Detection of second-stage juveniles of *Anguina agrostis* using TaqMan Real-time PCR

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Summary. *Anguina agrostis* is one of the most economically damaging plant-parasitic nematodes. In this paper, a real-time PCR system for rapid identification of juveniles of *A. agrostis* was developed based on ITS-DNA with species-specific TaqMan probe and primers. Four related species, *A. agrostis* (two populations), *A. tritici, A. wevelli* and *Ditylenchus destructor*, were used to verify the specificity of this detection. Both of the two *A. agrostis* populations were positively detected and all of the non-*A. agrostis* populations were negative. One tenth of the DNA from a single second-stage juvenile of *Anguina agrostis* was detectable in this assay.

Key words: *Anguina*, bent grass nematode, second stage juveniles, TaqMan Real-time PCR.

Anguina species, the seed gall nematodes, have remarkably rich and convoluted taxonomic histories (Brzeski, 1981; Fortuner & Maggenti, 1987; Krall, 1991; Siddiqi, 2000). There are 11 valid species of *Anguina* according to the classification of Siddiqi (2000). Undoubtedly, host records of different anguinid species are compromised by misidentification. The large range of reported hosts for *Anguina agrostis* most likely includes records for several *Anguina* species (Krall, 1991).

The bent grass nematode, *A. agrostis* (Steinbuch, 1799) Filpjev, 1936, was dispersed by redistribution of galls by threshing machines during harvest (Courtney & Howell, 1952). Newly planted fields also could be infested by seed stock contaminated with galls (Pinkerton & Alderman, 1994). On bent grass (*Agrostis capillaris*), *A.agrostis* was reported to cause 50-75% yield loss in the Pacific northwest region of the USA (Pinkerton & Alderman, 1994). In addition to direct yield loss, *Agrostis capillaris* seed contaminated with galls is prohibited from export to countries that have a zero tolerance restriction for *Anguina* spp. (Pinkerton & Alderman, 1994; Alderman *et al.*, 2003).

Other nematode species belonging to the genus *Anguina* are of economic importance as agricultural and quarantine pests in various countries. The wheat gall nematode, *A. tritici* (Steinbuch, 1799) Chitwood, 1935 has the ability to carry *Rathayibacter toxicus* into wheat (Chizhov & Subbotin, 1990; Krall, 1991; Riley, 1992; Karakaş, 2004; Subbotin *et al*., 2004). *Anguina wevelli* (Van den Berg, 1985) Siddiqi, 2000, found on weeping lovegrass also has quarantine status (Chizhov & Subbotin, 1990; Powers *et al*., 2001).

All plant-parasitic nematode species can generally be identified reliably and sensitively using female morphological characters (Grenier *et al.,* 1997; Abrantes *et al*., 2004). In the case of *Anguina*, often, only juveniles are found in seed galls, which complicates identification (Powers *et al.*, 2001).

The development of PCR technology has opened new opportunities for nematode diagnostics (Subbotin *et al*., 2001). The effect of life stage or environmental factors is avoided by using PCR technology that directly targets polymorphisms in the genomic DNA (Williamson *et al*., 1997; Wishart *et al*., 2002). The internal transcribed spacer region (ITS), located between the repeating array of nuclear 18S and 28S ribosomal DNA genes, is a versatile genetic marker (Powers *et al*., 1997). Most species of nematodes can be analysed using a molecular approach based on the rDNA ITS region. The application of the ITS to identification has received most attention by nematologists (Orui, 1996; Powers *et al*., 1997, 2001; Subbotin *et al*., 2001; Ma *et al.*, 2004, 2006).

The real-time PCR offers significant advantages for the detection of nematodes. The method allows an accurate and unambiguous identification and

quantification of nucleic acid sequences. Cross-contamination is reduced, and high throughput and automation can be achieved in real-time PCR. The real-time PCR technology has been widely used to detect nematodes (Gao *et al.*, 2005; Madani *et al.*, 2005; Wang *et al.*, 2005; Zhang *et al.*, 2005).

To detect and quantify juveniles of *Anguina agrostis*, a real-time PCR method using TaqMan probes was developed during this study. The assay enables us to detect a single second-stage juvenile (J2) of *A. agrostis* in a sample. To date, there is no such assay for the detection of *A. agrostis*.

MATERIAL AND METHODS

Nematode populations. A total of six nematode populations were used in this study (Table1). Species determinations were made by morphological examination of nematodes extracted from nematode galls or plant, host association, and geographic location (Southey, 1973; Price *et al.,* 1979; Stynes *et al.*, 1980; Krall, 1991; Wendt *et al.*, 1993; Riley et al., 2001). Additional determinations were made by RFLP-PCR and multiple-PCR as described in previous studies (Ma *et al.*, 2004, 2006; Wang *et al.*, 2005). The populations of *Ditylenchus destructor* Thorne, 1945 were isolated from infested sweet potato samples in China. The populations of *A. agrostis* (population 1), *A. tritici*, and *A. wevelli* were isolated from seed galls in merchandise intercepted by Tianjin Entry–Exit Inspection and Quarantine Bureau, Tianjin, P. R. China (TJCIQ). The second population of *A. agrostis* (population 2) was isolated from seeds intercepted by TJCIQ in another lot of plant seeds. Single nematodes were washed three times with double distilled water and then were processed by placing them in 10 µl double distilled water before subjecting to DNA extraction.

DNA extraction. One single nematode, juvenile of *Anguina* nematodes or one female of *Ditylenchus destructor*, was moved into a drop of double distilled water (8 µl) and cut into several pieces with a sterilised scalpel under stereoscope. The nematode pieces along with 8 µl double distilled water was transferred into an Eppendorf tube containing 1 µl 10× PCR-buffer and 1 µl proteinase K (1 µg µl-1). After freezing at –20°C for at least 1 h, the nematode debris suspension was incubated at 65°C for 1 h and 95°C for 10 min consecutively. After centrifuging at 5000 rpm for 1 min, the DNA suspension was ready for PCR amplification, real-time PCR assay or was stored at -20°C for further study (Ma *et al*., 2004, 2006). The process of DNA extraction for each sample was repeated 5 times.

PCR amplification and Sequencing. The forward primer F194 (5'-CGT AAC AAG GTA GCT GTA G-3') and the reverse primer 5368 (5'-TTT CAC TCG CCG TTA CTA AGG-3') used for amplification of the internal transcribed spacer (ITS) regions of the ribosomal DNA were described by Ferris *et al*., (1993) and Vrain (1993), respectively. The PCR amplification was performed as described by Ma *et al.* (2004). The amplicon generated in each sample was purified using the QIAquick purification kit (supplied by Eastwin Life Sciences, Inc., China) according to the manufacturer's instructions and sequenced with an ABI-377 DNA sequencer (PE Applied Biosystems, Foster City, USA).

Plasmids containing PCR products amplified from *Anguina agrostis* population 1 were obtained by cloning (cloned and purified by Shanghai Boya Biotechnology Co., Ltd, China). The concentration of the plasmid template was quantified by spectrophotometer (APL instrument (Shanghai) Co. Ltd); it was about 100 ng μl^{-1}.

Primers and TaqMan probe. The software Primer Express 2.0 was used for primers and probe design (Chase *et al.*, 2005; Hibbeler *et al.*, 2008). The primers and probe were designed based on the ITS sequences of 13 nematode populations from the NCBI database and our sequences, i.e. *A. agropyri* (AF396355), *A. agrostis* (AM888391), *A. australis* (AF396334), *A. caricis* (AF396311), *A. funesta* (AF396349), *A. graminis* (AF396351), *A. microlaenae* (AF396333), *A. phalaridis* (AF396352), *A. tritici* (AF396354), *A. wevelli* (AM888393), *Ditylenchus destructor* (EF418003), *D. dipsaci* (AM232235) and *D. destructor* (AM232230). The TaqMan probe, Pb (5'-FAM-TCA TGT CTT GGC TAT TGT AGA CGT ATC TGA-TAMRA-3') was designed to anneal to the target sequence between the set of primers, PF (5'-GTT TGC CTA CCG GTT GTT TAC G-3') and PR (5'-CCA CAT GCA GTC GGT GTG AA-3') (Fig. 1). Figure 1 shows *A. agrostis* partial 5.8S rRNA gene (1-47), ITS2 (48-235) and partial 28S rRNA gene (236-250). The set of primers was used in the PCR reaction to amplify a fragment of the ITS2 region. The probe was labeled at the 5' end with a reporter fluorochrome 6-carboxyfluorescain (6-FAM) and at 3' end with a quencher fluorochrome 6-carboxytetramethyl-rhodamine (TAMRA). The primers and the probe were synthesized by Shanghai Boya Biotechnology Co. Ltd.

Detection of real-time PCR. The concentration of the probe Pb was optimised with 0.1 µM, 0. 2 µM, 0.4 µM and 0.6 µM concentrations. The annealing temperature was optimised with 57°C,

60°C and 62°C and the MgCl2 concentrations with 1 mM, 1.25 mM, 2.5 mM and 5 mM.

One µl of the DNA suspension was added to 9 µl PCR mixture containing 1 µl 10× PCR buffer, 0.5 µl 25 mM MgCl2, 0.2 µl 10 mM dNTPs, 0.2 µl each primer of PF and PR (20 µM), 0.1 µl Taq Polymerase (5 U μl^{-1}), 0.1 µl Pb (20 µM) and 6.7 µl double distilled water. The amplification reaction was performed in a real-time PCR assay using the Roche lightCycler (Roche). The real-time PCR program consisted of denaturation 3 min at 94°C; 45 cycles of 10 s at 94°C and 30 s at 60°C (Øster & Höllsberg, 2002). One tube containing the PCR mixture without DNA template was added as the negative controls. One tube containing sequential dilution T6 (0.0001 ng μl^{-1}) of the plasmid template was used as positive control. The experiment was repeated three times. Also, the experiment was performed in an ABI 7700 real-time PCR system to verify the result.

Sensitivity of *Anguina agrostis* detection. In order to test the sensitivity of the real-time PCR assay, nematode DNA was then serially diluted with water. One plasmid template was serially diluted with 9× double distilled water (Zou et al., 2006; Yakubu, 2007). The sequential concentrations of the diluted plasmid are T1 (10 ng μl^{-1}), T2 (1 ng μl^{-1}), T3 (0.1 ng μl^{-1}), T4 (0.01 ng μl^{-1}), T5 (0.001 ng μl^{-1}), T6 (0.0001 ng μl^{-1}), T7 (0.00001 ng μl^{-1}), T8 (0.000001 ng μl^{-1}) and T9 (0.0000001 ng μl^{-1}). All the sequential dilutions of the plasmid template were used as template for real-time PCR sensitivity detection and conventional PCR.

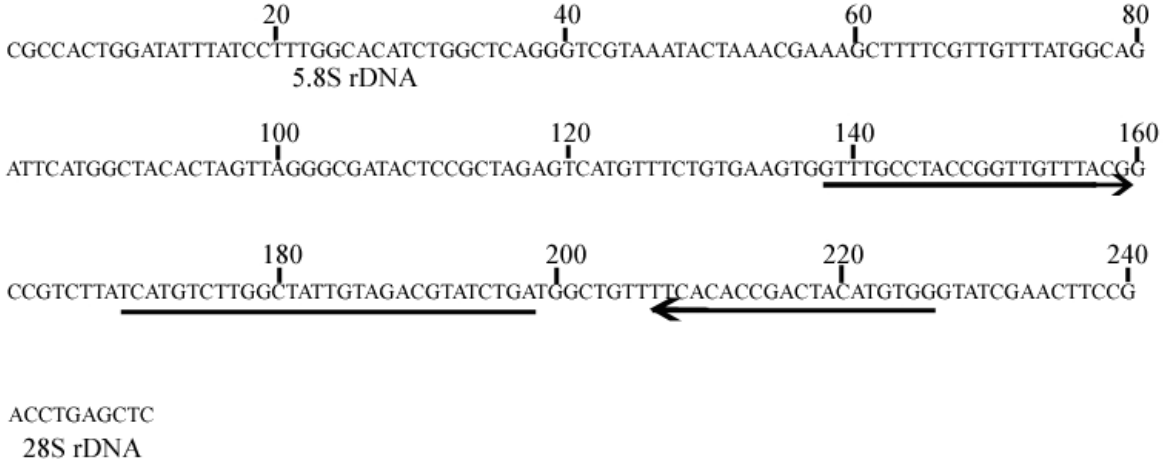

Fig. 1. The sequences and location of the two primers and a probe used in the real-time PCR assay. The forward primer is marked as a black bar with an arrow towards right; the reverse primer is marked as a black bar with an arrow towards left; and the probe is marked as a black bar without any arrows. The sequence is from our *Anguina agrostis*[1] population with NCBI accession number AM888391.

RESULTS

Sequencing assay. The DNA was extracted effectively for single nematodes in every repetition.

The DNA sequences obtained from *A. agrostis* (population 1), *A. tritici, A. wevelli,* and two populations of *D. destructor* , included partial DNA sequences of 18S rDNA, internal transcribed spacer one (ITS1), 5.8S rDNA, internal transcribed spacer two (ITS2) and partial DNA sequences of 28S rDNA. The NCBI accession numbers for these sequences are AM888391, AM888392, AM888393, AM232227 and AM232230.

There is no difference between sequences of samples of the two populations of *A agrostis*. There are differences among the sequences of *A. agrostis*, *A. tritici, A. wevelli*, and the two populations of *D. destructor*. All the nematodes can be distinguished by their sequences because the rDNA internal transcribed spacer region can be a taxonomic marker for nematodes (Powers *et al*., 1997; Hoyer *et al.,* 1998). Some methods based on PCR, such as PCR-RFLP and Multiple-PCR, have been used to identify *A. agrostis* (Powers *et al.*, 2001; Ma *et al*., 2004, 2006). However, a more rapid, precise and time-saving method is needed at inspection and quarantine service.

Specificity of real-time PCR assay. For the detection of *A. agrostis* by real-time PCR, we found the optimal conditions to be 0.2 µM probe Pb, the annealing temperature 60°C and 1.25 mM MgCl2.

In the initial cycles of the real-time PCR, there was little change in fluorescence signal. This defined the baseline for the amplification plot. An increase in fluorescence above the baseline indicated the detection of the accumulated target (Fig. 2 A).

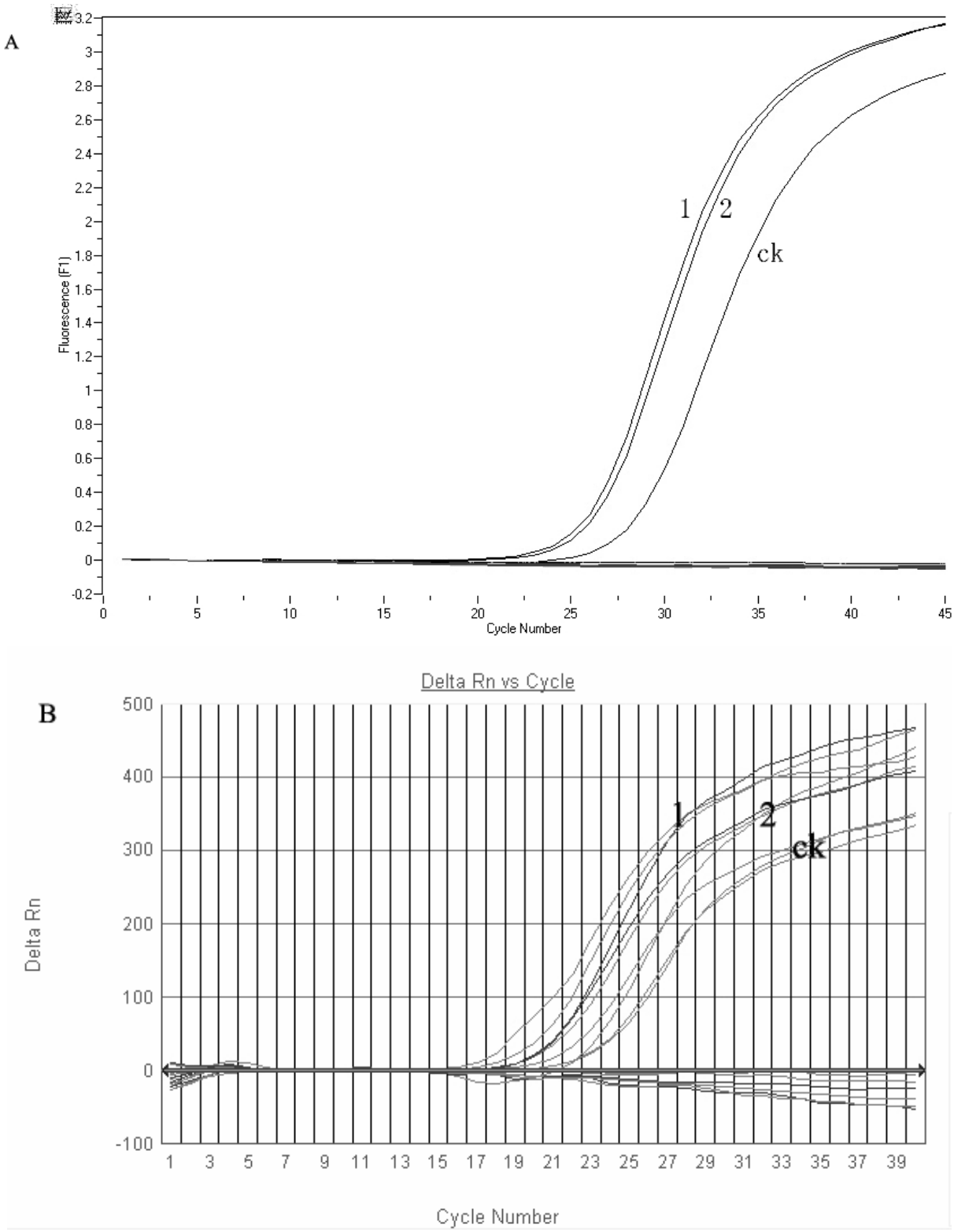

Fig. 2. A: - Specificity of real-time PCR detection amplified with Roche lightCycler. B: - Specificity of real-time PCR detection amplified with ABI 7700. Both the *Anguina agrostis* samples and the positive control had a positive reaction in the assay. All the other samples, including non-DNA-containing control, did not show any fluorescent signal. Curve 1, the fluorescent signal curve of *A*. *agrostis*; curve 2, the fluorescent signal curve of *A*. *agrostis*; ck, the fluorescent signal curve of the positive control.

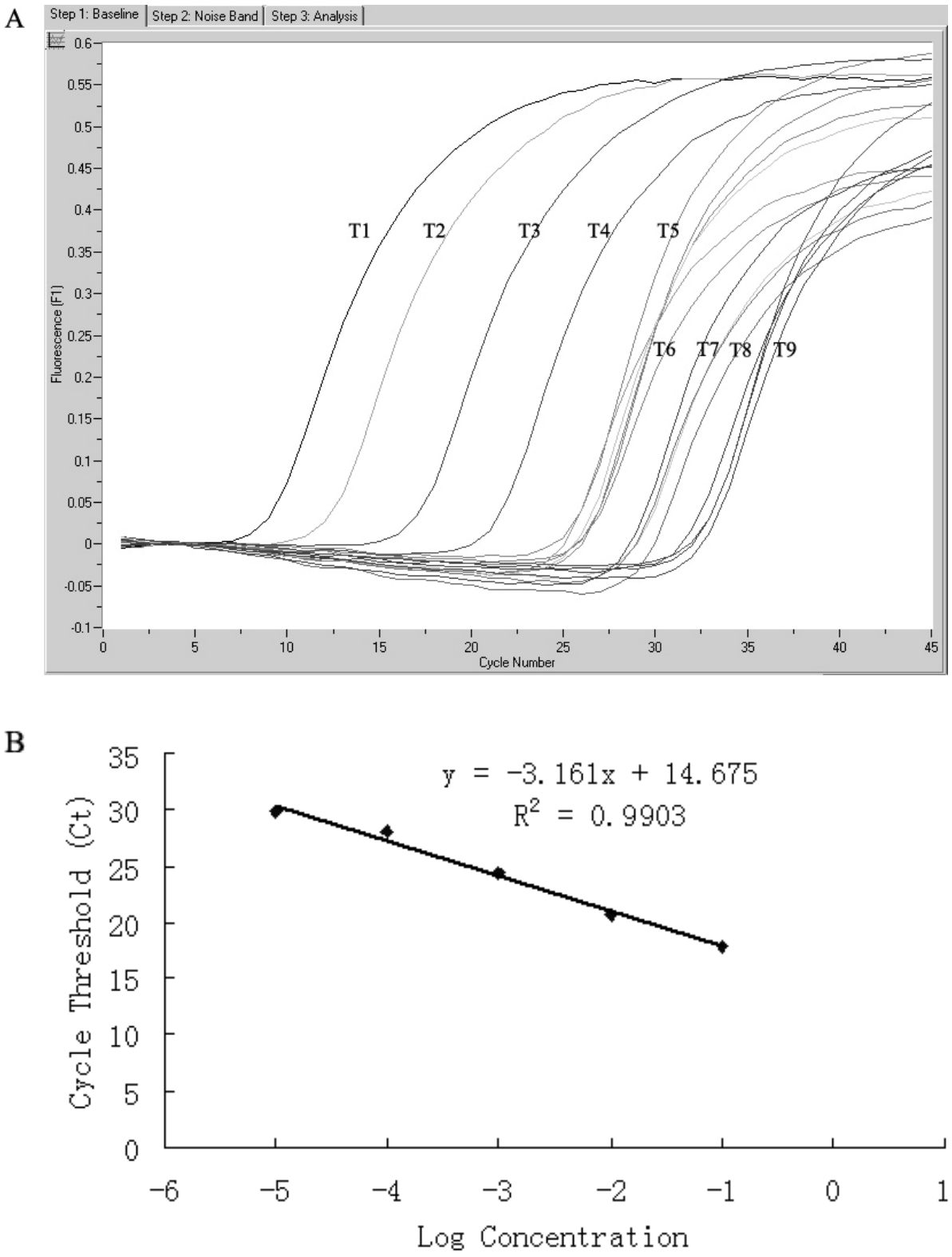

Fig. 3. **A -** *Anguina agrostis* plasmid DNA, which had been serially diluted 10-fold, were amplified with real-time PCR. **B -** Standard curve was created with the log starting quantity and threshold cycle of the 10-fold serially diluted *Anguina agrostis* plasmid DNA.

In the analyses, an amount equivalent to one tenth of the DNA from a single juvenile of *A. agrostis* was used. The result of real-time PCR showed that the two populations of *A. agrostis* and the positive control (ck) generated fluorescence signals, while all the other samples, including the negative controls, generated no signals (Fig. 2 A). The result demonstrates that the probe in the real-time PCR assay is specific for the detection of *A. agrostis*.

Similar results were obtained in the three repeat experiments. A similar result was obtained when the experiment was performed in an ABI 7700 real-time PCR system (Fig. 2 B).

No inhibitors, which have an strong effect on the efficiency of the real-time PCR, were found in this analysis with the unpurified DNA.

Sensitivity of real-time PCR assay. The diluted plasmid templates were used in the real-time PCR to determine the maximum dilution of the DNA detectable by the assay. The parameter CT (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed, and the smaller is the CT value (Fig. 3 A). All the five templates, T4, T5, T6, T7 and T8 were amplified with a good Ct value (18-30). All the other four templates, T1, T2, T3 and T9 were amplified without a good Ct value. The fluorescent signal in the present assay observed at various dilutions corresponded to a calculated minimal amount of detectable DNA of T8 (0.000001 ng μl^{-1}) of the plasmid templates (Fig. 3 A). A standard curve was created with the log starting quantity and threshold cycle of T4, T5, T6, T7 and T8 (Fig. 3 B).

The results in Figure 3A show that the detection limit of the TaqMan probe assay was about 0.000001 ng μl^{-1} plasmid template DNA per tube approximately in 30 cycles.

The sensitivity was also compared by electrophoresis in agarose gel (Fig. 4). As shown in Figure 4, the serially diluted *A. agrostis* plasmid DNA was amplified by conventional PCP and the PCR production could always be found in the lane corresponding to the dilutions with 10 ng μl^{-1}, 1 ng μl^{-1}, 0.1 ng μl^{-1}, 0.01 ng μl^{-1}, 0.001 ng μl^{-1} and 0.0001 ng μl^{-1}; however, the DNA bands corresponding to the dilutions 0.00001 ng μl^{-1} and 0.000001 ng μl^{-1} were unclear, which suggested that the TaqMan real-time PCR was about 100 times more sensitive than PCR gel electrophoresis detection.

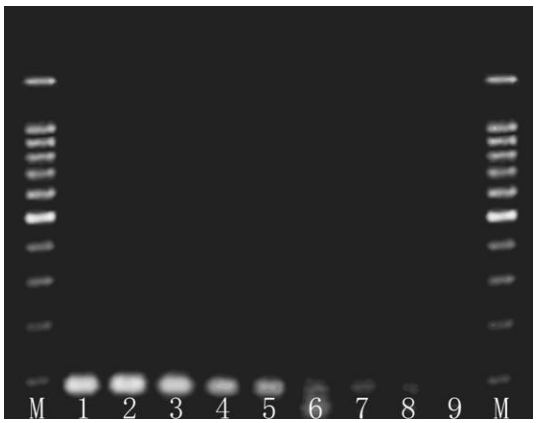

Fig. 4. Electrophoresis of PCR products. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel. Lanes M, molecular weight marker; lane 1, DNA dilution of 10ng μl^{-1}; lane 2, DNA dilution of 1 ng μl^{-1}; lane 3, DNA dilution of 0.1ng μl^{-1}; lane 4, DNA dilution of 0.01ng μl^{-1}; lane 5, DNA dilution of 0.001ng μl^{-1}; lane 6, DNA dilution of 0.0001ng μl^{-1}; lane 7, DNA dilution of 0.00001ng µl; lane 8, DNA dilution of 0.000001ng μl^{-1}; lane 9, DNA dilution of 0.0000001ng μl^{-1}.

DISCUSSION

The unpurified DNA of a single *Anguina* nematode can be used directly for real-time PCR. No mixed DNA of nematodes was used in this test because this paper only studied the real-time detection of single juveniles of *A. agrostis*. The assay is only suitable for detection of second-stage juveniles stage *A. agrostis*. This assay still needs to be optimised. Further testing for *A. agrostis* populations from different stages, different regions and other nematode species is still needed before being applied in routine identification of *A. agrostis.*

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Резюме. *Anguina agrostis* – важная в экономическом отношении нематода-вредитель. Предложен метод быстрого выявления и определения личинок *A. agrostis* на основе ПЦР в реальном времени, при использовании видоспецифичных TaqMan-меток и праймеров для ITS rDNA. Для проверки метода использовали 4 родственных вида: две популяции *A. agrostis*, *A. tritici, A. wevelli,* а также *Ditylenchus destructor.* Удалось успешно выявить присутствие *A. agrostis* из обох популяций, ответа на присутствие иных, не относящихся к *A. agrostis* нематод не было. Удавалось выявлять присутствие лишь одной десятой части ДНК, выделенной от единственной личинки *Anguina agrostis*.