Oak aphids *Tuberculatus annulatus* (Hartig, 1841) and *Thelaxes dryophila* (Schrank, 1801) (Hemiptera: Aphididae) endosymbiont microbiome diversity alters between natural and artificial oak forest regeneration

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Abstract

In the present study, the bacterial symbionts of two species of oak aphid, *Thelaxes dryophila* and *Tuberculatus annulatus* were tested as indicators between naturally and artificially regenerated oak forests. In total, 195 bacterial taxa were identified using the Ion Torrent PGM system. Here, we report for the first time differences in bacterial symbiont diversity between oak forests natural and artificial regeneration sites, and aphid species. Differences in the abundance of the primary obligate endosymbiont *Buchnera aphidicola* were also detected between the two study aphid species. In the ant attended *Thelaxes dryophila*, the species was found to harbour a higher density of the facultative symbionts *Serratia symbiotica* and *Wolbachia*, while in the non-ant attended *Tuberculatus annulatus*, the dominant facultative symbionts were *Hamiltonella* and *Rickettsia*. In addition, we report the presence of *Serratia marcescens* and *Enterobacter cloacae* as symbionts of *Thelaxes dryophila* and *Shigella boydii* as a symbiont of *Tuberculatus annulatus*. We conclude that the two aphid species harbour different facultative symbionts between oak forest regeneration types; these results might have importance in oak forest pest management via aphid adaptation through their endosymbions.

Keywords Adaptation · Diversity · Oak forests · Obligate endosymbionts · Facultative endosymbionts

1 Introduction

In European forests, pedunculate oak (*Quercus robur*) and sessile oak (*Quercus petraea*), are among the most valuable sources of high-quality hardwood timber in European forests (Spiecker 2021). Hence, why the attention of foresters to the care of these species should be increased. Developing oak forest regeneration techniques that reduce stand disturbance with the goal of producing high-quality timber remains a crucial challenge (Mölder et al. 2019).

The pedunculate oak is one of the most valuable deciduous tree species in the lowland and hill forests throughout Europe, including Romania. Although the species occupies only some 2% of the country's forest stands, it forms

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valuable pure stands and mixed stands in lowland and hill forests. The regeneration of these stands is to a large extent natural, but it is often necessary to provide artificial regeneration to maintain or increase yields (Bobiec et al. 2018).

Naturally regenerated oak stands have a much higher ecological stability compared with artificially regenerated oak stands. In terms of the costs of care and management of stands, the costs for natural regeneration are lower than for artificial regeneration. Still, for artificial regeneration, bare-root seedlings are frequently used, which affects the vitality of future trees in the short, medium and long term (Długosiewicz et al. 2019).

The use of containerized seedlings for afforestation work has started to be used because of the advantages they offer and it is hoped by those interested and/or involved in the sustainability of hardwood forestry that in the future containerized oak seedlings will be employed on a much larger scale in forest regeneration. In the case of pedunculate oak seedlings, containerization ensures better root development (Reho et al. 2022), and the larger the container, the better the results in terms of growth and vigour of the seedlings obtained (Dey et al. 2012).

The production of healthy pedunculate oak planting material and ensuring good phytosanitary conditions of young stands is a major objective of the production activity. Not infrequently, however, the planting material is either diseased, a common disease being oak powdery mildew (*Erysiphe alphitoides*), or attacked by insect pests, of which aphids form a major part (Marcais and Desprez-Loustau 2013). In recent years, mainly due to mild winters, little and scarce rainfall, aphids have been favoured and the frequency and intensity of damage has increased considerably (Zanfelici and Murányi 2023).

Heavy rainfall and variable temperatures have the opposite effect, disrupting aphid activity (Crossley et al. 2022). Aphids are known to feed by penetrating the phloem vessels with their stylets and sucking up the sap of both the leaves and shoots (Wari et al. 2021). Colonies that form on pedunculate oak consist of numerous mainly asexual (parthenogenetic) individuals at different stages of development, negatively influencing the annual growth and vitality of attacked plants (Mrazova et al. 2023).

The rate of generation succession is very high (Simon and Peccoud 2018), so that in about a week, under favorable conditions of temperature and humidity, a completely new asexual generation can result. As a result of their feeding activity, aphids can transmit viruses from diseased to healthy plants, which amplifies their undesirable effect and can cause oak die back (Ray and Casteel 2022).

Two species of monophagous aphids are important pests with expanding populations on pedunculate oak, *Tuberculatus annulatus* (Hartig, 1841) (Skvarla et al. 2017) and the *Thelaxes dryophila* (Schrank, 1801) (Lubiarz 2009).

Both are of economic interest having spread to oak in nurseries, young plantations and natural regenerations. *T. annulatus* propagates asexually mainly on the back of leaves, near the main vein and secondary veins, places where they find favorable feeding conditions (Oğuzoğlu et al. 2023). In contrast, *T. dryophila*, develops both on the bark of the shoots, on the back of the leaves or around the buds (Kaliuzhna 2020).

Unlike *Tuberculatus annulatus*, which is not ant-attended, *Thelaxes dryophila*, is attended by red wood ants, *Formica rufa* L., which guard the aphid colony members against arthropod predators and hymenopterous wasp parasitoids, whilst at the same time harvesting the sugars produced by them (honeydew), thus developing a mutual relationship (Kindlmann et al. 2007). Both species are present in both natural and artificial regenerations and are affected by environmental conditions but also by the diversity of facultative symbiont bacteria present in their bodies.

Considering these various aspects, in the present paper we asked the following questions: Are there any differences in bacterial symbionts species diversity between the two aphid species colonizing the same host plant?, Are there any differences in symbionts diversity when aphids from natural and artificial regenerations are compared; Are there also differences in terms of the influence of ant attendances on the success of aphid colonies under both ecological scenarios? Altogether, can differences in bacterial symbionts be an indicator between natural and artificial oak forests regenerations?

2 Materials and methods

2.1 Site presentation and data collection

Field collecting was performed in the forests managed by the National Forestry Regia "Romsilva", Timis Forestry Department. For both *T. annulatus* and *T. dryophila*, three sites were selected of natural and artificial pedunculate oak regeneration, respectively, with an average age of five years and at an altitude between 90 and 250 m above sea level. Thus, a total of 12 sites were chosen (three replicates from each aphid species and forest regeneration type), the description of which are given in Fig. 1. These sites and ecosystems were chosen because their environment, climate conditions and pest problems (presence of the two aphid species) represents oak forests throughout the temperate region of the European continent.

Samples of aphid nymphs of both aphid species were collected at each site under sterile conditions with the biological material placed in 1.5 ml. Eppendorf tubes containing absolute ethanol. The samples thus obtained were stored in a deep freezer at −70℃ prior to molecular analysis.
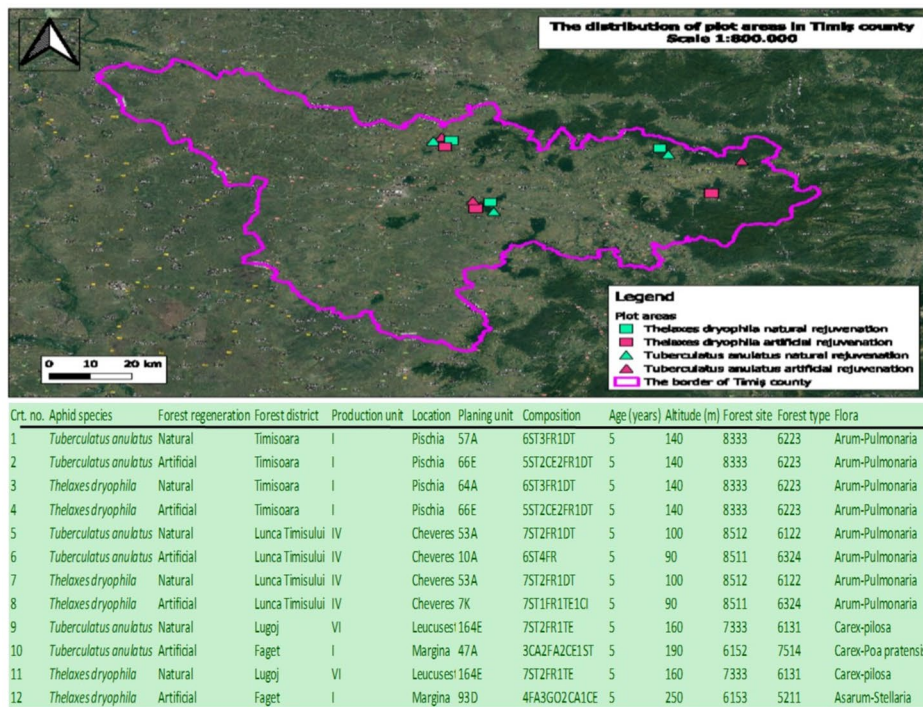
Note: ST - *Quercus robur*; CE - *Quercus cerris*; GO - *Quercus petraea*; FA - *Fagus sylvatica*; FR - *Fraxinus excelsior*; TE - *Tilia cordata*; CA - *Carpinus betulus*; DT - various hardwood species; 6152 - Hill cvercete hilly scrub with Bm, brown and brown podzolit edaphic large and medium hardwoods; 6153 - Oak woodland (oak and woodland/woodland) Bs, brown +/- weakly podzolite-pseudogleistate, high edaphic; 7333 - Oak woodland with oak Bs, strongly podzolite-pseudogleistate brown, high edaphic; 8333 - Oak woodland Bm, strongly podzolite-pseudogleistate broad depression, medium edaphic; 8511 - Forest plain, Bm shale meadow, wet gleissed or semi-gleissed brown, medium to high edaphic; 8512 - Forest plain, Bs shale meadow, wet gleissed or semi-gleissed brown, high edaphic; 5211 - Goruneto-forest with mull flora (s); 6122 - Lowland lowland steppe (s); 6131 - High yield hill plateau steppe (s); 6223 - Lowland lowland steppe-steppe (m); 6324 - Lowland steppe-steppe (i); 7514 - Low yield hill steppe-steppe (i).

Crt. no.	Aphid species	Forest regeneration	Forest district	Production unit	Location	Planing unit	Composition	Age (years)	Altitude (m)	Forest site	Forest type	Flora
1	*Tuberculatus anulatus*	Natural	Timisoara	I	Pischia	57A	6ST3FR1DT	5	140	8333	6223	Arum-Pulmonaria
2	*Tuberculatus anulatus*	Artificial	Timisoara	I	Pischia	66E	5ST2CE2FR1DT	5	140	8333	6223	Arum-Pulmonaria
3	*Thelaxes dryophila*	Natural	Timisoara	I	Pischia	64A	6ST3FR1DT	5	140	8333	6223	Arum-Pulmonaria
4	*Thelaxes dryophila*	Artificial	Timisoara	I	Pischia	66E	5ST2CE2FR1DT	5	140	8333	6223	Arum-Pulmonaria
5	*Tuberculatus anulatus*	Natural	Lunca Timisului	IV	Cheveres	53A	7ST2FR1DT	5	100	8512	6122	Arum-Pulmonaria
6	*Tuberculatus anulatus*	Artificial	Lunca Timisului	IV	Cheveres	10A	6ST4FR	5	90	8511	6324	Arum-Pulmonaria
7	*Thelaxes dryophila*	Natural	Lunca Timisului	IV	Cheveres	53A	7ST2FR1DT	5	100	8512	6122	Arum-Pulmonaria
8	*Thelaxes dryophila*	Artificial	Lunca Timisului	IV	Cheveres	7K	7ST1FR1TE1Cl	5	90	8511	6324	Arum-Pulmonaria
9	*Tuberculatus anulatus*	Natural	Lugoj	VI	Leucusesti	164E	7ST2FR1TE	5	160	7333	6131	Carex-pilosa
10	*Tuberculatus anulatus*	Artificial	Faget	I	Margina	47A	3CA2FA2CE1ST	5	190	6152	7514	Carex-Poa pratensis
11	*Thelaxes dryophila*	Natural	Lugoj	VI	Leucusesti	164E	7ST2FR1TE	5	160	7333	6131	Carex-pilosa
12	*Thelaxes dryophila*	Artificial	Faget	I	Margina	93D	4FA3GO2CA1CE	5	250	6153	5211	Asarum-Stellaria

Fig. 1 Sites locations and forests structure of natural and artificial regeneration of pedunculate oak forests

## 2.2 Bacterial symbionts identification and bioinformatics assessments

We utilized the Ion Torrent platform, which influences the choice of sequence processing pipeline. The amplified partial sequences of the 16 S rRNA gene from aphid DNA extracts were performed using primer pairs designed for the hypervariable regions of the gene (V2-4-8 and V3-6, 7–9). The resulting PCR products targeting specific regions of the 16 S rRNA gene were purified with Agencourt AMPure beads. Subsequently, libraries were constructed using the Ion Plus Fragment Library kit (Applied Biosystems) and their concentration determined with the Ion Universal Library Quantitation kit (Cat no. A26217). Template preparation was completed using the ION PGM Hi-Q View OT2 kit-400, followed by sequencing of the amplicon libraries on a 318-chip using the Ion Torrent PGM system (Fig. 2).

The sequence reads obtained were filtered by the Ion Reporter PGM software to eliminate polyclonal and low-quality reads. The resulting sequencing data were analyzed using Quantitative Insights Into Microbial Ecology (QIIME). Operational Taxonomic Units (OTUs) of the 16 S rRNA gene were defined at a threshold of ≥ 97% sequence homology to calculate downstream diversity measures. Classification of all reads was performed utilizing reference datasets (Curated Greengenes v13.5; Curated MicroSEQ(R)

16 S Reference Library v2013.1). The number of reads was an average of 537 per sample.

2.3 Data analyses

Analysis of the community composition of the most frequent bacterial symbionts for each aphid species and site were computed separately. Alignment of all sequences was performed using ClustalW, and the results visualized in EMPeror (Vázquez-Baeza et al. 2013). Non-metric multidimensional scaling (NMDS) based on beta diversity analysis using three-dimensional principal coordinates analysis was then performed involving the Bray–Curtis dissimilarity index. Bacterial communities were next classified in terms of Family, Genus and Species level, and the most dominant taxa from each community were presented at these three levels for both aphid species. Diversity indices for the bacterial symbiont community sampled were also computed separately for the two aphid species. Principal component analyses (PCoA) was used to test the effect of regeneration and site on bacterial symbiont abundances at species level for each aphid species, where regeneration type (natural vs. artificial) and location (site) were considered as main components and symbiont species abundances as variables and visualized using the computer program PAST 4.02. (https://past.en.lo4d.com/windows)
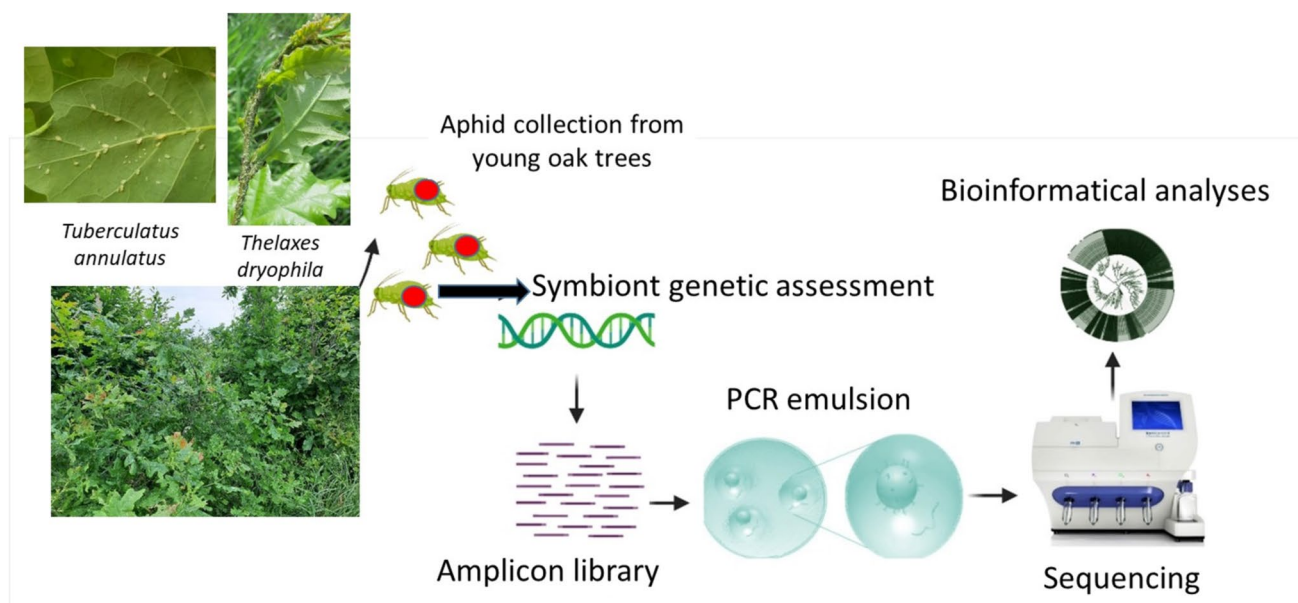
Fig. 2 Aphid sample collection, symbiont genetic analyses using the Ion Torrent PGM system

3 Results

In total, 195 bacterial taxa were identified in naturally and artificially regenerating pedunculate oak forests for the two aphid species tested, *T. dryophila* and *T. annulatus*. The community composition analyses clearly represents a snapshot of the frequency of the two aphid species microbiomes at the level of family, genus and species. Enterobacteria, Proteobacteria and Gammaproteobacteria communities dominated in both species and sites, whilst Alphaproteobacteria and Rickettsiales were also frequently detected in *T. dryophila* in site 3 of the artificial regeneration stand (Fig. 3). Similar trends for *T. annulatus* were detected in natural regeneration site 2, whereas the presence of Alphaproteobacteria and Rickettsiales was higher near to the other aforementioned groups, i.e. Enterobacteria, Proteobacteria and Gammaproteobacteria than in *T. dryophila* (Fig. 4).

The ant-attended aphid species, *T. dryophila* was dominated in terms of it symbionts by Enterobacteriaceae and *Buchnera*, whilst a lower incidence of *Rickettsia*, *Serratia* and *Wolbachia* were also detected; however, a higher diversity of these particular symbionts were detected in aphids from the site 3 artificial regeneration stand (Fig. 5A). Interestingly, the abundance of the facultative endosymbionts *Rickettsia*, *Serratia* and *Wolbachia* were higher for the non-ant attended *T. annulatus*, and revealed a lower diversity in the site 3 artificial regeneration stand (Fig. 5B). The dominance of the obligate primary symbiont *B. aphidicol*a was detected at genus and species level for both aphid species, however again, differences in terms

of diversity were observed. *T. dryophila* was almost completely dominated by *B. aphidicola* in all natural regeneration sites and in site 1 and 2 artificial regeneration sites. In contrast, the abundance of the facultative symbiont *S. symbiotica* and *Wolbachia* was higher at site 3 artificial regeneration, whilst the presence of *S. marcescens* and *Enterobacter cloacae* were detected (Figs. 6A and 7A).

The bacterial diversity detected in *T. annulatu* was less dominated by *B. aphidicol*a at all sites tested, whilst interestingly, the presence of *Hamiltonella* and *Rickettsia* were almost always observed at high frequency in natural regeneration sites 2. In addition, the endosymbiont *Shigella boydii* was here detected (Figs. 6B and 7B). Overall, the highest endosymbiont diversity was found in artificial regeneration site number 3 for *T. dryophila*, while in the case of *T. annulatus*, a high symbiont diversity was identified in natural regeneration site 2. As we observed, differences in diversities of endosymbionts between sites, regeneration types and aphid species was clear (Table 1).

Lastly, principal coordinate analyses revealed the effect of regeneration type on the two aphid species in terms of their endosymbiotic bacterial abundance. Regeneration type determined more than 84% of the symbionts distribution for *T. dryophila*, whilst site effects (without regeneration types), also considered as locations, showed only around 15% effect on endosymbiont distribution (Fig. 8A). No such clear effect on *T. annulatus* was detected, but here regeneration types also determined more than 74% of its symbiont distribution, whilst site effects had some 24% influence on this (Fig. 8B).
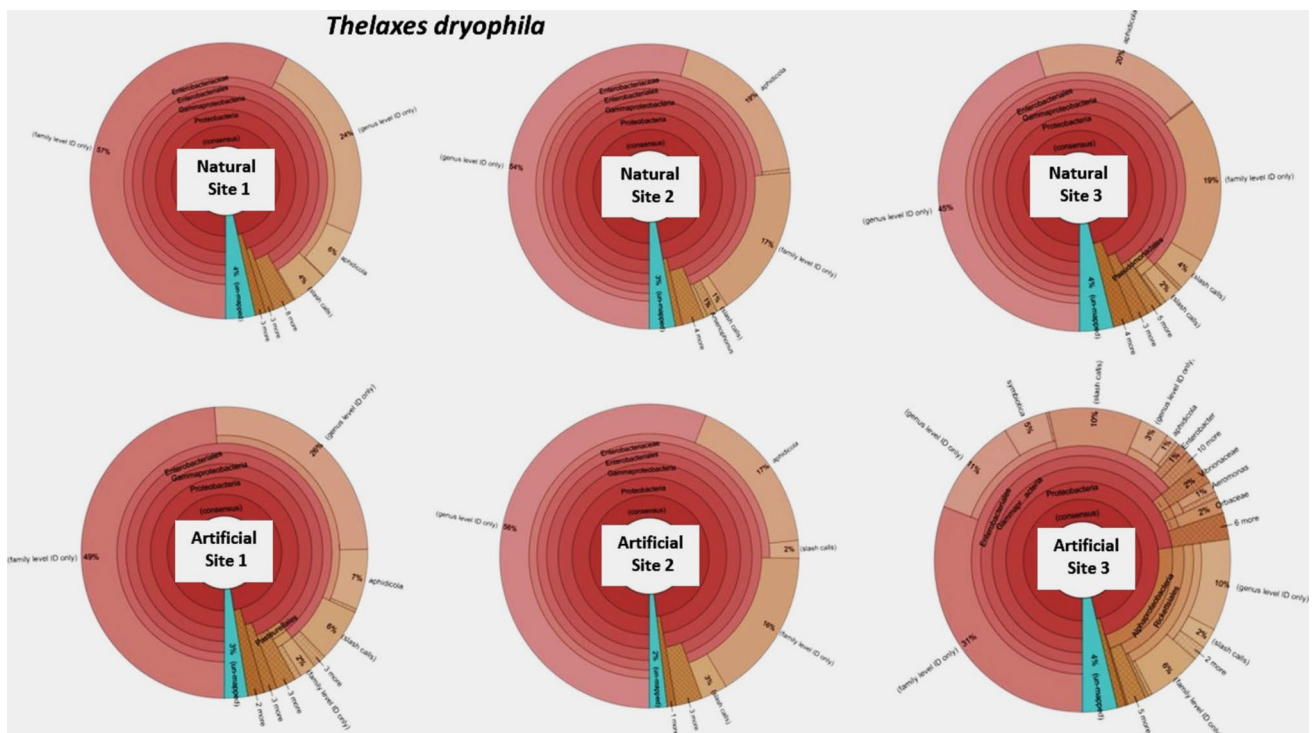
Fig. 3 The community composition of the most frequent bacterial symbionts of *Thelaxes dryophila* aphid under natural and artificial regeneration sites visualized in Emperor. Alignment of all sequences was performed using ClustalW

4 Discussion

In the present study, we report for the first time the endo-symbiotic bacterial diversity of the oak-feeding aphids *T. dryophila* and *T. annulatus* under natural and artificial regeneration of pedunculate oak forests, representing temperate forests from the European continent. Several previous studies have demonstrated the presence of these endosymbionts in other aphid species (Zytynska and Weisser 2016; Csorba et al. 2022) that we now detect in two forest aphid pests; however, their potential role in different forests regeneration types is novel and here discussed for the first time. The obligated primary bacterial symbiont *B. aphidicola* (family Enterobacteriaceae) dominated all sites and regeneration systems, but its frequency differed between regeneration systems and aphid species, and almost completely dominated sampled populations of the ant-attended aphid *T. dryophila*. The primary symbiont *B. aphidicola* has hitherto been reported as an essential symbiont required for the survival of aphids, providing essential amino acids that are rare in their phloem sap diet (Shigenobu et al. 2000), and in addition to this, also has a significant role in aphid heat tolerance (Zhang 2019a, b).

Studies with other aphid species have revealed long evolutionary relationships between aphids and *B. aphidicola* (Pérez-Brocal 2011), more especially a close relationship between aphid subfamilies Eriosomatinae and Lachninae compared with the subfamily Aphidinae. These authors also revealed that the effect of lifecycle and generation time of these particular aphid groups could explain the accelerated rate of *B. aphidicola* evolution as found in the subfamily Lachninae. No evidence was found in the present study however concerning the frequency of *B. aphidicola* in the aphid subfamilies Thelaxinae and Calaphidinae of the family Aphididae.

Other studies have also reported that the predominantly obligate parthenogenetic Russian wheat aphid, Diuraphis noxia Kurdjumov (cf. Puterka et al. 2012) only harbors a single endosymbiont, *B. aphidicola*. The authors conclude that the high fitness advantages offered by *B. aphidicola* could lead to the loss of other bacterial strain through genetic drift, and preserves uptake into developing embryos (Burger et al. 2024). In our study, the species *T. dryophila* was almost completely dominated by *B. aphidicola* in all natural regeneration sites and in site 1 and 2 artificial regeneration, which can also be explained by high fitness benefits (also detected under sample collections) and consequently the loss of other facultative symbionts.

The ant-attended *T. dryophila* harboured facultative symbionts *S. symbiotica* and *Wolbachia* in higher abundance in site 3 artificial regeneration. The presence of *S. symbiotica* has previously been reported by other authors from 54
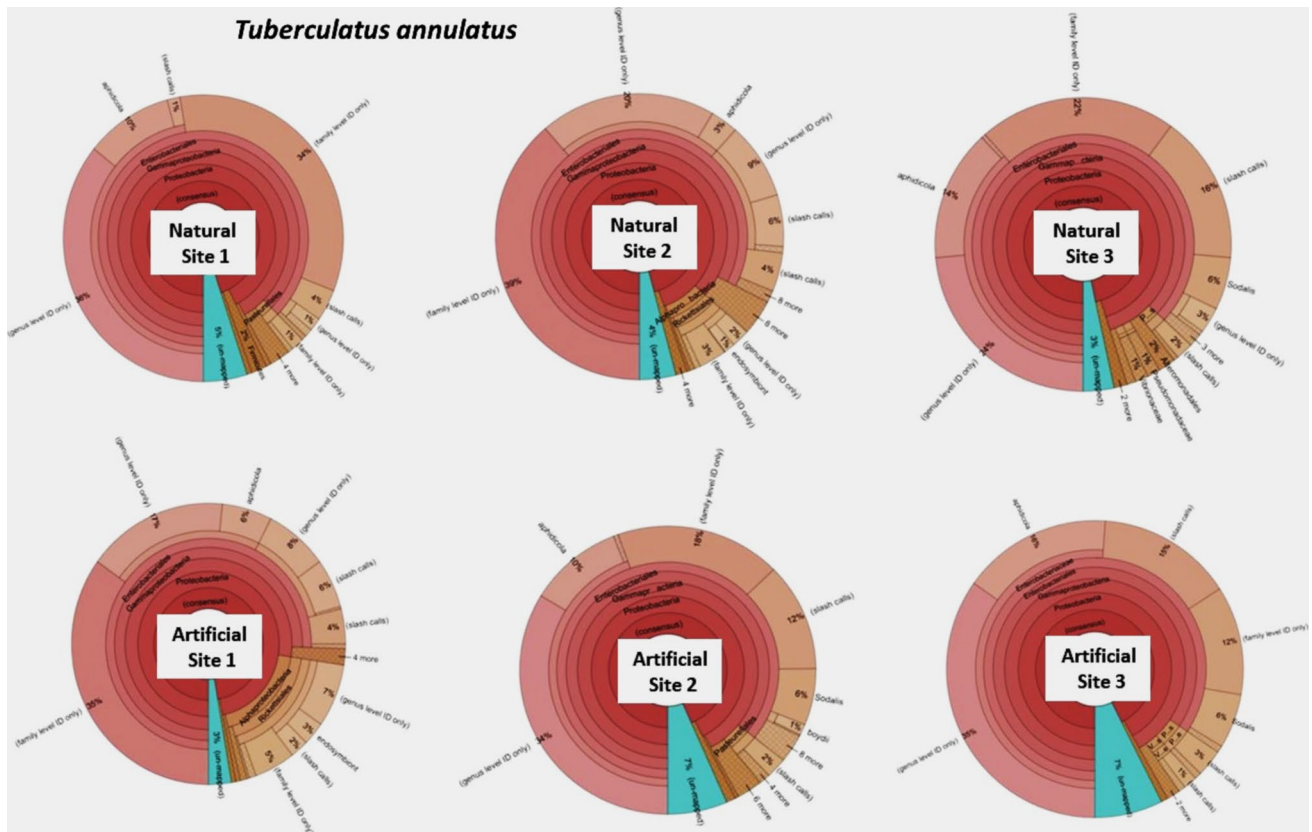
Fig. 4 The community composition of the most frequent bacterial symbionts of *Tuberculatus annulatus* aphid under natural and artificial regeneration sites visualized in Emperor. Alignment of all sequences was performed using ClustalW

species of the family Aphididae, including the tribes Macrosiphini (31 species) and Aphidini (23 species) (Pons et al. 2022). *Serratia* are pathogenic bacterial species. Thus, *S. symbiotica* has been described as a gut pathogen of aphids; however having said that, *Serratia* includes strains ranging from obligate, heritable symbionts that can be maternally transmitted (Perreau et al. 2021). Because *Serratia* is a gut-associated symbiont, it is possible that the host plant's permanent environment is required to maintain the infection across aphid generations (Pons et al. 2022). *S. symbiotica*has also been reported to be involved in defense against heat stress and potentially in aphid nutrition, being capable of producing proteases such us metalloproteases, which may facilitate aphid development and help in plant protein digestion as well as suppressing plant defenses (Skaljac et al. 2019).

In the present study, *S. marcescens* was also found to be present in relatively high density between *T. dryophila* symbiont communities in oak forests under artificial regeneration. To our knowledge, this is the first report of a relationship between this particular bacterial species with aphids. In previous studies the opportunist *S. marcescens* has been isolated from roots and stems of different rice varieties (Gyaneshwar

et al. 2001). This highly resistant bacteria can utilize a large variety of nutrients, which gives it the ability to survive under extreme conditions, whilst furthermore, antibiotic resistance has been reported in this species (Sleigh 1983).

The presence of *Wolbachia* in aphids (e.g. *Cinara cedri* Mimeur) was first detected by (Gómez-Valero et al. 2004) and reported to have importance in the prevalence and persistence of asexual lineages. In our case,*Wolbachia* was present at higher density in artificial regeneration sites compared with natural ones. Previous researches have reported this facultative endosymbiont to be one of the most frequently found in aphids, i.e. in 82 species (Zytynska and Weisser 2016). The role of *Wolbachia* is diverse, previous studies reporting that this symbiont can protect aphids (and other arthropods) against several pathogens and abiotic stresses .(Brownlie et al. 2009; Iturbe-Ormaetxe et al. 2011)). *Wolbachia* are also essential in aphids and other arthropods (e.g. bed bugs, parasitic wasps and collembolan species) for egg development (Kremer et al. 2009; Hosokawa et al. 2010), and can also enhance the fecundity of the female insect host (Ou et al. 2023). In our previous research involving the testing of corn-leaf aphid, *Rhopalosiphum maidis* (Fitch) infesting maize for symbiont diversity,
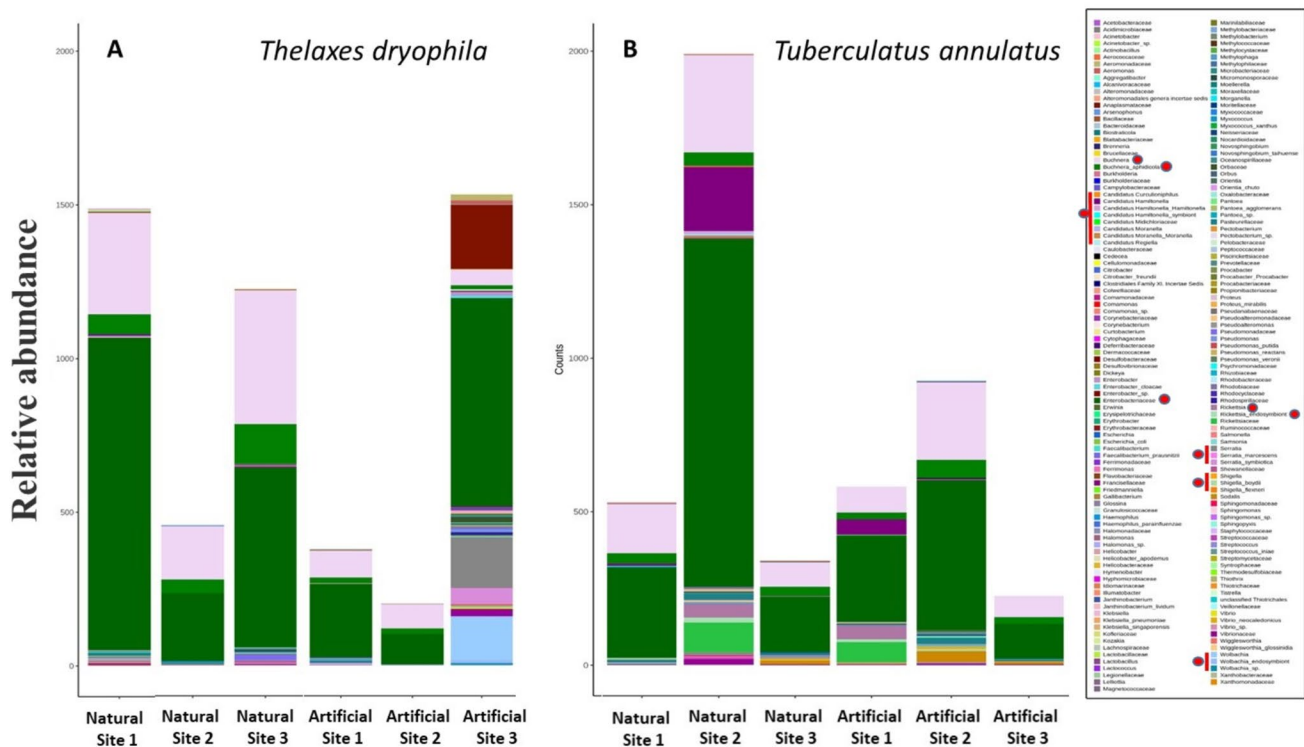
Fig. 5 *Thelaxes dryophila* (**A**) and *Tuberculatus annulatus* (**B**) bacterial symbiont frequency at family level under natural and artificial regeneration of pedunculate oak forests. Red dots represent the most frequent taxonomic groups

Wolbachia was present in large-scale field corn crops. We also reported that the presence of this symbiont in natural populations of these aphids was probably not influenced by management systems (intensive vs. extensive) or climate (hot vs. cold summer), but rather, due to infection by horizontal gene transfer from wasp parasitoids (Gómez-Valero et al. 2004a).

The presence of *Enterobacter cloacae* in artificially regeneration sites can also be considered as a new observation. *E. cloacae* strains were first isolated from other insects, i.e. fruit flies *Bactrocera tryoni* (Froggatt) (Dipetra: Tephritidae) (Murphy et al. 1994). Such aphid symbionts may play a role in growth and survival of the host aphid in the absence of suitable nutrition by facilitating nutrients (Woodbury and Gries 2013)). Furthermore, it appears that *E. cloacae* living within the gut cells of adult silverleaf whitefly, *Bemisia argentifolii* Bellows, Perring, Gill and Hedrick (a synonym of *B. tabaci* (Gennadius)) (Homoptera: Aleyrodidae) is mildly pathogenic and hence has control potential (Davidson et al. 2000)).

The bacterial diversity of *T. annulatus* was found to be less dominated by the obligate symbiont *B. aphidicola* at all sites (these results), probably due to its interaction with ants; therefore, we conclude that its role in this species'

survival is lower in pedunculate oak regeneration, in comparison with the other oak aphid species studied.

Other facultative symbionts such as *Hamiltonella* and *Rickettsia*, either not found or only in low density in *T. dryophila*, were present in higher density in *T. annulatus*. The presence of these symbionts was almost always detected at high frequency in natural regeneration sites, especially in site 2. Other studies reported that the presence of *Hamiltonella* serves as a defense against parasitoid wasps, effectively inhibiting the development of wasp larvae inside the body of the pea aphids (*Acyrthosiphon pisum*Harris) tested (Oliver et al. 2005). It has also been shown in a separate study (McLean et al. 2011) that artificial infection with *H. defensa* decreased the fitness of pea aphids on peavine or vetchling plants, *Lathyrus* spp., but not on broad bean, *Vicia faba*. The same study also revealed that the absence of *Hamiltonella* in natural aphid populations decreased aphid fecundity by up to 20%. From this it was concluded that *Hamiltonella* serves as a universal rather than a 'plant-species-specific' symbiont (McLean et al. 2011). Interactions with ants together with infection by *Hamiltonella* may provide high evolutionary benefits for *T. annulatus*, the reason why, perhaps, the importance (and abundance) of *B. aphidicola* is found to be lower in this aphid species.
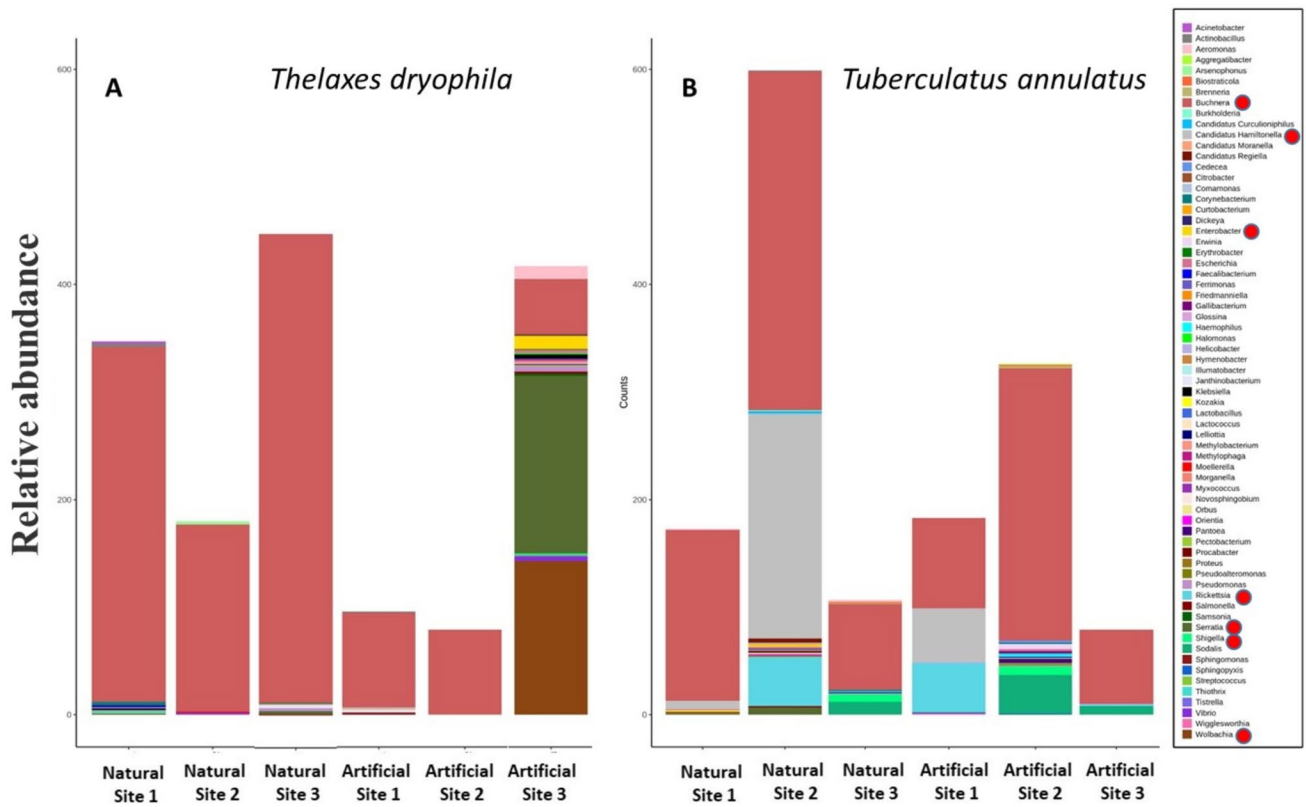
Fig. 6 *Thelaxes dryophila* (**A**) and *Tuberculatus annulatus* (**B**) bacterial symbiont frequency at genus level under natural and artificial regeneration of pedunculate oak forests. Red dots represent the most frequent taxonomic groups
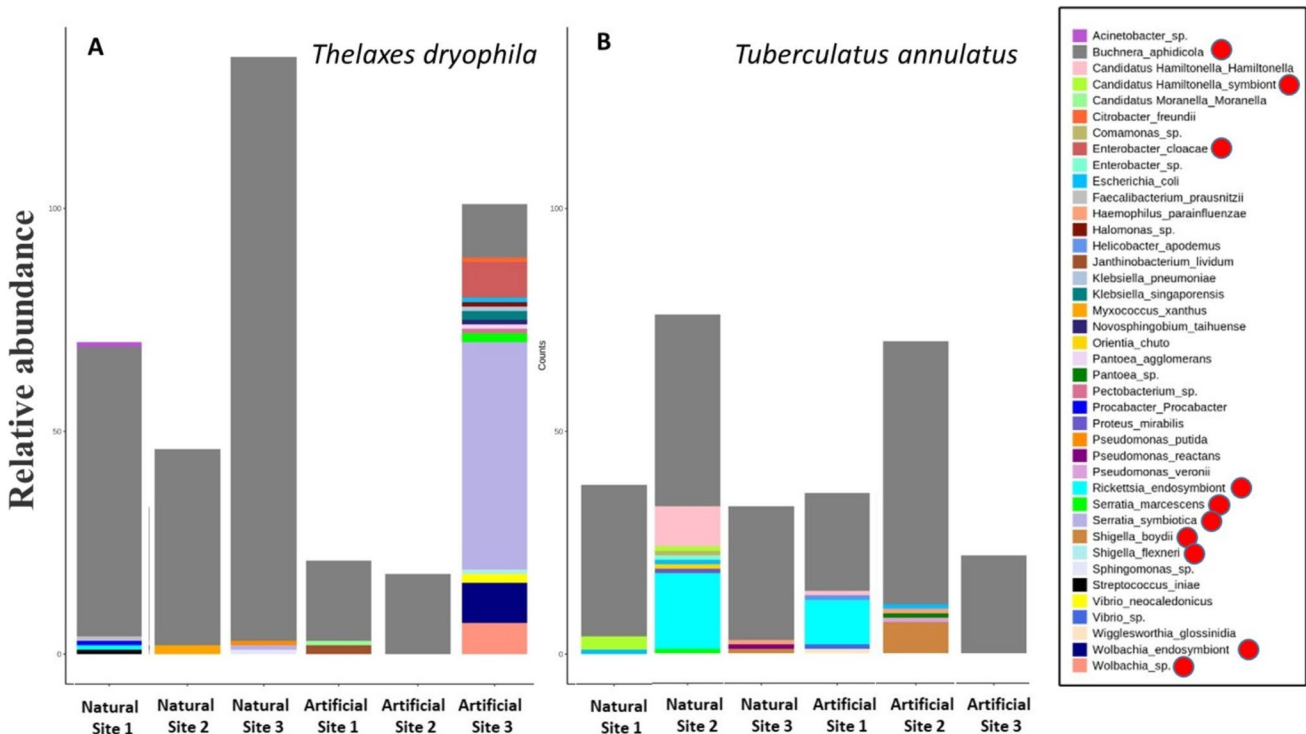

Fig. 7 *Thelaxes dryophila* (**A**) and *Tuberculatus annulatus* (**B**) bacterial symbiont frequency at species level under natural and artificial regeneration of pedunculate oak forests. Red dots represent the most frequent taxonomic groups

Table 1 Diversity indices of the most frequent bacterial symbionts of the aphid species *Thelaxes dryophila* and *Tuberculatus annulatus* under natural and artificial regeneration of pedunculate oak forests

Thelaxes dryophila

Bacterial symbionts diversity	Natural_Site1	Natural_Site2	Natural_Site3	Artificial_Site1	Atificial_Site2	Artificial_Site3
Taxa_S	6	2	4	3	1	16
Sequence abundance	70	46	134	21	18	101
Dominance_D	0.86	0.91	0.95	0.74	1	0.29
Simpson_1-D	0.13	0.08	0.04	0.25	0	0.7
Shannon_H	0.37	0.17	0.13	0.5	0	1.79
Fisher_alpha	1.57	0.42	0.77	0.95	0.22	5.35
Berger-Parker	0.92	0.95	0.97	0.85	1	0.5

Tuberculatus annulatus

Bacterial symbionts diversity	Natural_Site1	Natural_Site2	Natural_Site3	Artificial_Site1	Atificial_Site2	Artificial_Site3
Taxa_S	3	10	4	6	6	1
Sequence abundance	38	76	33	36	70	22
Dominance_D	0.8	0.38	0.82	0.45	0.72	1
Simpson_1-D	0.19	0.61	0.17	0.54	0.27	0
Shannon_H	0.39	1.3	0.4	1.05	0.61	0
Fisher_alpha	0.76	3.08	1.19	2.05	1.57	0.21
Berger-Parker	0.89	0.56	0.9	0.61	0.84	1

Fig. 8 Principal component analyses (PCoA) testing the effect of regeneration and site on bacterial symbiont abundances at species level for each aphid species tested (*Thelaxes dryophila*) (**A**) and (*Tuberculatus annulatus*) (**B**), under different regeneration types (natural vs. artificial) and location (Sites). Green colour represents natural regeneration sites, red artificial regeneration sites
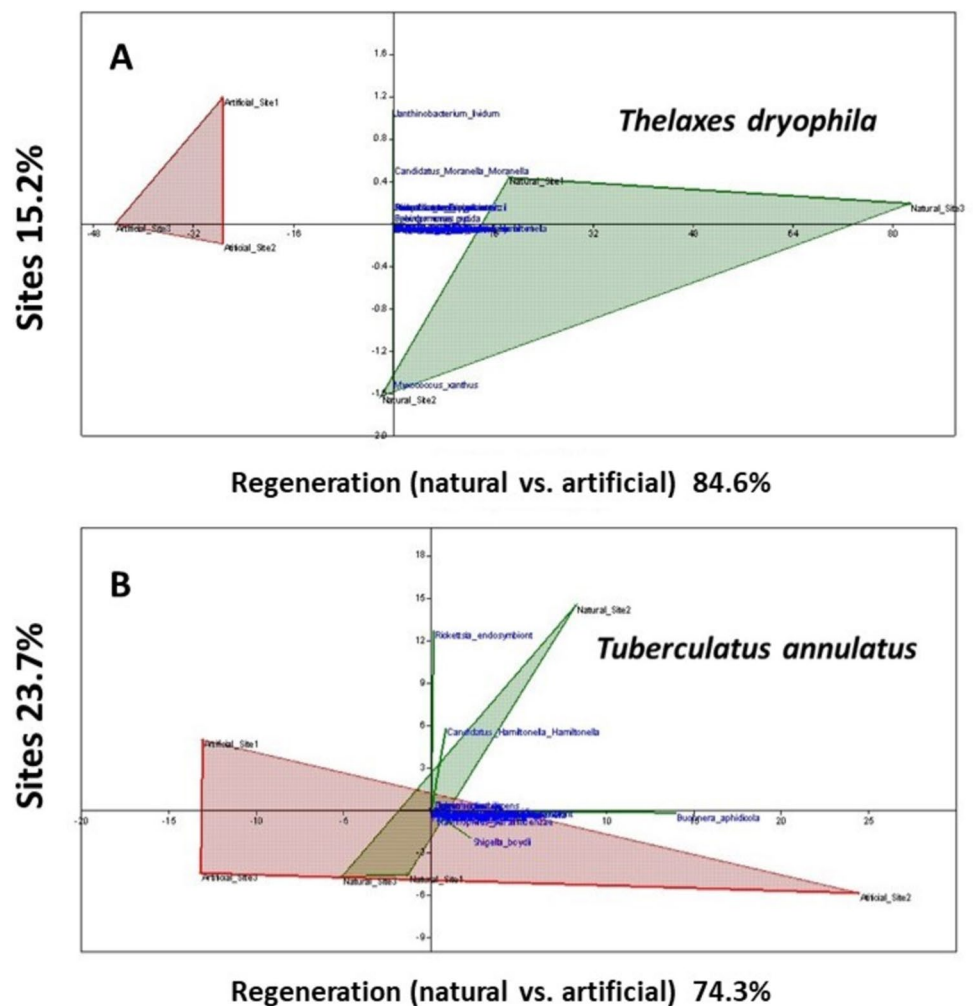
Rickettsia was also detected under natural regeneration conditions, the presence of this facultative symbiont in oak aphids perhaps reducing their fecundity and longevity (effect similar with *Serratia*) as has been reported in the blue alfalfa aphid, *Acyrthosiphon kondoi* Shinji (Chen et al. 2000).

In oak aphids collected from the natural oak sites sampled, the presence of the Gram-negative bacterium *Shigella boydii* was also detected. *Shigella*, which is known to infection non-human primates, e.g. *S. flexneri* in the rhesus macaque, *Macaca mulatta* (Zimmermann) (Lee et al. 2011), was also detected at different levels inside the leaves of the mouse-ear cress plant, *Arabidopsis thaliana* and was seen to damage the plant cells themselves (Jo et al. 2019). Its presence in aphids was recorded for the first time in our present study, the microorganism probably being harbored through plant cells.

As our study clearly shows, the two most frequent aphid species infecting oak forests, the ant-attended *T. dryophila* and non–attended *T. annulatus*, harbour both obligate and facultative bacterial symbionts at different frequencies. While the role of *B. aphidicola* appears to more important for the ant-attended *T. dryophila*, *S. symbiotica* and *Wolbachia* are frequently found in the body of this aphid as well. In contrast, *T. annulatus* is seemingly less dependent on *B. aphidicola*, but the presence of the facultative symbionts *Hamiltonella* and *Rickettsia* are also important for this aphid species. This differences can be explained in relation to the effect of oak regeneration type, natural or artificial. Such regeneration types determined more than 84% of the symbionts distribution in *T. dryophila*, whereas for *T. annulatus*, regeneration type determined some 74% of its symbionts. The effect of sites (without regeneration types) considered as location, had only around 15% effect on endosymbiont distributions for the first, and 23.7% influence on the second, oak aphid species.

5 Conclusions

Here we report for the first time the endosymbiont bacterial diversity of two oak feeding aphid species, the ant-attended *Thelaxes dryophila* and the non-attended *Tuberculatus annulatus* from both natural and artificial regeneration of pedunculate oak forests. Differences in *B. aphidicola* obligate endosymbiont abundance were also detected between the two aphid species. Also for the first time, we report the presence of *Serratia marcescens* and *Enterobacter cloacae* as symbionts of *T. dryophila*, and the presence of *Shigella boydii* as a symbiont of *T. annulatus*. Difference between the effect of natural and artificial regeneration sites on symbiont diversity were detected. Altogether, we conclude that the two oak aphid species harbour qualitatively and

quantitatively different facultative symbionts between different oak forest regeneration types that might have effects in adaptation of the aphid hosts through the presence of these various microorganisms. New pest management methods based on aphid facultative symbionts control – i.e. application of bacteriophages – has to be considered as specific tool for aphid control.

Declarations

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