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Short communication

The use of real-time polymerase chain reaction with high resolution melting (real-time PCR-HRM) analysis for the detection and discrimination of nematodes *Bursaphelenchus xylophilus* and *Bursaphelenchus mucronatus*

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ABSTRACT

The real-time PCR-HRM analysis was developed for the detection and discrimination of the quarantine nematode *Bursaphelenchus xylophilus* and *Bursaphelenchus mucronatus*. A set of primers was designed to target the ITS region of rDNA. The results have demonstrated that this analysis is a valuable tool for differentiation of these both species.

The pinewood nematode, *Bursaphelenchus xylophilus*, is the causal agent of pine wilt disease, an important disease of conifer trees [1]. This nematode is a serious threat to pine forests as it lead to sudden death of tree [2]. *B. xylophilus* is considered to be a native species to North America and it was introduced to Japan at the beginning of 20th century [3]. After 1980th, it has spread to other Asian countries. In 1999 *B. xylophilus* was detected in Portugal, and in 2008 in Spain [4,5]. The epidemic spread of *B. xylophilus* was very rapid and it resulted in serious damage to pine forests over a large area both in Asia and in Europe [6].

Due to the difficulties in controlling *B. xylophilus*, the first crucial step to prevent the introduction of this pathogen is to provide an effective diagnostic tool. However, detection of *B. xylophilus* is complicated due to its close relation to species, *Bursaphelenchus mucronatus*. *B. mucronatus* is widely distributed in Asia and Europe and it is practically harmless to forest ecosystem. These both species can be transmitted by the same beetles and they occupy the same niche in the pine forest ecosystem in their original habitats. *B. xylophilus* can be distinguished from the *B. mucronatus* by the shape and size of a mucro on the tail of female *B. mucronatus*. However, females of *B. xylophilus* isolates with a mucronate tail have been reported from Japan, Portugal and the USA [7–12]. Thus, it is very difficult to differentiate between these species based solely on morphological features. Reliable methods of taxonomic identification of these species are therefore of particular interest for plant quarantine services.

A number of molecular techniques have been developed and used to identify *Bursaphelenchus* species, namely: ITS-RFLP [13], RAPD [14], PCR-based methods with species-specific primers [15,16], ITS nested PCR [17], real-time PCR [18,19] and loop-mediated isothermal amplification (LAMP) [20,21].

The real-time polymerase chain reaction with high resolution melting analysis (real-time PCR-HRM) has been introduced as a rapid, efficient and high-throughput post-PCR analytical technique to investigate the variance in nucleic acid sequences and to scan genetic mutations in the population without requirement for specific fluorescent probes [22]. HRM analysis has been successfully used to detect and differentiate plant and animal pathogens

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Table 1
Origin of nematode species and isolates used in the study and their GenBank accession numbers.

Species	Isolate name	Geographical origin	GenBank accession no
Bursaphelenchus xylophilus	China	Nanjing, China	KM657966
	C14-5	Chiba, Japan	AB277203
	OKD-1	Okayama, Japan	AB277205
	S10	Shimane, Japan	AB277206
	T4	Iwate, Japan	AB277207
	BxPt67OL	Portugal	JN684843
	US10	Minnesota, USA	GU206790
	KR-3(w)	Korea	KT581988
	Ne 21/02	Nanjing, China	KT581989
Bursaphelenchus mucronatus	LIT-01[a]	Lithuania	HM590216
	Mdz-01[a]	Miedzychod, Poland	HM590212
	Rad-03[a]	Dobropol, Poland	HM590217
	Rad-04[a]	Wlodawa, Poland	HM590218
	UKR-02[a]	Ukraine	HM590215
	Wro-01[a]	Wronki, Poland	JF912332
	DE-1(w)[b]	Bavaria, Germany	AM400246
	DE-5(w)[b]	Thuringia, Germany	AM400247
	DE-7(w)[a]	Saxony, Germany	KT581990
	RU-DE-30(w)[a]	Germany	KT581991

[a] E – the European type of *B. mucronatus*.
[b] EA – the East Asian type of *B. mucronatus*.

[23–27]. HRM proved to be a simple, rapid and efficient method to detect DNA polymorphism [28].

In this study, the real-time PCR-HRM analysis was developed and evaluated to detect and distinguish *B. xylophilus* from a morphologically and genetically similar nematode species, *B. mucronatus*. Nine isolates of *B. xylophilus* and ten isolates of *B. mucronatus* were examined. The origin of particular nematode isolates used in the reported research is shown in Table 1.

For the real-time PCR-HRM analysis, the ITS of rDNA regions were selected. The nucleotide sequences of ITS regions of the examined isolates were retrieved from the GenBank database. Based on the sequence dissimilarity between *B. xylophilus* and *B. mucronatus*, the four primer sets were selected to produce short amplicons.

Genomic DNA of *B. xylophilus* and *B. mucronatus* (from 1 to 100 specimens at various developmental stages, *i.e.*, females, males and juveniles) were isolated with a QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) according to the protocol provided by the manufacturer. DNA concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA).

The obtained DNA was adjusted to 100 ng/μl. To optimize the reaction that would result in the lowest average threshold cycle (Cq) value (*i.e.*, the highest level of sensitivity), several combinations of primers (300 nM, 500 nM, 600 nM) and concentrations of DNA (100 ng/μl – 1 fg/μl) were tested. PCR amplifications, DNA melting and end point fluorescence level acquiring PCR amplifications were performed in a Rotor–Gene™ 6000 Thermocycler (Corbett Life Science, Sydney, Australia) The optimized reaction components consisted of: 1 μl of DNA template, 0.6 μM of each primer (Table 2), 0.25 mM dNTP, 1 μl of 10× PCR buffer, 1× of dye EvaGreen (Biotum), and 1 U *Taq* polymerase (Novazym, Poznan, Poland) in a total volume of 20 μl. The thermocycling parameters were as follows: pre-incubation for 3 min at 95 °C followed by 40 cycles of quantitation with denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72 °C for 20 s. After the amplification process was completed, the HRM analysis was performed by increasing the temperature in steps of 0.1 °C over the range from 75 °C to 95 °C, monitored in real-time simultaneously with fluorescence changes. The obtained data was analysed using the Rotor–Gene™ Software Version 1.7.

To evaluate the specificity and effectiveness of the HRM analysis, DNA extracted from *B. mucronatus*, was used. To analyze the sensitivity of the method ten-fold dilutions of DNAs were prepared ranging from 100 ng/μl to 100 fg/μl. Moreover, in order to determine whether the life stages of nematodes have an impact on HRM analysis, the reaction was conducted with DNA isolated both from the nematode population and from single specimens (*i.e.*, females, males and juveniles). HRM analysis was conducted as described previously, and the experiment was repeated three times.

The designed primer pairs efficiently amplified DNA from the all tested populations of *B. xylophilus* and *B. mucronatus*. However, not all primer sets allowed us to definitely distinguish the examined species. Among the designed primers, the primer pair: HRM-F1 and HRM-R1 was the most effective and allowed us to clearly distinguish the tested populations (Table 2). The primers generated PCR products of 107 bp in length, encompassing the mutations GC versus AA in positions 328–329 nt (according to the T4 isolate of *B. xylophilus*) present in all the examined populations of *B. xylophilus* and *B. mucronatus*, respectively, and additionally the mutations T versus C and A versus G in positions 288 and 327 nt, respectively, in all the examined populations of *B. xylophilus* and in the East Asian type of *B. mucronatus*. The conducted study revealed that 500 fg/μl was the lowest concentration of DNA detected in real-time PCR-HRM analysis. The analysis was the most effective with primer concentrations of 600 nM. The study revealed that the designed primers detected *B. xylophilus* and *B. mucronatus*,

Table 2
Primers used for the HRM analysis.

Primer name	Primer sequence (5′–3′)	Amplification region	Position (nt)[a]	Amplicon size (bp)	Tm of Bx	Tm of Bm		References
HRM-F1	CTGCCCTGACAAAACATTCA	ITS-1	282–301	107	82.44	80.74	82.34[b]	This study
HRM-R1	GCAATTCACTGCGTTCTTCA	5.8S	369–388					

[a] Corresponding to the sequence of *B. xylophilus* isolate T4 (GenBank accession no. AB277207).
[b] Additional peak in the case of the East Asian type of *B. mucronatus*.