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Brenneria nigrifluens Isolated from *Aesculus hippocastanum* L. Bark in Hungary

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Abstract: In Hungary, from the beginning of the 19th century, horse-chestnut trees have been planted widely and are popular ornamental trees in public parks, along streets, and in gardens. In the summer of 2015, longitudinal cracks on the trunk and branches and the intensive oozing of brown liquid were observed from a wound in a horse-chestnut tree in a park in Budapest. Some years later, in 2018 and 2019, the same symptoms were found in trees in other locations in Budapest. Several bacteria were reported that induce similar symptoms, including cracks and cankers on the bark of trunks and branches and sticky, white, red, brown, or black oozing. These pathogens belong to the genera *Brenneria* and *Lonsdalea*. Bark and exudate samples were taken with the aim of identifying the causal agent by conventional and molecular methods. Our results confirmed that the bacteria isolated from *Aesculus hippocastanum* trees belong to the genus *Brenneria* and phylogenetic analysis of the 16S rRNA gene region proved to have the closest phylogenetic relation with the *Brenneria nigrifluens* strains.

Keywords: *Brenneria* species; *Aesculus hippocastanum*; horse chestnut; bleeding canker; 16S rRNA

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1. Introduction

The horse chestnut was previously mentioned as one of the most significant urban trees in the cities of Hungary [1]. The Hungarian Ornamental Horticulturist Association publishes a list of urban trees every two years, which reports much valuable information (e.g., tolerance to urban environments, major pests). *Aesculus hippocastanum* L. has not been included since 2018, since it is sensitive to the dry and warm climate of the cities, to air pollution, and, particularly, to de-icing salting [2].

Within a Hungarian game reserve there is a horse-chestnut stand (22.5 hectares), located in Gyarmatpuszta, that was established at the end of the 19th century [3]. The purpose was to provide food for wild animals during autumn and winter without incurring any damage to oak regrowth caused by them—big game often causes severe damage when looking for food. In these forests, such game can no longer find the feed it previously had in its natural forest stands (primarily acorn and beechnut), with which they stabilized themselves before the harsh winter [3,4]. Among others, the fruits of horse chestnuts and acorns are significant for them [5]. Therefore, horse-chestnut stands, which usually produce their crops every year, are best suited for feeding big game. Besides, the horse chestnut has a higher nutritional value than acorn, and is also preferable to these animals—especially to deer [3,4]. However, experts very often have a negative attitude toward forests that have

been established from non-native tree species; yet Lakatos et al., in their study, pointed out that forests planted with horse chestnuts do not have significantly lower diversity of birdlife and soil fauna than in its natural state. Eighteen, 16, and 14 bird species were found associated with isolated chestnut stands and hornbeam and sessile oak stands, respectively. In the soil of the studied area, 45 species of *Collembola* were found, a high population of species. Based on the *Collembola* species investigated, no significant differences were found between hornbeam–sessile oaks and alien horse-chestnut stands in terms of number of species or individuals inhabiting them. The first occurrence of *Jevania weinerae*, in 14 horse-chestnut soil samples in Hungary, was a faunistic specialty; this species was previously known only to riparian alder forests in Poland [6].

1.1. Members of Brenneria Genus

The genus *Brenneria* belongs to the family *Pectobacteriaceae* (*Gammaproteobacteria*, *Enterobacterales*), which contains four further genera: *Pectobacterium*, *Dickeya*, *Lonsdalea* and *Sodalis* [7]. Members of the *Brenneria* genus affect several deciduous tree species (Table 1) and cause similar symptoms, including cracks; shallow or deep cankers on the trunk and branches; white, black, or brown, watery, or sticky substance oozing on the bark; and, in some cases, wilting and drying [8–12].

Table 1. Summary of *Brenneria* species, host plants, and sequence information.

Species	Host Plant(s)	Location	Type Strain	Culture Collection Numbers	Accession Numbers (GenBank)	Sequence Length (bp)
Brenneria salicis (syn. *Erwinia salicis*)	*Salix alba* L. [13], *Populus* sp., *Alnus glutinosa* (L.) Gaertn. [14]	Belgium [15], Hungary [16], Italy [17], Iran [18], Japan [19], Netherlands [20], United Kingdom [13]		ATCC 15712, CIP 105204, CCUG 48855, CFBP 802, DSM 30166, LMG 2698, NCPPB 447	AJ233419 U80210 Z96097	1499 1455 1471
Brenneria nigrifluens (syn. *Erwinia nigrifluens*)	*Juglans regia* L. [8]	France [21], Hungary [22], Iran [23], Italy [24], Serbia [25], Spain [26], United States [8]	EN 101	ATCC 13028, CCUG 48853, CFBP 3616, CIP 105198, DSM 30175, ICMP 1578, LMG 2694, NCPPB 564	AJ233415 FJ611884 U80203 Z96095	1500 1387 1456 1484
Brenneria rubrifaciens (syn. *Erwinia rubrifaciens*)	*Juglans regia* L. [27]	Spain [28], United States [27]	533 C	ATCC 29291, DSM 4483, ICPB ER103, NCPPB 2020, PDDCC 1915	AJ233418 FJ611885 Z96098 U80207	1491 1387 1484 1457
Lonsdalea quercina (syn. *Erwinia quercina*, *Brenneria quercina*, *Lonsdalea quercina* subsp. *quercina*)	*Quercus agrifolia* Née., *Quercus wislizenii* A. de Candolle [29]	Spain [30], United States [29]		ATCC 29181, ATCC 29281, CCUG 48867, CFBP 3617, CFCC 10717, CIP 105201, DSM 4561, ICMP 1845, LMG 2724, NCPPB 1852	AJ233416 AJ223469 KX537749 NR_041975 NR_114706	1509 1531 1323 1509 1531
Brenneria alni (syn. *Erwinia alni*)	*Alnus glutinosa*, *Alnus cordata* Loiseleur [9]	Italy [9]	PVFi20	ATCC 700181, CCUG 48887, CIP 104916, DSM 11811, ICMP 12481, NCPPB 3934	AJ233409 AJ223468 EU490603	1496 1502 1224
Brenneria goodwinii	*Quercus robur* L. [31], *Q. petraea* (Mattuschka) Lieblein, *Q. castaneifolia* von Meyer, *Q. macranthera* Hohenacker [32]	United Kingdom [31], Iran [32]	R-43656	BCC 845, DSM 27058, FRB 141, LMG 26270, NCPPB 484	KM032271 JN544202	1450 1344
Brenneria roseae subsp. *roseae*	*Quercus cerris* L. [33], *Q. castaneifolia*, *Q. macranthera* [32]	United Kingdom [33], Iran [32]	FRB 222	FRB 222, LMG 27714, NCPPB 4581	KF308291 NR_125698	1344 1344
Brenneria roseae subsp. *americana*	*Quercus kelloggii* Newb. [31]	United States [31]	FRB 223	FRB 223, LMG 27715, NCPPB 4582	KF308292 NR_125699	1344 1344
Brenneria populi Hauben et al. 1998a	*Populus* x *euramericana* (Dode) Guinier (unpublished)	Spain		NCPPB 4299	JX392892	
Brenneria populi subsp. *brevivirga*	*Populus* x *euramericana* [34]	China [34]	D8-10-2-5	CFCC 11935, KTC 42841	KX987135 NR_158058	1450 1450
Brenneria populi subsp. *populi* (syn. *Brenneria populi* Li et al. 2015.)	*Populus* x *euramericana* [34]	China [34]	D9-5	CFCC 11963, KCTC 42088	KJ632518 NR_144604	1450 1450
Brenneria corticis	*Populus* x *euramericana* [35]	China [35]	gBX10-1-2	CFCC 11842, KCTC 42840	MH206610 NR_171515	1463 1463

The *Brenneria* genus contains eight species and four subspecies. However, these species once belonged to the genus *Erwinia*. The first species was described as *Erwinia salicis* by Day (1924) as causing watermark disease on willow [13]. In 1957, Wilson et al. reported a new bacterial disease, shallow bark cankers on Persian walnut trees, that was caused by *Erwinia nigrifluens* [8]. Ten years later, deep bark cankers in walnut (*Erwinia rubrifaciens*) and drippy nut disease in oak (*Erwinia quercina*) were described [27,29]. *Erwinia alni* was published as the causal agent of bark canker on alder trees [9]. In 1998, based on molecular methods, six *Erwinia* species were transferred to the *Brenneria* genus: *B. alni*, *B. nigrifluens*, *B. quercina*, *B. paradisiaca*, *B. rubrifaciens*, and *B. salicis* [36]. Later, in 2012, Brady et al. reclassified *Brenneria quercina* and proposed a new genus, *Lonsdalea* [37]. Two further *Brenneria* species were described that are associated with acute oak decline (AOD), *B. goodwinii* and *B. roseae* [31,33], and two species were isolated from *Populus* × *euramericana* bark cankers (*B. populi*, *B. corticis*) [12,35]. From the above-mentioned species, *B. salicis* and

B. nigrifluens are reported in Hungary as well [16,22]. Watermark disease in willow was first described in the counties Győr, Moson, and Sopron and significant tree death was observed [16]. In 2013, cracks and black ooze appeared on the bark of walnut trees, from which *Brenneria nigrifluens* was isolated and found to be the causal agent [22].

1.2. Diseases Associated with Bleeding Canker on the Bark of Horse-Chestnut Trees

Two pathogens are known to cause bleeding canker disease on *Aesculus hippocastanum*. Since the 1970s, bark necrosis and gummy substance exudation have been observed from the bark of horse-chestnut trees. Further symptoms are crown dieback, water-soaked lesions under the necrotised bark on the trunk, and red or yellow-brown liquid oozing from the dead bark, under which the colour of wood turned to blue-black. *Phytophthora cactorum* (Lebert and Cohn) J. Schröt., *P. citricola* Sawada, and *P. citrophthora* were found to be the causal agents [38–40]. Later, another pathogen, *Pseudomonas syringae* pv. *aesculi* was isolated from *Aesculus hippocastanum* trees, causing similar symptoms. The bacterium causes wilting, cankers, necrotic areas on the main stem and branches, rust or black coloured liquid oozing on the bark and dieback [41–44]. McEvoy et al. (2016) isolated bacterial species present in bark of diseased horse-chestnut trees. Among them, three isolates were identified as *Brenneria nigrifluens* [45].

Characteristic symptoms were observed on horse-chestnut trees in parks and public areas in several locations of Budapest between 2015 and 2019. Symptoms included cracks on the trunks and branches, and a sticky substance was oozing from them, which stained the bark of the trees brown. The aims of this study were to isolate and identify the pathogen that induced the previously observed symptoms.

2. Materials and Methods

In several locations of Budapest, samples were collected from symptomatic inner bark and the exudate of horse-chestnut trees (*Aesculus hippocastanum*). The selected trees were in parks and public areas and showed a dark brown substance oozing on their trunks and branches (Table 2). Samples were transferred to the laboratory of Department of Plant Pathology (Hungarian University of Agriculture and Life Sciences, Buda Campus, Institute of Plant Protection, Budapest) for isolation and were examined by conventional and molecular identification methods. Pure cultures were obtained on King's medium B (KB) [46] and colonies were characterised.

Table 2. Summary of isolates with details of locations and dates of sampling.

Strain	Accession Number	Location	Date of Collection
BpAes1	MW662111	Budapest, Hungary	2015
BpAes2	MW662109	Budapest, Hungary	2018
BpAes3	MW662110	Budapest, Hungary	2019

Biochemical tests were performed on all horse-chestnut isolates using an API 20E Kit (BioMérieux) according to the manufacturer's instructions. After inoculation of the test strips, they were incubated for 24 h at 26 °C. Gram testing was done with a 3% KOH solution [47]. Hypersensitive reaction was assayed on *Nicotiana tabacum* L. 'Xanthi' plants, according to the method described by Klement (1963) [48]. Tobacco leaves were inoculated with bacterial suspension (10^7 cfu/mL) of cultures of the three horse-chestnut strains and an *Erwinia* amylovora strain, as a positive control, which were grown on KB medium for 24 h. Hypersensitive response was evaluated 24–48 h after inoculation.

Pathogenicity tests were conducted with all horse-chestnut isolates on one-year-old *Aesculus hippocastanum* saplings. Two methods were performed with bacterial suspensions (10^7 cfu/mL) of 24-h-old cultures grown on KB medium. Control plants were treated with sterile distilled water. The first method was the following: the bases of leaves on the top of shoots were inoculated with the bacterial suspension using a sterile syringe with a needle. After inoculation, plants were covered with transparent plastic bags for 72 h and

sprayed with water to provide humidity for the infection. Three days later, plastic bags were removed. In the second method, 1–1.5 cm long wounds were made with a sterile scalpel on the trunks of the saplings and were inoculated with the bacterial suspension. After inoculation, the wounds were sealed with Parafilm, and symptoms were monitored. Three months later, symptoms were evaluated in the case of both methods.

Conventional methods were followed for molecular-based identification. DNA was extracted from colonies of 24 h cultures and the 16S rRNA gene was amplified using the primer sets 63F/1389R [49]. The PCR reaction mixture contained (50 µL final volume) 5 µL of 10 × PCR buffer, 3 µL of 25 mM MgCl2, 2 µL of dNTP's (5 mM), 1 µL of forward primer (20 µM) (63f–5′-CAG GCC TAA CAC ATG CAA GTC-3′), 1 µL of reverse primer (20 µM) (1389r–5′-GGG CGG WGT GTA CAA GGC-3′), 1 µL Taq DNA polymerase (5 U/µL), and 3 µL of template DNA. The amplification parameters were an initial denaturation at 95 °C for 5 min, followed by 35 cycles at 94 °C for 15 s, 55 °C for 30 s and 72 °C for 1 min 30 s. Final elongation was accomplished at 72 °C for 10 min. Gel electrophoresis was performed on 1% agarose gel (*w*/*v*) to visualize amplicons. PCR product purification was carried out using a High Pure PCR Product Purification Kit (Roche, Basel, Switzerland) following the manufacturer's instructions. Purified PCR products were ligated into pGEM-T Easy vector and transformed into competent cells of Escherichia coli DH5α strain. Plasmid DNA was purified by Quantum Prep Plasmid Miniprep kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's protocol. Nucleotide sequences were compared to members of *Pectobacteriaceae* family available at the National Center for Biotechnology Information (NCBI) database using Nucleotide BLAST. Then, 16S rRNA gene sequences of the *Brenneria* and *Lonsdalea* species, originating from Hungary and other countries known to cause bark cankers and exudate oozing on various tree species, were downloaded from the GenBank sequence database. Nucleotide sequences were aligned by CLUSTAL W [50]. Determination of the best fit substitution model and maximum likelihood analysis were conducted by using MEGA version 7 [51] with the following settings: Hasegawa–Kishino–Yano model [52], bootstrap method supported by 1000 replicates, gamma distribution with invariant sites (G + I), and partial deletion of gaps/missing data treatment with a 95% cut-off. The 16S rRNA gene sequences of BpAes1, BpAes2 and BpAes3 were deposited in the NCBI GenBank (MW662111, MW662109, MW662110).

3. Results

In 2015 and since, vertical cracks were observed on the trunks and branches of *Aesculus hippocastanum* trees in Budapest. From these cracks and pruning wounds, brown-coloured, watery liquid has been oozing in humid and warm conditions (Figure 1). These symptoms were similar to those caused by bacteria isolated from willow (*Brenneria salicis*), walnut, plane (*B. nigrifluens*), and poplar (*Lonsdalea populi*) trees in Hungary [16,22,53].

All strains isolated from horse-chestnut trees grew on King's medium *B.*, colonies were convex, round, white, slightly bluish, and had entire margins. Biochemical characterisation of the horse-chestnut isolates was done by using an API 20E biochemical test and the results were compared with a Hungarian *Brenneria nigrifluens* strain isolated from walnut (Bn–WalnutZa–Hun1) and a type strain for *Brenneria nigrifluens* (LMG 2694T). Differences between strains in phenotypic characteristics are shown in Table 3.

All isolates were Gram-negative and a hypersensitive response was not induced on the tobacco leaves. Pathogenicity tests were evaluated three months after inoculation. In the case of the first method, shoots of horse-chestnut saplings were inoculated; around the inoculation point, small, necrotic lesions were observed, but without oozing exudate. In the case of the second method, wounds were made on the stems of horse-chestnut trees. Around these wounds, necrotic tissues were observed but brown, liquid ooze did not appear. On the control plants, no symptoms were detected. From all inoculated saplings necrotic tissues were visible, and the same bacteria were successfully reisolated.

Figure 1. Cracks and brown ooze on horse-chestnut trees in Budapest. (Imola Tenorio-Baigorria, Rita Gyuris).

Table 3. Phenotypic characteristics of type strain and Hungarian strains of *Brenneria nigrifluens* and horse-chestnut isolates.

Characteristics	1	2	3	4	5
β-Galactosidase	−	−	−	−	−
arginine dihydrolase	−	−	−	−	−
lysine decarboxylase	−	−	−	−	−
ornithine decarboxylase	−	−	−	−	−
citrate utilization	+	−	−	−	−
h_2s production	+	−	−	−	−
urease	+	−	−	−	−
tryptophane deaminase	−	−	+	+	+
indole production	−	−	−	−	−
acetoin production	+	+	+	−	−
gelatinase	−	−	−	−	−
acid production from					
D-glucose	+	+	+	−	−
D-mannitol	−	+	+	+	+
Inositol	+	+	+	+	+
D-sorbitol	−	+	−	−	−
L-rhamnose	−	+	+	+	+
D-sucrose	+	+	+	+	+
D-melibiose	−	+	−	−	−
Amygdalin	−	+	+	+	+
L-arabinose	+	+	+	+	+

Strains 1—*Brenneria nigrifluens* (Bn–WalnutZa–Hun1); 2—*Brenneria nigrifluens* (LMG 2694T); 3—BpAes1; 4—BpAes2; 5—BpAes3; +—positive; −—negative.

In the PCR assay, a 1294-bp-long DNA fragment was amplified by the 63F/1389R primer pair spanning the partial 16S rRNA gene fragments of the isolates. The nucleotide sequences of the amplified PCR fragments of the16S rRNA gene of horse-chestnut isolates were determined. BLAST search (using MEGA Nucleotide BLAST) results showed that the determined sequences were 99.36% similar to *Brenneria nigrifluens* strains sequences

(LR735272, MG950413, LN875281, LN875278) isolated in Hungary, 97.28%, 96.89%, and 96.65% similar to *B. corticis* (MH206610), *B. roseae* subsp. *roseae* (KY231168), and *B. populi* subsp. *brevivirga* (NR_158058), respectively. A maximum likelihood tree was created from the nucleotide sequences of the three horse-chestnut isolates, as well as from *Brenneria*, *Lonsdalea*, *Dickeya*, and *Pectobacterium* type strains (obtained from NCBI GenBank), with *Cronobacter sakazakii* as an outgroup (Figure 2). The isolates analysed in the present study formed a separate branch and were found to be the closest phylogenetic relationship to the Hungarian *Brenneria nigrifluens* strain which was described on walnut (HF936707) trees. Besides, they showed lower similarity to other *B. nigrifluens* strains: the type strain (AJ233415) and a strain (JX484738) which originates in Serbia.

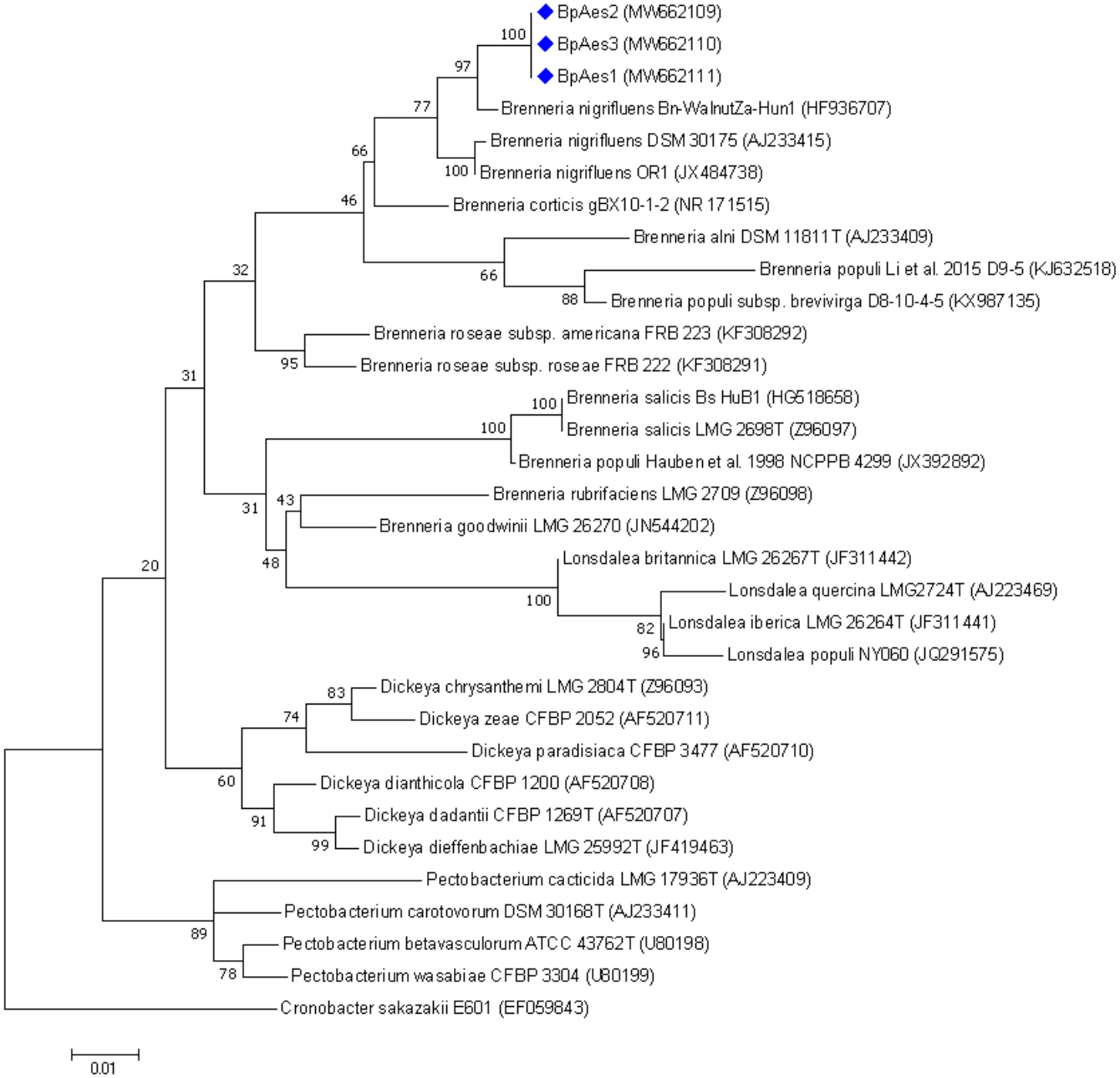

Figure 2. Maximum likelihood tree based on 16S rRNA gene nucleotide sequences of the *Aesculus hippocastanum* strains, as well as from the *Brenneria*, *Lonsdalea*, *Dickeya*, and *Pectobacterium* species. An outgroup is included, *Cronobacter sakazakii*. Bar—0.01 substitutions per nucleotide position; rhombus—indicates the isolates characterized in this study.

4. Discussion

The present study contributes to the knowledge of phytopathogenic bacteria in horse-chestnut trees, being an important tree species because *Aesculus hippocastanum* is on the Red List of the International Union for Conservation of Nature and Natural Resources (IUCN) as a "vulnerable" species [54]. Since 2015, cracks on the bark and brown exudate oozing have been observed on *Aesculus hippocastanum* trees in several places, streets, parks, and public places of Budapest. The symptoms were similar to those caused by the *Brenneria* and *Lonsdalea* species isolated from other tree species in previous years [12,17,18,31–33,35]. However, horse chestnut was not originally known as a host plant of these pathogens. In Europe, bleeding canker disease of horse-chestnut trees is an increasing problem [43,44,55,56]. In some cases, *Pseudomonas syringae* pv. *aesculi* was isolated from symptomatic horse-chestnut trees [44], but *Phytophthora* species could cause similar symptoms as well [38–40]. McEvoy et al. isolated and identified several bacterial species from diseased *Aesculus hippocastanum* trees such as *Brenneria nigrifluens*, *Pseudomonas marginalis* and *P. putida* [45].

Based on the results presented in this study, the three horse-chestnut isolates are Gram-negative, did not induce hypersensitive response and are grouped to *Brenneria nigrifluens* strains and isolates (closest to a walnut isolate from Hungarian) formed a single branch on a maximum likelihood phylogenetic tree, but differences in phenotypic characteristics were observed. Further characterization of isolates could be performed as by DNA–DNA hybridisation, fatty acid analysis, and MLSA trees based on housekeeping genes (*atpD*, *gyrB*, *infB*, *rpoB*).

However, the pathogenicity tests demonstrated that the isolated strains were not able to induce cankers or bacterial oozing on the samplings, though they provoked small necrotic lesions at the inoculation point. Since the pathogenicity trials were carried out in the field and at the time of and after inoculation, the temperature and moisture content were not high enough to produce the entire infection. Probably, it could take years for the typical symptoms to develop.

On the other hand, other bacterial and non-bacterial pathogens also could have contribution to the formation and development of symptoms, but, in the present study, neither were investigated. There are many questions that are awaiting answers, e.g., What kind of impact could *Brenneria* species have? Could one or more other pathogens play a role in the development of symptoms? What are the long-term consequences of the infection? Is the disease present in other countries?

Four years after sampling, the diseased tree seemed alive but was in weaker condition than the horse-chestnut trees around it. Damage caused by *Cameraria ohridella* was visible on all of them. In the parks and streets of Budapest, plant protection of *Aesculus hippocastanum*—and of other ornamental trees—is a significant challenge because of the legislation in force in public areas, a lack of plant protection products, the height of the trees, and the lack of sterilisation during pruning. In some cases, plant protection experts try to control horse-chestnut leaf miners with the injection method, by making a small hole in the trunk of the tree and using insecticide. The problem with this method is that the hole created is an open door to plant pathogens—occasionally bacterial oozing was observed from these holes.

5. Conclusions

To the best of our knowledge this is the first report on *Brenneria* species isolated from horse-chestnut trees in Hungary.

Studies from previous years demonstrate that the number of hosts of *Brenneria* and *Lonsdalea* species is increasing in the number of countries reporting its appearance. It is important to have better knowledge of these pathogens, to inhibit their spread and find a way to protect these valuable trees. Deciduous ornamental trees play an important role in cities; they give shade in summer heat; provide proper habitat for birds, squirrels, and insects; clean the air; and, it is proven, they have stress-relieving effect. These are just a few arguments in favour of their protection.

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