A scientific note on the lactic acid bacterial flora within the honeybee subspecies Apis mellifera (Buckfast), A. m. scutellata, A. m. mellifera, and A. m. monticola

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Scientific note

A scientific note on the lactic acid bacterial flora within the honeybee subspecies *Apis mellifera* (Buckfast), *A. m. scutellata*, *A. m. mellifera*, and *A. m. monticola*

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***Lactobacillus* / *Bifidobacterium* / *Apis mellifera* / co-evolution / 16S rRNA genes**

It was discovered by Olofsson and Vásquez (2008) that a novel lactic acid bacteria (LAB) microbiota with numerous LAB, comprising the genera *Lactobacillus* and *Bifidobacterium*, live in a symbiotic relationship with honeybees (*Apis mellifera*) in their honey stomach. The study was conducted in Sweden with bees bred according to the Buckfast system. A repetitive study by Vásquez et al. (2009a, b) on *A. mellifera* and *Apis mellifera scutellata* was conducted in the USA near the Carl Hayden Honeybee Research Centre (Tucson, AZ), showing a microbiota composed of the exact same bacterial phylotypes.

An erratum was later published on the latter study, stating that trinomial subspecies epithets of collected honeybees should refer to populations sampled within the endemic range of a recognized subspecies, which was not done in that case. The correct denomination for this bee source should have been African-derived *A. mellifera*, frequently also referred to as Africanized honeybees or New World African bees, instead of *Apis mellifera scutellata*. In theory, a honey stomach LAB microbiota in the African-derived *A. m. scutellata* could be different from the original *A. m. scutellata* sampled in Africa. In the present study, a comparison of the LAB microbiota in the honey stomachs of the subspecies *A. m. scutellata*, *Apis mellifera mellifera*, *Apis mellifera monticola*, and the subspecies mix *A. mellifera* bred according to the Buckfast system was conducted. The purpose was to investigate whether subspecies of *A. mellifera* that evolved on different continents share the same phylotypes or strains of indigenous LAB microbiota in their honey stomachs. All of the bee subspecies were sampled within their respective endemic range of the recognized subspecies, except for the Buckfast bee (which is a mixture of many *A. mellifera* subspecies, primarily *A. m. mellifera* and *Apis mellifera ligustica*).

Samples of bees, fresh bee bread, bee pollen, and unripe honey from the subspecies *A. m. scutellata* and *A. m. monticola* were collected from the Kakamega rainforest, Kenya, and express shipped to Sweden. Samples from the native bee *A. m. mellifera* were retrieved from a conservation project in Sweden called Nordbi. We analyzed this variety of samples

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since the bees add their LAB microbiota from their honey stomach to bee pollen, bee bread (Vásquez and Olofsson 2009), and nectar during honey production (Olofsson and Vásquez 2008). From each colony, the bacterial cultivation was conducted from ten pooled honey stomachs of incoming foragers, 1.0 g of honey, 1.0 g of bee bread, and from bee pollen of the legs of five foragers. At the laboratory in Sweden, a total of 173 bacterial isolates from *A. m. scutellata*, *A. m. mellifera*, and *A. m. monticola* (Table I) were identified by their 16S rRNA genes (methods of Olofsson and Vásquez 2008). Seventy-three were previously identified honey bee LAB, and 27 of those were unique (Figure 1).

Previous results from 16S rRNA gene sequencing of the honey stomach LAB from the Buckfast bees were included, but not the sequences of the American samples from the same study Vásquez et al. (2009a, b) since they were nearly identical. Additionally, the American bees were not regarded as endemic *A. mellifera* subspecies.

The results revealed that all studied honeybee subspecies of *A. mellifera* probably share the same *Lactobacillus* and *Bifidobacterium* phylotypes (Figure 1). Presently, 13 phylotypes have been identified from the most extensively studied *A. mellifera*, the Buckfast bee (clusters 1 to 12 in Figure1). Phylotypes Bin2 and Hma3 in cluster 3 are not readily separated since the main differences in the 16S rRNA gene occur in the second half of the gene, which is not used in this study. All phylotypes are represented by bacterial strains derived from two to four *A. mellifera* subspecies (Figure 1). Nevertheless, absent strains may be recovered by a more extensively performed study since the different phylotypes of the bees' LAB microbiota vary in numbers by the bees' health conditions, sample times, and food sources. In this study, a new *Lactobacillus* phylotype (cluster 11) was isolated and includes strains Fhon13 (Buckfast, Sweden) and bbt1 (*A. m. scutellata*, Kenya). Another novel *Lactobacillus* phylotype (bbr24, cluster 7) was found only from samples of *A. m. scutellata*. As expected, the similarities and divergences of the bacterial strains among the discovered LAB phylotypes seem to reflect the similarities and divergences among the bee subspecies included in this study. It points in the direction that the composition of bacterial symbionts, in this case the LAB microbiota, reflects the changes in evolution of the *A. mellifera* subspecies.

In the study of Vásquez et al. (2009a, b), phylotypes belonging to the *Pasteurellaceae* family were obtained from both Swedish and American honeybee samples. However, such phylotypes were not found in the present study from samples of *A. m. scutellata*, *A. m. mellifera*, or *A. m. monticola*.

One of the key issues in the present study was to clarify whether subspecies of the honeybee *A. mellifera* harbored a similar LAB microbiota within their honey stomach. As we have shown, subspecies of *A. mellifera* share exactly the same LAB phylotypes in their honey stomach microbiota despite the fact that Buckfast is a mix of many different *A. mellifera* subspecies and that bee subspecies from different continents were compared. Furthermore, the results indicate that *A. mellifera* subspecies may host subspecies-specific LAB strains. Our results display—for the first time—a plausible co-evolution of LAB symbionts as the strains within phylotypes that represent the divergences and similarities within the investigated *A. mellifera* subspecies. Yet, a more extensive sampling must be undertaken to confirm these results.

Table I. Number of identified bacterial isolates sampled from bees or bee food from the different *A. mellifera* subspecies either from colonies in an apiary or from wild colonies.

Bee subspecies	Honey stomach	Honey	Bee pollen	Bee bread	Number of colonies
A. m. mellifera	25	1	5	0	1
A. m. scutellata	33	21	0	24	1[a]
A. m. monticola	32	0	32	0	1[a]

[a] Wild colonies

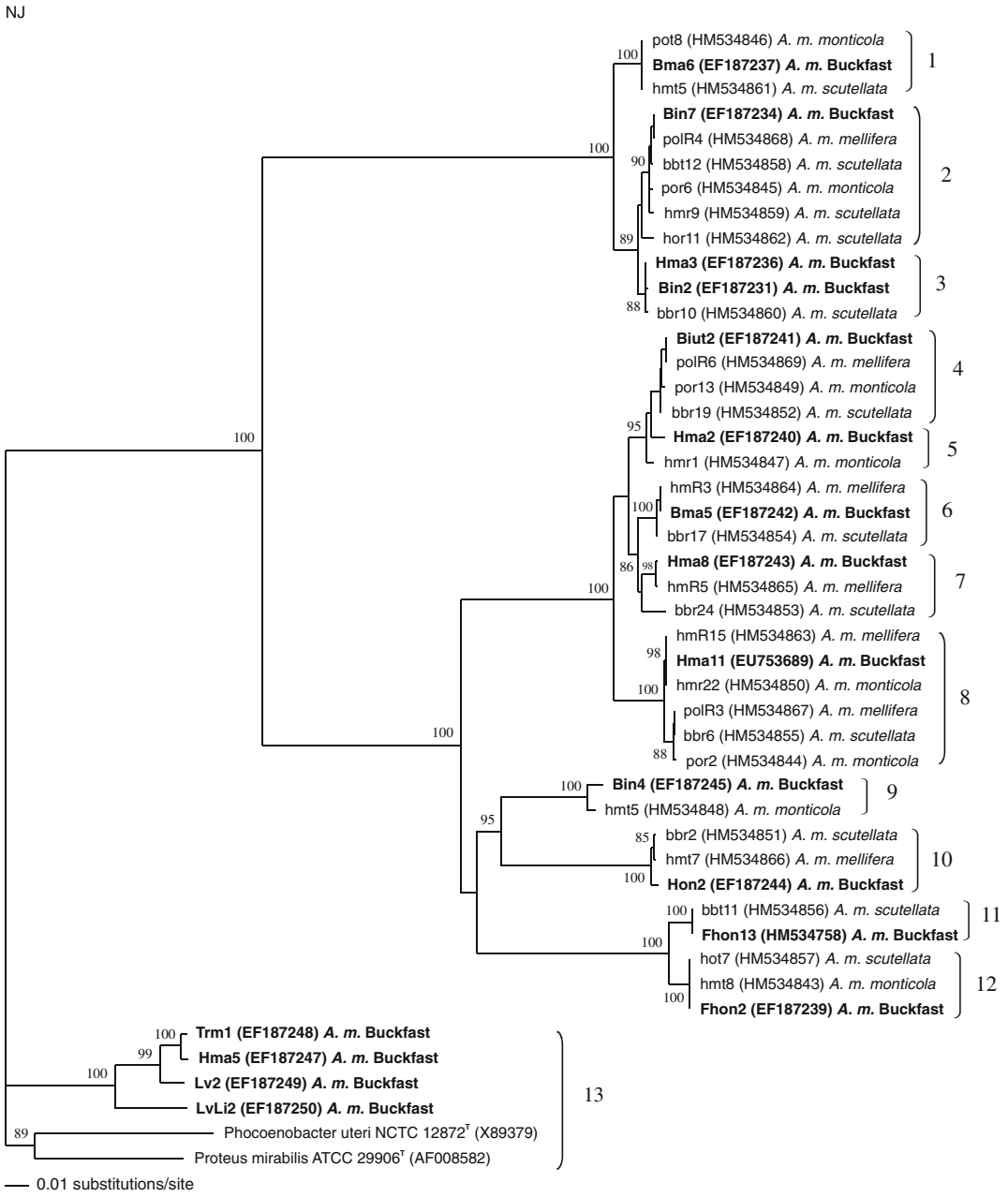

Figure 1. Phylogenetic tree based on a distance matrix analysis of 748 positions in the 16S rRNA gene. The phylotypes characterized in the previous study are displayed in *bold print*. Clusters 1–13 display the LAB phylotypes. Bifidobacteria is represented in clusters 1–3 and lactobacilli in clusters 4–12. The *Pasteurellaceae* group (cluster 13) served as the out-group. The characteristic trinominal subspecies epithets are in *bold print*, the accession numbers in *parenthesis*. *Bar* 5-bp changes. The bacterial 16S rRNA gene sequences were deposited in GenBank using the displayed accession numbers.

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Flore bactérienne de l'acide lactique chez les sous-espèces d'abeilles, *Apis mellifera* (Buckfast), *A.m. scutellata, A. m. mellifera* and *A. m. monticola* (Note scientifique)

Eine wissenschaftliche Notiz über die Milchsäurebakterienflora in Unterarten der Honigbiene, *Apis mellifera* (Buckfast), *A. m. scutellata*, *A. m. mellifera* und *A. m. monticola*

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