Journal of Apicultural Research and Bee World 48(4): 233-237 (2009)
DOI 10.3896/IBRA.1.48.4.02

ORIGINAL RESEARCH ARTICLE

Phylogenetic comparison of bacteria isolated from the honey stomachs of honey bees *Apis mellifera* and bumble bees *Bombus* spp.

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Received 3 December 2008, accepted subject to revision 22 April 2009, accepted for publication 24 July 2009.

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Summary

It has recently been discovered that the honey bee *Apis mellifera* has a large flora of symbiotic lactic acid bacteria in its honey stomach, belonging to the genera *Lactobacillus* and *Bifidobacterium*. It appears that the flora may protect the honey bees, their larvae and their food against harmful microorganisms. Since bumble bees (*Bombus* spp.) are related to honey bees and have a honey stomach where they store nectar during their flight we investigated whether their honey stomachs also contain symbiotic lactic acid bacteria. Bacterial isolates cultivated from both the surface and from the honey stomachs of bumble bees were identified using 16S rRNA gene analyzes. The results showed that bumble bees also possess lactic acid bacteria in their honey stomachs but in fewer phylotypes and only belonging to the genus *Lactobacillus*. In contrast to honey bees, bumble bees do not produce honey or bee bread but feed their larvae directly with nectar and pollen, and their society does not survive the winter in temperate climates. It was therefore concluded that bumble bees have less need than honey bees of an extensive lactic acid bacterial flora.

Comparación filogenética de bacterias aisladas del estómago de la miel de abejas *Apis mellifera* y abejorros *Bombus* spp.

Resumen

Recientemente se ha descubierto que la abeja *Apis mellifera* tiene una gran flora de bacterias simbióticas del ácido láctico en su estómago de la miel, pertenecientes a los géneros *Latobacillus* y *Bifidobacterium*. Parece que la flora protege a las abejas, a sus larvas y a su comida de microorganismos peligrosos. Como los abejorros (*Bombus* spp) están relacionados con las abejas y tienen estómago de la miel donde conservan néctar durante sus vuelos, investigamos si sus estómagos de la miel contienen también bacterias simbióticas del ácido láctico. Se identificaron cultivos bacterianos aislados de la superficie y del estómago de la miel de abejorros mediante análisis del gen ribosomal ARNr 16S. Los resultados mostraron que los abejorros también poseen bacterias del ácido láctico en su estómago de la miel pero pocos filotipos y pertenecientes únicamente al género *Lactobacillus*. Al contrario que las abejas, los abejorros no producen miel o pan de abeja si no que alimentan directamente a sus larvas con néctar y polen, y sus sociedades no sobreviven al invierno en climas templados, Por tanto se concluye que los abejorros tienen menor necesidad que las abejas de una extensa flora bacteriana del ácido láctico.

Keywords: lactic acid bacteria, LAB, honey stomach flora, bumble bees, honey bees, *Lactobacillus*, *Bifidobacterium*

Introduction

Lactic acid bacteria (LAB) of the genera *Lactobacillus* and *Bifidobacterium* have recently been discovered in the honey stomach of honey bees *Apis mellifera* (Olofsson and Vásquez, 2008; Vásquez *et al*., 2009). The phylogenetic analyzes performed in both studies showed the LAB flora in the honey stomach to be composed of twelve different phylotypes. It appeared that honey bees and the novel LAB flora evolved in mutual dependence on one another, the LAB obtaining a niche in which nutrients are available; the honey bees and their honey in turn being protected by the LAB from harmful microorganisms.

The honey bee brood pathogen *Paenibacillus larvae* has also been detected by sampling house bees and honey stomachs (Olofsson and Vásquez, 2008). Moreover, large numbers of three bacterial phylotypes (LvLi2, Lv2 and Hma5) were found by sampling larvae and honey stomachs during the time that a bee colony was infected with *P. larvae*. These phylotypes were most closely related to the genera *Actinobacillus* and *Phocoenobacter*, belonging to the family *Pasteurellaceae* (Fig. 1, cluster IV; Table 1). A fourth *Pasteurellaceae* phylotype (Trm1) was found only in the hindgut of honey bees (Fig. 1).

The social behaviours of honey bees (Winston, 1987) and bumble bees (Goulson, 2003) differ, but both types of bee collect nectar, a sweet liquid composed of varying amounts of sucrose, glucose and fructose, temporarily stored in their honey stomach during flight. The honey stomach is an enlargement of the oesophagus that can expand to a rather large volume, ending with a structure called the proventriculus, which ensures that the nectar is never contaminated by the contents of the ventriculus (midgut), which is the functional stomach of honey bees and bumble bees.

Both honey bees and bumble bees collect pollen from flowers and store it on their legs during flight in order to feed their larvae and themselves. They maintain a colony temperature of about 35°C. The major differences between them are that a honey bee colony lives through the winter in temperate zones but with bumble bees only the queen survives. Honey bees makes honey by reducing the water content of the nectar from around 50-80% to below 20%, enabling it to be stored through the winter. In contrast bumble bees store their nectar more or less as it is when collected and only for a few days as it is fed directly to their larvae. Honey bees utilise lactic acid bacteria to produce a fermented “bee bread” from pollen, nectar, saliva and honey, which is then fed to their larvae (Vásquez and Olofsson, 2009) whilst bumble bees feed their larva separately with pollen and nectar.

Honey bees and bumble bees are closely related, belonging to the same subfamily *Apinae* (family *Apidae*), and are derived from a common ancestor, so the question prompting this work was whether their bacterial flora in terms of symbionts and pathogens are also related.

Materials and methods

Sampling

Bacteria compared in this study were mainly sampled from the surface of the bees and from their honey stomachs. Bacterial sampling of honey bees *Apis mellifera* was carried out as previously described (Olofsson and Vasquez, 2008; Vásquez *et al*., 2009). For comparison, a variety of bumble bees (*Bombus* spp.) and their honey stomachs were sampled over two years. The bumble bees were collected from Kullaberg, a nature reserve in southern Sweden. They were sampled as they collected nectar and pollen from wild raspberry (*Rubus idaeus* L) and from heather (*Calluna vulgaris*). Samples from bumble bees were retrieved at different occasions from ten foraging bumble bees and from five honey stomachs by aseptic excision according to Olofsson and Vasquez (2008). Only bees whose honey stomachs were full of nectar were selected for this purpose.

Procedure for isolates

For the identification of the bacterial isolates 16S rRNA gene analysis was performed using pure-culture techniques. The bees that were sampled were placed in separate sterile 10 ml tubes, each containing 5 ml sterile physiological saline (0.9% w/v NaCl, 0.1% w/v Tween 80 and 0.1% w/v peptone). The honey stomachs were placed in 1.5 ml sterile micro-tubes, each containing 0.9 ml physiological saline. The tubes with bees were shaken gently and the tubes with honey stomachs were shaken vigorously following immediate transportation to the laboratory in Lund, 70 km from Kullaberg. Pure cultures were obtained on media containing Tryptone Soy Broth agar (TSB) (Oxoid; Basingstoke, Hampshire, UK), tomato juice agar (TJ) (Oxoid), an all-purpose medium containing Tween® (APT) (Merck; Darmstadt, Germany) and Rogosa agar (Merck). The isolates were cultivated both aerobically and anaerobically at 37ºC for 3-4 days. Ten to thirty colonies were picked randomly from each of the media involved, containing 30-300 colonies each, and were re-cultivated for purity isolates. DNA purification from isolates and the following PCR amplification were performed as previously described (Olofsson *et al*., 2007).

Sequencing and identification of DNA

PCR products originating from the bacterial samples were sequenced by a sequencing Company (MWG Biotech; Ebersberg, Germany) using the universal primers ENV1 and ENV2 (Olofsson and Vásquez, 2008). These 16S rDNA sequences were searched against GenBank (National Centre for Biotechnology Information; Rockville Pike, Bethesda, MD, USA) using the Advanced BLAST similarity search option (Altschul *et al*., 1997), accessible from the homepage of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The 16S rRNA gene sequences were also checked using the software RDP

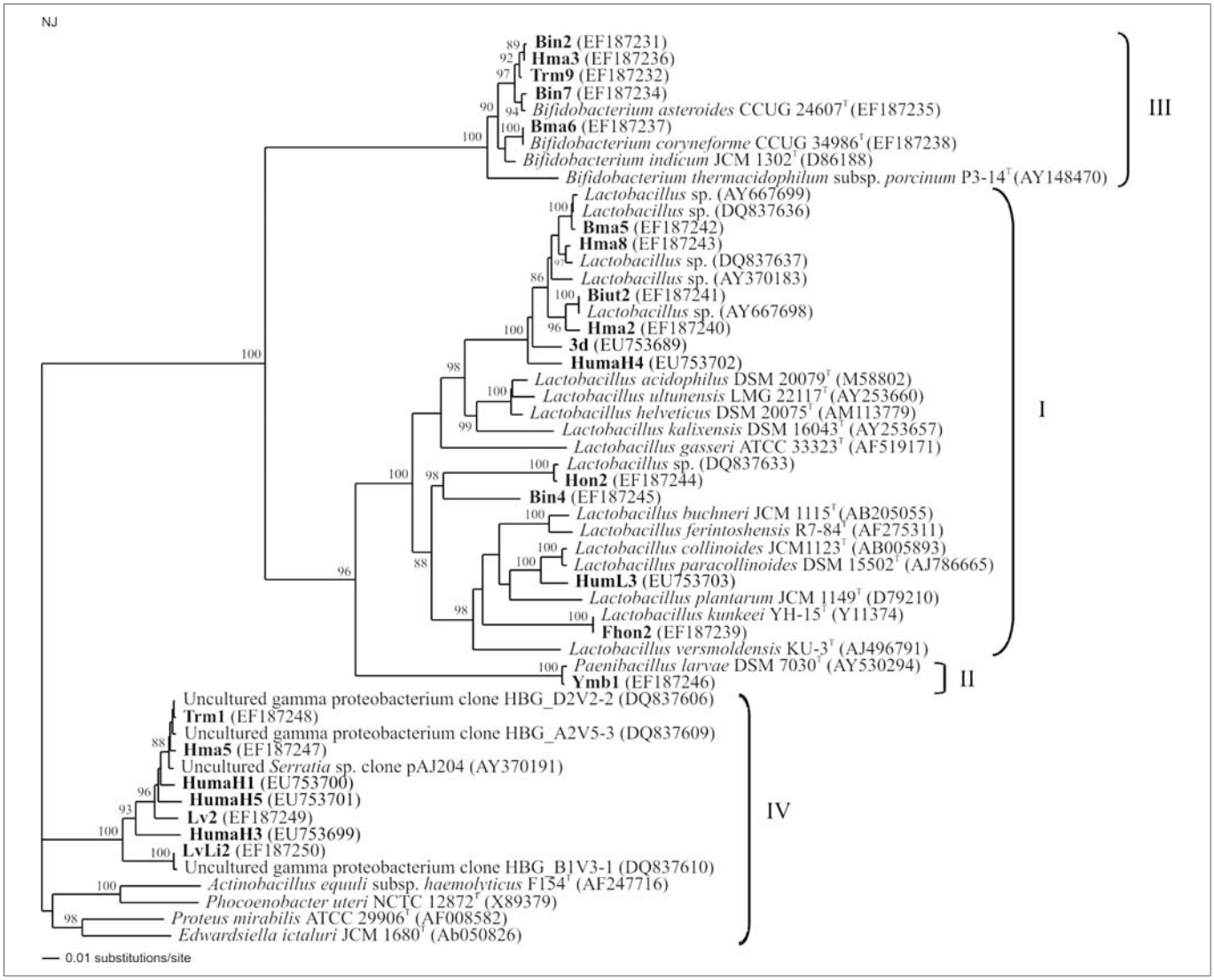

Fig. 1. A phylogenetic tree based on a distance matrix analysis of 745 positions in the 16S rRNA gene. Closely related type strains and reference strains are indicated in parenthesis, together with accession numbers from GenBank. Cluster I displays the *Lactobacillus* group, cluster II *Paenibacillus larvae*, cluster III the *Bifidobacterium* group, and cluster IV the *Pasteurellaceae* group, which served as the out-group. The phylotypes characterised in the study are in bold print, the accession numbers being included. Bar: 0.10 substitutions per nucleotide position.

(Ribosomal Database Project II) (Cole *et al.*, 2005), accessible from the homepage (http://rdp.cme.msu.edu/). The partial sequences were approximately 750 base pairs (range 50-800 bp).

Phylogeny

The phylogenetic tree shown in Fig. 1 was obtained using the computer software programs: Clustal X (version 1.81) (Thompson *et al.*, 1997) for alignment; BioEdit (version 6.0.7) (Hall, 1997) for editing; and PAUP (version 4.0 beta) (Swofford, 2003) for computing the phylogenetic tree. The tree was constructed using the neighbour-joining method (Saitou and Nei, 1987) in PAUP by Distance Matrix. Evolutionary distances were estimated using the LogDet/Paralinear method. Bootstrap values were computed using 1,000 re-samplings, evolutionary distances being estimated using the LogDet/Paralinear method. The bacterial 16S rRNA gene sequences were deposited in GenBank using the accession numbers EF187231-EF187250 and EU753688- EU753698 for the honey bee isolates and EU753699-EU753703 for the bumble bee isolates.

Results

Distribution of bumble bee derived isolates

A total of 45 sequences from bacteria were identified from isolates picked from both aerobic and anaerobic plate counts, all showing a similarity of >85% to type strains in RDP. The identity of bacteria that did not originate from bumble bees is not indicated.

The bacterial flora of bumble bees

The samples isolated from the surface of the bumble bees and from their honey stomachs were dominated by two different *Lactobacillus*

Table 1. Bacterial phylotypes originating from honey bees and their larvae. The identity of 16S rRNA gene sequences generated from isolates and clones. [*]The sequence lengths are shown in parentheses; the number of identical sequences found are shown in brackets. [**] GenBank accession numbers are shown in parentheses; taxonomic affiliation was established by comparing the sequence in the database of the Ribosomal Database Project II (http://www.rdp.cme.msu.edu/) with the entry labelled "sequence match'' and the options ''type'' and ''NCBI.'' [***] The similarity to the closest Type strain sequence is shown as a percentage within parentheses.

Isolates[*]	**Most closely related type strain[**]**	**Sequence lengths and similarity[***]**
HumL3 (490-800) [11]	*Lactobacillus collinoides* JCM1123^T (AB005893)	800 (97.0)
HumaH4 (590-966) [12]	*Lactobacillus kalixensis* DSM 16043T^T (AY253657)	960 (91.6)
HumaH1 (700-920) [4]	*Actinobacillus equuli* subsp. *haemolyticus F154T (AF247716)*	920 (85.9)
HumaH3 (730-930) [2]	*Actinobacillus equuli* subsp. *haemolyticus F154T (AF247716)*	930 (85.4)
HumaH5 (1000) [1]	*Actinobacillus equuli* subsp. *haemolyticus F154T (AF247716)*	1000 (86.8)

phylotypes (HumL3 and HumaH4) (Fig. 1). The phylotype HumL3 was isolated from the surface of the bumble bees when foraging on heather and the phylogenetic analysis (Fig. 1) indicated that it was most closely related to the *Lactobacillus* genus (Cluster I in Fig. 1 and Table 1). The phylotype HumaH4 was isolated from the honey stomach of the bumble bees when foraging on wild raspberry flowers and the phylogenetic analysis indicated that it was distant but most closely related to the *Lactobacillus* genus (Cluster I in Fig. 1 and Table 1). In addition, three bacterial phylotypes (HumaH1, HumaH3 and Huma5) were found by sampling the honey stomach when bumble bees were foraging on wild raspberry flowers. These phylotypes were most closely related to the genera *Actinobacillus* and *Phocoenobacter*, belonging to the family *Pasteurellaceae* (Fig. 1, cluster IV and Table 1).

Discussion

The aim of this study was to investigate whether the two related insects, honey bees and bumble bees, have closely related bacterial symbionts or pathogens although their social behaviours and life cycles differ. In the previous studies of Olofsson and Vásquez (2008) and Vásquez *et al.* (2009), the honey stomach of honey bees was identified as a niche for a bacterial flora composed of lactic acid bacteria from the genera *Lactobacillus* and *Bifidobacterium*. In addition, the honey bee pathogen *P. larvae* responsible for American foulbrood disease (AFB) and novel bacterial phylotypes belonging to the *Pasteurellacea* family were detected in the honey bee honey stomach. In the present study, the honey stomach of bumble bees was dominated by only one *Lactobacillus* phylotype (HumaH4). This phylotype is distant related to the *Lactobacillus* genus and showed a 91.6 % similarity of the partially sequenced 16S rRNA gene to the type strain of *Lactobacillus kalixensis* (Fig. 1). Phylotype HumL3, isolated from the surface of bumble bees, showed a 97.0% similarity of the partially sequenced 16S rRNA gene to the type strain of *Lactobacillus collinoides* (Fig. 1). Although HumL3 was retrieved from the surface of bumble bees, when shaking them in a liquid medium, it is possible that this phylotype may originate from the honey stomach. These two phylotypes that are most closely related to the *Lactobacillus* genus may comprise novel species, since their sequences only resembled the closest known taxon by 91.6 – 97.0% (Table 1), which is below the threshold level generally used to define a genus (95 - 97%) (Ludwig *et al.*, 1998). It is feasible that both phylotypes HumaH4 and HumL3 have their niche in the honey stomach of bumble bees like phylotypes Hon2, Hma2, Fhon2, Hma8, Bma5, Biut2, Bin4 and 3d (Fig. 1) discovered in the honey stomach of honey bees. In fact, the bumble bee phylotype HumaH4, derived from the bumble bee honey stomach, is distant but more closely related to the honey bee honey stomach *Lactobacillus* phylotypes 3d, hma2, Hma8, Bma5 and Biut2 than to any previously described *Lactobacillus* type strain (Fig. 1). The phylotype HumL3 from bumble bees was distant but closest related to *Lactobacillus paracollinoides* and *L. collinoides*. Interestingly, it was phylogenetically situated in the same cluster as the previously described honey bee honey stomach *Lactobacillus* phylotype Fhon2 (Fig. 1).

No bifidobacteria were isolated from the bumble bees in this work. The flora within the bumble bee honey stomach, that showed one or perhaps two LAB phylotypes, differed in numbers compared to the honey stomach LAB flora found in honey bees in which eight different *Lactobacillus* and four different *Bifidobacterium* phylotypes were isolated.

We have previously suggested (Olofsson and Vásquez, 2008; Vásquez *et al.*, 2009), that the newly discovered lactic acid bacterial flora members living in the honey stomach of honey bees are probably honey bee symbionts that have evolved together with the bees. We strongly believe that the honey stomach LAB serves to protect the production of honey from spoilage microorganisms during

its transformation from nectar to honey. This is a process that can take several days to reduce the water content from 50 – 80% in the nectar to below 20% in the ripened honey. We furthermore suggested that the honey stomach LAB takes part in protecting the bees themselves and their larva against pathogens. We suggested (Vásquez and Olofsson, 2009) that honey bee bread is probably fermented and preserved by the honey stomach LAB flora that has been added to bee pollen via regurgitated nectar from the honey stomach.

The significant difference between the LAB floras found from honey bees and bumble bees could be explained by the smaller samples collected from bumble bees in the present study compared to the previous honey bee studies. It may be, however, that bumble bees do not need such an extensive LAB flora for protection and production because they do not produce honey or bee bread and they feed their larva with fresh nectar and pollen (Goulson, 2003). The *Lactobacillus* phylotypes found in bumble bees could, however, serve for pathogen protection, a hypothesis that needs further investigation.

Large numbers of four bacterial phylotypes (LvLi2, Lv2, Hma5 and Trm1) were found in the studies of Olofsson and Vásquez (2008) and Vásquez *et al*. (2009). These bacteria were most closely related to the genera *Actinobacillus* and *Phocoenobacter* that belong to the family *Pasteurellaceae* (Fig. 1, cluster IV) and were supposed to comprise a novel genus. They were found in the larva, in the honey stomach and in the hindgut of the honey bees. Notably, when sampling the bumble bee honey stomachs bacterial phylotypes (HumaH1, HumaH3 and Huma5) closely related to the honey bee phylotypes were detected (Fig. 1, cluster IV and Table 1). Phylotypes HumaH1 and HumaH5 isolated from the honey stomachs of bumble bees were closest related to honey bee phylotype Hma5. Phylotype HumaH3 isolated from the honey stomachs of bumble bees were closest related to phylotype Lv2 isolated from both the honey bee honey stomach from larvae. The impact of these bacteria for honey bee and bumble bee health has to be investigated, as they may constitute an opportunistic bacterial genus (Olofsson and Vásquez, 2008). The *Pasteurellaceae* phylotypes within honey bees were mostly detected when the bacterial pathogen *P. larvae* was present in the honey bee colony, but *P. larvae* was not found when sampling bumble bees, thus supporting the consensus in the literature that bumble bees do not seem to be exposed to this pathogen.

Acknowledgements

This study was financed by Gyllenstiernska Krapperupstiftelsen, Ekhagastiftelsen and Sparbankstiftelsen Skåne. We are grateful for the help of the beekeeper Tage Kimblad, late ass. Prof. Sten Ståhl and others for their knowledge and experience, and for their comments and reflections on our work.

References

ALTSCHUL, S F; MADDEN, T L; SCÄFFER, A A; ZHANG, J; ZHANG, Z; MILLER, W; LIPMAN, D. J (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25(17): 3389–3402.

COLE, J R; CHAI, B; FARRIS, R J; WANG, Q; KULAM, S A; MCGARRELL, D M; GARRITY, G M; TIEDJE J M (2005) The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Research* 33: 294-296. DOI:10.1093/nar/gki038

GOULSON, D (2003) *Bumble bees; their behaviour and ecology*. Oxford University Press; Oxford, UK.

HALL, T (1997) *BioEdit Sequence Alignment Editor. Copyright 1997–2004.* Isis Pharmaceuticals, Inc.; Carlsbad, CA, USA.

LUDWIG, W; STRUNK, O; KLUGBAUER, S; KLUGBAUER, N; WEIZENEGGER, N; NEUMAIER, J; BACHLEITNER, M; SCHLEIFER K H (1998) Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* 19(4): 554-568. DOI: 10.1002/elps.1150190416

OLOFSSON, T C; AHRNE´, S; MOLIN, G (2007) The bacterial flora of vacuum-packed cold-smoked salmon stored at 7 degrees C, identified by direct 16S rRNA gene analysis and pure culture technique. *Journal of Applied Microbiology* 103(1): 109–119. DOI: 10.1111/j.1365-2672.2006.03216.x

OLOFSSON, T C; VÁSQUEZ, A (2008) Detection and identification of a novel lactic acid bacterial flora within the honey stomach of the honey bee *Apis mellifera*. *Current Microbiology* 57(4): 356-363. DOI: 10.1007/s00284-008-9202-0

SAITOU, N; NEI, M (1987) The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4(4): 406-425.

SWOFFORD, D (2003) *PAUP 4.0 Beta version*. Sinauer Associates Inc. Publishers; Sunderland, Massachusetts, USA.

THOMPSON, J D; GIBSON, T J; PLEWNIAK, F; JEANMOUGIN, F; HIGGINS, D G (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25(24): 4876–4882.

VÁSQUEZ, A; OLOFSSON, T C; SAMMATARO, D (2009) A scientific note on the lactic acid bacterial flora in honey bees in the USA - a comparison with bees from Sweden. *Apidologie* 40(1): 26-28. DOI: 10.1051/apido:2008063

VÁSQUEZ, A; OLOFSSON, T C (2009) The lactic acid bacteria involved in the production of bee pollen and bee bread. *Journal of Apicultural Research* 48: 189-195. DOI: 10.3896/IBRA.1.48.3.07

WINSTON, M L (1987) *The biology of the honey bee*. Harvard University Press; Cambridge, MA, UK.