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Does *Unaspis euonymi* (Comstock) (Hemiptera: Diaspididae) host *Serratia symbiotica* Moran (Bacteria: Enterobacteriaceae)?

ABSTRACT

The euonymus scale *Unaspis euonymi* (Comstock) (Hemiptera: Diaspididae) is a pest of spindle that exhibits a strong preference for *Euonymus*, although it has been detected on at least 18 genera in 13 plant families (*Buxus, Camellia, Celastrus, Daphne, Eugenia, Euonymus, Hibiscus, Ilex, Jasminum, Ligustrum, Lonicera, Olea, Pachistima, Pachysandra, Perychmenum, Prunus* and *Syringa*) (Salisbury *et al.,* 2013). Heavy infestation by this pest may lead to the death of the host plant and consequential loss of income from the cultivation of ornamental plants (Kaygin *et al.,* 2008). *U. euonymi* is an armored scale insect originally from mild Eastern Asia and probably introduced into Europe in the 20th century (Pellizzari & Germain, 2010). Its lifecycle, depending on climate conditions, comprises two-three generations a year and the control measures to limit its diffusion mainly rely on the use of insecticides or the growing of resistant cultivars.

The insects can engage mutualistic interactions or symbioses with a variety of bacteria that can profoundly affect the host's biology. Apart from obligate symbionts (maternally transmitted), a growing number of facultative or secondary symbionts (that can be horizontally transmitted) have been identified (Sandstrom *et al.,* 2001, Moran *et al.,* 2008). Despite not being essential for the host's life cycle, this last type of symbiont can strongly influence their fitness (Oliver *et al.,* 2003, Jaenike & Brekke, 2011). Additionally, the mutualistic association between insects and bacteria may play a role in the evolution of the latter as described for some groups of *Entereobacteriaceae* (Moran *et al.,* 2005). A number of genomic and phylogenetic studies on mutualistic associations between *Enterobacteriaceae* and aphids, psyllids, scale insects, whiteflies, weevils and other insects have been reported (Lefevre *et al.,* 2004, Thao & Baumann, 2004).

Here we report the identification of *Serratia symbiotica* (strain UESS2016) in *U. euonymi* adult females collected from Sofia (Bulgaria) in 2013.

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U. euonymi samples were collected from a single euonymus plant and immediately stored at 4°C in absolute ethanol until use. Genomic DNA was extracted from four single adult females and from a pool of 20 adult individuals, following purification with the DNA stool kit (Qiagen, Milan, Italy). The identification of *S. symbiotica* was first achieved by sequencing PCR amplicons of an internal region of the16S rRNA bacteria gene (standard universal primers 357f and 907r) (Yu & Morrison, 2004), resolved by electrophoresis of a 35% to 60% linear DNA denaturing gradient gel (DGGE). The 536-nucleotide sequence (KX373616. 1) was found to be identical in both singles and pool of adult females. The sequence was then subjected to NCBI BLAST-N (http://www. ncbi. nlm. nih. ov/blast/) to search for closely related sequences. An identity of 100% spanning the 536 bp was identified with the 16S rRNA gene of the *S. symbiotica* strains CWBI-2. 3 (NR_117512. 1) isolated from the black bean aphid *Aphid fabae* (Sabri *et al.,* 2011) and AFFBNST (KT176016. 1), also isolated from *A. fabae*. These matches showed the highest score in all three of the available NCBI databases we searched (prokaryotic_16S-ribosomal_RNA; nr and wgs) (tab. 1). The identification of UESS2016 as *S. symbiotica* was further confirmed by amplification and nucleotide sequence analysis of three housekeeping genes (namely *rpoB*, *groEL* and *gyrB*, KX387568 to KX387570). Primers and PCR conditions were as described previously (Scrascia *et al.,* 2016). An identity of at least 99% was found with the *S. symbiotica* strains CWBI-2. 3 and SCt-VLC for all three housekeeping genes (tab. 1). CWBI-2. 3 was identified as a natural clone, isolated and characterized from *A. fabae* collected in Belgium in 2009 and proposed as a novel secondary symbiont. The *S. symbiotica* strain SCt-VLC was identified in *Cinara tujafilina* (collected in Spain) and also reported as a secondary symbiont that shows a variety of metabolic, genetic and architectural features consistent with an intermediate stage from a facultative to a reduced intracellular endosymbiont (Manzano-Marin & Latorre, 2014).

Following our detection in *U. euonymi* we searched the Genbank database for records of *S. symbiotica* with either host or organism annotation finding more than 600 sequences. We filtered out sequences with a percentage of identity versus NR_117512. 1 lower than 97% (a standard threshold below which bacteria species are clearly different) (Mizrahi-Man *et al.,* 2013), then selected the best matching sequence for each of the remaining host species (fig. 1; tab S1). Within the *Aphididae familia* of the phylum *Arthropoda*, *S. symbiotica* was only reported in *Formica cinerea* isolated in Finland in 2001 (tab. S1) (Sirvio & Pamilo, 2010). This species known as the velvet or silky ant, is distributed from South Western Asia to Central Europe and locally in Northern Europe too. The 16S rRNA nucleotide sequence reported for *S. symbiotica* identified in *F. cinerea* was 99. 78% identical to that identified in the strain CWBI-2. 3. Detection of *S. symbiotica* in both *F. cinerea* and aphids may be explained by the habit of ants to breed them for honeydew (Sirvio & Pamilo, 2010). This would supply a potential transmission route of this bacterium either as a true facultative symbiont in ants or as a transient bacterium in their gut.

The presence and horizontal transmission of facultative symbionts, like *S. symbiotica*, is a matter of growing ecological and evolutionary interest. In *Aphididae* (e. g. in pea aphids) they may confer putative fitness benefits such as a wider tolerance to the detrimental effects of temperature variations (Burke *et al.,* 2009, Oliver *et al.,* 2010). Additionally, it has been suggested that facultative symbionts may constitute a "horizontal gene pool" among different host lineages, which influences the adaptation and distribution of their insect hosts (Henry *et al.,* 2013). The carriage of identical or closely related symbiont genotypes seems, at least for aphids, related to the same shared ecological niche.

In this study we report the identification of *S. symbiotica* in *U. euonymi* samples collected in Bulgaria from heavy infested euonymus plant. The potential role played by this bacterium as facultative symbiont in *U. euonymi*, at the present time, needs further investigations although such a possibility seems consistent with the findings of this study.

Tab. 1 - Blast analysis

A) Best match in database

Query	**Subject**										
Gene	**Accession**	**size (bp)**	**NCBI Database**	**Identities**	**Species**	**Strain**	**Source**	**Country**	**Accession**	**Start**	**End**
16S rDNA	KX373616. 1	536	prok_16S_rRNA	100%	*S. symbiotica*	CWBI-2. 3	*Aphis fabae*	Belgium	NR_117512. 1	360	895
			nr	100%	*S. symbiotica*	clone AFFBNS7	*Aphis fabae*	India	KT176016. 1	376	911
			wgs	100%	*S. symbiotica*	CWBI-2. 3T SYMBAF_Contig_9	*Aphis fabae*	Belgium	CCES01000032. 1	3902	4437
rpoB	KX387568. 1	783	nr	99%	*S. symbiotica*	SCt-VLC genomic scaffold 09	*Cinara tujafilina*	Spain	FR904238. 2	92720	93502
			wgs	99%	*S. symbiotica*	CWBI-2. 3T SYMBAF_Contig_17	*Aphis fabae*	Belgium	CCES01000009. 1	14688	15470
groEL	KX387569. 1	781	nr	99%	*S. symbiotica*	SCt-VLC genomic scaffold 01	*Cinara tujafilina*	Spain	FR904230. 1	178859	179639
			wgs	99%	*S. symbiotica*	CWBI-2. 3T SYMBAF_Contig_18	*Aphis fabae*	Belgium	CCES01000010. 1	41368	32148
gyrB	KX387570. 1	746	nr	99%	*S. symbiotica*	SCt-VLC genomic scaffold 04	*Cinara tujafilina*	Spain	FR904233. 1	137707	138449
			wgs	100%	*S. symbiotica*	CWBI-2. 3T SYMBAF_Contig_14	*Aphis fabae*	Belgium	CCES01000006. 1	47562	48307

B) Best match in database, limited to non-symbiotica organisms

Query	**Subject**										
Gene	**Accession**	**size (bp)**	**NCBI Database**	**Identities**	**Species**	**Strain**	**Source**	**Country**	**Accession**	**Start**	**End**
16S rDNA	KX373616. 1	536	prok_16S_rRNA	98%	*S. proteamaculans*	568	*P. trichocarpa* (root)	-	NR_074820. 1	376	911
			nr	98%	*S. plymuthica*	HC27	soil	China	KJ605413. 1	348	883
			wgs	98%	*S. plymuthica*	A30 sply. contig. 15	*S. tuberosum*	Netherlands	AMSV01000014. 1	676	1211
rpoB	KX387568. 1	783	nr	92%	*S. proteamaculans*	568	*P. trichocarpa* (root)	-	CP00082. 1	313621	314403
			wgs	93%	*S. ficaria*	NBRC 102596 SFI01_CON0039_0001	*H. sapiens*	Belgium	BCTS01000045. 1	4365	5147
groEL	KX387569. 1	781	nr	90%	*S. proteamaculans*	568	*P. trichocarpa* (root)	-	CP00082. 1	458622	459402
			wgs	90%	*S. fonticola*	LMG 7882 serratia7882_scaffold27	*freshwater*	Portugal	AVAH01000027. 1	33244	34024
gyrB	KX387570. 1	746	nr	88%	*S. marcescens*	S8	*R. ferrugineus Oliver*	Italy	KT992362. 1	163	910
			wgs	89%	*S. ficaria*	NBRC 102596 SFI01_CON0039_0001	*H. sapiens*	Belgium	BCTS01000045. 1	13081	13828

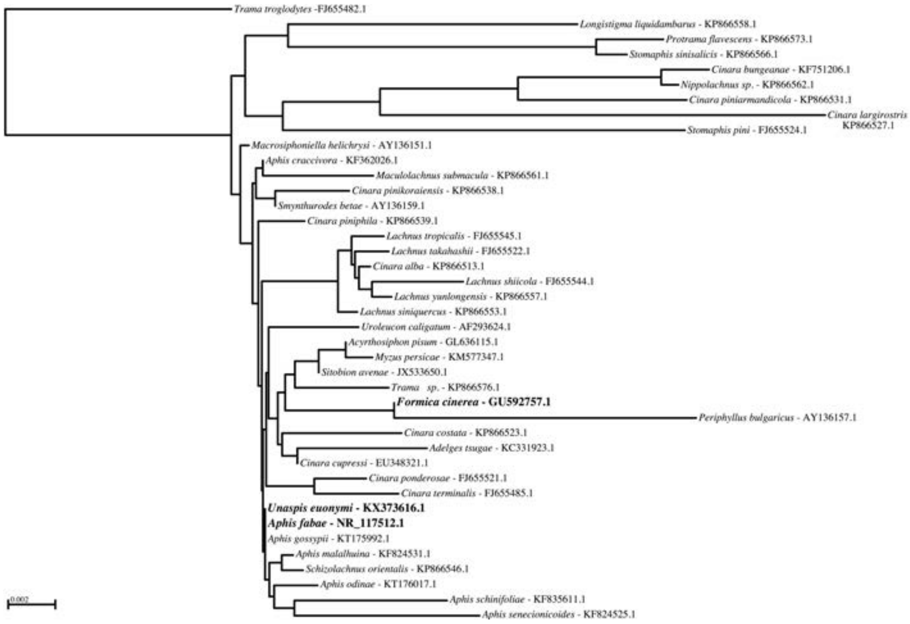

Fig. 1 - Phylogenetic tree
The branches report the host species and accession number of each *S. symbiotica* 16S rRNA sequence (see tab. S1 for more details).

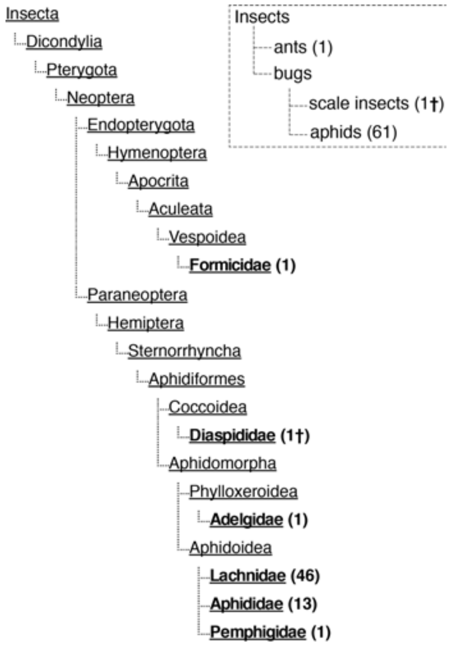

Fig. 2 - Taxonomy tree of *familiae* that are known to host *S. symbiotica*
The number of species in each *familia*, which are recorded to host these bacteria, is reported between brackets. A simplified tree, using common names, is in the upper right box.
† indicates position of the *Unaspis euonymi* in both trees.

The high percentage of 16S rRNA identity shared between *S. symbiotica* found in UESS2016 and the 16S rRNA of *S. symbiotica* detected in evolutionary distinct species of insects (e. g. *A. fabae* and *F. cinerea*) (fig. 2) is an interesting datum. Further studies in this direction may lead to the identification of ecological niches (e. g. ornamental plants like Euonymus) where the horizontal transfer of facultative symbionts could take place.

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