

## Facultative endosymbionts of aphid populations from coastal dunes of the North Sea

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**ABSTRACT.** Aphids establish symbiotic associations with a diverse assemblage of mutualistic bacteria. Some of them are not required for the host's survival but still have a crucial impact on the biology and ecology of their host. Facultative symbionts may modify important host-life-history traits and affect the interactions of aphids with other members of the community. So far several species of aphid have been reported to occur in coastal dunes. Given the extreme environmental conditions of this type of habitat and the wide distribution along the European coast of some aphid species, these aphids would be expected to show variation in their facultative endosymbionts. However, there is currently no information available for these species. To address this question, we collected specimens from different populations of aphids (i.e. *Schizaphis rufula*, *Laingia psammae* and *Rhopalosiphum padi*) associated with the dune grass *Ammophila arenaria* in several locations of the North and the Irish Sea. By means of specific diagnostic PCR's we checked for the presence of facultative bacterial endosymbionts in these populations. Results of this explorative assessment showed variation in the endosymbiont community according to species and location. All populations sampled along the North Sea coast were associated with the facultative endosymbiont *Serratia symbiotica*. *Hamiltonella defensa* was only detected in some specimens coming from the population in Het Zwin, Belgium. *Regiella insecticola* and the  $\gamma$ -proteobacteria X-type were only found associated with the population of *Schizaphis rufula* in De Panne, Belgium. Although further experiments are necessary to characterize the nature of these symbiotic relationships, our correlation analyses showed a significant co-occurrence of *S. symbiotica* with *H. defensa* and *R. insecticola* with X-type proteobacteria suggesting reciprocal regulatory functions. No significant correlation was detected between the number of mummies (i.e. carcasses of aphids parasitized by wasps) and the occurrence of bacterial symbionts. The potential role of these symbionts in coastal dune ecosystems is discussed.

**KEY WORDS:** wasps, parasitoids, heat shock, specific primers, top-down, bottom-up control

### INTRODUCTION

Aphids are one of the most common insect groups studied for symbiotic associations (OLIVER et al., 2010; MORAN et al., 2008). Aphids engage in symbiotic associations with a diverse assemblage of heritable bacteria. In addition to the obligate endosymbiont *Buchnera aphidicola*, aphids may carry one or more facultative bacterial symbionts. Although these symbionts are not required for the survival of the aphid, they may transfer beneficial features to their

hosts such as increased resistance against natural enemies and pathogens, protection from heat shocks, and more importantly influence survival and fitness on specific host plants (OLIVER et al., 2010; LEONARDO & MONDOR, 2006).

Several aphid species have been reported to occur along the Western European Atlantic coast and the North Sea, including *Schizaphis rufula* (WALKER, 1849), *Laingia psammae* (THEOBALD, 1922), *Metapolophium sabiahe* (PRIOR, 1976) and *Rhopalosiphum padi* (1758) (BRÖRING

& NIEDRINGHAUS, 1989; VANDEGEHUCHTE et al., 2010). These species are usually found on *Ammophila arenaria* (L.) Link but also on other dune grasses thriving in pioneer dunes e.g. *Elymus farctus*, *Festuca rubra* and *Leymus arenaria*. From laboratory observations we know that these aphid species reproduce freely on young *Ammophila arenaria* shoots and spikes (VANDEGEHUCHTE et al., 2009; VANDEGEHUCHTE et al., 2010) but the factors underlying their ecology and population dynamics in the field remain relatively unexplored. Aphid populations colonizing coastal dunes do not commonly reach high densities as they are controlled either by natural enemies or by constitutive and induced plant defenses regulated by plant mutualists such as fungal endophytes (VANDEGEHUCHTE et al., 2013; DE LA PEÑA et al., 2006). Moreover, for some aphid species the endosymbiont community plays an important role in defining the host-plant range and the ability to exploit

certain plant species (LUKASIC et al., 2013, MORAN et al., 2008). Therefore, to understand aphid-plant interactions in coastal dunes the endosymbiont community in dune aphids needs to be characterized.

Coastal dunes are extreme environments, where both plant and animal species have to cope with several environmental stresses such as sand accretion, salt spray, extreme temperature variability, wind, etc (MAUN 2009). In addition to these abiotic factors, aphids have to deal with the host-plant defences, other herbivore competitors exploiting the same host-plants, and their natural enemies. Mutualism with facultative (i.e. non-essential) heritable bacteria may influence the biology of these insects, and can have major (positive and negative) effects on the host's fitness (MORAN et al., 2008). Facultative symbionts of aphids can confer protection against insect parasitoids and also

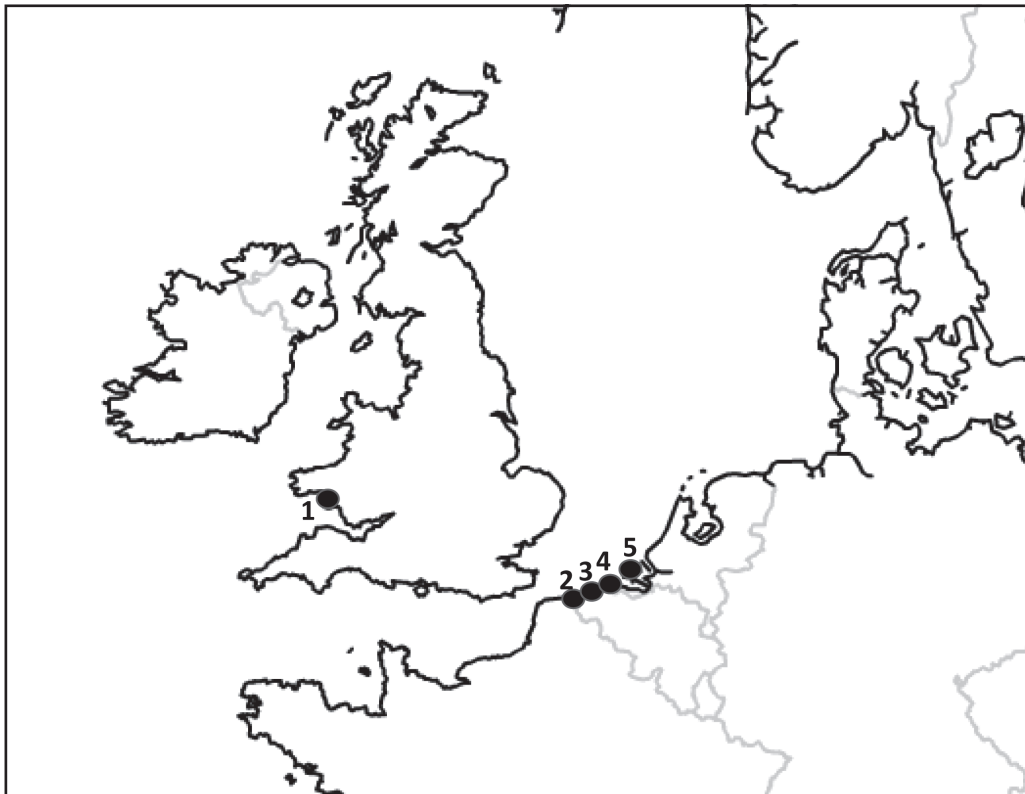


Fig. 1. – The five geographic locations sampled, and the aphid species found, in this study. 1. Ynyslas (Wales, UK): *Schizaphis rufula*. 2. Westhoek (Belgium): *S. rufula* and *Rhopalosiphum padi*. 3. Ter Yde: *S. rufula*. 4. Sluis-Het Zwin (the Netherlands): *S. rufula* and *Laingia psammae*. 5. Duinnoord (the Netherlands): *S. rufula*.

increase resistance to extreme temperatures. Recent research suggests that in the pea aphid, *Acyrtosiphon pisum*, the population structure of some species of facultative symbionts is mostly influenced by climate. In particular, symbiont species that confer resistance to heat shocks have been found to be commonly associated with aphids from arid regions (HENRY et al., 2013).

With such *a priori* knowledge it is not far-fetched to assume that dune aphids rely on such mutualistic interactions to better survive and we report here the first records on dune aphid-endosymbiotic bacteria associations in several locations of the Atlantic and North Sea coast in Western Europe.

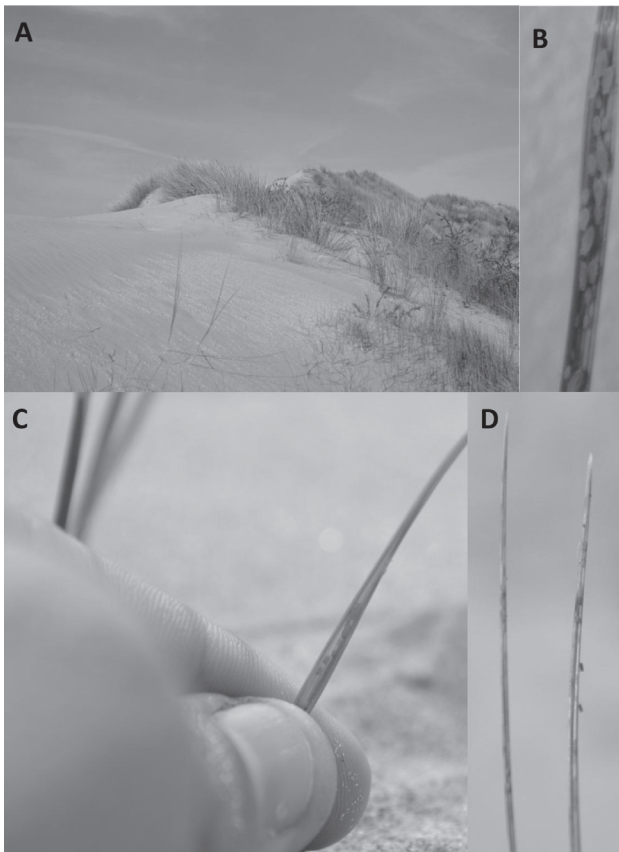


Fig. 2. – A. Foredunes with vigorous *Ammophila arenaria* where the different populations of aphids were collected. B. Infestation of marram leaves by the aphid *Schizaphis rufula*. C-D. Symptoms of aphid multiplication: yellowing of leaf tips.

## MATERIAL AND METHODS

### Sampling surveys and establishment of cultures of aphid isolates

In total, five locations with active dune systems dominated by *A. arenaria* were sampled along the coast of the North Sea and the Irish Sea (Fig. 1). The first sampling survey took place in June 2011. In the field, plants were visually inspected to detect aphid populations feeding on *A. arenaria* shoots (Figs 2-3). Once aphids were detected, they were manually collected and transferred to an eppendorf tube filled with 100% ethanol. From each site we collected aphids from at least four different plants. During this first sampling survey individuals of the species *S. rufula* were retrieved from De Panne, Ter Yde and Het Zwin. In De Panne (Belgium), individuals of the species *R. padi* were also detected and sampled. Once in the lab, the identity of 10-15 aphids from each location was double-checked and this bulk sample was further used for DNA extraction.

Since the preliminary assessment based on bulk samples revealed the presence of bacterial endosymbionts, in a second sampling survey, aphids were individually screened for symbiont infection to assess the frequency of infection by different endosymbiotic bacteria within a site. In October 2011, the same populations were revisited and aphids were taken to the laboratory alive in order to establish cultures of the different isolates. Also in October 2011, parasitoid impact was assessed by counting the number of mummies (i.e. carcasses of aphids parasitized by parasitoid wasps) and healthy aphids on the surveyed plants and locations. Once in the lab, leaves infested with aphids were transferred to *A. arenaria* seedlings that had been previously prepared as in DE LA PEÑA et al. (2010). To ensure aphids were kept in conditions as natural as possible, we reared them in sympatric *A. arenaria* plants. In our second survey, we did not detect *R. padi* as in the preliminary survey, and instead we detected *L. psammae* (Fig. 3) in plants from the location sampled in the Netherlands. From each site and species, we established between ten to

fifteen aphid lines (i.e. from a single female), which we kept in the laboratory under long photoperiod (16/8h light/dark regime) to ensure continuous asexual reproduction. In order to assess the degree of incidence of endosymbionts per species and population we checked for the presence of the different endosymbionts in 10 aphid-lines per population.

With the data for *S. rufula* from the second sampling survey, a Spearman correlation analysis was conducted to infer patterns in the simultaneous occurrence of the different facultative endosymbionts and the number of mummies (i.e. carcasses of aphids that have been parasitized by wasps) detected in the field.

#### Extraction of DNA and PCR for molecular identifications

Genomic DNA was extracted using the NucleoSpin® Tissue Kit (Macherey-Nagel). The facultative endosymbiont communities of

the different aphid populations were assessed with diagnostic PCRs (Polymerase Chain Reaction)s using specific primers for the 16S ribosomal RNA genes for the following bacterial species (commonly found in the pea aphid, *Acyrtosiphon pisum* model system): *Hamiltonella defensa*, *Regiella*, *Serratia symbiotica*, *Rickettsia*, *Spiroplasma* and the and the bacterial complement X-type- a  $\gamma$ -Proteobacteria- (see FERRARI et al., 2012 and MCCLEAN et al., 2011 for further information). The amplification of the 16S ribosomal RNA gene was done using a universal bacterial primer 10F, 35R. These primers are able to detect a wide range of Eubacteria. This initial amplification was followed by a diagnostic PCR using specific primers (Table 1) to detect the specific bacterial endosymbionts. PCRs were performed in a final volume of 10  $\mu$ L containing  $\leq 20$ ng/ $\mu$ l of genomic DNA, 1 x PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.2mM each dNTP, 0.25mM each primer and 1U of *Taq* DNA polymerase. Thermal profile for amplification included an initial denaturation step at 95 °C for 5 min, followed by 30 cycles

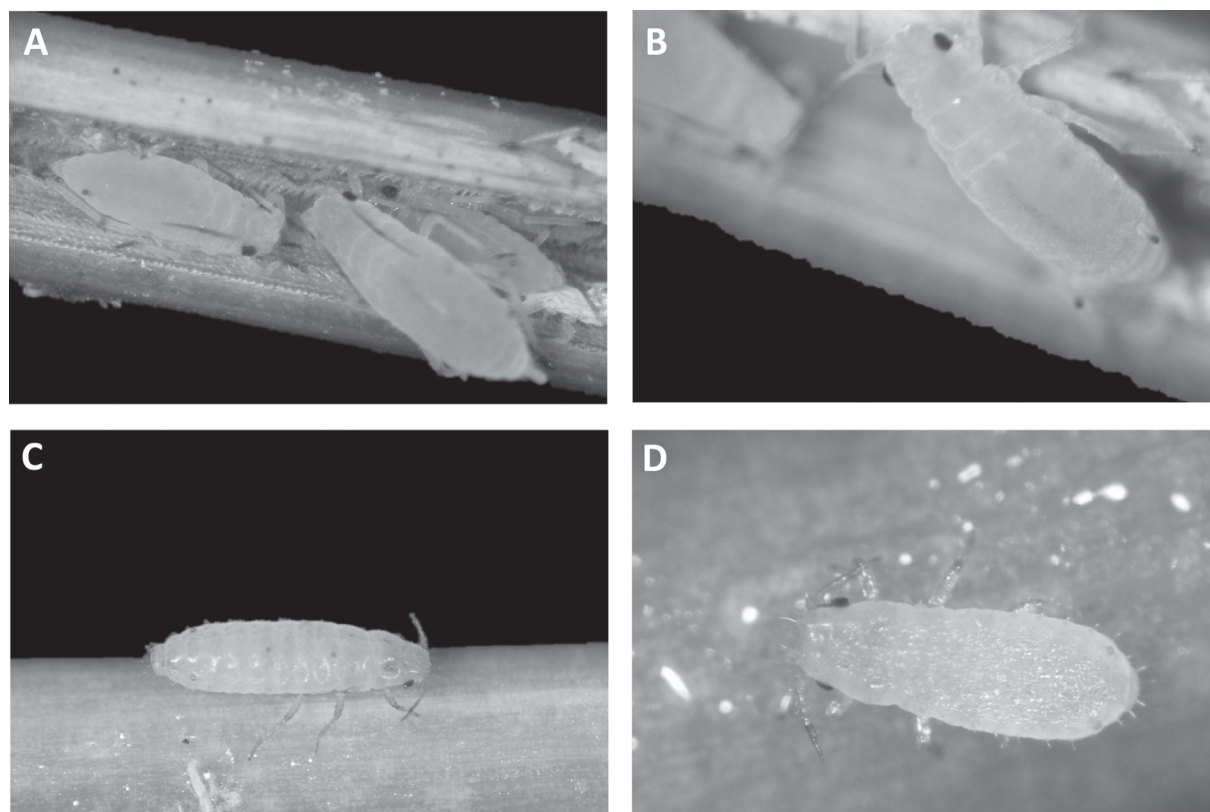


Fig. 3. – *Schizaphis rufula* (A, B) and *Laingia psammae* (C, D) on leaves of *Ammophila arenaria*.

TABLE 1

Specific primers and PCR conditions for diagnostic symbiont detection. From MCLEAN et al., 2010 and FERRARI et al., 2011.

Symbiont species	Forward primer	Reverse primer	PCR programm
<i>Hamiltonella defensa</i>	10F 5'- AGTTTGATCATGGCTCA- GATT-3'	T419R 5'- AAATGGTATTCGCATT- TATCG-3'	1
<i>Regiella insecticola</i>	10F	U443R 5'- GGTAACGTCAATCGATAAG- CA-3'	1
<i>Serratia symbiotica</i>	10F	R443R 5'- CTTCTGCGAGTAACGTCAA- TG-3'	1
X-Type	10F	X420R 5'- GCAACACTCTTTGCAT- TGCT-3'	1
<i>Rickettsia</i>	16SA1 5'- AGAGTTTGATCMTGGCT- CAG-3'	Rick16SR 5'- TTTGAAAGCAATTCCGAG- GT-3'	1
<i>Spiroplasma</i>	10F	TKSSsp 5'- ATCATCAACCCTGCCTTT-3'	2

Cycling conditions:

Programm 1: 94°C 2 min, 10 cycles of (94°C 1min, 65°Cà55°C in 1°C steps each cycle 1min, 72°C 2min), 25 cycles of (94°C 1min, 55°C 1min, 72°C 2min), 72°C 6min.

Programm 2: 94°C 2 min, 35 cycles of (94°C 1 min, 54°C 1 min, 72°C 2 min), 72°C 6 min.

of 30 s at 94°C, 30 s at Ta ( $\gamma$ -Proteobacteria 50°C, *Hamiltonella defensa* 57°C, *Regiella insecticola*, *Serratia symbiotica* 57°C, X-type 57°C, *Rickettsia* 45°C, *Spiroplasma* 45°C) and 1 min at 72°C; a final step at 72 °C for 10 min was used to complete primer extension. PCR products were visualized after electrophoresis on a 1.2% agarose gel stained with GelRed. Since some PCR reactions produced faint bands, all PCR reactions were repeated twice to discard potential false positives. Furthermore, some of the PCR products were sequenced to confirm their identity based on sequence homologies (from GenBank) (BENSON et al., 2013). For

this purpose, PCR products were purified using Exonuclease I and the purification kit FastAP™ (Fermentas). The purified PCR products were sequenced on both strands by Macrogen (Seoul, Korea) using the PCR primers.

## RESULTS

The results of the assessment of the aphid populations collected during the sampling surveys showed that facultative endosymbionts are common and widespread in aphid populations occurring in coastal dunes (Table 2). PCR

TABLE 2

Overview of endosymbionts in *Schizaphis rufula*, *Rhopalosiphum padi* and *Laingia psammae* based on the results of PCR amplifications using specific primers and posterior confirmation through sequence blasting.

Species	Location	<i>Hamiltonella defensa</i>	<i>Regiella</i>	Type-X	<i>Serratia symbiotica</i>	<i>Rickettsia</i>	<i>Spiroplasma</i>
<i>S. rufula</i>	Duin-Noord, Netherlands	No	No	No	<b>Yes</b>	No	No
<i>S. rufula</i>	Belgium, Het Zwin	<b>Yes</b>	No	No	<b>Yes</b>	No	No
<i>S. rufula</i>	Belgium, Ter Yde	No	No	No	<b>Yes</b>	No	No
<i>S. rufula</i>	Belgium, De Panne	No	<b>Yes</b>	<b>Yes</b>	<b>Yes</b>	No	No
<i>S. rufula</i>	Wales (UK), Ynyslas	No	No	No	No	No	No
<i>R. padi</i>	Belgium, De Panne	No	No	No	<b>Yes</b>	No	No
<i>L. psammae</i>	Belgium, De Panne	No	No	No	<b>Yes</b>	No	No

amplifications using specific primers for *H. defensa* yielded positive results (i.e. with an amplification band of *ca.* 490 bp) in one population, i.e. Het Zwin (the Netherlands). *Serratia symbiotica* was detected in all specimens tested except for the population of *S. rufula* from Ynyslas (Wales). The population of *R. padi* was found to be only associated with *S. symbiotica*. In all cases amplification bands had a size of *ca.* 890bp.

In the second assessment, using 10 aphid-lines, a different pattern in the results was observed (Fig. 4). Again, only the *S. rufula* population from Het Zwin was infected with *H. defensa*. *Serratia symbiotica* was once more the most common facultative endosymbiont although this time, the bacterium was not detected in specimens of *S. rufula* from Ter Yde. In this second study, we also detected *R. insecticola* and the  $\gamma$ -protobacteria X-type in some specimens from the population in De Panne, yielding amplification bands near 470 bp and 450bp respectively.

The identity of some of the positive samples was further confirmed by sequencing the PCR products and DNA blasting (BENSON et al., 2013). These sequences are available in GenBank and correspond with accession numbers KJ943256-KJ943268.

The Spearman correlation analysis (Table 3) showed a significant co-occurrence of *S. symbiotica* with *H. defensa*, and *R. insecticola* with the endosymbiont X-type. The incidence of mummies in the field was not correlated with any of the endosymbionts detected.

## DISCUSSION

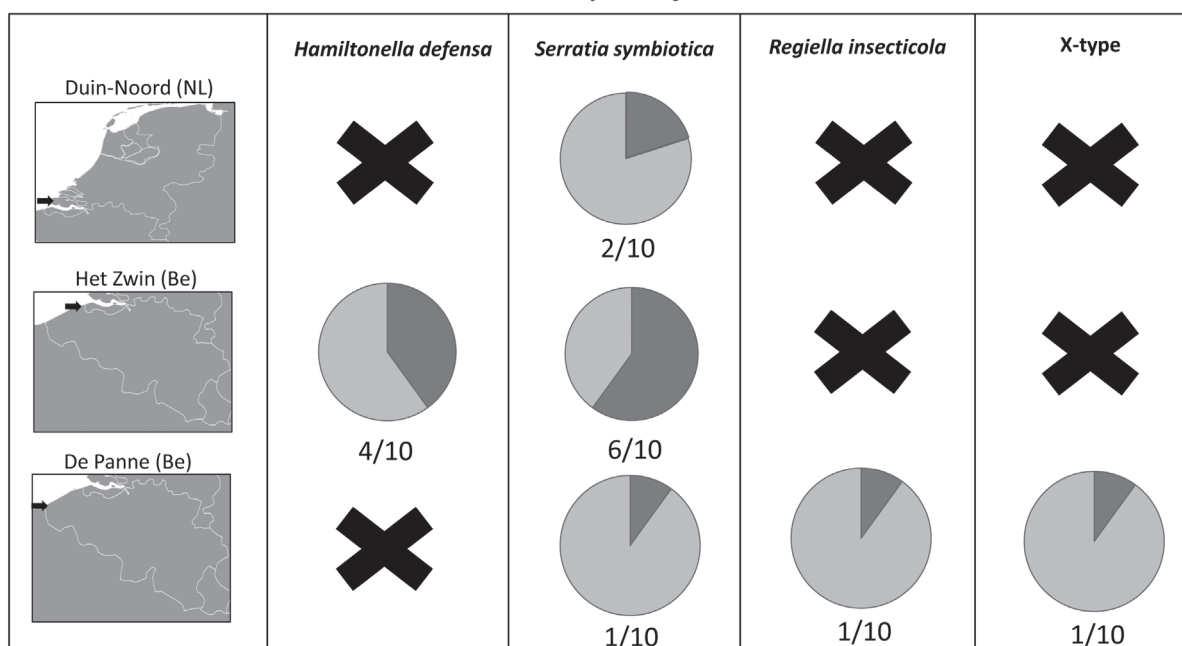
By means of diagnostic PCRs we assessed the occurrence of facultative endosymbionts in different species and populations of aphids from coastal dunes. The results of this first assessment not only show that facultative endosymbionts are common elements in these aphids, but also showed variation in the endosymbiont community according to species and location. All populations, except *S. rufula* from Wales, were associated with facultative endosymbionts and by combining the results of the two sampling surveys, four different taxa of facultative endosymbionts were detected: *H. defensa*, *S. symbiotica*, *R. insecticola* and the  $\gamma$ -protobacteria X-type. Based on a relatively small sample (i.e. 10 aphid-lines per population/species) we have shown that even within a population, there may be abundant variation in the occurrence of facultative endosymbionts; specimens coming from different *A. arenaria*

TABLE 3

Spearman coefficients for the correlation between facultative endosymbionts (i.e. *Hamiltonella defensa*, *Serratia symbiotica*) and the number of mummies observed in *Schizaphis rufula*.

	<i>H. defensa</i>	<i>S. symbiotica</i>	<i>R. insecticola</i>	X-type	Mummies
<i>H. defensa</i>		<b>0.61</b> <b>&lt;0.0001</b>	-0.05 0.74	-0.05 0.74	0.14 0.35
<i>S. symbiotica</i>	<b>0.61</b> <b>&lt;0.0001</b>		-0.08 0.59	-0.08 0.59	0.08 0.62
<i>Regiella insecticola</i>	-0.05 0.74	-0.08 0.59		<b>0.99</b> <b>&lt;0.0001</b>	0.21 0.18
X-type	-0.05 0.74	-0.08 0.59	<b>0.99</b> <b>&lt;0.0001</b>		0.21 0.18
Mummies	0.14 0.35	0.08 0.62	0.21 0.18	0.21 0.18	

*Schizaphis rufula*



*Laingia psammae*

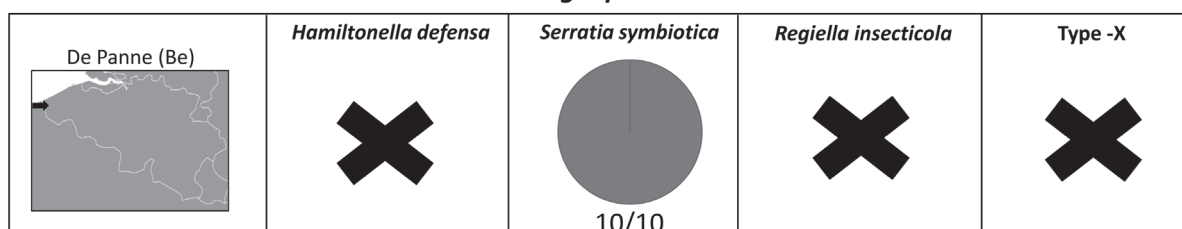


Fig 4. – Frequency of facultative endosymbionts in two species of aphids found in coastal dunes: *Schizaphis rufula* and *Laingia psammae*.

plants, only separated a few meters, showed dissimilar endosymbiotic profiles. Moreover, the results showed that different species of dune aphids shared facultative endosymbionts. For instance, *L. psammae*, *R. padi* and *S. rufula* were all infected with *S. symbiotica*. Also temporal variation in the occurrence of endosymbionts cannot be excluded since different endosymbionts were detected in the samples collected in June and in October.

These findings open interesting future research avenues. For instance, *H. defensa* is an endosymbiont already reported in aphids and other sap-feeding insects as protecting its hosts from parasitoid wasps (OLIVER, 2010; NYABUGA et al., 2010; MCCLEAN et al., 2011; LUKASIK et al., 2013). As observed in the field, the sampled populations were frequently parasitized by parasitoid wasps but only a few individuals from Het Zwin revealed the presence of *H. defensa*. Since not all *H. defensa* strains are known to confer resistance to parasitoids and the effect also varies among aphid species (DEGNAN et al., 2009; VORBURGER et al., 2009) the meaning of the interaction for this Belgian aphid population needs further experimental examination. The correlation analysis did not show any relationship between *H. defensa* and the number of mummies detected in the field. However, these results should be taken with caution due to the limited number of observations and also because a lack of correlation cannot exclude causality. The same type of ideas can be put forward regarding *R. insecticola* as this endosymbiont is known to protect aphids from different natural enemies. Preliminary experimental manipulations with *R. insecticola* in other aphid species have demonstrated that it confers resistance against aphid fungal pathogens (FERRARI et al., 2004; SCARBOROUGH et al., 2005). Nonetheless, it has also been shown that this endosymbiont protects *Myzus persicae* and *Aphis fabae* against parasitoids (VORBURGER et al., 2009).

*Serratia symbiotica* was consistently detected in the three species (*S. rufula*, *R. padi*, *L. psammae*) collected in coastal dunes from both sampling

dates. Dune habitats are not only harsh for the plant community but also for the associated insects. For example, oscillations in temperature of ca. 25°C are common during the summer and dune sand can easily reach temperatures above 50°C (MAUN 2009). Besides different life history traits and behavioral adaptations to such rapid environmental changes, symbiotic relationships with facultative bacteria may ameliorate such harsh environmental conditions. The facultative endosymbiont *S. symbiotica* has been reported to be pivotal in protection against heat shocks in several species of aphids; in arid areas a relatively high proportion of aphids are found to be carrying this symbiont (HENRY et al., 2013; BRUMIN et al., 2011; BURKE et al., 2010). Whether this is the case for dune species requires further investigation. Nonetheless, it is noteworthy that the three aphids species do carry this symbiont.

A regulatory role of some endosymbionts has been reported in the literature. For instance, in the pea aphid comparison of strains with similar genetic background with or without *Rickettsia* showed a remarkable interaction between this endosymbiont and other endosymbiotic bacteria, including *Buchnera aphidicola* (SAKURAI et al., 2005). From other systems, we also know that the  $\gamma$ -proteobacteria X-type plays a pivotal role in the regulation of resistance against parasitoid wasps during abiotic stress (GUAY et al., 2009). Whether this applies to the species included in this study is a question that needs further analysis. In this direction points the correlation of *R. insecticola* with the endosymbiont X-type and *H. defensa* with *S. symbiotica*.

Regarding the aphid species studied here, we still have very limited knowledge on the factors determining their population dynamics on dune grasses. Experimental evidence shows that population growth in *S. rufula* and *R. padi* is negatively affected by the presence of plant parasitic nematodes in grass roots (DE LA PEÑA et al., 2009; VANDEGEHUCHTE et al., 2011). Other experiments have shown that fungal endophytes are also involved in the control



of aphid populations through belowground / aboveground plant-mediated interactions (JABER & VIDAL 2009; DE LA PEÑA unpublished). In the field, we found significant numbers of aphid mummies, indicating that aphid control by parasitoid wasps occurs in the dunes. Preliminary research addressing the host-range of *S. rufula* has also shown that the species is able to feed on several grasses (PETTERSSON 1971), but given the differences in the composition of the endosymbiont community it would be interesting to address the question of whether aphid host-range is influenced by these endosymbionts. Putting all these pieces together, it is clear that in order to understand the ecology of aphids on dune grasses a multitrophic perspective needs to be taken to further understand how all these players modulate each other (VANDEGEHUCHTE et al., 2013).

More and more empirical evidence reveals the pivotal role of facultative bacteria in mediating indirect interactions in insect communities through changes in plant physiology (FRAGO et al., 2013). The results of our initial assessment showed an unexpected diversity of facultative endosymbionts in dune aphids. The further study of these inconspicuous organisms in coastal dunes will provide new insights into the functioning of these ecosystems. Finally, it is important to highlight that the aphid species included in this study are not only relevant for the dune system; they offer an interesting system to address general eco-evolutionary questions regarding bacterial endosymbionts, the community in which they are embedded, and their abiotic environment.

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