PDF2 specific for '*Ca.* P. pyri' (d). (D) Male gonads of *C. pyri*. a) Interferential contrast micrograph of *C. pyri* testes. b–d) CLSM image of FISH of the same testes after hybridization with the FITC-labelled universal Eub338 probe (b), the Cy5-labeled probes Lib223 and Lib451 specific for '*Ca*. L. europaeus' (c) and the Cy3-labeled probes PDF1 and PDF2 specific for '*Ca.* P. pyri' (d). (E) Female gonads of *C. pyri*. a) Interferential contrast micrograph of *C. pyri* ovary. b–d) CLSM image of FISH of the same ovary after hybridization with the FITC-labelled universal Eub338 probe (b), the Cy5-labelled probes Lib223 and Lib451 specific for '*Ca*. L. europaeus' (c) and the Cy3-labelled probes PDF1 and PDF2 specific for '*Ca*. P. pyri' (d).

Table 1. Identification of microorganisms associated with *C. pyri* adults according to DGGE profiles. Band ID is defined according to Fig. 1.

Band ID	Most related species	Accession No.	Nucleotide identity (%)	Putative classification	No. positives[a]
A1	'*Ca.* C. ruddii'	AF211131	100 (570/570 bp)	Gammaproteobacteria	30/36
A2	'*Ca.* P. pyri'	AJ542543	99 (519/520 bp)	Mollicutes, Acholeplasmatales	7/36
A3	S-endosymbiont of *C. pyricola*	AF286125	98 (527/533 bp)	Gammaproteobacteria, Enterobacteriales	24/36
A4	'*Ca.* L. asiaticus'	AB480102	98 (499/507 bp)	Alphaproteobacteria; Rhizobiales	13/36
A5	*Ralstonia* sp.	AB503703	100 (530/530 bp)	Betaproteobacteria, Burkholderiales	16/36
A6	*Sodalis*-related secondary endosymbiont of the weevil *Curculio sikkimensis*	AB517595	96 (511/528 bp)	Gammaproteobacteria, Enterobacteriales	4/36

[a] Number of individuals positive for the presence of the specific band in DGGE analysis compared to the total number of individuals analyzed.

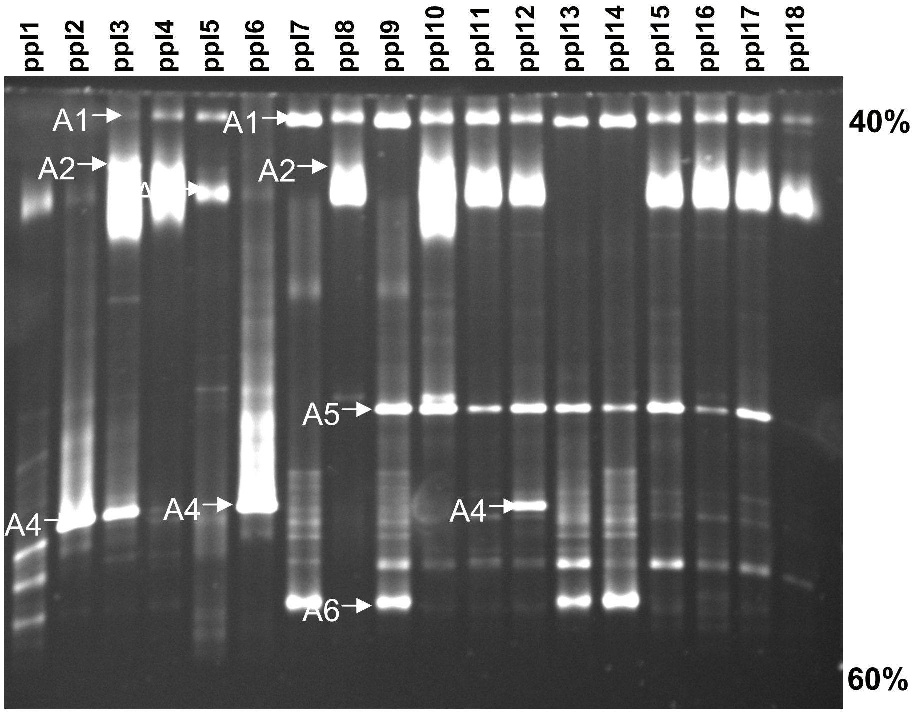

Fig.1. - Raddadi et al.2010-Ms.: *'Candidatus* Liberibacter europaeus' …

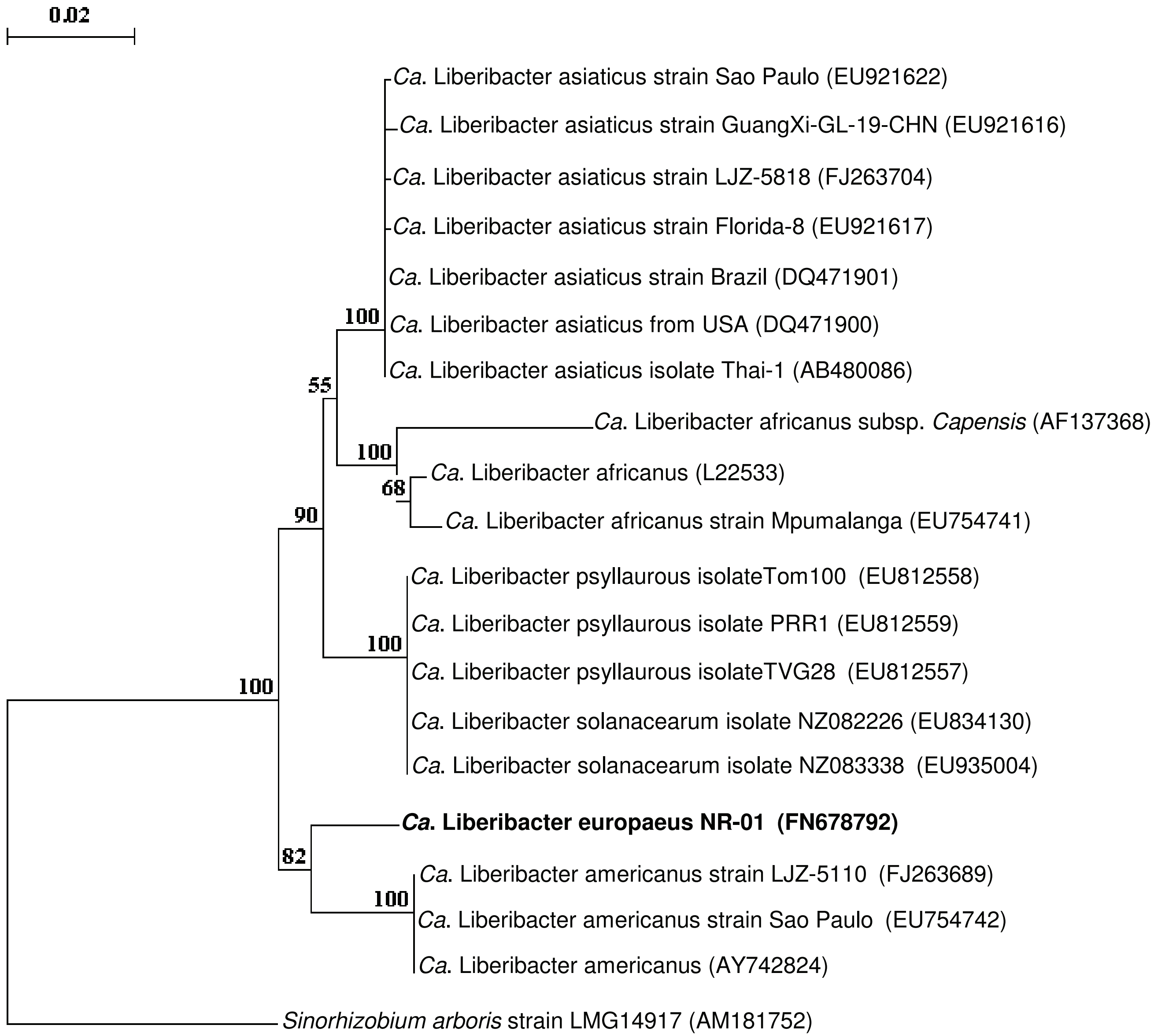

Fig.2. - Raddadi et al.2010-Ms.: ‘*Candidatus* Liberibacter europaeus’ …

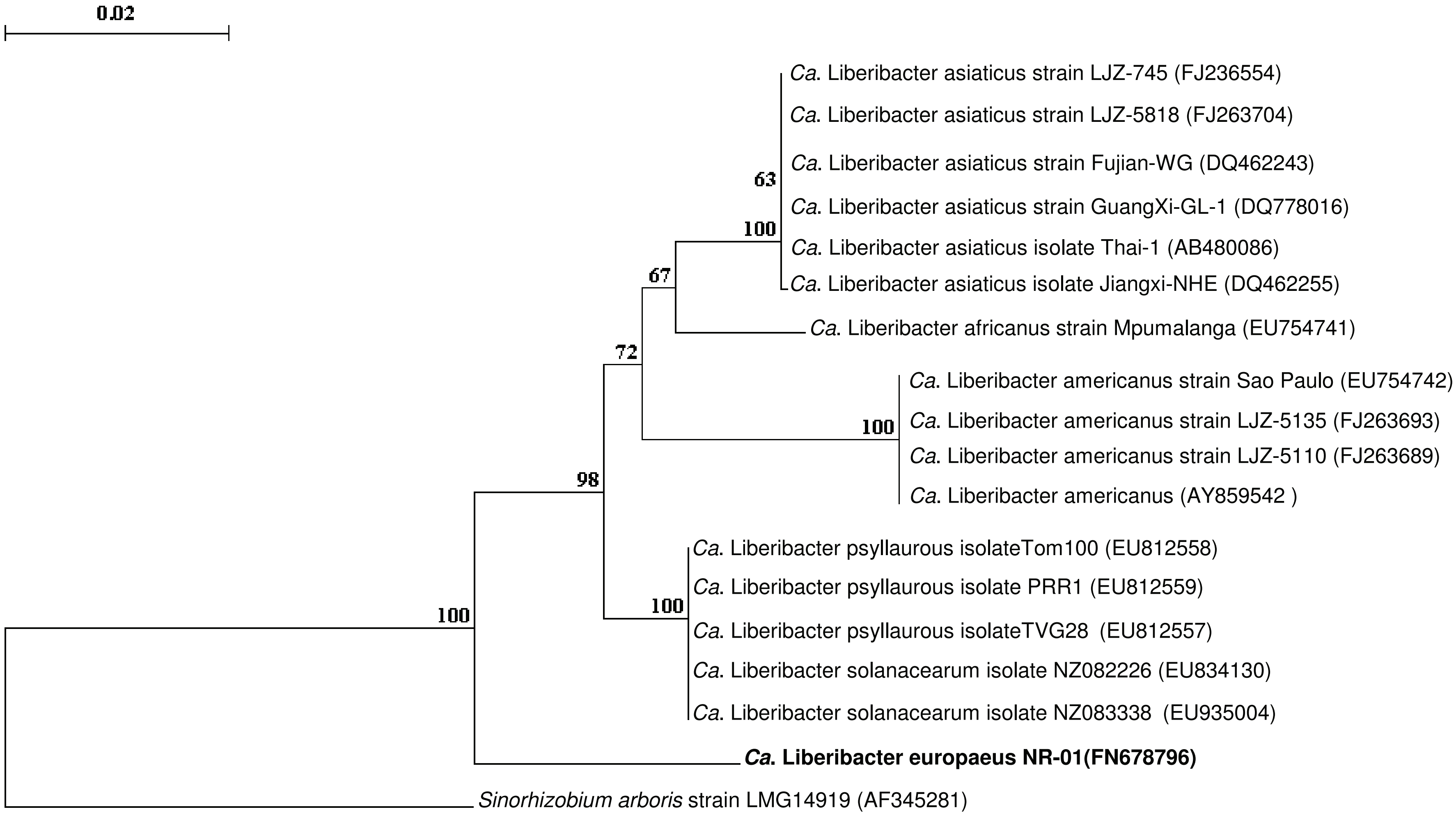

Fig.3. - Raddadi et al.2010-Ms.: ‘*Candidatus* Liberibacter europaeus’ …

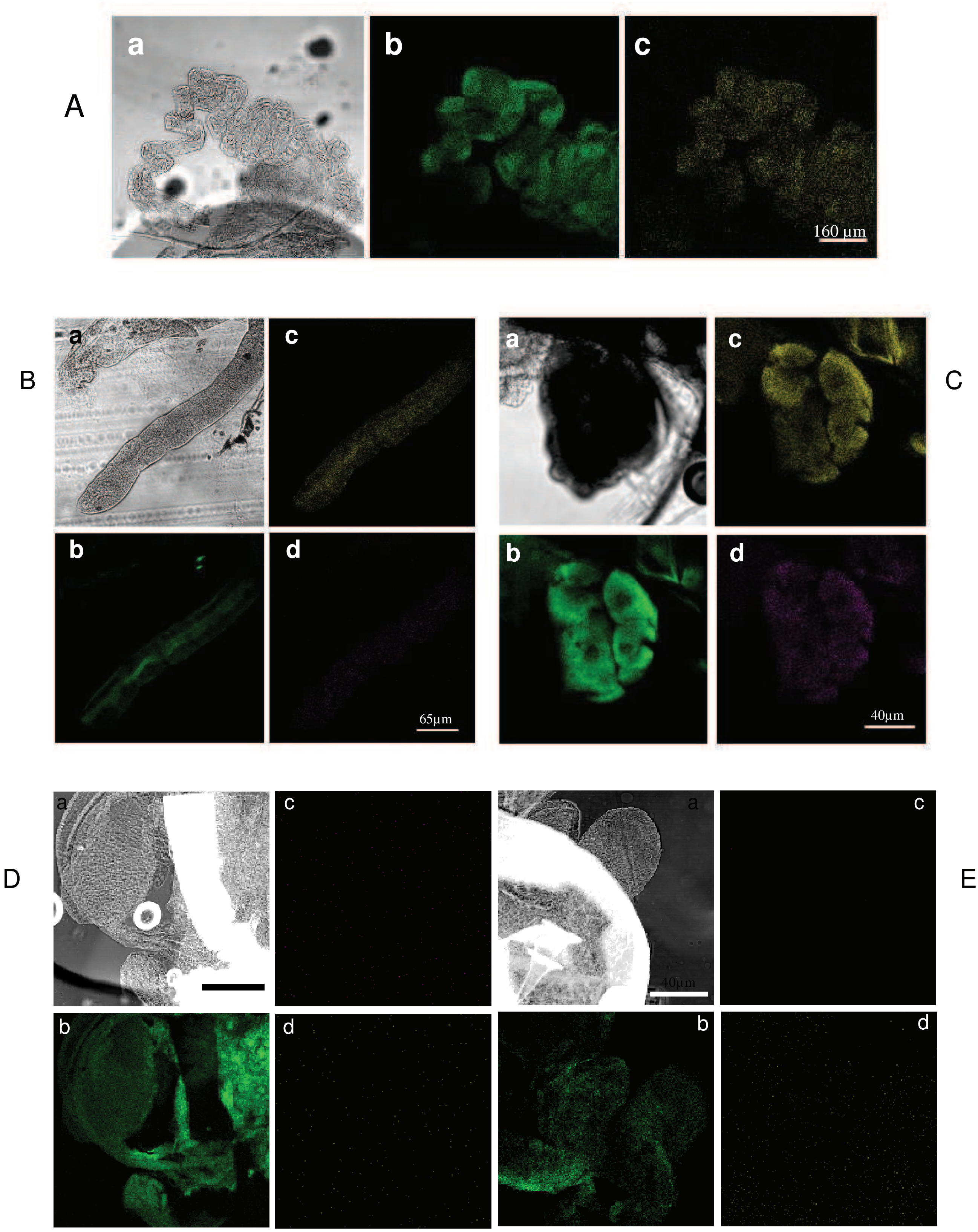

Fig.4. - Raddadi et al.2010-Ms.: *'Candidatus* Liberibacter europaeus' …