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Antibacterial activity of bryophyte species against *Paenibacillus* larvae isolates

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Abstract: This study was performed to determine the antibacterial activity of methanol extracts of 23 bryophyte species against *Paenibacillus larvae* isolates that cause American foulbrood diseases in honeybee larvae. The honey and larva samples were collected from nine different locations of Rize in Turkey. A total of 22 gram-positive spore-forming bacteria were isolated from the larva and honey samples. According to the results of morphological, biochemical, and molecular (16S rRNA gene sequencing) tests, 10 isolates of the 22 gram-positive spore-forming bacteria were identified as *P. larvae*. A total of 10 bryophyte species (*Polytrichum formasum*, *Polytrichum commune*, *Calliergonella cuspitada*, *Calliergonella lindbergi*, *Metzgeria conjugata*, *Isothecium alopecuroides*, *Syntrichia calcicola*, *Syntrichia intermedia*, *Tortella densa*, and *Grimmia alpestris*) among 23 bryophytes showed good antimicrobial activity against *P. larvae* isolates according the results of agar-well diffusion method and minimal inhibition concentration experiments.

Key words: Bryophytes, antibacterial activity, *Paenibacillus larvae*, American foulbrood

1. Introduction

In natural ecosystems, honeybees (*Apis mellifera*) have an important role as pollinators. Bee diseases caused by parasites and pathogens including mites, beetles, fungi, protozoa, viruses, and bacteria are the most important problems for domestic and feral honeybees (1). American foulbrood (AFB), caused by *Paenibacillus larvae*, which is a gram-positive and spore-forming bacterium, is a common bacterial disease of the honeybee brood. The endospores of *P. larvae* are extremely infectious and only infect honeybee larvae (2). Environmentally stable spores produced by *P. larvae* are resistant to heat, desiccation, and disinfectants. Therefore, the prevention of AFB is quite difficult for beekeepers (3). In apiculture, AFB can be considered as a global threat. In most European countries, the combs infected by *P. larvae* are burned for the prevention of this disease. The use of antibiotics such as sulfonamides, oxytetracycline, streptomycin, tylosin, and erythromycin is another strategy for the prevention of AFB (4,5). The use of these antibiotics causes a series of problems for the environment and human health such as the appearance of resistance in bacteria and the accumulation of antibiotic residues in beehive products. These decrease the quality of beehive products and marketing opportunities (6).

In recent years, many researchers have begun to investigate new strategies that will be alternatives to the use of antibiotics. One of the most important strategies is the application of plant extracts that are known to inhibit or retard the growth of bacteria, molds, and yeast (7). In many studies, it has been shown that substrates of bryophytes have antimicrobial activity against bacteria and fungi (8–11). The bryophytes, including the subgroups Bryophyta (mosses), Marchantiophyta (liverworts), and Anthocerotophyta (hornworts), are a diverse group of land plants. Bryophytes produce secondary metabolites such as aromatics, phenolic compounds, and flavonoids. These secondary metabolites are known to be toxic to bacteria and fungi (12).

In this study, we aimed to investigate nonantibiotic natural sources against *P. larvae* causing serious problems and economic losses for beekeepers and to find potential plant sources that can be used in the prophylaxis of disease in bees. For this purpose, the antibacterial effects of methanol extracts of 23 bryophyte species were tested against *P. larvae* isolates for the first time in this work.

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2. Materials and methods

2.1. Plant materials

The 23 bryophyte species used in this study were collected from different localities of Turkey. Detailed information about the bryophyte species is given in Table 1. The bryophytes were identified by Nevzat Batan and Turan Özdemir. The voucher specimens are deposited at the herbarium of the Department of Biology, Faculty of Science, Karadeniz Technical University, Turkey (KTUB).

2.2. Isolation of *Paenibacillus larvae* isolates

The larvae and honey samples that were taken from nine hives showing AFB clinical symptoms were obtained from the Samsun Veterinary Control Institute. Five larva samples and approximately 10 g of honey were taken from each hive to isolate *P. larvae*. The larva samples were surface-sterilized with ethanol (70%) three times to prevent possible contamination (13). After that, the surface-sterilized broods were homogenized in 3 mL of sterile distilled water using a tissue grinder. Honey samples (5 g) were solubilized in 50 mL of sterile water overnight at 37 °C. Serial dilutions from 10^{-1} to 10^{-8} were prepared from larval homogenates and honey solutions. The prepared dilutions were incubated at 80 °C for 10 min to eliminate nonspore-forming bacteria. From each dilution, 100 µL was plated on MYPG agar and incubated at 35 °C for 3–4 days. At the end of the incubation period, *P. larvae*-like colonies were checked with 3% (v/v) H_2O_2 for catalase activity. Catalase-negative colonies were selected and identified using morphological and biochemical tests as well as 16S rRNA gene sequencing.

2.3. Identification of *Paenibacillus larvae* isolates

P. larvae isolates were identified based on their morphological, biochemical, and molecular characteristics. Morphological characterizations of the bacterial isolates were performed by evaluating properties of colony morphologies, Gram staining, endospore staining, motility of isolates, and growth rate in MYPG broth. To determine biochemical characteristics of the bacterial isolates, some tests such as nitrate reduction, starch hydrolysis, Voges–Proskauer, citrate, esculin hydrolysis, and sugar fermentation (glucose, maltose, lactose, trehalose, and xylose) tests were performed (14). For 16S rRNA gene sequencing, genomic DNA was extracted according to Sambrook's procedure using a standard phenol:chloroform protocol (15). A fragment of about 1450 bp of the 16S rRNA gene was amplified from all isolates using primers of 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The PCR conditions were adapted according to the study of Demirci et al. (16). PCR products were sent to Macrogen (Amsterdam, the Netherlands) for sequencing. Phylogenetic analysis was performed using neighbor-joining phylogenies with p-distance correction, gap omission, and 1000 bootstrap pseudoreplicates. All analyses were performed with MEGA 6.0 phylogenetic software (17).

2.4. Antimicrobial susceptibility test

Antimicrobial susceptibility tests were performed according to the disk diffusion method (18). Disks with gentamicin (10 µg), streptomycin (25 µg), erythromycin (15 µg), tylosin (15 µg), tetracycline (30 µg), oxytetracycline (30 µg), chloramphenicol (30 µg), lincomycin (2 µg), ampicillin (10 µg), norfloxacin (10 µg), and furazolidone (10 µg) were used.

2.5. Preparation of methanol extracts of 23 bryophyte species

Powdered air-dried samples of all parts of the plant materials (30 g for each sample) were extracted using analytical grade methanol (3 × 100 mL) in a shaking incubator for 24 h at 37 °C and 110 rpm. The extracts were then combined, filtered, and concentrated under vacuum with an evaporator (19) and 10 mg of each sample was dissolved in 1 mL of methanol.

2.6. Antibacterial assay

Antibacterial activities of methanol extracts of 23 bryophytes were tested against *P. larvae* isolates using the agar-well diffusion method (20). Each bacterial sample was suspended in 3 mL of MYPG broth, and then the bacterial suspensions were diluted to 10^6 CFU/mL. The bacterial suspensions were plated on the surface of MYPG agars and then dried. Wells of 5 mL in diameter were cut from the agar using a sterile cork-borer and each well was filled with 50 µL of the methanol extracts. The plates were incubated at 35 °C for 48 h. Antibacterial activity was evaluated by measuring the zone of inhibition. An ampicillin disk (10 µg) was used as a standard antibacterial agent. Analytical-grade methanol was used as solvent control.

The minimum inhibition concentrations (MICs) of ten bryophyte extracts showing good activity against *P. larvae* isolates were determined by microdilution technique (21). The microdilution technique was performed in 96-well microtiter plates in MYPG broth. Each extract was serially diluted (1.2, 2.5, 5, 10, 25, 50, 100, 125, 250, 500, 750, and 1000 µg/ml). *Paenibacillus larvae* isolates were adjusted to 0.5 McFarland turbidity and added to wells including serially diluted extracts. The plates were incubated at 35 °C for 48 h in order to determined MIC values. The MIC values were defined as the lowest concentration that showed no growth. Ampicillin (10 µg/mL) was used as a standard antibacterial agent. Analytical-grade methanol was used as solvent control.

2.7. GenBank accession numbers

The 16S rRNA sequences for *P. larvae* isolates used in this study were deposited in GenBank (NCBI, Bethesda,

Table 1. Localities and other properties of bryophytes used in this study.

	Species	Localities	Altitude (m)	GPS coordinates
1	*Polytrichum commune* Hedw.	Turnasuyu Valley, Ordu	720	40°55′52″N 38°01′08″E
2	*Polytrichum formosum* Hedw.	Turnasuyu Valley, Ordu	720	40°55′52″N 38°01′08″E
3	*Isothecium alopecuroides*	Maçka district, Trabzon	720	40°45′06.36″N 39°37′37.54″E
4	*Eurhynchium striatum* (Schreb. ex Hedw.) Schimp.	Dernekpazarı district, Trabzon	368	40°46′31.11″N 40°13′05.63″E
5	*Tortella humilis* (Hedw.) Jenn.	Dernekpazarı district, Trabzon	368	40°46′31.11″N 40°13′05.63″E
6	*Tortella inclinata* var. *densa* (Lorentz & Molendo) Limpr.	Perşembe district, Ordu	525	40°56′53″N 37°40′19″E
7	*Tortella tortuosa* (Hedw.) Limpr.	Perşembe district, Ordu	525	40°56′53″N 37°40′19″E
8	*Syntrichia virescens* (De Not.) Ochyra.	Yeşilova district, Burdur	1180	37°30′38.51″N 29°41′33.31″E
9	*Syntrichia laevipila* (Milde) Mönk.	Yeşilova district, Burdur	1180	37°30′38.51″N 29°41′33.31″E
10	*Syntrichia ruralis* var. *ruraliformis* (Besch.) Delogne.	Altınyayla district, Burdur	1418	36°56′23.58″N 29°27′02.49″E
11	*Syntrichia intermedia* Brid.	Altınyayla district, Burdur	1418	36°56′23.58″N 29°27′02.49″E
12	*Syntrichia calcicola* J.J.Amann	Uğurlu Mountain, Ardahan	1100	40°00′18″N 42°34′07″E
13	*Grimmia orbicularis* Bruch ex Wilson	Altınyayla district, Burdur	1436	36°56′09.14″N 29°27′00.31″E
14	*Grimmia alpestris* (F.Weber & D.Mohr) Schleich.	Altınyayla district, Burdur	1436	36°56′09.14″N 29°27′00.31″E
15	*Schistidium papillosum* Culm.	Ulubey, Ordu	240	40°49′50″N 37°45′40″E
16	*Schistidium trichodon* (Brid.) Poelt.	Ulubey, Ordu	240	40°49′50″N 37°45′40″E
17	*Leucobryum glaucum* (Hedw.) Angstr.	Gülyalı district, Ordu	300	40°56′52″N 38°02′32″E
18	*Dicranum majus* Turner	Gülyalı district, Ordu	300	40°56′52″N 38°02′32″E
19	*Hedwigia ciliata* (Hedw.) P. Beauv.	Akyayla, Burdur	1435	37°30′05.84″N 30°20′06.83″E
20	*Calliergonella cuspidata* (Hedw.) Loeske	Araklı, Trabzon	474	40°47′23.73″N 39°58′04.88″E
21	*Calliergonella lindbergii* (Mitt.) Hedenäs	Araklı, Trabzon	474	40°47′23.73″N 39°58′04.88″E
22	*Diplophyllum taxifolium* (Wahlenb.) Dumort	Sürmene district, Trabzon	249	40°54′39.92″N 40°13′33.47″E
23	*Metzgeria conjugata* Lindb.	Sürmene district, Trabzon	249	40°54′39.92″N 40°13′33.47″E

MD, USA) under accession numbers KU598688 (Pb3.1a), KU598689 (Pb3.2a), KU598690 (Pb3.2b), KU598691 (Pb3.2b2), KU598692 (Pb3.3a), KU598693 (Pb4), KU598694 (Pb5a), KU598695 (Pb5b), KU598696 (Pb6a), and KU598697 (Pb6b).

3. Results

3.1. Identification of *Paenibacillus larvae* isolates

Twenty-two gram-positive and spore forming-bacteria were isolated from larvae and honey samples that were taken from nine hives showing AFB clinical symptoms in Rize, Turkey. No bacteria were isolated from the larvae or honey samples taken from two hives. Although eight spore-forming bacteria were isolated from honey samples taken from three different hives, 14 spore-forming bacteria were isolated from larva samples taken from seven different hives. Among the isolated 22 gram-positive and spore-forming bacteria, 10 bacteria were identified as *P. larvae* according to the results of morphological, biochemical, and molecular tests (16S rRNA sequencing) (Table 2). Colonies of *P. larvae* isolates have a cream, transparent, and semitransparent appearance (Figure 1). It was observed that all isolates were nonmotile and showed slow growth in MYPG broth. All isolates had subterminal spores, except for Pb3.2b2. All isolates were negative with respect to catalase production, starch hydrolysis, and the citrate test. All isolates were able to metabolize glucose and trehalose sugars but none of them were able to metabolize maltose and lactose sugars. All isolates were negative in terms of metabolizing xylose, except for Pb4 and Pb5b. Morphological and biochemical properties of the *P. larvae* isolates are given in Table 3.

Table 2. *P. larvae* isolates isolated from honey and larva samples.

No.	Locations	Samples	İsolate name (n=22)	İsolate name of *P. larvae* (n=10)
1	Fındıklı-Rize	Honey	Pb1a.1, Pb1a.2	-
		Larva	Pb1b, Pb1c	-
2	Rize	Honey	Pb2a, Pb2b, Pb2c	-
		Larva	Pb2d	-
3	Güneysu-Rize	Honey	Pb3.2a, Pb3.2b, Pb3.2b2	Pb3.2a, Pb3.2b Pb3.2b2
		Larva	Pb3.1a, Pb3.3a Pb3.3b, Pb3.3c	Pb3.1a, Pb3.3a
4	İkizdere-Rize	Honey	-	-
		Larva	Pb4	Pb4
5	İyidere-Rize	Honey	-	-
		Larva	Pb5a, Pb5b	Pb5a, Pb5b
6	İkizdere-Rize	Honey	-	-
		Larva	Pb6a, Pb6b	Pb6a, Pb6b
7	Fındıklı-Rize	Honey	-	-
		Larva	-	-
8	Fındıklı-Rize	Honey	-	-
		Larva	-	-
9	Pazar-Rize	Honey	-	-
		Larva	Pb9a, Pb9b	-

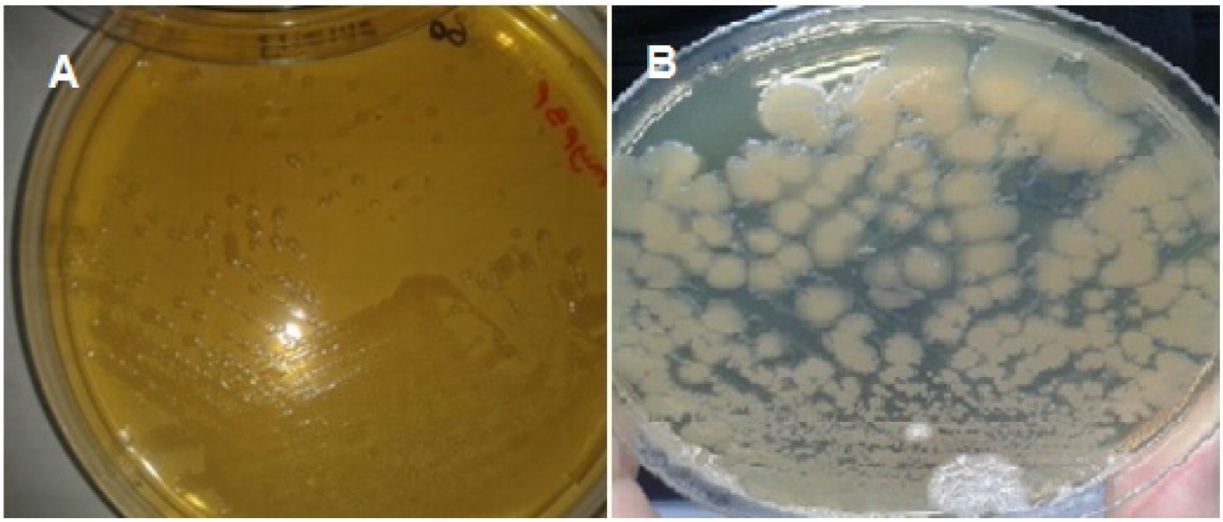

Figure 1. The appearance of *P. larvae* isolates on MYPG agar: A) the appearance of transparent colonies (Pb3.1a); B) the appearance of cream colonies (Pb4).

The partial sequences of the 16S rRNA gene were used for further characterization of the bacterial isolates. For all isolates, a fragment of the 16S rRNA gene region of approximately 1400 bp was amplified and sequenced. The obtained sequences were used for a Blast search in the NCBI database and phylogenetic analysis. Based on all identification studies, 10 isolates were identified as *P. larvae* and named as Pb3.1a, Pb3.2a, Pb3.2b, Pb3.2b2, Pb3.3a, Pb4, Pb5a, Pb5b, Pb6a, and Pb6b. This identification was also supported by a dendrogram, which was constructed using 16S rRNA gene sequences (Figure 2).

3.2. Antibiotic resistance profile of *Paenibacillus larvae* isolates

The antibiotic susceptibility of 10 *P. larvae* isolates was investigated by disk diffusion method. All *P. larvae* isolates were sensitive to chloramphenicol, erythromycin, oxytetracycline, streptomycin, tylosin, norfloxacin, lincomycin, and furazolidone. Gentamicin resistance was determined in six isolates (Pb3.1a, Pb3.2a, Pb3.3a, Pb5a, Pb6a, and Pb6b). Additionally, strain Pb3.1a was resistant to tetracycline, ampicillin, and gentamicin (Table 3).

3.3. Antibacterial activity of plant extracts

It was observed that all plant extracts showed antibacterial activity against at least one *P. larvae* isolate. Among 23 bryophytes extracts, 10 plant extracts (*P. formosum*, *P. commune*, *C. cuspidata*, *C. lindbergii*, *M. conjugata*, *I. alopecuroides*, *S. calcicola*, *S. intermedia*, *T. densa*, and *G. alpestis*) produced good activity against all *P. larvae* isolates. The remaining 13 bryophyte extracts had antimicrobial activity against some *P. larvae* isolates. Strains Pb5b and Pb6a were sensitive to all bryophyte extracts (Table 4).

3.4. Minimal inhibition concentrations of plant extracts

The MICs of ten bryophyte extracts showing good antimicrobial activity based on the agar-well diffusion method were determined. The methanol extract of *C. lindbergii* was the most effective extract against all *P. larvae* isolates with 10–1.2 µg/mL MIC values. MIC values of 1.2 µg/mL were obtained from *P. commune* and *G. alpestis* against strains Pb3.3a1, Pb5a, Pb5b, Pb6a, and Pb6b. The MIC values are given in Table 5.

4. Discussion

Rize Province, located in the Eastern Black Sea Region of Turkey, is a very important place for beekeeping. The region has a rich flora and the use of chemical fertilizers and pesticides is quite low in agricultural areas of the region. Therefore, the region has an important potential for the production of organic bee products. In this region, one of the most important factors affecting beekeeping is bee diseases. AFB caused by *P. larvae* is one of the most important bee diseases. In this study, honey and larva samples suspected to be contaminated with *P. larvae* were obtained from different regions of Rize. However, *P. larvae* was isolated from only three different districts (Güneyse, İyidere, and İkizdere).

AFB, caused by *P. larvae*, a spore-forming bacteria, is a common bacterial disease of honeybees. This cosmopolitan disease affects honeybees in the larval and pupa stages and is highly contagious (2). The morphological, biochemical, and molecular characteristics (16S rRNA sequencing) of the isolates were used for identification. *P. larvae* colony morphology on bacteriological agar is described as whitish to grayish, somewhat transparent and slightly

Table 3. Morphological and biochemical properties of *P. larvae* isolates.

	Morphological properties						Biochemical properties											
Isolate no.	Color	Shape	Pigmentation	Spore location	Motility	Growth in MYPG	Cat	Nit	Vp	Cit	Esc	Stc	Glu	Mal	Lac	Tre	Xyl	Patterns of antibiotic resistance
Pb3.1a	T	R	-	Subterminal	-	S	-	+	+	-	-	-	+	-	-	+	-	CN, TE, AMP
Pb3.2a	T	R	-	Subterminal	-	S	-	+	-	-	-	-	+	-	-	+	-	CN
Pb3.2b	C	R	-	Subterminal	-	S	-	+	+	-	-	-	+	-	-	+	-	-
Pb3.2b2	C	R	-	Terminal	-	S	-	+	+	-	-	-	+	-	-	+	-	-
Pb3.3a	C	R	-	Subterminal	-	S	-	+	-	-	-	-	+	-	-	+	-	CN
Pb4	C	R	-	Subterminal	-	S	-	+	+	-	-	-	+	-	-	+	+	-
Pb5a	St	R	-	Subterminal	-	S	-	+	+	-	+	-	+	-	-	+	-	CN
Pb5b	T	R	-	Subterminal	-	S	-	+	+	-	+	-	+	-	-	+	+	-
Pb6a	St	R	-	Subterminal	-	S	-	+	+	-	-	-	+	-	-	+	-	CN
Pb6b	T	R	-	Subterminal	-	S	-	+	+	-	-	-	+	-	-	+	-	CN

T: Transparent; C: cream; St: semitransparent; R: rough; S: slow; Cat: catalase; Nit: nitrate reduction; Vp: Voges–Proskauer; Cit: citrate; Esc: esculin hydrolase; Stc: starch hydrolase; Glu: glucose; Mal: maltose; Lac: lactose; Tre: trehalose; Xyl: xylose; +: positive, -: negative; CN: gentamycin; TE: tetracycline; AMP: ampicillin.

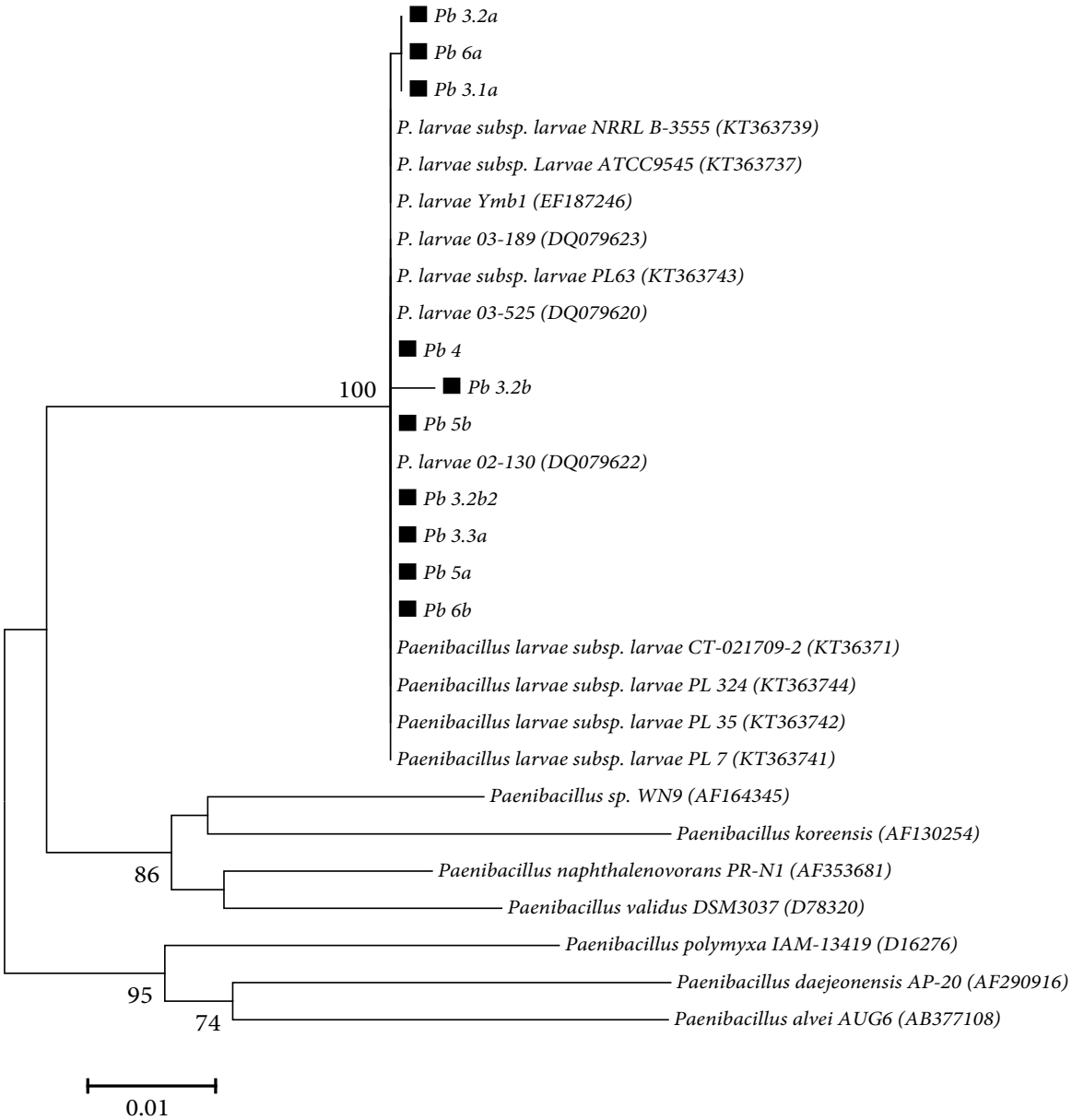

Figure 2. Phylogenetic analysis of the *P. larvae* isolates based on partial sequencing of the 16S rRNA gene. Neighbor-joining analysis with p-distance method was used to construct the dendrogram. Bootstrap values shown next to nodes are based on 1000 replicates. Bootstrap values C ≥ 70% are labeled. *P. larvae* isolates are indicated with black squares. The scale on the bottom of the dendrogram shows the degree of dissimilarity.

glistening; some colonies produce an orange pigment. *P. larvae* is catalase-negative or weak-delayed positive and it has a typical carbohydrate acidification profile with acid from glucose and trehalose, not from arabinose and xylose (22,23). In this study, we isolated 22 gram-positive bacteria from larva and honey samples collected from Rize Province. Among the 22 isolates, 10 were identified as *P. larvae* according to the generated phylogenetic tree using 16S rRNA sequences of the isolates and the results of biochemical and morphological tests. Among the *P. larvae* isolates, isolates Pb3.1a, Pb3.2a, Pb5a, Pb5b, and Pb6b have a transparent appearance. None of *P. larvae* isolates have pigmentation. All of *P. larvae* isolates are catalase-negative and produced acid from glucose and trehalose.

In beekeeping, AFB is considered as a very serious problem worldwide (2). The spores of the pathogen are resistant to environmental conditions and this situation makes the control of the disease more difficult.

Table 4. The tested bryophyte species and the antimicrobial activity of methanol extracts against *P. larvae* isolates

Taxonomic position			Strain numbers and inhibition zones (mm)									
Division	Class	Species	3.1a	3.2a	3.2b2	3.2b2	3.3a1	4	5a	5b	6a	6b
Bryophyta	Polytrichopsida	*Polytrichum commune*	>25	>25	>25	>25	29	24	>25	>25	>25	>25
		Polytrichum formasum	>25	>25	>25	15	15	15	15	>25	>25	15
	Bryopsida	*Isothecium alopecuroides*	>25	>25	>25	10	10	15	15	>25	>25	15
		Eurhynchium striatum	15	0	0	15	0	18	15	>25	25	25
		Syntrichia calcicola	>25	>25	>25	16	15	15	15	>25	>25	12
		Syntrichia virescens	15	0	15	15	0	15	15	>25	25	25
		Syntrichia laevipila	0	0	18	15	0	15	25	18	25	25
		Syntrichia intermedia	8	6	5	12	15	8	8	18	12	14
		Syntrichia ruralis var. *ruraliformis*	0	0	12	12	0	10	0	20	25	20
		Tortella densa	>25	>25	>25	10	10	12	14	>25	>25	12
		Tortella tortuosa	0	0	10	0	0	10	0	12	15	0
		Tortella humilis	0	0	10	12	0	10	0	25	18	10
		Grimmia orbicularis	0	0	10	10	0	15	15	>25	25	15
		Grimmia alpestris	20	12	18	12	15	15	30	>25	25	25
		Schistidium popillosum	0	0	10	12	0	12	0	18	>20	15
		Schistidium trichodon	0	0	10	10	0	10	0	22	24	18
		Leucobryum glaucum	0	0	5	8	0	12	0	12	>20	15
		Dicranum majus	0	0	0	0	0	18	0	18	>20	10
		Hedwigia ciliate	0	0	0	10	0	0	0	22	12	15
Marchantiophyta	Jungermanniopsida	*Calliergonella cuspitada*	>25	>25	>25	15	15	15	15	>25	>25	15
		Calliergonella lindbergi	>25	>25	>25	>25	20	25	>25	>25	>25	>25
		Diplophyllum taxifolium	15	0	0	20	0	15	10	>25	25	20
		Metzgeria conjugate	>25	>25	>25	18	17	16	16	>25	>25	>25

Table 5. The minimal inhibition concentrations of bryophyte methanol extracts against *P. larvae* isolates.

	Isolate numbers and minimal inhibition concentrations (µg/mL)									
Species	3.1a	3.2a	3.2b2	3.2b2	3.3a1	4	5a	5b	6a	6b
Polytrichum commune	2.5	2.5	2.5	2.5	1,2	2.5	2.5	1.2	1.2	1.2
Polytrichum formasum	2.5	2.5	2.5	25	25	25	25	2.5	2.5	25
Isothecium alopecuroides	5	5	5	125	125	75	75	5	5	75
Syntrichia calcicola	2.5	2.5	2.5	100	25	25	25	2.5	2.5	125
Syntrichia intermedia	500	500	500	250	125	500	500	25	250	250
Tortella densa	5	5	5	250	250	250	100	5	5	250
Grimmia alpestris	500	250	50	250	100	100	1.2	1.2	5	5
Calliergonella cuspitada	2.5	2.5	2.5	50	50	50	50	2.5	2.5	50
Calliergonella lindbergi	1.2	1.2	1.2	1.2	10	2.5	1.2	1.2	1.2	1.2
Metzgeria conjugata	5	5	5	10	10	25	25	5	5	5

In many European countries, hives infected by AFB are burned. Furthermore, the use of antibiotics as an alternative application is available (4). Many antibiotics such as tetracycline derivatives (oxytetracycline and chlortetracycline), streptomycin, sulfonamides, tylosin, erythromycin, chloramphenicol, and lincomycin are used to control AFB worldwide (5). However, recent studies have shown the development of resistant strains in *P. larvae* isolates (1,24,25). In this study, gentamycin resistance was found in 6 of 10 *P. larvae* isolates. Isolate Pb3.1a is resistant against ampicillin, tetracycline, and gentamycin. The high gentamycin resistance might be due to the neo-terramycin-vitamin soluble powder (containing 55 mg neomycin sulfate, 55 mg oxytetracycline HCl, and vitamin complex; Pfizer, USA) commonly used by local beekeepers.

Plants synthesize many aromatic substrates that have strong antimicrobial activity and most of these are secondary metabolites. These substrates mostly act as plant protective substances against microorganisms, insects, and herbivores (26). It is suggested that bryophytes including the subgroups Marchantiophyta (liverworts), Anthocerotophyta (hornworts), and Bryophyta (mosses) are among the most interesting and promising sources of antibiotics and biologically active compounds in nature (27). Bryophytes chemically synthesize many terpenoids, phenolic compounds, and rare aromatic substances and the antimicrobial activity of bryophytes against fungi and prokaryotic cells has been demonstrated in many studies (10,11,28,29). In this study, the antimicrobial activities of some bryophytes that were collected from Turkey were tested for the first time in the literature against *P. larvae*. The results of antimicrobial activity tests showed that some bryophyte extracts (*Polytrichum commune*, *Polytrichum formasum*, *Isothecium alopecuroides*, *Syntrichia calcicola*, *Tortella densa*, *Grimmia alpestris*, *Calliergonella cuspitada*, *Calliergonella lindbergi*, and *Metzgeria conjugata*) are very effective against *P. larvae* isolates. In addition, the MIC results showed that many of the bryophyte extracts were highly effective against *P. larvae* isolates at low concentrations. The secondary metabolites of these bryophytes may be used against *P. larvae* as an alternative to antibiotic use.

The antimicrobial activity of various extracts of *Polytrichum juniperium* and *Tortella tortuosa* against six bacteria and three fungi was investigated by Savaroglu et al. (29). The methanol extracts of mosses were observed to not be effective against the microorganisms, except for the methanol extracts of *Polytrichum juniperium*, which were effective against *B. subtilis*. In this study, two species of the genus *Polytrichum* (*Polytrichum commune* and *Polytrichum formosa*) were used and the methanol extracts of these mosses were found to be highly effective on *P. larvae* isolates. However, among three species of the genus *Tortella* (*Tortella densa*, *T. tortuosa*, and *T. humilis*), only the methanol extracts of *Tortella densa* were found to be very effective on all of the *P. larvae* isolates.

Elibol et al. (30) investigated the antimicrobial activities of ethanol, methanol, acetone, and chloroform extracts of some mosses against six bacteria and two yeasts. They found that the methanol extract of *S. ruralis* was effective on *Salmonella* sp. and *S. cerevisiae*, while the methanol extract of *T. tortuosa* was not effective on any microorganisms. These results are similar to ours. In our study, the methanol extracts of *S. ruralis* and *T. tortuosa* were observed to not be very effective on *P. larvae* isolates.

In conclusion, the antimicrobial activities of many bryophyte species were investigated in several studies. Some extracts have been found to be effective. In our

study, the antimicrobial activity of some bryophyte species against *P. larvae* isolates were investigated for the first time and some bryophytes were found to be remarkably effective on *P. larvae*, which causes AFB disease. In light of these results, it is thought that secondary metabolites obtained from these bryophytes could be an alternative therapy to replace the antibiotics used in the treatment of AFB disease.

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