

Pollen microbiomes of solitary bees (*Osmia rufa* L. syn. *O. bicornis*) from seed orchards

Mateusz Kęsy (✉ matkesy@gmail.com)

Poznań University of Life Sciences

Tomasz Cłapa

Poznań University of Life Sciences

Dorota Narożna

Poznań University of Life Sciences

Research Article

Keywords: Solitary bee, pollen, seed orchards, mortality, genomic DNA analyses

Posted Date: October 3rd, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-3382180/v1>

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Additional Declarations: No competing interests reported.

Abstract

The red mason (*Osmia rufa* L. syn. *O. bicornis*) is a solitary bee that is commonly used for pollination of fruit crops. Given the declining numbers of pollinators in various environments, the mason bee has not only become an effective insect as an additional pollinator, but sometimes it becomes the main species that is used to increase the yield of crops. Due to easy rearing and not showing aggressiveness towards people. The individual features of the mason bee have made the interest in this species constantly growing. Currently, the wild bees is more important for the forestry and production the seeds. So far, only the honeybee (*Apis mellifera mellifera*) has been successfully used to work in forest areas, especially with the flowers of *Tilia cordata* Mill. The rearing of mason bee (*Osmia rufa* L.) in forest areas is new. The paper presents information on the microbiome of pollen collected from the brood chambers of the solitary bee *Osmia rufa* L. in various forest biotopes: *Tilia cordata* Mill, *Quercus petraea* Liebl. and *Prunus avium* L. The presence of five groups of bacteria was found in all analyzed pollens were rich in α -*Proteobacteria*, *Actinobacteria* and *Firmicutes*, while β -*Proteobacteria* and *Bacteroides* were present in a lower level. Despite the presence of bacteria in the pollen, no increased bee mortality was observed in any of the bee nests of forest biotopes. It was found that the forest environment does not pose a threat to the development of *Osmia rufa* L. bees.

Introduction

Solitary bees (*Osmia rufa* L.) are reared in a controlled manner [29, 12]. Research with use of the masonry had been carried out for many years. So far, they have focused on assessing the possibility of using the species for pollinating horticultural, agricultural and seed crops, and the biology of bees has been studied in detail [9, 11]. The food base used by this species is quite broad, therefore it is described as a polylectic species, consuming pollen and nectar from various plant species [20, 28]. In the forest environment it uses the pollen or nectar of such tree species to the greatest extent as: oak (*Quercus* sp.) and cherry (*Prunus* sp.) [21]. *Osmia rufa* L. can also be used as a species supporting the pollination of other tree and shrub species [10]. Forest areas are an environment for bees in which they can properly develop and pollinate selected plant species [10]. *Osmia rufa* L. is characterized by the fact that it carries bee pollen on a specially designed brush located on the underside of its abdomen [2]. The bee's weight does not exceed 100 mg and its body is 12 mm long [2], and its occurrence in nature occurs in April, when the temperature exceeds 15°C [11]. They most willingly nest in plant stems, the diameter of which is between 5–8 mm inside the stems [26]. In the mason's nests, you can often find other insects, they are so-called nest parasites that are attracted by chemical signals, which means that they are using pollen collected in the breeding chambers of females of this species [2]. Among species belonging to nest parasites, there are representatives of families closely related to masonry, e.g., *Megachiliade*, *Drosophiliade* and *Chrysidadae* [2]. They are usually building linear nest cells, that they place in dry and hollow stems [6, 19]. Female bees deliver a mixture of clay, mud and salivary gland secretions to the nests. It enables the creation of breeding chambers for the larvae developing inside the plant stems [19], where the female assembles first the fertilized eggs (female diploid offspring) and then the unfertilized eggs (male haploid offspring) [11,

12]. Unfertilized eggs are usually closer to the stem exit. The last cell built in the stem remains empty, protecting the remaining cells from parasites and sudden changes in ambient temperature [25]. These insects can be introduced into the cultivation areas outside their natural period of occurrence (early-spring) - even up to 3 months later. For this purpose, it is enough to cool the bees' organisms to the temperature corresponding to the hibernation time (about 5°C) [27], which allows for later than usual use of the bees for pollination, e.g., small-leaved linden (*Tilia cordata* Mill.) [1]. So far, the bee species *Osmia rufa* L. has also been the subject of research in relation to the pollen that is carried into its nests. Splitt et al. [20] and Pearson et al. [17] showed that the species of this bee willingly uses the pollen of plant species that occur in the environment, including oak trees, that moreover are recommended by the same authors for planting to support the development of bees [3, 4]. In their research, they revealed that the pollen composition referring to the participation of individual groups of plants, which undoubtedly increases our knowledge about the species. Other researchers have shown that the richness and quantity of pollen in the nest can be influenced by land use and the landscape of the structure [14, 23, 18]. Eckhardt et al. [7] suggested in research that it is common for bees to mix different pollen that accumulate in the nests, and in the case of predominance of some of them, larvae mortality may be higher [17, 8]. The phenomenon described in our study concerning the presence of bacteria and other microorganisms in the pollen of solitary and honeybees that already has been written off by various researchers [22, 13, 24, 14, 15, 16].

Materials and methods

Biological material

The research material was pollen from the rearing chambers of the *Osmia rufa* L. bees. The pollen content was assessed in terms of the presence of microorganisms. The area with nests of *Osmia rufa* L. were forest monocultures - forest seed plantations of three tree species. Small-leaved lime (*Tilia cordata* Mill.), Sessile oak (*Quercus petraea* (Matt.) Liebl., and wild cherry (*Prunus avium* L.) seed orchards located in western Poland were selected for the research. *T. cordata* was cultivated in 4 forest districts (Fig. 1): Jastrowie (53°39"N; 116°74"E), Pniewy (52°48"N; 16°26"E), Łopuchówko (52°57"N; 17°09"E) and Świerczyna (53°42"N; 16°21"E). The size of these orchards was as follows: 4.00; 4.32; 2.20 and 4.43 ha. *Q. petraea* cultivated areas were located in Tuczno (53°22"N; 16°09"E) and Złocieniec (53°45"N; 16°07"E) and covered the area of 7.89 and 4.76 ha, while *P. avium* orchards covered by the experiment were located in Łopuchówko (52°57"N; 17°10"E) and Świerczyna (53°46"N; 16°30"E). Their area was 2.18 and 3.98 ha respectively. These seed orchards were established at different times: Jastrowie (1987), Łopuchówko (*Prunus* 2007, *Tilia* 1996), Pniewy (1996), Świerczyna (*Prunus* 2010, *Tilia* 2000), Tuczno (2005), Złocieniec (2005).

The nests of the insects from which the pollen originated were made of common reed stalks, which are popularly used to breed these insects. The pollen was extracted from the bees' nests in the fall and then stored in a refrigerator at 4 °C.

Nests with bees were placed in selected forest monocultures (three different species of deciduous forest trees) in such a way that the flying distance of the insects was maximally appropriate to their capabilities. Naturally, solitary bees collect food at a distance of about 300–400 meters from the nest, which is why forest areas made it possible to use the bees' work to a high extent.

Bee nests were built in the area of 8 forest crops (monocultures), which collected pollen from the forest area throughout their lives for the larvae (new generation).

The bees collected pollen from forest plants, which also contained microorganisms (naturally living in the forest environment). The effect of this phenomenon shows that bees brought foreign microorganisms together with pollen (food for bee larvae). Thanks to the pollen collected from the nests, it was possible to better understand the process taking place between bees, forest microorganisms and the interior of bee nests, to which microorganisms on the bees' bodies reached.

Genomic DNA extraction

To extraction of total DNA from the investigated pollen samples from the breeding chambers of the *Osmia rufa* L. bees had been done by using the protocol of Zhou, Bruns, Tiedje, 1996 (with modifications). One gram of the sample materials was placed into a sterile Eppendorf tube and 200 µL of DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% CTAB) was added. The samples were ground off. Next, the 20 µL of lysozyme (10 mg/mL) and 20 µL of proteinase K (10 mg/mL) were added and incubated for 1.5 h at 65°C on a horizontal shaker at 100 rpm/min. After incubation, samples were centrifuged at 6000 rpm for 10 min. at room temperature (RT). The supernatant was transferred to a new Eppendorf tube and extracted with an equal volume of chloroform and vortex vigorously. The aqueous phases were transferred into fresh Eppendorf tubes.

After extraction, the samples were clean thoroughly as follows: 300 µL of each sample was incubated for 15 min. at RT with an equal volume of isopropanol. Furthermore, the samples were centrifuged at 14.000 rpm for 10 min. the supernatant was discarded, and the pellet was washed with 70% ethanol, dried and resuspended in 50 µL of autoclaved double distilled water. DNA prepared in this way from individual pollen samples was subjected to 1% agarose gel electrophoresis (to remove PCR inhibitors), appropriate gel fragments with pollen genomic DNA were excised and DNA was recovered using the Wizard® SV Gel and PCR Clean-Up System (Promega). The DNA from the samples were precipitated by adding 2.5 volume of cold ethanol (to increase DNA concentration). After gentle mixing, the samples were left overnight at -20°C. Precipitates were collected by centrifugation at 14.000 rpm for 10 min. at 4°C. The pellets were washed with 70% ethanol, dried and resuspended in 35 µL of autoclaved double distilled water. DNA concentrations in individual pollen samples were determined using the spectrophotometric method, DNA at a concentration of 10 ng/µL was used for molecular analyzes.

Amplification of 16S rDNA of selected groups of bacteria

The sets of primers for major phylogenetic groups of microorganisms had been used (see Table 1.), based on the work of [8], except the primers for 16S rDNA (this study). A volume of 1.0 µL of the genomic DNA was PCR-amplified using Allegro Tag Polymerase DNA (Novazym Polska s.c., Poznań, Poland) in a 25 µL reaction mixture. The conditions of the reactions were as follows: initial denaturation at 94°C for 2 min., which was followed by 30 cycles of denaturation (94°C for 30 s), annealing (see Table 1. for 30 s), elongation (72°C for 2 min.) and final elongation (72°C for 4 min.).

Restriction analysis of 16S rDNA sequences (ARDRA).

The amplified 16S rDNA products were characterized by hydrolysis with endonuclease *CfoI* at 37°C for 2 h. The digestion products were separated in 1.7% agarose gel electrophoresis for 90 min. at 80 V. Bands were visualized by staining with ethidium bromide and UV illumination.

RAPD-PCR pollen samples collected from *Osmia rufa* L. bee breeding chambers.

The diversity of DNA from pollen samples collected from *Osmia rufa* L. bee breeding chambers was analyzed using PCR with primer RP01 (5'-AATTTTCAAGCGTCGTGCCA-3') as described by Bosch, Sgolastra & Kemp [2008]. The PCR was carried out using Allegro Taq DNA polymerase (Novazym, Poznań, Poland) under the following conditions: initial denaturation at 95°C for 2 min.; 30 cycles of 30 s at 95°C, 30 s at 45°C, and 1 min. at 72°C; and a final elongation step of 5 min. at 72°C. The PCR products were analyzed on 1.5% agarose gels.

Results and discussion

Molecular analysis of selected groups of microorganisms inhabiting pollen samples collected from *Osmia rufa* L. bee breeding chambers.

Genomic DNA was isolated from 8 pollen samples collected from *Osmia rufa* L. bee breeding chambers. The isolated DNA was the template for the amplification of 16S rDNA gene fragments in order to track quantitative changes in the populations of *α-Proteobacteria*, *β-Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and all species of fungi (except basidiomycetes) inhabiting individual samples. Equalized concentrations of DNA templates in the PCR reaction allowed to determine the relative intensity of the amplified PCR products for all tested groups of microorganisms. The composition of taxonomic groups were very similar between the samples, most dominant groups were *α-Proteobacteria*, *Firmicutes* and *Actinobacteria*. In addition to these groups, *β-proteobacteria* and *Bacteroidetes* were also found (Fig. 4).

As can be observed, all tested pollens were particularly rich in *Firmicutes* (all at similar level), *Actinobacteria* (particularly pollens 2, 3 and 8) and *α-Proteobacteria* (6, 7 and 8), which may be related to the presence of various plant species near solitary bees nests. The diversity of plant species from which solitary bees collected pollen is confirmed by the profiling of DNA isolated from pollen by the RAPD method Fig. 2. The other two groups of bacteria, *β-Proteobacteria* and *Bacteroides*, are present in all pollen at much lower levels (especially *β-Proteobacteria*).

Profiling of DNA isolated from pollen collected from brood chambers using the RAPD method Fig. 5A indicates a significant diversity of individual pollens resulting from the diversity of plant species found in the vicinity of the brood chambers of the solitary bee (Table 1).

Profiling of DNA isolated from pollen collected from brood chambers using the RAPD method Fig. 5. indicates a significant diversity of individual pollens resulting from the diversity of plant species found in the vicinity of the brood chambers of the solitary bee (Table 1).

On the other hand, a comparison of the profiles of bacterial 16s rDNA gene fragments digested with restriction endonuclease *CfoI*, Fig. 5B allows to conclude that pollen collected from breeding chambers set up in seed orchards of *Q. petraea* Liebl., *T. cordata* Mill. and *P. avium* L. have different DNA profiles, which indicates that the microbiomes of individual pollens are similar but not identical.

In all analysed pollens, apart from bacteria from various groups, the presence of fungi Fig. 6, that are colonizing pollen from *Q. petraea* Liebl. and *P. avium* L. seed orchards collected from breeding chambers were at a similar level, while for pollen collected from *T. cordata* Mill. seed orchards, the pollen collected from the Jastrowie site was particularly rich in fungi.

Conclusion

The solitary bee *Osmia rufa* L., due to its biology and the possibility of introducing it to forest environment, also outside its natural period of occurrence in nature, has a chance to become one of the main pollinating insects used into increasing the yield of forest crops in the future. Our study examined the microbiome of pollen collected from solitary bee nests located in various forest monocultures. Collected pollen stocks had been found to contain a variety of microbial community groups, including being a major source of food for the development of bee larvae. The composition of taxonomic groups of microorganisms were similar between the tested samples. The most dominant groups were α -*Proteobacteria*, *Firmicutes* and *Actinobacteria*, in addition to these groups β -*Proteobacteria*, *Bacteroidetes* and fungi were also found. Solitary bee nests were located in various monocultures of forest seed orchards, which were accompanied by other plants within a radius of less than 1 km (Table 2). DNA profiling by RAPD method by using primer RP04 shows differences in the DNA pool of individual pollens, which may be a result of presence of pollen from plants present in the forest monocultures collected by solitary bees. However, narrowing the DNA analysis of individual pollens to bacterial communities using the ARDRA method indicates that bacterial populations are similar within the same forest seed orchards from different locations (Fig. 4B).

Pollen-associated microbes are critical to bee health, not only as nutrients but also as a major component of the diet. In times of global bee decline, interactions between bees and microbes could be an important aspect of pollinator conservation strategies. Most of the diverse microorganisms present in solitary bee nests come from environmental sources, not only from stored pollen but also from the nests building material. Such diversity of microorganisms, both bacterial and fungal, may affect the survival rate of solitary bee larvae. Our research conducted in forest areas of various seed orchards indicates that the

mortality rate of solitary bee larvae ranged from 11 to 41%. Higher mortality of larvae (41%) was observed on *Tilia cordata* Mill. plantations (Łopuchówko) (based on authors' research; results not published yet).

The conducted research on bee pollen also provides evidence of the diverse biodiversity of forest communities, that are a natural habitat for pollinating insects, including bees of the species *Osmia rufa* L. at the same time. Laboratory analyzes shows which groups of microorganisms participate in the natural breeding process of bees. Results presented in this work also provide new facts that can be used when planning bee rearing and selecting sites for effective insect reproduction.

Declarations

Author Contributions

M.K. conceptualize the review and wrote the manuscript, collect the data for analysis; T.C. wrote the manuscript and analyze the data; D.N. analyze the data and wrote the manuscript.

Acknowledgments

This work and research was founded by the authors.

Conflict of Interest

The authors have no financial conflicts of interests to declare.

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Tables

Table 1. Primers used for PCR.

Target group	Primers	Amplicon length (bp)	Annealing temp. (°C)	Primer sequence (5' – 3')
All bacteria	Eub338F	200	53	ACT CCT ACG GGA GGC AGC AG
	Eub518R			ATT ACC GCG GCT GCT GG
<i>α-Proteobacteria</i>	Eub338F	365	60	ACT CCT ACG GGA GGC AGC AG
	Alf685R			TCT ACG RAT TTC ACC YCT AC
<i>β-Proteobacteria</i>	Eub338F	360	60	ACT CCT ACG GGA GGC AGC AG
	Bet680R			TCA CTG CTA CAC GYG
<i>Firmicutes</i>	Lgc353F	180	60	GCA GTA GGG AAT CTT CCG
	Eub518R			ATT ACC GCG GCT GCT GG
<i>Bacteroidetes</i>	Cfb319F	220	65	GTA CTG AGA CAC GGA CCA
	Eub518R			ATT ACC GCG GCT GCT GG
<i>Actinobacteria</i>	Actino235F	300	60	CGC GGC CTA TCA GCT TGT TG
	Eub518R			ATT ACC GCG GCT GCT GG
All fungi	5.8sF	300	53	CGC TGC GTT CTT CAT CG
	ITS1fR			TCC GTA GGT GAA CCT GCG G

Table 2. Diversity of plant species found in the vicinity of the brood chambers of the solitary bee

Species and area	Plants growing around seed orchards (<1 km)*	Breeding chambers with parasites	Dead bee larvae	Live bees	Total number of brood chambers built by insects
<i>Quercus petraea</i> Liebl.	BK, BRZ, CZM, DB, JRZ, SO	2 0%	848 26%	2393 73%	3273
Tuczno					
<i>Quercus petraea</i> Liebl.	BK, BEZ.C, BRZ, CZM, JRZ, KL, OS, SO, SW	68 1%	657 11%	4478 86%	5212
Złocieniec					
<i>Tilia cordata</i> Mill.	BK, BRZ, CZM, DB, DG, GB, JD, JS, LP, MD, SO, SW, WZ	49 2%	464 17%	2067 78%	2656
Świerczyna					
<i>Tilia cordata</i> Mill.	BK, CZM, DB, DG, GB, GŁG, JD, JW, LP, MD, SO, SW, TP, WZ	222 15%	208 14%	925 64%	1455
Jastrowie					
<i>Tilia cordata</i> Mill.	BK, CZM, GB, JS, JW, KL, LP, LSZ, OL, OS, PB.S, SO, WZ	-	329 28%	828 71%	1166
Pniewy					
<i>Tilia cordata</i> Mill.	AK, BRZ, CZM, DB.S, DG, JW, LP, MD, SO, SW	192 9%	875 41%	1001 47%	2135
Łopuchówko					
<i>Prunus avium</i> L.	BK, BRZ, CZM, DB, MD, OS, SO, SW	-	586 15%	3139 82%	3830
Świerczyna					

<i>Prunus avium</i> L.	BK, BRZ, CZM, DB.S, GB, MD, SO	-	567 13%	3822 86%	4427
Łopuchówko					

* AK- *Acacia* Mill., BK- *Fagus sylvatica* L., BRZ- *Betula* L., DB.S- *Quercus robur* L., DB.B- *Quercus petraea* Liebl., DBC- *Quercus rubra* L., DG- *Pseudotsuga menziesii* (Mirb.) Franco, GB- *Carpinus betulus* L., JD- *Abies alba* Mill., JS- *Fraxinus* L., JW- *Acer pseudoplatanus* L., KL- *Acer platanoides* L., LP- *Tilia cordata* Mill., MD- *Larix decidua* Mill., OL- *Alnus glutinosa* (L.) Gaertn., OS- *Populus tremula*, SO- *Pinus sylvestris* L., SW- *Picea abies* (L.), TP- *Populus* L., WB- *Salix* L., WZ- *Ulmus minor* Mill., JRZ- *Sorbus aucuparia*, CZM- *Prunus avium* L., LSZ- *Corylus avellana* L., Bez.C- *Sambucus nigra* L.

Figures

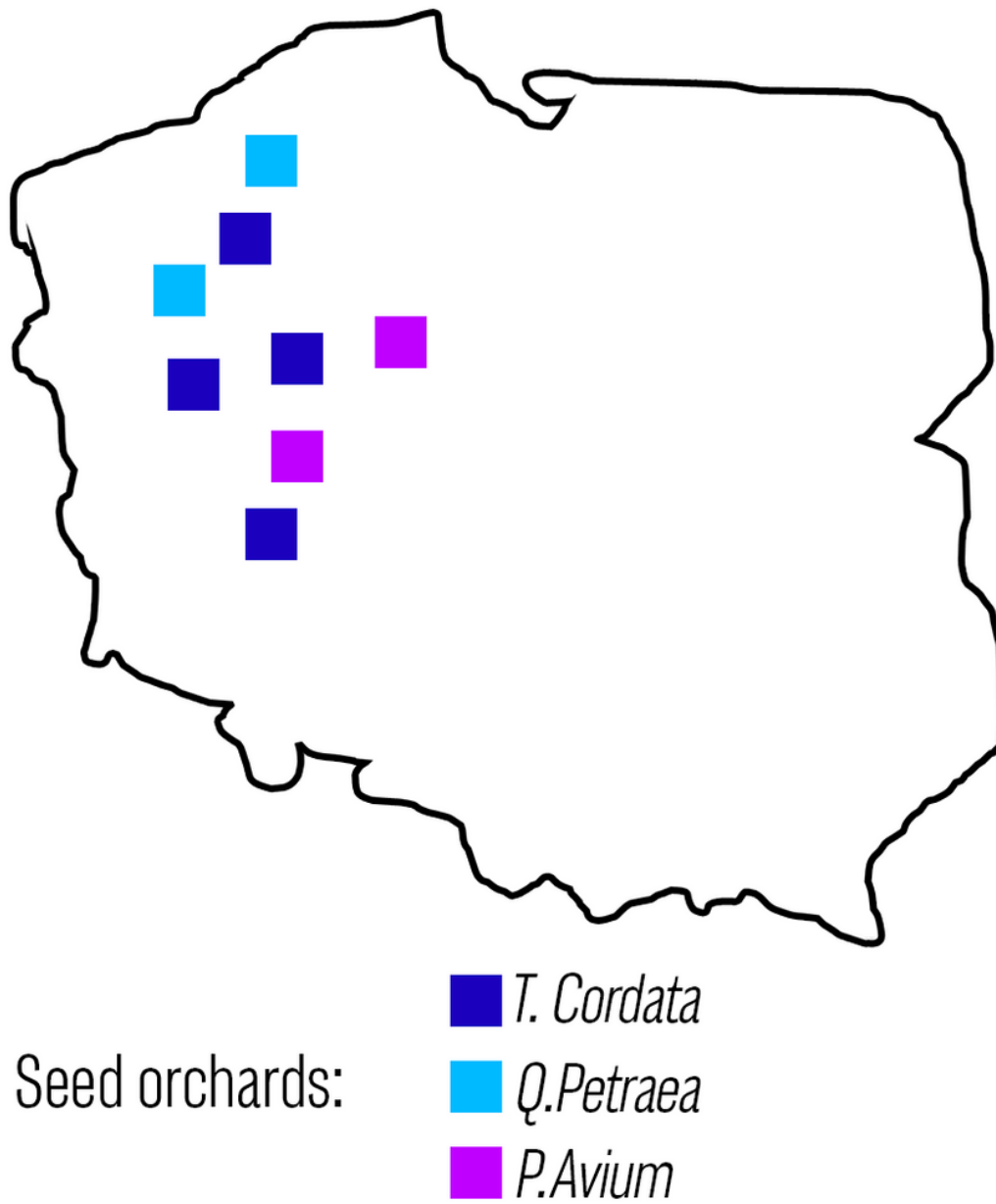


Figure 1

Research area showing the distribution of forest areas (monocultures) where nests with bees *Osmia rufa* L.

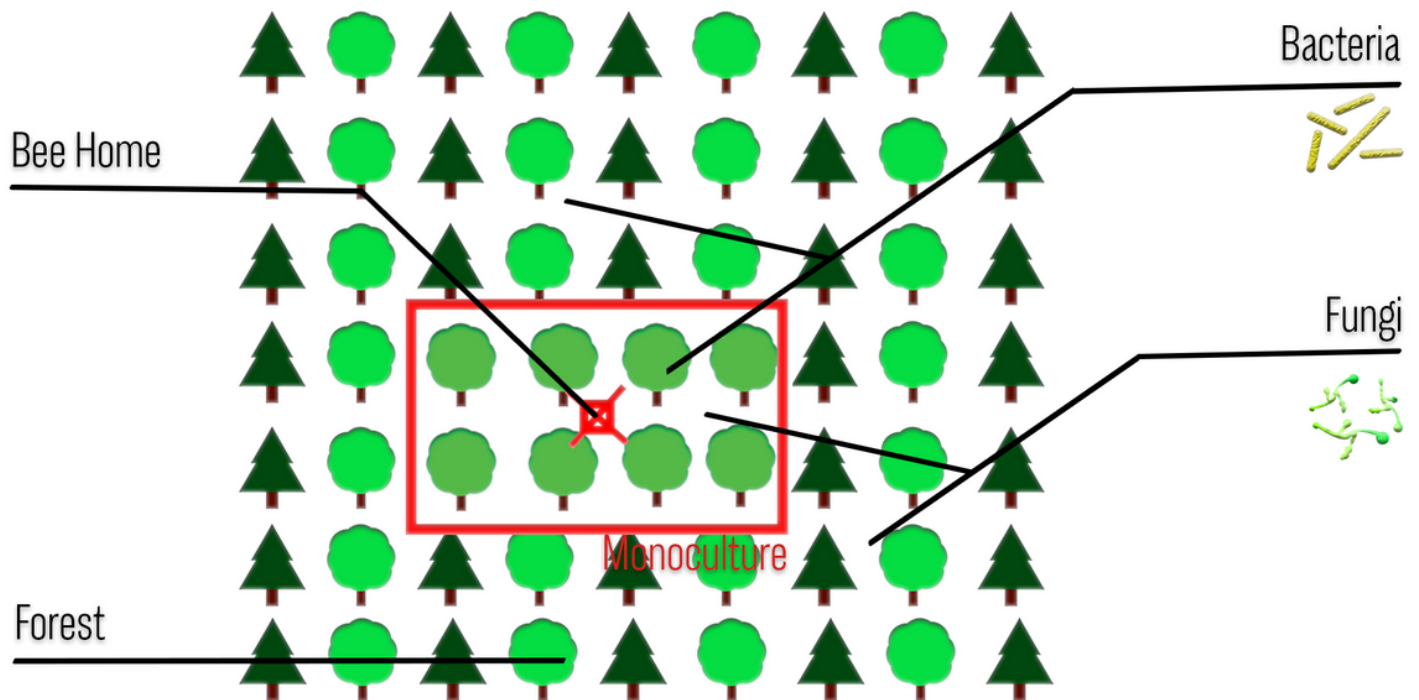


Figure 2

Situation between bees and forest trees (monoculture) in the forest environment

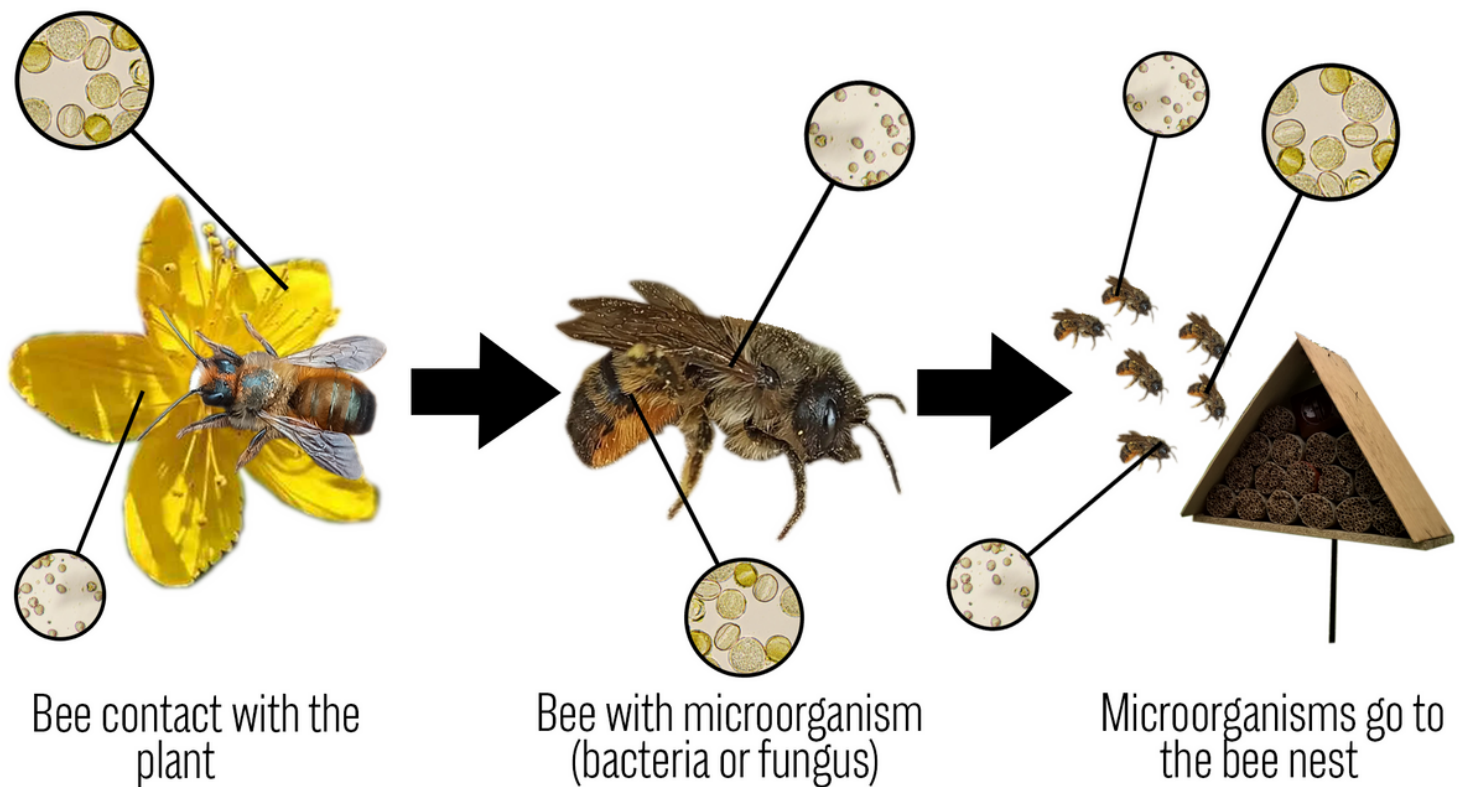


Figure 3

Bee homes and microorganism

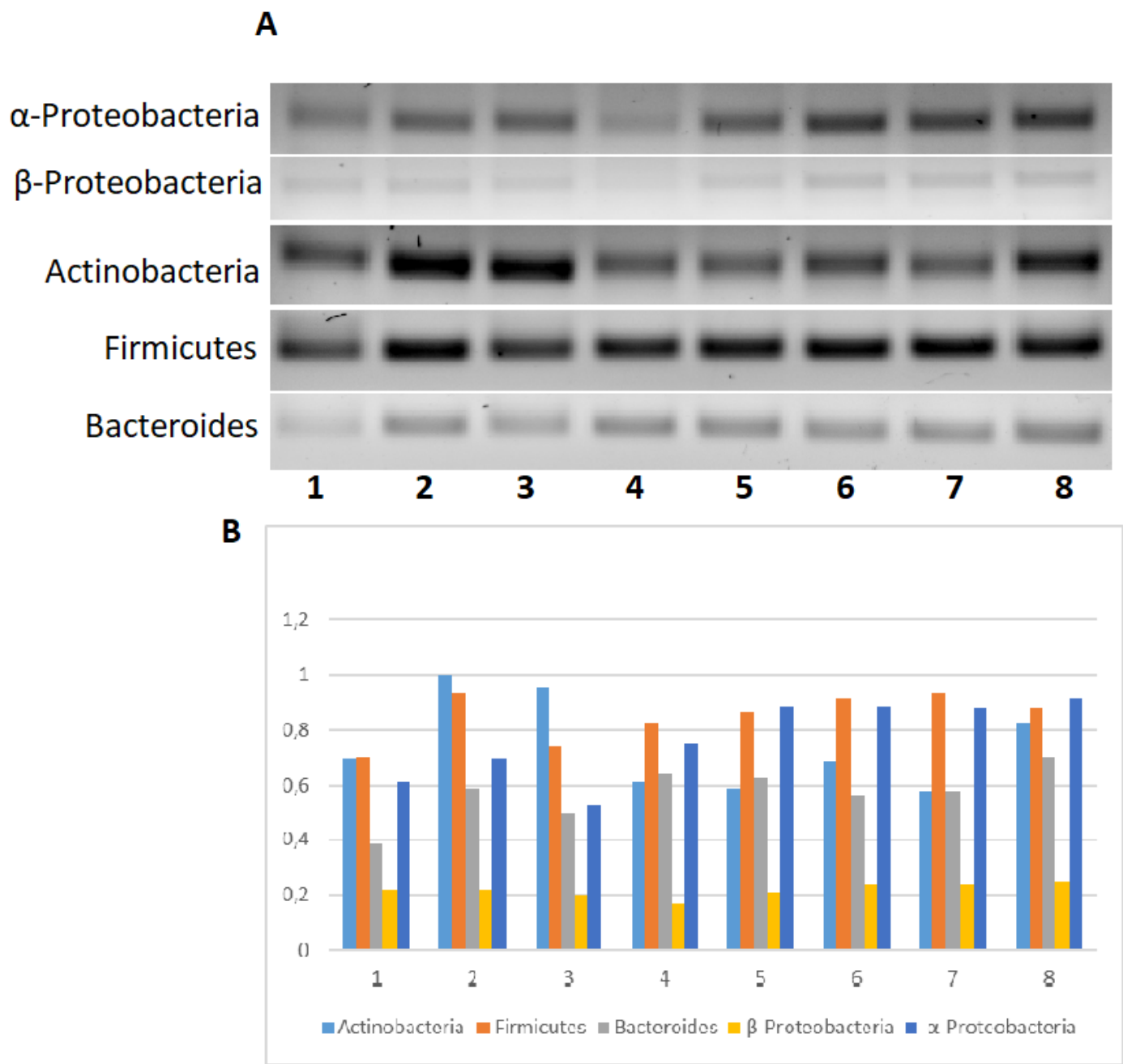


Figure 4

Bacteria colonizing pollen samples collected from the breeding chambers of *Osmia rufa* L. The individual lanes in the agarose gel (A) and the bars in the graph (B) correspond to 1 and 6 seed orchards (*Q. petraea* Liebl.); 2, 3, 4 and 5 seed orchards (*T. cordata* Mill.) 7 - 8 seed orchards (*P. avium* L.).

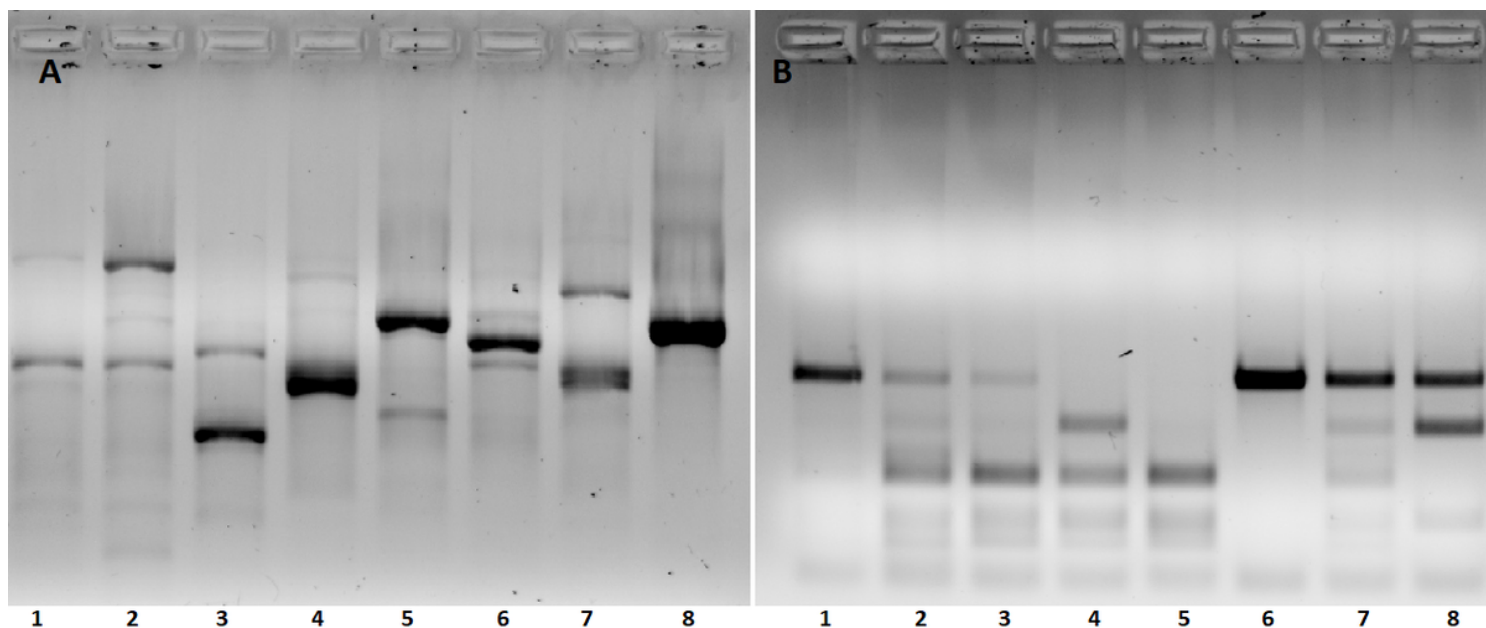


Figure 5

Profiling of DNA isolated from pollen using ARDRA and RAPD method.

The lanes of the gels contain the amplification products obtained with RP04 primer (A) and digestion products of a DNA fragment amplified with 16sF and 16sR primers digested with *Cfo*I restriction endonuclease (B): 1, 6 seed orchards (*Q. petraea* Liebl.), 2, 3, 4 and 5 seed orchards (*T. cordata* Mill.) 7, 8 seed orchards (*P. avium* L.).

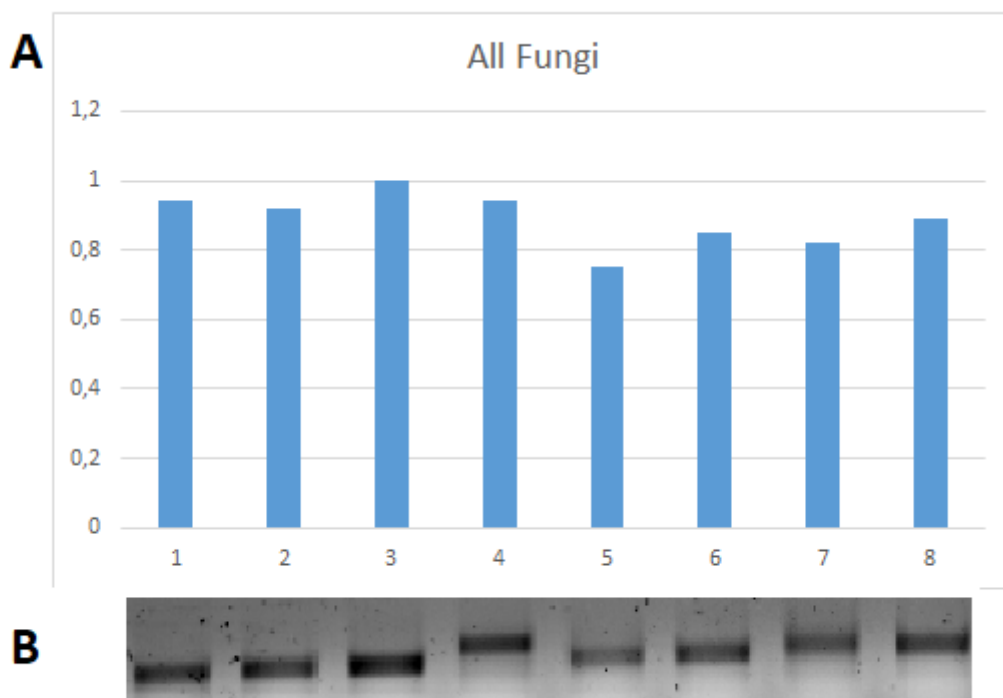


Figure 6

Fungi colonizing pollen samples collected from the breeding chambers of *Osmia rufa* L. The individual lanes in the agarose gel (A) and the bars in the graph (B) correspond to 1, 6 seed orchards (*Q. petraea* Liebl.); 2, 3, 4 and 5 seed orchards (*T. cordata* Mill.) 7 - 8 seed orchards (*P. avium*).