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# Development and validation of species-specific primers that provide a molecular diagnostic for virus-vector longidorid nematodes and related species in German viticulture

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**Abstract**

The nematode species *Longidorus attenuatus*, *L. elongatus*, *L. macrosoma* and *Paralongidorus maximus* are economically important pests to the viticulture industry due to their ability to vector two nepoviruses (Raspberry Ringspot Virus and Tomato Black Ring Virus) to grapevines. In Germany, these species occur in vineyard soil with other non-vector but morphologically similar longidorid species, *L. helveticus*, *L. profundorum* and *L. sturhani*.

Species-specific primers were designed from ribosomal DNA for all seven species to facilitate taxonomic identification for non-specialists. Primers were assessed for their reliability by screening, where possible, a number of populations of each species. Furthermore, their selectivity and sensitivity were determined when challenged with closely related longidorid species and general nematode communities typical of vineyard soil. A multiplex approach using a common forward primer combined with species-specific reverse primers enabled three target nematode species to be detected in the same PCR reaction. All primers were highly specific, detecting all nematode developmental forms from disparate populations and were sufficiently sensitive to detect a single target nematode within a whole nematode community typical of a vineyard soil comprising of a range of non-target species.

Given their specificity, sensitivity and reliability, these diagnostic primers should be of great benefit to both phytosanitary/quarantine services related to the viticulture industry and also as a decision management tool for growers.

## Introduction

In German vineyard soils, as in most other viticultural regions around the world, several species of longidorid nematodes occur, some of which are of economical importance as virus vectors (Rüdel, 1985; Brown and Taylor, 1987; Brown and Trudgill, 1989). In addition to *Xiphinema diversicaudatum* and *X. index*, three *Longidorus* and one *Paralongidorus* species are known to be vectors of nepoviruses in Germany. In particular, *L. attenuatus* has been shown to transmit tomato black ring virus (English and German strain) (Harrison, 1964), *L. elongatus* vectors both tomato black ring (Scottish strain) (Harrison et al., 1961) and raspberry ringspot virus (Scottish strain) (Taylor, 1962) and both *L. macrosoma* and *P. maximus* are vectors of raspberry ringspot virus (English and Palatinate strain, respectively) (Harrison, 1964; Jones et al., 1994). Apart from these species,

several other longidorids occur in German vineyards, for example *L. profundorum* is a relatively common species in the Palatinate region. This species was reported to transmit raspberry ringspot virus (Fritzsche and Kegler, 1968), but the record does not fulfil the criteria for assessing transmission of viruses by members of the Longidoridae reported by Trudgill et al. (1983). Other longidorid species reported from German vineyard soils include *L. helveticus* (Lamberti et al., 2001) and *L. sturhani* (Rubtsova et al., 2001) that are morphologically similar to the vector species *L. macrosoma* and *L. elongatus*, respectively.

It is important to distinguish between recognised virus vector and non-vector longidorid species, as German viticulture regulations in particular stipulate that fields on which grapevines used for multiplication are grown must be free of virus-transmitting nematodes. Species identification, which is currently based on morphological features of adult specimens, requires a high degree of specialist knowledge, is often complicated by the low number of specimens found in soil samples and further compounded by the occurrence of mixed populations. Thus, a method allowing identification of the vector species, even if just a single adult, juvenile or a mixture of species are present, is required.

Increasingly, molecular diagnostics are providing a powerful alternative to morphological identification for a few predominantly (semi) endoparasitic nematode genera such as *Globodera* (for example, Mulholland et al., 1996; Shields et al., 1996; Fleming et al., 1998), *Heterodera* (Subbotin et al., 2000; Zheng et al., 2000; Amiri et al., 2002), *Pratylenchus* (Uehara et al., 1998, 1999; Waeyenberge et al., 2000) and especially *Meloidogyne* (for example, Zijlstra et al., 1995, 1997, 2000; Zijlstra, 1997, 2000; Dong et al., 2001; Wishart et al., 2002), although recently a number of molecular diagnostics have focussed on entomopathogenic (Reid et al., 1997; Nguyen et al., 2001; Stock et al., 2001) and *Bursaphelenchus* (Hoyer et al., 1998; Iwahori et al., 1998; Liao et al., 2001) species. The highly variable ITS regions of ribosomal DNA have been used for molecular characterisation of nematodes, predominantly using two-step PCR-based restriction fragment length polymorphisms (RFLPs) methodologies (for example, Vrain et al., 1992; Zijlstra et al., 1995, 1997; Powers et al., 1997; Fleming et al., 1998; Hoyer et al., 1998; Iwahori et al., 1998; Knoetze et al., 2000; Waeyenberge et al., 2000; Nguyen et al., 2001) and only occasionally for the development of a single-step species-specific diagnostic (Uehara et al., 1998; Liao et al., 2001; Wang et al., 2003).

Recently, Wang et al. (2003) published species-specific primers derived from ITS1 sequence for two of the six virus vector longidorid species known to occur in German viticulture, namely *X. diversicaudatum* and *X. index*. These primers have been assessed for their reliability, specificity and sensitivity to confirm their potential as a routine phytosanitary test (Hübschen et al., in press).

Currently no similar molecular diagnostics are available for species of either *Longidorus* or *Paralongidorus*. Lamberti et al. (2001) characterised six *Longidorus* species using superoxide dismutase, esterase isozymes and ITS-RFLPs. Rubtsova et al. (2001) presented a phylogenetic tree for seven *Longidorus* species based on sequences of the D2–D3 expansion region of rDNA, but did not provide any molecular means to distinguish between the species.

The objective of this study was to develop species-specific primers located in both the 18S and ITS regions of rDNA for the virus-transmitting nematode species *L. attenuatus*, *L. elongatus*, *L. macrosoma* and *P. maximus* and for three other *Longidorus* species, namely *L. profundorum*, *L. helveticus* and *L. sturhani*, that either commonly occur in vineyard soil or are morphologically very similar to the virus-vector species.

## Materials and methods

### *Nematodes*

Soil samples were taken from different vineyards in the Palatinate and surrounding area (Table 1). Nematodes were extracted by a decanting and sieving technique (Schaaf, 1999) and longidorid specimens were hand-picked and stored in 1 M NaCl at −20 °C or used directly for DNA extraction. If possible, nematodes of different developmental stages and from different populations were used to investigate whether there was any sequence variability. In total, 16 populations of seven species were used for initial sequencing and primer design (Table 1), with a further thirteen popula-

*Table 1.* Longidorid populations used for sequencing of ITS1 region of ribosomal DNA

| Nematode species | Population | Origin |
|---|---|---|
| *L. attenuatus* | Gau Algesheim | Rheinhessen |
| *L. attenuatus* | Mettenheim | Rheinhessen |
| *L. elongatus* | Bad Dürkheim | Pfalz |
| *L. elongatus* | Breeding Pot[1] | The Netherlands |
| *L. elongatus*[2] | Heppenheim | Hessische Bergstrasse |
| *L. macrosoma*[3] | Freiburg | Baden |
| *L. macrosoma*[2] | Heppenheim | Hessische Bergstrasse |
| *L. macrosoma* | Landau | Pfalz |
| *P. maximus* | Kallstadt | Pfalz |
| *P. maximus* | Mußbach | Pfalz |
| *L. helveticus* | Horrweiler | Nahe |
| *L. helveticus*[4] | Roche d'Or | Schweiz |
| *L. profundorum* | Gau Algesheim | Rheinhessen |
| *L. profundorum* | Horrweiler | Nahe |
| *L. sturhani* | Gau Algesheim | Rheinhessen |
| *L. sturhani* | Ockenheim | Rheinhessen |

[1] Scottish Crop Research Institute glasshouse culture.
[2] Nematodes provided by S. Schütz (Pflanzenschutzdienst Hessen).
[3] Soil samples provided by G. Bleyer (Weinbauinstitut Freiburg).
[4] Soil samples provided by P. Kunz (Eidgenössische Forschungsanstalt Wädenswil).

*Table 2.* Longidorid populations used to determine specificity of species-specific primers

| Nematode species | Population | Origin (winegrowing area) |
|---|---|---|
| *L. macrosoma* | Edesheim | Pfalz |
| *L. macrosoma* | Herxheim | Pfalz |
| *L. macrosoma* | Oberhausen | Pfalz |
| *L. profundorum* | Flemlingen | Pfalz |
| *L. profundorum* | Schimsheim | Rheinhessen |
| *L. profundorum* | Stadecken | Rheinhessen |
| *P. maximus* | Bad Dürkheim | Pfalz |
| *P. maximus* | Deidesheim | Pfalz |
| *P. maximus* | Hambach | Pfalz |
| *X. diversicaudatum* | Schweigen-Rechtenbach | Pfalz |
| *X. index* | Bissersheim | Pfalz |
| *X. pachtaicum* | Landau | Pfalz |
| *X. vuittenezi* | Westhofen | Rheinhessen |
| Nematode community 1 | Deidesheim | Pfalz |
| Nematode community 2 | Landau | Pfalz |

tions and two total nematode communities used to validate the specificity and reliability of the designed primers (Table 2).

### *DNA extraction*

DNA from at least two individual nematodes per species was extracted by a modification of the method described by Stanton et al. (1998). A single nematode was placed in 20 µl of 0.25 M NaOH, incubated overnight at room temperature, thereafter heated to 99 °C for 3 min. Afterwards 10 µl of 0.25 M HCl, and 5 µl each of 0.5 M Tris–HCl (pH 8) and 2% Triton X-100 were added and the mixture was incubated for another 3 min at 99 °C. The DNA was either used directly after extraction or stored at −20 °C.

### *ITS sequencing and primer design*

Each PCR reaction comprised of a single PuRe-Taq™ Ready-To-Go™ PCR Bead (Amersham Pharmacia Biotech), 0.5 µl of template nematode DNA, 1 µl each of 10 µM primers UNIVERSAL (5′ CCCGTCGMTACTACCGATT 3′) and rDNA1.58S (5′ ACGAGCCGAGTGATCCACCG 3′) described by Boutsika et al. (2004) and Cherry et al. (1997), respectively and sterile, distilled water to a total volume of 25 µl. Amplification was done using a GeneAmp® PCR System 2700 thermocycler (Applied Biosystems) with the following conditions: 94 °C for 2 min 45 s followed by 40 cycles at 94 °C for 1 min, 57 °C for 45 s, and 72 °C for 2 min, followed by a 10 min cycle at 72 °C. PCR products were separated on a 1% agarose gel stained with ethidium bromide in 1x TBE buffer and visualised under UV light.

If PCR products proved to be of high quality, they were purified directly using a NucleoSpin® Extraction Kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. Direct bi-directional sequencing on the purified DNA used the aforementioned primers UNIVERSAL and rDNA1.58S. For each sequencing reaction 4 µl Big Dye Terminator Ready Reaction Mix (Applied Biosystems), 1 µl of one of the primers (3.4 µM) and 5 µl of purified DNA were added to a 0.2 ml micro-centrifuge tube. Cycle sequencing conditions were as recommended by the manufacturer of the Big Dye Mix. Samples were sequenced by GENterprise (Mainz, Germany).

Sequenced ITS1 regions were analysed using Lasergene Version 5 software (DNASTAR, Inc., Madison, Wisconsin, USA). Consensus sequences

*Table 3.* Species-specific primers for each longidorid species

| Name | Associated nematode species | Length (bp) | Primer sequence (5′–3′) | Sense |
|---|---|---|---|---|
| Latten3 | *L. attenuatus* | 30 | TTC CCT TTT CCC TGA TTA TAA TTT TCT ATC | Reverse |
| Lelong1 | *L. elongatus* | 23 | TTA TCG TAC GTA TTC CCA GTT CT | Reverse |
| Lmacro2 | *L. macrosoma* | 21 | GTT CCC GAC GAT TAT TTT TGT | Reverse |
| Pmax1 | *P. maximus* | 22 | TGC ATT TCA CCA CTT CTC ACT C | Reverse |
| Lhel1 | *L. helveticus* | 29 | CCG CAT CTC TTT ATT TCC GAC CAT CAA CC | Reverse |
| Lprof2 | *L. profundorum* | 28 | TTA TTA TTT TTC AGG CTC TAC CTT TCG C | Reverse |
| Lstur | *L. sturhani* | 25 | TTT TCC CCA CTA ATA CTC CCT CGT T | Reverse |
| GenF | Universal | 23 | TTG ATT ACG TCC CTG CCC TTT GT | Forward |

for each species were constructed and compared to identify possible species-specific primer sites. A universal forward primer (GenF, Table 3) located in the 18S gene was identified from full length 18S gene rDNA (Hübschen et al., unpublished) and was used with a number of potential species-specific reverse primers located in the ITS1 region characterised for each species using the UNIVERSAL/ rDNA1.58S primer pair.

*Primer testing*

Potential species-specific primers were initially tested against the different nematode populations that provided template DNA for sequencing (Table 1). After initial screening, successful primers, i.e. those that produced a single potential species-specific PCR product were tested with additional populations (Table 2) to determine their reliability. Specificity was determined by using DNA of non-target longidorids including four *Xiphinema* species and DNA of two nematode communities containing saprophagus, predatory and other plant-parasitic nematode species (Tables 1 and 2). Primer sensitivity was tested by adding a single specimen of the four target vector species, *L. attenuatus, L. elongatus, L. macrosoma* and *P. maximus* to that of total nematode community DNA from two sites, Westhofen and Biebelnheim. The potential of multiplexing species-specific primers was investigated.

## Results

Primers UNIVERSAL and rDNA1.58S yielded PCR products of variable lengths for the different nematode species investigated (Figure 1) ranging between 950 and 1250 bp. Potential primer combinations (different reverse primers with the universal primer GenF) were screened and those found to be species-specific (Figure 2) are listed in Table 4. Reliability of the species-specific primer pairs was confirmed when tested with a number of different populations of each species and different developmental stages (Figure 3A and B). Multiplexing proved possible for several combinations of primers (Figure 4) due to the variable size of the

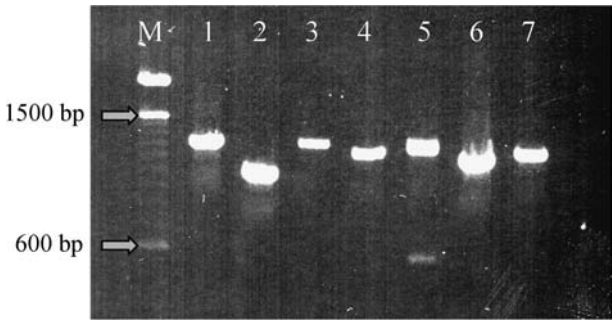


*Figure 1.* Electrophoresis of the amplification products of DNA from seven longidorid species with primers UNIVERSAL and rDNA1.58S. M corresponds to a 100 bp DNA ladder (GIBCO BRL, Life Technologies). Lane 1: *L. attenuatus*; lane 2: *L. elongatus*; lane 3: *L. macrosoma*; lane 4: *P. maximus*; lane 5: *L. helveticus*; lane 6: *L. profundorum*; lane 7: *L. sturhani*.

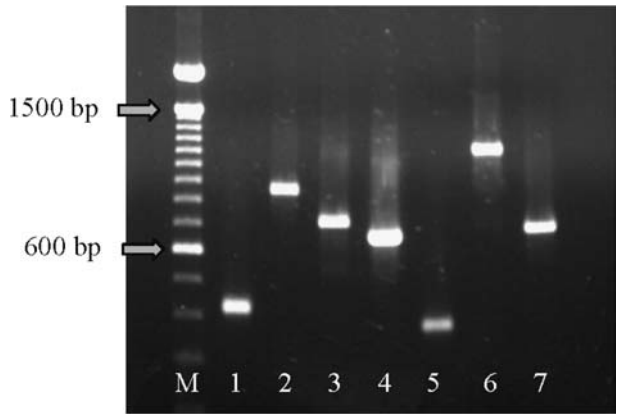


*Figure 2*. Electrophoresis of the amplification products of DNA isolated from seven different longidorid species using the species-specific primers listed in Table 4 and the universal primer GenF. M corresponds to a 100 bp DNA ladder (GIBCO BRL, Life Technologies). Lane 1: *L. attenuatus*; lane 2: *L. elongatus*; lane 3: *L. macrosoma*; lane 4: *P. maximus*; lane 5: *L. helveticus*; lane 6: *L. profundorum*; lane 7: *L. sturhani*.

*Table 4*. Primer pairs and length of corresponding PCR fragments

| Primer pairs | Fragment length (bp) |
|---|---|
| GenF/Latten3 | 419 |
| GenF/Lelong1 | 847 |
| GenF/Lmacro2 | 705 |
| GenF/Pmax1 | 649 |
| GenF/Lhel1 | 369 |
| GenF/Lprof2 | 1071 |
| GenF/Lstur | 667 |

PCR products (Table 4). The sensitivity of the species-specific primers was demonstrated when template DNA equivalent to a single target nematode was detected amongst total nematode community DNA (Figure 5A and B). Furthermore, multiple target nematode species could be simultaneously detected amongst the total nematode community of field soil although not all four target longidorid nematode species were detected (Figure 5A and B).

## Discussion

Longidorid nematodes are considered one of the ten most economically important nematode groups globally (Sasser and Freckman, 1987). Coupled with the fact that the number of recognised longidorid species has increased since 1960 (Taylor and Brown, 1997), there are surprisingly few published molecular diagnostics (Vrain et al., 1992; Vrain, 1993; Molinari et al., 1997; Knoetze et al., 2000; Wang et al., 2003). Of those diagnostics, only Wang et al. (2003) are considered of value as a routine diagnostic in a phytosanitary laboratory (Hübschen et al., in press).

In this study, species-specific primers have been developed that reliably discriminate seven longidorid nematode species, belonging to the genera *Longidorus* and *Paralongidorus*, typical of grapevine soil in Europe (Alphey and Taylor, 1986; Brown and Taylor, 1987; Brown and Trudgill, 1989; Taylor and Brown, 1997), of which four are recognised vectors of plant viruses (Trudgill et al., 1983). The remaining three diagnostic primers are for non-vector longidorid species that are easily mistaken as vector species due to their morphological and morphometric similarity (Robbins et al., 1995; Chen et al., 1997; Lamberti et al., 2001; Rubtsova et al., 2001).

Primer reliability was confirmed by screening where possible with different populations and a range of developmental stages of each target species. Furthermore, selectivity and sensitivity were also confirmed by challenging the primer combinations with both non-target longidorid species and the nematode communities comprising a range of different trophic groups (Yeates et al., 1993)

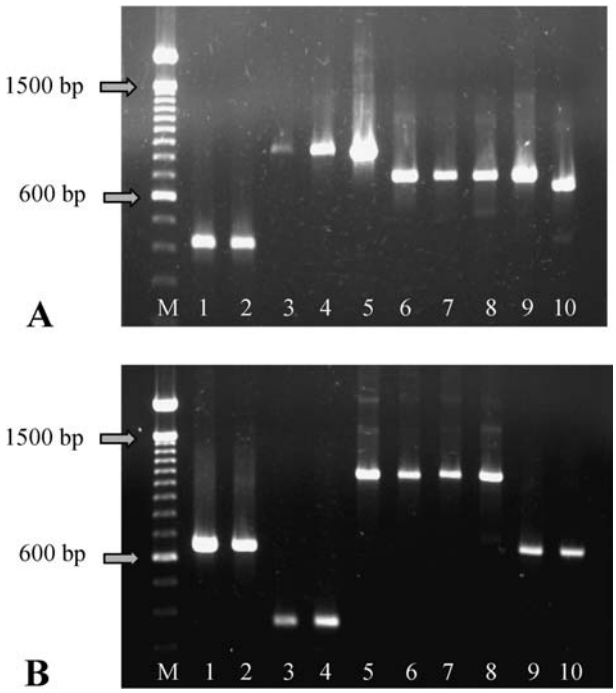


*Figure 3*. Electrophoresis of the amplification products of DNA isolated from several populations of seven longidorid species to determine compatibility of the species-specific primers. M corresponds to a 100 bp DNA ladder (GIBCO BRL, Life Technologies). (A) lanes 1 and 2: *L. attenuatus* (Gau Algesheim, juvenile, Mettenheim, juvenile); lanes 3–5: *L. elongatus* (Heppenheim, female, Bad Dürkheim, female, The Netherlands, juvenile); lanes 6–9: *L. macrosoma* (Heppenheim, female, Landau, juvenile, Freiburg, male, Herxheim, juvenile); lane 10: *P. maximus* (Mußbach, juvenile). (B) lanes 1 and 2: *P. maximus* (Kallstadt, female, Deidesheim, juvenile); lanes 3 and 4: *L. helveticus* (Horrweiler, juvenile, Roche d'Or, female); lanes 5–8 *L. profundorum* (Horrweiler, female, Gau Algesheim, juvenile, Landau, female, Schimsheim, male); lanes 9 and 10: *L. sturhani* (Gau Algesheim, female, Ockenheim, juvenile).

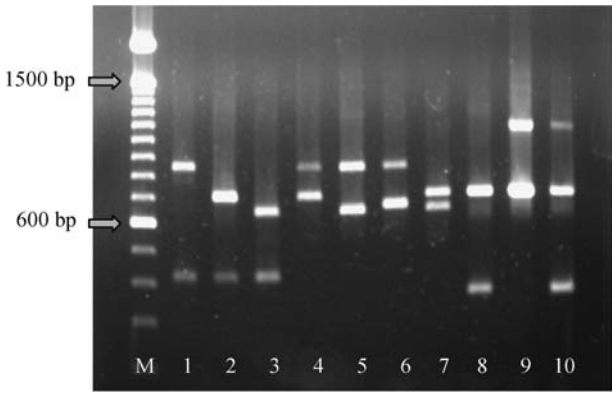


*Figure 4*. Electrophoresis of the amplification products of DNA isolated from seven longidorid species using a multiplex test with primer combinations using the appropriate species-specific primer (Table 4) and the universal primer GenF. M corresponds to a 100 bp DNA ladder (GIBCO BRL, Life Technologies). Lane 1: *L. attenuatus* + *L. elongatus*; lane 2: *L. attenuatus* + *L. macrosoma*; lane 3: *L. attenuatus* + *P. maximus*; lane 4: *L. elongatus* + *L. macrosoma*; lane 5: *L. elongatus* + *P. maximus*; lane 6: *L. elongatus* + *L. sturhani*; lane 7: *L. macrosoma* + *P. maximus*; lane 8: *L. macrosoma* + *L. helveticus*; lane 9: *L. macrosoma* + *L. profundorum*; lane 10: *L. macrosoma* + *L. helveticus* + *L. profundorum*.

from two vineyard soils thus fulfilling the criteria outlined by Hübschen et al. (in press) for a single-step PCR diagnostic test.

As with other longidorid nematodes, the length of the ITS1 was relatively large (950–1250 bp) and varied between species (Vrain et al., 1992; Powers et al.,

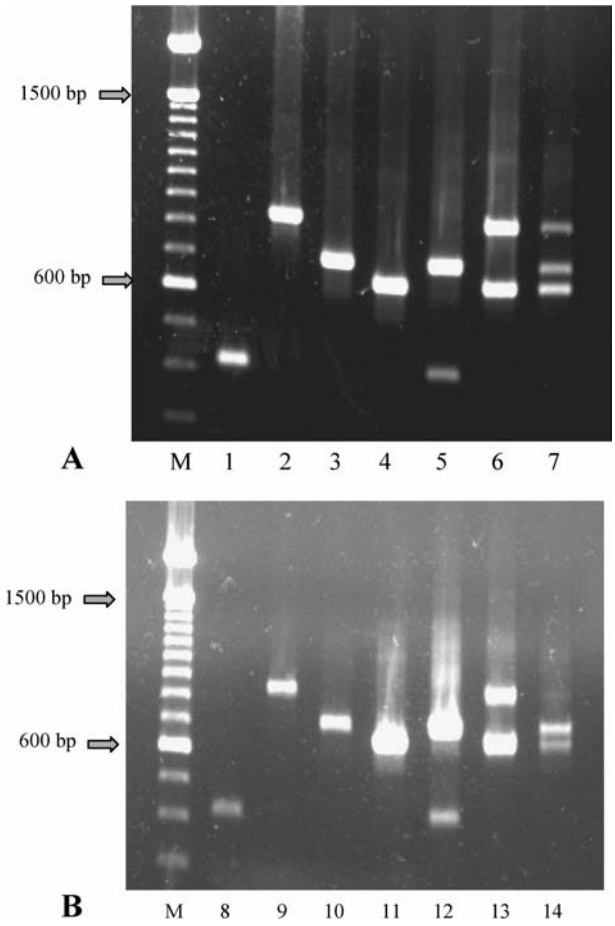


*Figure 5*. Electrophoresis of the amplification products of DNA extracted from (A) nematode community Biebelnheim plus one individual each of *L. attenuatus*, *L. elongatus*, *L. macrosoma* and *P. maximus* and (B) DNA isolated from nematode community Westhofen plus DNA of *L. attenuatus*, *L. elongatus*, *L. macrosoma* and *P. maximus*. M corresponds to a 100 bp DNA ladder (GIBCO BRL, Life Technologies). Lanes 1 and 8: *L. attenuatus*; lanes 2 and 9: *L. elongatus*; lanes 3 and 10: *L. macrosoma*; lanes 4 and 11: *P. maximus*; lanes 5 and 12: *L. attenuatus* + *L. macrosoma*; lanes 6 and 13: *L. elongatus* + *P. maximus*; lanes 7 and 14: *L. attenuatus* + *L. elongatus* + *L. macrosoma* + *P. maximus*.

1997; Wang et al., 2003). This inter-specific variability of the ITS1 region allowed for the design of primers that produced differing fragment sizes with a view of establishing a multiplex PCR protocol. Such an approach has been previously described for *Xiphinema* (Wang et al., 2003) and *Meloidogyne* (Zijlstra, 1997) species. A multiplex approach had limited success in this study, with a maximum of three target nematode species (*L. macrosoma*, *L. helveticus* and *L. profundorum*) detected in the same PCR reaction.

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