Intraspecific Variability within *Globodera tabacum solanacearum* and Selection for Virulence Against Flue-Cured Tobacco

by

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(ABSTRACT)

The tobacco cyst nematode (TCN), *Globodera tabacum solanacearum* [(Miller and Gray, 1972) Behrens 1975] Stone 1983, is one of the most economically important pests of flue-cured tobacco (*Nicotiana tabacum* L.) in Virginia. Although TCN has been reported from other countries, the geographical distribution of *G. t. solanacearum* within the United States is limited to Virginia, North Carolina, and one county in Maryland. Approximately 30% of the tobacco acreage in Virginia is infested; average yield reduction is 15%, but complete crop failure can occur. The objectives of this research were to examine intraspecific variability within *G. t. solanacearum* and to evaluate the relative adaptability of *G. t. solanacearum* on a resistant (NC567) and a susceptible (K326) flue-cured tobacco cultivar.

Nineteen geographic isolates of *G. t. solanacearum*, one isolate each of *G. t. virginiae* and the Mexican cyst nematode (*G. "mexicana"*), two isolates of *G. t. tabacum*, and five *Heterodera* species were characterized by DNA fingerprinting using the RAPD-PCR technique. Reproducible differences in fragment patterns allowed similar differentiation of the isolates and species with each primer. Hierarchical cluster analysis was performed to illustrate the relatedness between nematode isolates and species. In contrast to reports in the literature, we found a Miller isolate of *G. "mexicana"* to cluster more closely with *G. t. solanacearum* than with *G. t. tabacum* or *G. t. virginiae*. Although no pathotype differences

have been found within *G. t. solanacearum*, the average Jaccard's similarity index among isolates of *G. t. solanacearum* was 74%, representing greater variation than that observed across different pathotypes of the closely related potato cyst nematode, *Globodera pallida*. This result suggests that the emergence of resistance-breaking biotypes is more likely than previous research suggests. If a new pathotype is reported, a RAPD marker associated with virulence against a specific host resistance gene could prove to be a valuable tool in population diagnosis, resistance screening, and overall TCN management.

One isolate of *G. t. solanacearum* was cultured on a resistant (NC567) and a susceptible (K326) flue-cured tobacco cultivar over five generations. Variable TCN reproduction was observed on both cultivars over each generation. This variability in reproduction could be attributed to differences among generations in the time interval between inoculation and cyst extraction, temperature, possible diapause effects, and/or daylength. Ninety-eight cysts were produced in the fifth and final generation compared to the 14 to 50 cysts produced during each of the previous four generations. Increased reproduction on the resistant variety suggests that increased virulence might be selected, but research involving additional generations would need to be carried out in order to conclude whether or not TCN virulence is being selected.

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History

In 1951, Lownsbery and Lownsbery described the tobacco cyst nematode (TCN) (*Heterodera tabacum* Lownsbery and Lownsbery 1954) parasitizing shade tobacco (*Nicotiana tabacum* L.) in Hazardville, Connecticut. A cyst nematode parasitizing horsenettle (*Solanum carolinense* L.) was discovered in 1959 on the coastal plain of Virginia (Miller *et al*., 1962), and Miller and Gray (1968) named it *Heterodera virginiae*. Dr. W. W. Osborne and H. M. Holmes of the Virginia Cooperative Extension Service discovered a cyst nematode parasitizing flue-cured tobacco cultivar Hicks in Amelia County in a 1.33-ha field that had been in continuous tobacco production for 7 years (Osborne, 1961; Miller and Gray, 1972; Komm 1983). Miller and Gray (1972) described this species as *Heterodera solanacearum.* Behrens (1975) established a new genus for the round cysts, and placed these three tobacco cyst nematodes, along with the potato cyst and others into *Globodera* (Baldwin and Mundo-Ocampo, 1991). In 1983, Stone considered the three tobacco cyst nematode species as a species complex and recognized them as subspecies based on morphological differences and host range. The three subspecies are probably allopatric. The Connecticut tobacco cyst nematode was named *Globodera tabacum tabacum* [(Lownsbery and Lownsbery, 1954) Behrens, 1975] Stone 1983; the horsenettle cyst nematode became *Globodera tabacum virginiae* [(Miller and Gray, 1968) Behrens 1975] Stone 1983; and Osborne's cyst nematode was named *Globodera tabacum solanacearum* [(Miller and Gray, 1972) Behrens, 1975] Stone, 1983.

From the initial discovery of TCN, Lownsbery (1951) was able to differentiate *G. t. tabacum* from *G. rostochiensis* with host range studies; potato cyst nematodes cannot

reproduce on tobacco. *G. t. virginiae* was differentiated from *G. rostochiensis* in the original description because of its ability to reproduce on tobacco (Miller and Gray, 1968). Differences in host range are sufficient for dividing the species into three sub-species (Miller and Gray, 1972). *Globodera tabacum tabacum* and *G. t. solanacearum* reproduce well on *Nicotiana* species (Lownsbery and Peters, 1955; Miller and Gray, 1972), but *G. t. virginiae* does not (Miller, 1978). The three subspecies can be differentiated morphologically using a combination of characters such as the shape of the stylet knobs, Granek's ratio (distance between anal pore and nearest edge of vulva), length of the stylet in second-stage juveniles, and terminal end patterns (Mota and Eisenback, 1993). By means of two-dimensional gel electrophoresis, Bossis and Mugniéry (1993) concluded that the genetic relationship of *G. t. virginiae*, *G. t. solanacearum,* and *G.t. tabacum* to be close enough to be considered as a single species, with *G. t. virginiae* and *G. t. solanacearum* being more closely related than *G. t. tabacum*.

In Mexico and Central America, a species was described, but not published, by Campos-Vela in a Ph.D. dissertation in 1967 (Baldwin and Mundo-Ocampo, 1991). This species has been termed "the Mexican cyst nematode" (MCN). In subsequent investigations, it was considered conspecific with *G. t. virginiae* on the basis of host range (Stone, 1983). Conversely, Ferris *et al.,* (1995) found MCN to be closely related to *G. rostochiensis* by using ribosomal DNA comparisons. In addition to *G. t. "mexicana"*, *G. t. virginiae* has been reported from Mexico (Sosa-Moss, 1986), and in 2001, Marché *et al*. used AFLPs to identify several putative *G. t. virginiae* populations (based on morphological characters) from Mexico as unique *G. t. solanacearum* populations, which they renamed *G. t. "azteca"*. *Globodera "mexicana"* appears to be morphologically indistinguishable from *G. pallida*, but does not

reproduce on potato or tobacco (Baldwin and Mundo-Ocampo, 1991). Because it does not occur on economically important crops, further research has not been warranted.

Economic Importance

Globodera tabacum solanacearum is one of the most economically important pests of flue-cured tobacco in Virginia. In 1990, *G. t. solanacearum* was first discovered outside of Virginia in Warren County, North Carolina (Melton *et al*., 1991). Since then, it has been found in 7 counties in North Carolina and in 1995, it was found on a tobacco farm in Charles County, Maryland (Rideout *et al*., 2000a). The infested acreage in the 14 counties in Virginia accounts for approximately 30% of the state's total flue-cured tobacco (Virginia Impacts, 2001). Average yield reductions have been estimated at 15% and can result in complete crop failures (Komm, 1983). In the year 2000, $979,000 was lost in flue-cured tobacco yield to *G. t. solanacearum* and an additional $1,000,000 was spent for control at $50 to $200 per acre (Johnson, personal communication, 2001). *Globodera tabacum virginiae* is found only in the Tidewater area of Virginia parasitizing horsenettle and other solanaceous weeds; no commercial problems or economic relevance have been reported.

Globodera tabacum tabacum has been reported from China, Pakistan, France, Italy, Yugoslavia, Morocco, Spain, and Argentina (Shepherd and Barker, 1990; Johnson, 2002, personal communication). In the United States, *G. t. tabacum* is an economically important parasite of shade and broadleaf tobacco in the Connecticut River Valley of Connecticut and Massachusetts; virtually all of the shade tobacco acreage is infested to some extent and the majority of the broadleaf acreage is also infested (LaMondia, 1995a). The combined value of the shade and broadleaf crop is 90 million dollars; the nematodes damage 20% of the crop, accounting for approximately 18 million dollars in losses. *Globodera tabacum tabacum* can

reduce yield by up to 45% (LaMondia, 2002; LaMondia, 1995b). Most shade tobacco growers fumigate at a cost of about $500 per acre annually (LaMondia, 2001, personal communication).

Life Cycle

The lifecycle of cyst nematodes includes the egg, four juvenile stages, and the mature adult. The first molt occurs in the egg; a vermiform second-stage juvenile (J2) hatches from the egg stored within the protective cyst formed from the tanned cuticle of the adult female. Hatching is stimulated by root exudates of host plants, as well as other factors including soil temperature, moisture, and aeration (LaMondia, 1995a; Wang *et al.*, 1997; Wang *et al*., 2001). The J2 enters a host root just behind the growing tip or where a lateral root emerges, and migrates until its head reaches the pericycle (Williams, 1982). After an initial syncytial cell (ISC) is established at the feeding site in the vascular tissue of the root, syncytia formation is initiated by injection of esophageal secretions through the stylet. Breakdown of cell walls between the ISC and adjacent cells occurs and results in the formation of a primary syncytium (Goverse *et al*., 1998; Jung and Wyss, 1999). The syncytia acts as a metabolic sink and allows the cyst nematode to become an effective parasite. After ISC establishment, the nematode immediately begins nutrient uptake from the plant. In the second molt, the vermiform J2 enlarges into a swollen third-stage juvenile (J3). In the J3 stage, the genitalia develop and the male elongates within the cuticle (Williams, 1982). After the third molt, the female J3 enlarges into a pyriform fourth-stage juvenile (J4), and the male further elongates within the cuticle. The male has three flexures, so it is about four times the length of the third-stage cuticle (Williams, 1982). The female becomes round in the adult stage with only the neck and head still in contact with the root; the male exits into the soil, vermiform in

shape, and is attracted to females. Many males may surround each female and multiple matings can occur (Green *et al*., 1970). When the female dies, the body, containing hundreds of eggs, breaks away from the root and becomes a brown leather-like sack (cyst), resistant to the harsh soil environment and breakdown by soil organisms (Lucas, 1975). Population densities reach their peak at the end of the growing season and drop during winter (Johnson, 1998).

Symptoms and Disease Complexes

Above ground symptoms of TCN damage include severe stunting and wilting (Lucas, 1975). Infected plants occur in clumped or random patches throughout the field and may show symptoms of nutrient deficiency (Johnson, 1998). Below ground symptoms include a greatly reduced root system with pearly white or dark brown females attached to the roots which are just barely visible by the unaided eye (Lucas, 1975).

There is often a synergistic relationship between TCN and other tobacco diseases, suspected to be due to the increased number of physical entry points for the secondary pathogen and alteration of the plant's physiology. Tomato plants inoculated with TCN together with Verticillium wilt (*Verticillium albo-atrum* Reinke and Berthier) resulted in plants with more *Verticillium* than the untreated control (Miller, 1975). This is suspected to be due to a change in physiology of the roots by TCN.

Fusarium wilt (*Fusarium oxysporum* Schlecht Wr.) is the most destructive disease of broadleaf tobacco in Connecticut (LaMondia, 1992). Tobacco cyst nematode increased occurrence and severity of Fusarium wilt on both the susceptible and wilt-resistant cultivars, even though resistant varieties were much less severely damaged than susceptible cultivars (LaMondia and Taylor, 1987). LaMondia (1992) also found that *G. t. tabacum* predisposed

broadleaf tobacco plants to *Fusarium* more than the northern root-knot nematode, *Meloidogyne hapla* Chitwood, 1949. *Globodera tabacum tabacum* may also suppress mycorrhizal fungi, which are important in the normal development of the tobacco plant (Fox and Spasoff, 1972).

The black shank fungus, *Phytophthora parasitica* Dastur var. *nicotianae* (Breda de Haan) Tucker is more relevant to tobacco in Virginia and interacts synergistically with *G. t. solanacearum* (Bower *et al.*, 1980). Black shank and TCN reacted synergistically under all inoculum levels and synergism was more pronounced under low soil moisture and high soil temperature conditions (Grant *et al.*, 1984). Tobacco cyst nematode development was accelerated on roots of plants that were infected with the black shank fungus (Grant *et al.*, 1984).

Control Methods

Cyst nematodes are difficult to control because of their ability to persist in the soil without a host, their exponential rate of reproduction, and their protective cyst. There are three management options for growers with infested fields: cultural practices, nematicides, and resistance. Once a field is infested, it is nearly impossible to eradicate cyst nematodes, however, utilizing one or more of these management strategies, it is possible to produce a successful tobacco crop in the presence of cyst nematodes.

The two cultural control practices most commonly used are crop rotation and sanitation. Crop rotation has many benefits in addition to cyst nematode management. It increases the efficiency of flue-cured tobacco production and is probably the most effective control method to suppress TCN populations (Reed *et al.*, 2001; Komm, 1983). Small grains, fescue, ryegrass, soybeans, and sorghum are commonly rotated with flue-cured tobacco (Reed

et al., 2001). Rotations longer than one year are most effective, but acreage to implement longer rotation schemes with less profitable crops limits its utility. Growers can substitute crop rotation with a TCN-resistant cultivar and achieve similar results (Johnson, 1990).

Sanitation as a cultural practice prevents the spread of TCN to non-infested fields. Spread can be avoided by use of transplants from TCN-free plant beds. Tobacco transplants are now commonly produced in greenhouses in soilless media (Reed *et al*., 2001). Spread can also be limited by avoiding the use of transplant or irrigation water from ponds or streams that drain from infested fields (Reed *et al*., 2001). After working in TCN infested fields, all soil from equipment and shoes of workers should be removed before entering uninfested fields. Early root and stalk destruction limits the number of generations TCN can complete by effectively removing the host from the field (Reed *et al*., 2001).

Although nematicides are highly toxic to the environment and the applicator, they are an effective nematode control method. A highly effective fumigant, methyl bromide, is scheduled to be phased out and farmers are looking for alternative nematicides. Commonly used fumigant nematicides for flue-cured tobacco in Virginia include Chlor-O-Pic®, Telone II®, and Telone C-17® (Reed *et al.*, 2001). The only non-fumigant nematicide is Temik 15G (Reed *et al.*, 2001). For shade tobacco, oxamyl, a systemic insecticide-nematicide, has been shown to reduce TCN population densities and increase plant yields (LaMondia, 1990). Johnson (1995) found that fosthiazate, although not currently registered for use on flue-cured tobacco, is an effective nematicide with increases in yield and quality that were as great or greater than those resulting from use of aldicarb (Temik®), fenamiphos (Nemacur®), methyl bromide, or 1,3-dichloropropene (Telone II®).

Resistant cultivars have many advantages over other control strategies: their use requires little or no technology, they are cost effective, rotations can be shortened, and there are no toxic residues (Trudgill, 1991). Resistance also improves the profitability of tobacco production by reducing time, labor, and money growers spend on nematicides. As in other field crops, resistance cannot be used alone, but rather it must be used in an integrated pest management program.

Baalawy and Fox (1971) showed that *N. glutinosa* L., *N. paniculata* L., *N. plumbaginifolia* L., and *N. longiflora* L. display differential forms of resistance to *G. t. solanacearum*. Penetrating nematodes did find a feeding site but they did not develop into adult females, which suggested the possibility of breeding resistance into *N. tabacum* for the first time. Spasoff *et al*. (1971) crossed *G. t. solanacearum* and wildfire-resistant burley BVA 523 with the *G. t. solanacearum* susceptible flue-cured cultivar NC 2326. A continuous range of mature females was observed in the F_2 generation, indicating that TCN resistance may be multigenic. A follow-up study by Miller *et al*. (1972) on inheritance of resistance in the dark fire-cured breeding line DVA 606 supported the evidence that resistance to *G. t. solanacearum* may be multigenic. In flue-cured tobacco, Crowder *et al*. (2000) found both a multigenic and a single dominant gene for resistance to *G. t. solanacearum*, depending on the source. A single dominant gene confers *G. t. solanacearum* resistance in 'Coker 371-Gold' and *G. t. solanacearum* resistance in 'Kutsaga 110' is multigenic. A single dominant gene for *G. t. tabacum* resistance has been confirmed in shade-grown tobacco, which was derived from flue-cured tobacco cultivars VA-81 and PD-4 (LaMondia, 1991; LaMondia, 2002). In addition, segregation ratios indicated that resistance to *G. t. tabacum* in the burley cultivars, B-21 and B-49, was inherited as a single dominant gene (LaMondia, 2002).

Komm and Terrill (1982) suggested that wildfire resistance derived from *N. longiflora* might be closely linked or pleiotropic to *G. t. solanacearum* resistance. Gwynn *et al*. (1986) found some lines resistant to both *G. t. solanacearum* and wildfire, but some lines were resistant to *G. t. solanacearum* and susceptible to wildfire; no line was resistant to wildfire but susceptible to *G. t. solanacearum*. Hayes *et al*. (1997) confirmed that screening for wildfire resistance is not a reliable method of screening for TCN resistance. *Globodera tabacum solanacearum* resistance is also associated with the single dominant gene, designated *Ph*, that confers a very high level of resistance to race 0 of *Phytophthora parasitica* var. *nicotianae* (black shank) (Carlson *et al*., 1997). Planting cultivars that have the *Ph* gene derived from Coker 371-Gold should enable tobacco producers to control black shank and TCN (Johnson, 2001).

Continuous cropping of a resistant cultivar can result in selection pressure that leads to development of new nematode biotypes that can reproduce on these cultivars (Elliott *et al*., 1986; Triantaphyllou, 1987; Young, 1992). In the potato cyst nematode, six genes have been reported to be involved in the resistance mechanisms: H1, K1, Fa-Fb, H2, and H3 (Phillips, 1994). Rideout *et al.* (2000b) looked for pathotypes among geographic isolates of *G. t. solanacearum* from Virginia, Maryland, and North Carolina. No differences were observed in development and reproduction of the nematode on a resistant and a susceptible flue-cured tobacco cultivar, suggesting that different *G. t. solanacearum* biotypes do not exist or exist at extremely low frequencies. Rideout *et al*. (2000b) also speculated that over more generations, resistance-breaking biotypes could be selected for due to selection pressure from the use of resistant cultivars.

Molecular Research

Historically, host range and morphology have been the primary means of differentiating nematode species. Molecular techniques are powerful taxonomic tools and their use in the identification of intraspecific groupings is becoming more prevalent (Curran, 1991; Williamson, 1991). Several biochemical techniques such as serology and electrophoresis of proteins and isozymes have been applied to identify and differentiate between the major species of *Meloidogyne* as early as 1971 (Dickson *et al*., 1971; Hussey, 1972; Hussey *et al*., 1972; Dalmasso and Berge, 1978). Curran *et al*. (1985) were the first to identify nematode species by restriction fragment length polymorphisms (RFLPs) across different genera. Curran *et al*. (1986) were also able to differentiate *Meloidogyne* species by detection of restriction fragment length differences in total DNA. Other early groupings of nematodes includes separation of species of *Meloidogyne* by the use of mitochondrial DNA probes (Powers *et al*., 1986); delineation of races of *Heterodera glycines* by restriction endonuclease digestions (Kalinski and Huettel, 1988); and differentiation of pathotypes of *Bursaphelenchus xylophilus* using RFLPs (Bolla *et al*., 1988).

DNA markers obtained in RFLP analysis are useful for identification and comparison of nematodes, but this technique requires large quantities of DNA and the use of radioactive isotopes. DNA polymorphism assays based on polymerase chain reaction (PCR) amplification of DNA segments do not require radioisotopes. Harris *et al*. (1990) was the first to amplify plant-parasitic nematode DNA using PCR by use of specific nucleotide sequences and mitochondrial DNA of *Meloidogyne* juveniles.

Random amplified polymorphic DNA (RAPD) is a technique, described by Williams *et al*. (1990) and Welsh *et al*. (1990), to amplify arbitrary sequences of genomic DNA by use

of an arbitrary primer. The primer is allowed to anneal at a relatively low temperature, priming the amplification of DNA fragments distributed randomly in the genome. These highly polymorphic fragments are useful as genetic markers to identify organisms and also to establish relative degrees of similarity between individuals, populations, and species. The greatest potential of RAPD analysis is in obtaining markers linked to genes of interest (Jones *et al*., 1997). The advantages of RAPDs over conventional RFLP-based techniques include speed, simplicity, elimination of radioisotopes, and ability to work with minute concentrations of DNA (Powers *et al*., 1991). Greater intraspecific variation may be revealed with RAPDs than with RFLP assays (Blok *et al*., 1997b).

The RAPD technique has been used successfully to differentiate the four most common *Meloidogyne* species (Cenis, 1993; Guirao, 1995; Blok *et al*., 1997b). Powers and Harris (1993) used a PCR method for identification of five major *Meloidogyne* species and Williamson (1997) used RAPD bands to develop a PCR assay to identify and distinguish single *Meloidogyne* second-stage juveniles. The RAPD technique is useful for cyst nematodes because cysts can be cultured in the greenhouse, adult females are relatively large and easy to manipulate, and several geographic populations are readily available. Caswell-Chen *et al*. (1992) were among the first to use RAPDs to differentiate *Heterodera cruciferae* Franklin 1945 from *H. schachtii* Schmidt 1871. These two species were easily distinguished by differences in fragment patterns with any of nineteen primers, which indicated that the RAPD technique is a useful diagnostic tool for nematode species identification. Geographic isolates of *H. schachtii* were compared and cluster analysis revealed that inter-continental geographic proximity of the populations did not necessarily correlate with genetic relatedness. RAPD analysis may help in defining relatedness among populations and the history of

introductions (Caswell-Chen *et al*., 1992). Roosien *et al*. (1993) performed a similar experiment with RAPDs to differentiate the two potato cyst nematodes *Globodera rostochiensis* (Wollenweber, 1923) Skarbilovich, 1959 and *G. pallida* Stone, 1973. Two discrete DNA fragments were consistently present in *G. rostochiensis* populations but absent in *G. pallida* populations.

The RAPD technique is able to delineate species, but separating races or biotypes is the true test of its power as a diagnostic tool. In potato cyst nematodes (PCN), five pathotypes of *G. rostochiensis* (Ro_1 - Ro_5) and three pathotypes of *G. pallida* (Pa_1 - Pa_3) are recognized in Europe (Kort *et al.*, 1977). Many researchers have used RAPDs to delineate species of PCN from numerous localities including Europe (Blok *et al*., 1997a; Burrows *et al*., 1996; Conceição *et al*., 1998; Fullaondo *et al*., 1997; Pastrik *et al*., 1995; Roosien *et al*., 1993), South America (Bendezu *et al*., 1997; Grenier *et al*., 2000), and Russia (Subbotin *et al*., 1999). Folkertsma *et al*. (1994) used the RAPD technique to examine inter- and intraspecific variation between nine populations of *G. rostochiensis* and 17 populations of *G. pallida* from the Netherlands. The two PCN species shared only nine of 250 RAPD fragments, showing a wide divergence on the molecular level, which had also been established on the basis of proteins (Bakker and Bouwman-Smits, 1988) and RFLPs (DeJong *et al*., 1989; Stratford *et al*., 1992; Gonzalez *et al*., 1995). The genetic variability within *G. pallida* populations seems to be larger than that of *G. rostochiensis*, confirming similar results from two dimensional gel electrophoresis of proteins (Bakker *et al*., 1992); isoelectric focusing and specific enzyme staining (Zaheer *et al*., 1996); and RFLPs (Schnick *et al*., 1990). Follow-up experiments using RAPDs confirmed more variability in *G. pallida* in Europe than in *G. rostochiensis* (Burrows *et al*., 1996; Bendezu *et al*., 1997; Blok *et al*.

1997a; Conceição *et al*., 1998). This may be due to the fact that original *G. pallida* populations introduced to Europe had wider genetic variability than those of *G. rostochiensis*; and/or fewer introductions of *G. rostochiensis* were made, thereby making the populations more genetically uniform.

Three processes predominantly determine variations in virulence: the genetic structure of the initial population, random genetic drift, and gene flow (Bakker *et al*., 1992; Bakker *et al*., 1993). For PCN, mutation can be excluded as a major source of genomic variation because of their low multiplication rate per potato crop and the fact that mutation rates vary from 10^{-4} to 10^{-6} per gene per gamete for most eukaryotic organisms (Ayala, 1976). Agricultural practices also impact the genetic structure of PCN; populations from a region characterized by wider crop rotation schemes and very limited use of nematicides resulted in relatively little variation within the population, but more variation between local nematode populations (Folkertsma *et al*., 2001). A more significant source of variation is gene flow from subsequent introductions of new populations of PCN either from overseas or over short distances (Burrows *et al*., 1996; Bendezu *et al*., 1997). Potato cyst nematode populations from Europe have a relatively narrow gene pool taken from the larger South American gene pool during the last 150 years (Jones, 1970; Baldwin and Mundo-Ocampo, 1991). Low genomic similarity of the South American populations indicates that genomic variation in PCN populations could be due to the co-evolution of PCN with solanaceous plants (Bendezu *et al*., 1997). Blok *et al*. (1997a) found that PCN populations were clustered clearly by continent, and found the indication of grouping by countries within Europe by comparing RAPD patterns. Fullaondo *et al*. (1997) associated one RAPD pattern with virulence against a resistance gene. An application of this finding is that the traditional testing for determining

virulence of PCN populations could be replaced by molecular analysis, as demonstrated in a follow-up experiment in which RAPD fragments were used to derive two primer combinations for PCR to amplify one specific band in each species of *G. rostochiensis* and *pallida* (Fullaondo, *et al*., 1999). For the cereal cyst nematode (*Heterodera avenae*), López-Braña *et al*. (1996) found no correlation between RAPD clustering and geographic locality within Europe.

Pastrik *et al*. (1995) used the RAPD technique to analyze a population of *G. pallida* selected for increased virulence and compared this selected population with an avirulent population, which revealed two RAPD products associated with virulence. A hybridization study showed this product would bind only to the DNA of virulent populations, suggesting that it is tightly linked to a gene involved in virulence.

Bossis and Mugniéry (1993) were the first to examine *Globodera tabacum* at a molecular level using two-dimensional gel electrophoresis. *Globodera tabacum virginiae* and *G. t. solanacearum* appeared to be more closely related to each other than to *G. t. tabacum*, but all three were distinctively different from *G. pallida*, *G. rostochiensis*, and the Mexican cyst nematode (MCN). Conversely, based on ribosomal DNA sequence data, Ferris *et al*. (1997) found that *G. t. tabacum* was 98% similar to the three MCN isolates, 97.6% similar to *G. t. rostochiensis*, 96.7% similar to *G. pallida*, and only 88.7% similar to the sequence of *G. t. virginiae*. By use of RAPDs, Thiéry *et al*. (1997) demonstrated that the three subspecies of the TCN complex clustered together with *G. rostochiensis* as the closest related species, distinctively different from *G. pallida* and MCN which are closely related to each other. This research confirmed the RFLP findings of Thiéry and Mugniéry (1996). These results are also in accord with the 2-DGE findings of Bossis and Mugniéry (1993).

By use of amplified fragment length polymorphism (AFLP), Marché *et al*. (2001) found a population of *G. t. solanacearum* in Mexico, although thought to be restricted to the United States, clustered close to the USA isolates *G. t. solanacearum*, but not within. This is supported by rDNA sequence results of Subbotin *et al*. (2000), showing that two Mexican tobacco cyst populations clustered close to *G. t. solanacearum*, but not within.

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Chapter 2: Intraspecific variability within *Globodera tabacum solanacearum* using Random Amplified Polymorphic DNA (RAPD).

Abstract: Random amplified polymorphic DNA (RAPD) were used to investigate the intraspecific variability among nineteen geographic isolates of *Globodera tabacum solanacearum* obtained from eight counties in Virginia and one county in North Carolina. *Globodera tabacum tabacum*, *G. t. virginiae*, the Mexican cyst nematode (MCN), and five *Heterodera* species were included as outgroups. Six primers were used and 140 amplification products were observed. Reproducible differences in fragment patterns allowed similar differentiation of the isolates and species with each primer. Hierarchical cluster analysis was performed to illustrate the relatedness between isolates and species. In contrast to reports in the literature, we found the Miller isolate of MCN to cluster more closely with *G. t. solanacearum* than with *G. t. tabacum* or *G. t. virginiae*. The average Jaccard's similarity index among isolates of *G. t. solanacearum* was 74%, representing greater variation than that observed across different pathotypes of the potato cyst nematode, *Globodera pallida*, also using RAPDs. The considerable variability indicated within *G. t. solanacearum* suggests that the existence or development of resistance-breaking biotypes is more likely than previous research suggests. If a pathotype difference is reported, a RAPD marker could be associated with virulence, which could prove to be a valuable tool in population diagnosis, resistance screening, and overall *G. t. solanacearum* management.

Key Words: Tobacco cyst nematodes, *Globodera tabacum solanacearum*, RAPD, Molecular nematology, DNA fingerprinting

Introduction

Tobacco cyst nematodes (TCN) are grouped into three sub-species: *Globodera tabacum tabacum* [(Lownsbery and Lownsbery, 1954) Behrens, 1975] Stone 1983, *G. t. virginiae* [(Miller and Gray, 1968) Behrens, 1975] Stone 1983, and *G. t. solanacearum* [(Miller and Gray, 1972) Behrens, 1975] Stone 1983. Tobacco cyst nematodes have been reported from China, Pakistan, France, Italy, Yugoslavia, Morocco, Spain, and Argentina (Shepherd and Barker, 1990; Johnson, 2002, personal communication). In the United States, the geographical distribution of *Globodera tabacum tabacum* is limited to Connecticut and Massachusetts, while *G. t. virginiae* and *G. t. solanacearum* occur in Virginia and North Carolina, as well as in one county in Maryland (Johnson, 1998; Miller, 1986). *Globodera tabacum tabacum* is an economically important parasite on shade-grown cigar wrapper and field-grown broadleaf cigar tobaccos in Connecticut and Massachusetts (Lownsbery and Peters, 1955). *Globodera tabacum solanacearum* is an important parasite of flue-cured tobacco cultivars (Komm *et al*., 1983; Johnson, 1998). Infested acreage accounts for 30% of Virginia's total flue-cured tobacco acreage; average yield reductions have been estimated at 15% and complete crop failure has been reported (Virginia Impacts, 2001; Komm *et al*., 1983). *Globodera tabacum virginiae* does not reproduce well on *Nicotiana* species (Miller, 1978).

Cultivars that suppress reproduction of *G. t. solanacearum* are widely planted in North Carolina and Virginia (Johnson, personal communication, 2002). Resistance breaking biotypes have been reported for *G. rostochiensis*, *G. pallida*, and the soybean cyst nematode (*Heterodera glycines* Ichinohe, 1952) (Arntzen *et al*., 1994; Price *et al*., 1978; Caviness, 1992). Selection pressure through continuous cropping of resistant cultivars may lead to

development of new TCN biotypes (Elliott *et al*., 1986; Triantaphyllou, 1987; Young, 1992). Elliot *et al*. (1986) found TCN resistance to be durable over a period of three years, but this study involved only one isolate of the pathogen over a relatively short period of time. Rideout *et al.* (2000) looked for pathotype differences among 15 geographic isolates of *G. t. solanacearum* from Virginia, Maryland, and North Carolina. No differences were observed in nematode development and reproduction on a resistant and a susceptible flue-cured tobacco cultivar, suggesting that different *G. t. solanacearum* biotypes do not exist or exist at extremely low frequencies. This study was conducted over only one or two generations and concluded that a longer-term study would more fully document the long-term effectiveness of resistance to *G. t. solanacearum* (Rideout *et al*., 2000).

RAPD fingerprinting of TCN isolates may reveal phenotypically neutral markers, a molecular characterization of the nematode population, which could indicate putative biotypes (Bakker *et al*., 1993). According to the gene pool similarity concept proposed by Bakker *et al*. (1993), a relatively high overall genetic variability within *G. t. solanacearum* would indicate a higher probability of the eventual emergence of resistance-breaking biotypes. An accurate characterization of the genetic variability among geographic isolates of *G. t. solanacearum* could predict the effectiveness of a resistance gene and would have important implications for population diagnosis, plant breeding, and engineering of resistant cultivars (Bakker *et al*., 1993). The research presented here used RAPD fingerprinting to compare the genetic profile of 23 geographically distinct populations of *G. t. solanacearum*.

Materials and Methods

Geographic Isolates - Soil was sampled from fields infested with *G. t. solanacearum* across Southside Virginia and one county in North Carolina. Table 2.1 and Figure 2.1 present

the location and identification for each site. Soil was collected randomly at each location using a standard soil probe taking cores 2 cm in diameter to a maximum depth of 20 cm. A criss-cross sampling procedure was used to obtain an adequate representation of the nematode population in the entire field (Barker *et al*., 1984). Soil was stored in polyethylene-lined paper bags at room temperature. Infested soil was also obtained through farmer-submitted samples from the nematode assay laboratory at the Virginia Tech Southern Piedmont Agricultural Research and Extension Center (SPAREC) in Blackstone, Virginia. Cysts were extracted from air-dried soil samples using a modified Fenwick can (Caswell *et al*., 1985), rinsed from a 0.25 mm sieve, and captured on filter paper, where they were allowed to dry. Cysts were stored at room temperature in 30 ml bottles with a snap cap until further use.

Culturing Geographic Isolates - The first geographic isolates obtained were cultured in the greenhouse using *Nicotiana tabacum* cultivar K326 to increase their numbers. Seeds were germinated in vermiculite and grown in 11 cm diameter clay pots for approximately four weeks. Individual tobacco seedlings were transplanted into 11 cm diameter clay pots containing approximately 300 cm^3 of a 1:1 soil conditioner (Schultz® Profile, St. Louis, MO):field soil (steam-sterilized sandy loam) mix. The soil conditioner was mixed with soil to improve soil moisture, aeration, and nutrient retention for optimal nematode reproduction. Seedlings were allowed to grow for two weeks prior to inoculation for adequate root establishment. Eggs for inoculum were obtained by crushing cysts in a blender (Oster Osterizer, Sunbeam® Products, Boca Raton, FL) and catching them on a 25 μm sieve. Inoculation was performed by introducing a tap water suspension of approximately 6,000 *G. t. solanacearum* eggs into a trench around each seedling. The trench was covered with approximately 100 cm^3 of a 1:1 Profile:soil mix. Plants were lightly hand-watered for the

first week after inoculation to ensure that unhatched nematodes were not lost through the bottom of pots; thereafter, plants were watered and fertilized using an automated system. Approximately sixty days after inoculation, cysts were extracted, dried, and stored for use.

DNA Extraction - Fifty cysts of each isolate were hand-picked using fine-tipped forceps under a dissecting microscope (Bendezu *et al*., 1997). Visible presence of a neck and the dark brown color was used to distinguish cysts from other objects extracted from soil. The selected cysts were placed in 1.5 ml microcentrifuge tubes and homogenized using micropestles (Kontes™ Pellet Pestle™). DNA was extracted using the DNeasy™ Tissue Kit (QIAGEN Inc., Valencia, CA) according to the manufacturers protocol. In short, nematode cells were lysed and loaded onto a DNeasy spin column where DNA selectively binded to the silica-gel membrane. Remaining contaminants were removed in two wash steps and pure DNA was eluted in low-salt buffer. DNA concentration was determined spectrophometrically using a SpartSpec™ 3000 (BioRad™, Hercules, CA).

RAPD-PCR Procedure - All RAPD reactions were carried out using the Ready-To-Go™ RAPD Analysis Kit (AP Biotech Inc., Piscataway, NJ). Each tube contained a room-temperature-stable bead consisting of buffer, dATP, dCTP, dGTP, dTTP, bovine serum albumin (BSA), AmpliTaq™, and Stoffel fragment (Anonymous, 1999). In a pilot test, the six 10-mer primers included with the kit were assessed for their ability to produce clear and reproducible RAPD profiles with *G. t. solanacearum* DNA as a template. The primer sequences are presented in Table 2.2. All RAPD reactions contained 5 µl primer, 15 ng template DNA, and molecular biology grade water (Sigma®) to a total volume of 25 µl. Reactions were overlaid with 50 µl light mineral oil (Fisher) to prevent evaporation. Amplification was performed in a PTC-100™ Programmable Thermal Controller (MJ

Research, Inc., Waltham, MA) programmed for an initial denaturization step at 95° C for 5 min followed by 45 cycles of 1 min at 95° C, 1 min at 36° C, and 2 min at 72° C (Anonymous, 1999). After amplification, DNA products were loaded on a 2% agarose gel in TAE running buffer and electrophoresed with a PowerPac™ 200 (BioRad™, Hercules, CA) for 5.75 hours at 70 volts. The gel was stained in a 0.325 μg/ml ethidium bromide-TAE buffer solution for 15 minutes, rinsed in distilled water for 15 minutes, and photographed with a Kodak DC120 Zoom digital camera under UV light. The RAPD fingerprint obtained for each isolate was replicated three times with all six primers.

Data Collection and Analysis - Digital photographs were adjusted in Adobe® Photoshop for optimal band visualization and printed on Kodak Premium high-gloss picture paper with a color ink-jet printer. Amplification products were visually scored as present or absent and recorded as 1 or 0, respectively. Only bands clearly present in 2 out of 3 replications were scored as being present. The statistical analysis involved computation of Jaccard's similarity coefficients, which were used to conduct a nearest neighbor, hierarchical cluster analysis (Gower, 1985) performed using the software SYSTAT (Wilkinson, 2002), as in Caswell-Chen *et al*. (1992). The similarity coefficient assesses the similarity between any two isolates on the basis of the number of shared bands, and the cluster tree illustrates the relatedness of isolates.

Results

The amplified DNA fragments were in the range of 150 to 2500 bp, with 19 to 25 amplification products per primer, yielding a total of 140 markers for the 28 nematode isolates (Figures 2.2 - 2.19). The Jaccard's distance matrix for all isolates is presented in Table 2.3, with average distances between species and genera in Table 2.4. The average inter-generic

distance was 0.244, the average intra-generic distance among isolates and species within *Globodera* was 0.683 and among species within *Heterodera* was 0.522. The average distance among isolates of *G. t. solanacearum* was 0.741. The nearest-neighbor cluster analysis, obtained from Jaccard's similarity coefficients (Figure 2.20), illustrates the distance between the *Globodera* and *Heterodera* species tested, the variability within the *Globodera tabacum* species complex, and the intraspecific variability within *G. t. solanacearum*. All *Heterodera* species (27 to 31) grouped together, separate from the *Globodera* isolates. Within the *G. t. solanacearum* group, isolates 8 and 23 were more distant from the other *G. t. solanacearum* isolates, but isolate 32 (the Mexican cyst nematode) clustered within the *G. t. solanacearum* group. Isolates 24 and 25 (*G. t. tabacum*) clustered close to *G. t. solanacearum*, with isolate 26 (*G. t. virginiae*), as the most distant from *G. t. solanacearum* and *G. t. tabacum*, with an average distance of only 0.372.

Isolates 1, 7, 14, and 19, from Amelia, Dinwiddie, Mecklenburg, and Pittsylvania, respectively, were considered outliers because their banding patterns were consistantly different from all other isolates and from each other across all primers. Their estimated genetic distance from all other isolates ranged from 0.101 to 0.392 (Table 2.5). Their estimated genetic distance from each other ranged from 0.214 to 0.311 (Table 2.6), which was considered too distant to represent a single organism. In the cluster tree (Figure 2.21), they clustered out before the *Heterodera* isolates, therefore, were eliminated from all analyses.

Discussion

This is the first report of intraspecific variability within *G. t. solanacearum* in which a large number of isolates were examined. Thiéry and Mugniéry (1996) found no intraspecific variability within three isolates of *G. t. solanacearum*, but this was by use of the less sensitive

RFLP fingerprinting technique. By use of RAPD-PCR, Thiéry *et al*. (1997) found very little intraspecific variability among three isolates of *G. t. solanacearum*. Using the more sensitive technique of AFLPs, Marché *et al*. (2001) found one isolate out of three *G. t. solanacearum* isolates to show the presence of a specific marker, as well as an isolate of *G. t. virginiae* that clustered with *G. t. solanacearum*. Marché *et al*. (2001) also concluded *G. t. virginiae* to be very closely related to *G. t. solanacearum*, as previously proposed based on the use of RFLPs (Thiéry and Mugniéry, 1996), RAPDs (Thiéry *et al*., 1997), and 2-DGE (Bossis and Mugniéry, 1993). Subbotin *et al*. (2000) found two isolates of *G. t. solanacearum*, two isolates of *G. t. virginiae*, and two isolates of the Mexican cyst nematode to cluster closely together, based upon rDNA similarities. Conversely, we found *G. t. tabacum* to be the next closest related sub-species to *G. t. solanacearum*, with *G. t. virginiae* only distantly related. The low number of isolates used is a plausible reason for this discrepancy; we used only one isolate of *G. t. virginiae*, thereby creating a greater probability for error compared to the use of several isolates.

We found *Globodera "mexicana"* to cluster within the *G. t. solanacearum* group. Thiéry *et al*., (1997) used six Mexican isolates in a RAPD analysis, and found one to cluster within *G. t. tabacum*, one to cluster within *G. t. virginiae*, and four to cluster distantly separate, close to *G. pallida*. This finding was perfectly in line with the RFLP findings of Thiéry and Mugniéry (1996), which showed two Mexican isolates within the *G. tabacum* cluster, and four isolates in their own cluster, close to *G. pallida*. Bossis and Mugniéry (1993) had previously demonstrated a MCN association with *G. pallida* by use of 2-DGE. Marché *et al*. (2001) used AFLPs to find four Mexican isolates to group separately from the *G. tabacum* complex, but another four Mexican isolates to cluster very close to three USA *G.*

t. solanacearum isolates. These were considered a new sub-species within the *G. tabacum* complex, and named *Globodera tabacum "azteca"*. The background of the Mexican isolate used in our RAPD analysis is unknown. Since *G. t. "azteca"* had not been proposed when our isolate was described, it is plausible that it may be *G. t. "azteca"*, and not *G. "mexicana"*. In addition, because we used only one isolate, an accurate depiction of the MCN species is not well represented.

Prior to DNA extraction, isolates 1, 7, 14, and 19 were not greenhouse-cultured, but rather extracted directly from field soil. All cysts may not have been of the same age, integrity, gravidity, and viability, thereby introducing unwanted variability into the RAPD analysis. A possible source of contamination could be a rotting or parasitizing organism such as bacteria (Sayre, 1980) or fungi (Mankau, 1980; Kerry, 1980; Morgan-Jones *et al*., 1984). Greenhouse culturing is a good method to standardize the age and integrity of all cysts before using them in a highly sensitive molecular analysis such as RAPD-PCR.

An association between geographic origin and genetic relatedness has been observed among isolates of *G. pallida* from South America and Europe (Bendezu *et al*., 1997; Blok *et al*., 1997), but geographical origin has not commonly been reported to be correlated with RAPD grouping on a continent-wide scale (Burrows *et al*., 1996; Caswell-Chen *et al*., 1992; Pinochet *et al*., 1994; Folkertsma *et al*., 1994). Some correlation of genetic distance and geographic location was observed in our RAPD analysis, but not consistently. Isolates 20, 21, and 22 were all from the same farm in Warren County, NC and clustered very closely; the two isolates from Lunenburg County (10 and 11) clustered closely; and the three isolates from Dinwiddie County (5, 6, and 8) clustered toward the edges of the *G. t. solanacearum* group. Conversely, the three isolates from Mecklenburg County (12, 13, and 15) were relatively

distant from each other, spread throughout the *G. t. solanacearum* cluster, and the two most related isolates (12 and 18) were from non-adjacent counties.

Baalway and Fox (1971) found resistance to *G. t. solanacearum* in wild *Nicotiana* species, which indicated the possibility of breeding resistance into *N. tabacum* for the first time. Resistance derived from *Nicotiana longiflora* was shown to be linked to wildfire (*Pseudomonas syringae* pv. *tabaci*) resistance, but the continuous range of mature females observed in the F_2 and F_3 generations indicated that *G. t. solanacearum* resistance may be multigenic (Spasoff *et al*., 1971; Miller *et al*., 1972; Hayes *et al*., 1997). However, a single dominant resistance has been confirmed in shade-grown tobacco against *G. t. tabacum*, which was derived from flue-cured tobacco cultivars VA-81 and PD-4 (LaMondia, 1991; LaMondia, 2002). In flue-cured tobacco, Crowder *et al*. (2000) found both a multigenic and a single dominant gene for resistance to *G. t solanacearum*, depending on the source. Coker 371 Gold, which demonstrates a single dominant resistance gene, is the parent of many recently introduced, commonly used hybrids. In general, single genes for resistance are considered less durable than multigenic resistance; selection for just one mutant biotype may render the single-gene resistance useless (Elliott *et al*., 1986; Triantaphyllou, 1987; Young, 1992).

Conversely, Bakker *et al*., (1993) states that a well-chosen form of resistance, including single dominant genes, can be very effective against plant parasitic nematodes because of their relatively low reproduction capacities and poor dispersal abilities. Accurate predictions of the effectiveness of a resistance gene may be achievable given the proper characterization of the nematode populations. According to Bakker's gene-pool similarity concept (1993), polymorphisms for virulence should be integrated with genomic variability revealed by molecular techniques to arrive at a more reliable and stable classification. Several

studies have shown that virulent populations of PCN were already present in Europe before the introduction of resistant cultivars (Bakker, *et al*., 1993). For *G. t. solanacearum*, there are currently no reported pathotype differences (Rideout, 2000), but high genetic variability between isolates could suggest the existence of a virulent pathotype (Bakker *et al*., 1993).

Through our study, the 74% intraspecific similarity within *G. t. solanacearum* is as low as the reported *G. pallida* and *G. rostochiensis* variability by use of RAPDs. Bendezu *et al*. (1997) and Folkertsma *et al*. (1994) found 82% and 92.2% similarity, respectively, within *G. rostochiensis* isolates from Europe across three different pathotypes. For *G. pallida*, intraspecific similarity ranged from 41% to 91.1%. Benduez *et al*. (1997) found only 41% and 73% similarity across three pathotypes of Peruvian and European isolates, respectively. Burrows *et al*. (1996) reported 77% similarity within three pathotypes, Blok *et al*. (1997) found 80.8% similarity within three pathotypes, and Folkertsma *et al*. (1994) reported 91.1% similarity between two pathotypes of *G. pallida*. Therefore, our reported intraspecific variability within *G. t. solanacearum* is less than most reported *G. pallida* and *G. rostochiensis* similarities across more than one pathotype. This suggests that the existence of resistance-breaking biotypes within *G. t. solanacearum* is more likely than previous research had suggested.

The use of RAPD fingerprinting is an important first step toward evaluating the variability of *G. t. solanacearum* geographic isolates in Southside Virginia. Relative to PCN, high variability found among *G. t. solanacearum* isolates indicates that pathotypes may exist. Agronomically desirable resistant cultivars have recently been deployed on a widespread basis, exerting selection pressure on any biotype present in commercial tobacco fields. If a resistance-breaking pathotypes arises, a RAPD marker may be associated with virulence,

which could have several implications. The RAPD marker could be sequenced and a specific primer could be developed for diagnosis of TCN populations, as in root-knot nematodes (Dong *et al*., 2001) and PCN (Fullaondo, 1997). Or, a unique RAPD fragment could be used as a molecular marker probe for use with dot blot tests for pathotype identification, as in soybean cyst nematodes (Li *et al*., 1996). A pathotype scheme would need to be implemented, as in PCN (Kort *et al*., 1977), and used in resistance screening of new cultivars. This would ensure that promising lines are screened against all known nematode virulence genes and would help ensure the longevity of resistance in newly developed tobacco cultivars. Growers could have their TCN populations identified, allowing them to make an appropriate cultivar selection, thereby preserving resistance and decreasing pesticide use in an integrated nematode management scheme.

Table 2.1. Isolate identifier, taxonomic classification, location, source, and isolate identification code for nematode isolates used.

Identifier	Genus and Species	Location	Source	Identification Code
1*	*Globodera tabacum solanacearum*	Amelia Co., VA	Hastings farm	1: Gts-AmH
2	*Globodera tabacum solanacearum*	Brunswick Co., VA	Jones farm	2: Gts-BrJ
3	*Globodera tabacum solanacearum*	Brunswick Co., VA	Wright farm	3: Gts-BrW
4	*Globodera tabacum solanacearum*	Campell Co., VA	Guthrie farm	4: Gts-CaG
5	*Globodera tabacum solanacearum*	Dinwiddie Co., VA	Wallace farm 1	5: Gts-DiW
6	*Globodera tabacum solanacearum*	Dinwiddie Co., VA	Wallace farm 2	6: Gts-DiW2
7*	*Globodera tabacum solanacearum*	Dinwiddie Co., VA	Bentley farm 1	7: Gts-DiB
8	*Globodera tabacum solanacearum*	Dinwiddie Co., VA	Bentley farm 2	8: Gts-DiB2
9	*Globodera tabacum solanacearum*	Halifax Co., VA	Smiley farm	9: Gts-HaS
10	*Globodera tabacum solanacearum*	Lunenburg Co., VA	Coffee farm	10: Gts-LuC
11	*Globodera tabacum solanacearum*	Lunenburg Co., VA	Parrish farm	11: Gts-LuP
12	*Globodera tabacum solanacearum*	Mecklenburg Co., VA	Warren farm	12: Gts-MeH
13	*Globodera tabacum solanacearum*	Mecklenburg Co., VA	Hudson farm 1	13: Gts-MeH
14*	*Globodera tabacum solanacearum*	Mecklenburg Co., VA	Hudson farm 2	14: Gts-MeH2
15	*Globodera tabacum solanacearum*	Mecklenburg Co., VA	Hudson farm 3	15: Gts-MeH3
16	*Globodera tabacum solanacearum*	Nottoway Co., VA	Davis farm	16: Gts-NoD
17	*Globodera tabacum solanacearum*	Nottoway Co., VA	SPAREC [a]	17: Gts-NoS
18	*Globodera tabacum solanacearum*	Prince Edward Co., VA	Forrest farm	18: Gts-PrF
19*	*Globodera tabacum solanacearum*	Pittsylvania Co., VA	Owen farm	19: Gts-PiO
20	*Globodera tabacum solanacearum*	Warren Co., NC	Hight farm 1	20: Gts-WaH
21	*Globodera tabacum solanacearum*	Warren Co, NC	Hight farm 2	21: Gts-WaH2
22	*Globodera tabacum solanacearum*	Warren Co., NC	Hight farm 3	22: Gts-WaH3
23	*Globodera tabacum solanacearum*	unknown	L. I. Miller [b]	23: Gts-M
24	*Globodera tabacum tabacum*	unknown	Mota/Eisenback [b]	24: Gtt-M/E
25	*Globodera tabacum tabacum*	Italy	L. I. Miller [b]	25: Gtt-M2
26	*Globodera tabacum virginiae*	unknown	Mota/Eisenback [b]	26: Gtv-M/E
27	*Heterodera carotae*	unknown	L. I. Miller [b]	27: Hc-M
28	*Heterodera glycines*	unknown	L. I. Miller [b]	28: Hg-M
29	*Heterodera schachtii*	unknown	L. I. Miller [b]	29: Hs-M
30	*Heterodera trifoli*	unknown	L. I. Miller [b]	30: Ht-M
31	*Heterodera rumicis*	unknown	L. I. Miller [b]	31: Hr-M
32	*Globodera "mexicana"*	Mexico	L. I. Miller [b]	32: Gm-M

* Isolates removed from analysis

[a] Southern Piedmont Agricultural Research and Extension Center

[b] Virginia Tech

Table 2.2. Random primers (Amersham Pharmacia Biotech) used in the RAPD-PCR analysis.

Primers Used[a]	Sequence 5' to 3'	GC Percentage
RAPD Analysis Primer 1	GGTGCGGGAA	70
RAPD Analysis Primer 2	GTTTCGCTCC	60
RAPD Analysis Primer 3	GTAGACCCGT	60
RAPD Analysis Primer 4	AAGAGCCCGT [b]	60
RAPD Analysis Primer 5	AACGCGCAAC [b]	60
RAPD Analysis Primer 6	CCCGTCAGCA [b]	70

[a] Each primer is a 10-mer of arbitrary sequence that is specifically designed and tested for use in RAPD analysis

[b] Akopyanz, N., N. O. Bukanov, T. U. Westblom, S. Kresovich, and D. E. Berg. 1992. DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. Nucleic Acids Research 20(19): 5137-5142.

Table 2.3: Jaccard's similarity matrix of 28 isolates[1], excluding the outlier isolates[1] 1, 7, 14, and 19.

Isolate Identifier	2: Gts-BrJ	3: Gts-BrW	4: Gts-CaG	5: Gts-DiW	6: Gts-DiW2	8: Gts-DiB2	9: Gts-HaS	10: Gts-LuC	11: Gts-LuP	12: Gts-MeW	13: Gts-MeH	15: Gts-MeH3	16: Gts-NoD	17: Gts-NoS	18: Gts-PrF	20: Gts-WaH	21: Gts-WaH2	22: Gts-WaH3	23: Gts-M	24: Gtt-M/E	25: Gtt-M2	26: Gtv-M/E	27: Hc-M	28: Hg-M	29: Hs-M	30: Ht-M	31: Hr-M	32: Gm-M
2	1.000																											
3	0.859	1.000																										
4	0.855	0.867	1.000.																									
5	0.725	0.681	0.723	1.000																								
6	0.803	0.785	0.778	0.657	1.000																							
8	0.623	0.580	0.591	0.514	0.557	1.000																						
9	0.806	0.761	0.781	0.662	0.735	0.542	1.000																					
10	0.696	0.754	0.719	0.629	0.701	0.597	0.681	1.000																				
11	0.731	0.738	0.758	0.638	0.687	0.582	0.716	0.734	1.000																			
12	0.846	0.918	0.852	0.696	0.773	0.594	0.776	0.797	0.754	1.000																		
13	0.736	0.794	0.761	0.627	0.768	0.533	0.722	0.714	0.700	0.809	1.000																	
15	0.836	0.846	0.785	0.690	0.765	0.592	0.821	0.761	0.696	0.891	0.775	1.000																
16	0.833	0.903	0.839	0.662	0.761	0.586	0.791	0.758	0.769	0.919	0.797	0.877	1.000															
17	0.797	0.891	0.800	0.681	0.754	0.583	0.757	0.750	0.710	0.906	0.764	0.866	0.922	1.000														
18	0.833	0.903	0.839	0.662	0.761	0.586	0.765	0.758	0.742	0.951	0.797	0.877	0.935	0.892	1.000													
20	0.833	0.873	0.839	0.639	0.788	0.563	0.818	0.731	0.716	0.859	0.771	0.794	0.875	0.836	0.875	1.000												
21	0.791	0.828	0.794	0.625	0.746	0.571	0.803	0.716	0.727	0.815	0.732	0.754	0.831	0.794	0.831	0.919	1.000											
22	0.806	0.844	0.839	0.639	0.761	0.542	0.818	0.706	0.716	0.831	0.746	0.768	0.846	0.809	0.846	0.935	0.951	1.000										
23	0.588	0.591	0.554	0.522	0.567	0.531	0.574	0.515	0.594	0.582	0.586	0.603	0.574	0.549	0.574	0.597	0.606	0.597	1.000									
24	0.473	0.536	0.522	0.514	0.452	0.414	0.459	0.507	0.493	0.529	0.493	0.507	0.543	0.521	0.565	0.500	0.507	0.521	0.377	1.000								
25	0.500	0.547	0.532	0.414	0.478	0.438	0.423	0.448	0.455	0.515	0.500	0.493	0.530	0.486	0.507	0.507	0.471	0.507	0.492	0.413	1.000							
26	0.348	0.343	0.323	0.304	0.343	0.383	0.314	0.375	0.381	0.358	0.352	0.343	0.353	0.357	0.353	0.333	0.338	0.333	0.339	0.290	0.281	1.000						
27	0.213	0.189	0.235	0.205	0.239	0.227	0.216	0.194	0.214	0.203	0.221	0.195	0.216	0.208	0.200	0.233	0.219	0.233	0.242	0.238	0.203	0.265	1.000					
28	0.213	0.190	0.184	0.205	0.175	0.243	0.171	0.211	0.230	0.203	0.205	0.210	0.231	0.222	0.215	0.215	0.218	0.215	0.221	0.217	0.167	0.259	0.269	1.000				
29	0.218	0.211	0.222	0.211	0.243	0.232	0.175	0.200	0.219	0.192	0.225	0.215	0.221	0.213	0.205	0.205	0.208	0.221	0.266	0.155	0.190	0.222	0.255	0.373	1.000			
30	0.218	0.211	0.222	0.227	0.195	0.214	0.190	0.216	0.203	0.208	0.195	0.185	0.221	0.228	0.205	0.237	0.240	0.253	0.227	0.206	0.190	0.179	0.231	0.346	0.333	1.000		
31	0.203	0.176	0.169	0.159	0.194	0.177	0.171	0.147	0.167	0.174	0.178	0.167	0.188	0.197	0.188	0.206	0.191	0.206	0.190	0.129	0.145	0.200	0.156	0.234	0.333	0.302	1.000	
32	0.638	0.642	0.631	0.528	0.571	0.493	0.623	0.565	0.574	0.632	0.589	0.606	0.623	0.597	0.623	0.672	0.632	0.647	0.547	0.408	0.476	0.400	0.242	0.222	0.194	0.211	0.213	1.000

[1] Abbreviations for isolates consist of the first letter of genus and species (Gts = *Globodera tabacum solanacearum*; Gtt = *Globodera tabacum tabacum*; Gtv = *Globodera tabacum virginiae*; Hc = *Heterodera carotae*; Hg = *Heterodera glycines*; Hs = *Heterodera schachtii*; Ht = *Heterodera trifoli*; Hr = *Heterodera rumicis*; Gm = *Globodera "mexicana"*), first two letters of county name (Br = Brunswick; Ca = Campell; Di = Dinwiddie; Ha = Halifax; Lu = Lunenburg; Me = Mecklenburg; No = Nottoway; Pr = Prince Edward; Wa = Warren), and first letter of source (grower name or culture collection).

Table 2.4: Average of Jaccard's similarity coefficients across different species and genera.

	G.t.s.	G.t.t.	G.t.v.	G.m.	G	H
G.t.s.	0.741					
G.t.t.	0.492	0.804				
G.t.v.	0.346	0.286	1.000			
G.m.	0.602	0.422	0.400	1.000		
G	0.685	0.501	0.372	0.597	0.683	
H	0.208	0.184	0.225	0.217	0.244	0.522

G.t.s. = *Globodera tabacum solanacearum*; G.t.t. = *Globodera tabacum tabacum*; G.t.v. = *Globodera tabacum virginiae*; G.m. = *Globodera "mexicana"*; G = *Globodera;* H = *Heterodera*

Table 2.5: Jaccard's similarity matrix of 32 isolates[1]. The outlier isolates (1, 7, 14, and 19) are indicated by bold-face font, and range from 0.10-0.39.

Isolate Identifier	1: Gts-AmH	2: Gts-BrJ	3: Gts-BrW	4: Gts-CaG	5: Gts-DiW	6: Gts-DiW2	7: Gts-DiB	8: Gts-DiB2	9: Gts-HaS	10: Gts-LuC	11: Gts-LuP	12: Gts-MeW	13: Gts-MeH	14: Gts-MeH2	15: Gts-MeH3	16: Gts-NoD	17: Gts-NoS	18: Gts-PrF	19: Gts-PiO	20: Gts-WaH	21: Gts-WaH2	22: Gts-WaH3	23: Gts-M	24: Gtt-M/E	25: Gtt-M2	26: Gtv-M/E	27: Hc-M	28: Hg-M	29: Hs-M	30: Ht-M	31: Hr-M	32: Gm-M
1	**1.00**																															
2	**0.12**	1.00																														
3	**0.11**	0.86	1.00																													
4	**0.13**	0.86	0.87	1.00																												
5	**0.15**	0.76	0.68	0.72	1.00																											
6	**0.12**	0.80	0.79	0.78	0.66	1.00																										
7	**0.31**	**0.28**	**0.24**	**0.29**	**0.24**	**0.28**	**1.00**																									
8	**0.18**	0.62	0.58	0.59	0.51	0.56	**0.34**	1.00																								
9	**0.12**	0.81	0.76	0.78	0.66	0.74	**0.30**	0.54	1.00																							
10	**0.14**	0.70	0.75	0.72	0.63	0.70	**0.27**	0.60	0.68	1.00																						
11	**0.17**	0.73	0.74	0.76	0.64	0.69	**0.27**	0.58	0.72	0.73	1.00																					
12	**0.11**	0.85	0.92	0.85	0.70	0.77	**0.26**	0.59	0.78	0.80	0.75	1.00																				
13	**0.15**	0.74	0.79	0.76	0.63	0.77	**0.27**	0.53	0.72	0.71	0.70	0.81	1.00																			
14	**0.21**	**0.18**	**0.18**	**0.16**	**0.13**	**0.17**	**0.23**	**0.24**	**0.15**	**0.22**	**0.19**	**0.20**	**0.22**	**1.00**																		
15	**0.10**	0.84	0.85	0.79	0.69	0.77	**0.25**	0.59	0.82	0.76	0.70	0.89	0.78	**0.19**	1.00																	
16	**0.12**	0.83	0.90	0.84	0.66	0.76	**0.27**	0.59	0.79	0.76	0.77	0.92	0.80	**0.21**	0.88	1.00																
17	**0.13**	0.80	0.89	0.80	0.68	0.75	**0.29**	0.58	0.76	0.75	0.71	0.91	0.76	**0.20**	0.87	0.92	1.00															
18	**0.12**	0.83	0.90	0.84	0.66	0.76	**0.27**	0.59	0.77	0.76	0.74	0.95	0.80	**0.23**	0.88	0.94	0.89	1.00														
19	**0.24**	**0.33**	**0.36**	**0.33**	**0.31**	**0.31**	**0.26**	**0.31**	**0.32**	**0.30**	**0.29**	**0.38**	**0.37**	**0.27**	**0.38**	**0.39**	**0.39**	**0.39**	**1.00**													
20	**0.12**	0.83	0.87	0.84	0.64	0.79	**0.32**	0.56	0.82	0.73	0.72	0.86	0.77	**0.20**	0.79	0.88	0.84	0.88	**0.39**	1.00												
21	**0.13**	0.79	0.83	0.79	0.63	0.75	**0.30**	0.57	0.80	0.72	0.73	0.82	0.73	**0.21**	0.75	0.83	0.79	0.83	**0.36**	0.92	1.00											
22	**0.14**	0.81	0.84	0.84	0.64	0.76	**0.33**	0.54	0.82	0.71	0.72	0.83	0.75	**0.21**	0.77	0.85	0.81	0.85	**0.39**	0.94	0.95	1.00										
23	**0.14**	0.59	0.59	0.55	0.52	0.57	**0.21**	0.53	0.57	0.52	0.59	0.58	0.59	**0.15**	0.60	0.57	0.55	0.57	**0.31**	0.60	0.61	0.60	1.00									
24	**0.15**	0.47	0.54	0.52	0.51	0.45	**0.23**	0.41	0.46	0.51	0.49	0.53	0.49	**0.19**	0.51	0.54	0.52	0.57	**0.34**	0.50	0.51	0.52	0.38	1.00								
25	**0.12**	0.50	0.55	0.53	0.41	0.48	**0.21**	0.44	0.42	0.45	0.46	0.52	0.50	**0.18**	0.49	0.53	0.49	0.51	**0.33**	0.51	0.47	0.51	0.49	0.41	1.00							
26	**0.15**	0.35	0.34	0.32	0.30	0.34	**0.23**	0.38	0.31	0.38	0.38	0.36	0.35	**0.14**	0.34	0.35	0.36	0.35	**0.22**	0.33	0.34	0.33	0.34	0.29	0.28	1.00						
27	**0.20**	0.21	0.19	0.24	0.21	0.24	**0.23**	0.23	0.22	0.19	0.21	0.20	0.22	**0.15**	0.20	0.22	0.21	0.20	**0.24**	0.23	0.22	0.23	0.24	0.24	0.20	0.27	1.00					
28	**0.22**	0.21	0.19	0.18	0.21	0.18	**0.21**	0.24	0.17	0.21	0.23	0.20	0.21	**0.26**	0.21	0.23	0.22	0.22	**0.26**	0.22	0.22	0.22	0.22	0.22	0.17	0.26	0.27	1.00				
29	**0.27**	0.22	0.21	0.22	0.21	0.24	**0.26**	0.23	0.18	0.20	0.22	0.19	0.23	**0.20**	0.22	0.22	0.21	0.21	**0.25**	0.21	0.21	0.22	0.27	0.16	0.19	0.22	0.26	0.37	1.00			
30	**0.19**	0.22	0.21	0.22	0.23	0.20	**0.28**	0.21	0.19	0.22	0.20	0.21	0.20	**0.27**	0.19	0.22	0.23	0.21	**0.21**	0.24	0.24	0.25	0.23	0.21	0.19	0.18	0.23	0.35	0.33	1.00		
31	**0.14**	0.20	0.18	0.17	0.16	0.19	**0.20**	0.18	0.17	0.15	0.17	0.17	0.18	**0.26**	0.17	0.19	0.20	0.19	**0.26**	0.21	0.19	0.21	0.19	0.13	0.15	0.20	0.16	0.23	0.33	0.30	1.00	
32	**0.11**	0.64	0.64	0.63	0.53	0.57	**0.26**	0.49	0.62	0.57	0.57	0.63	0.59	**0.17**	0.61	0.62	0.60	0.62	**0.34**	0.67	0.63	0.65	0.55	0.41	0.48	0.40	0.24	0.22	0.19	0.21	0.21	1.00

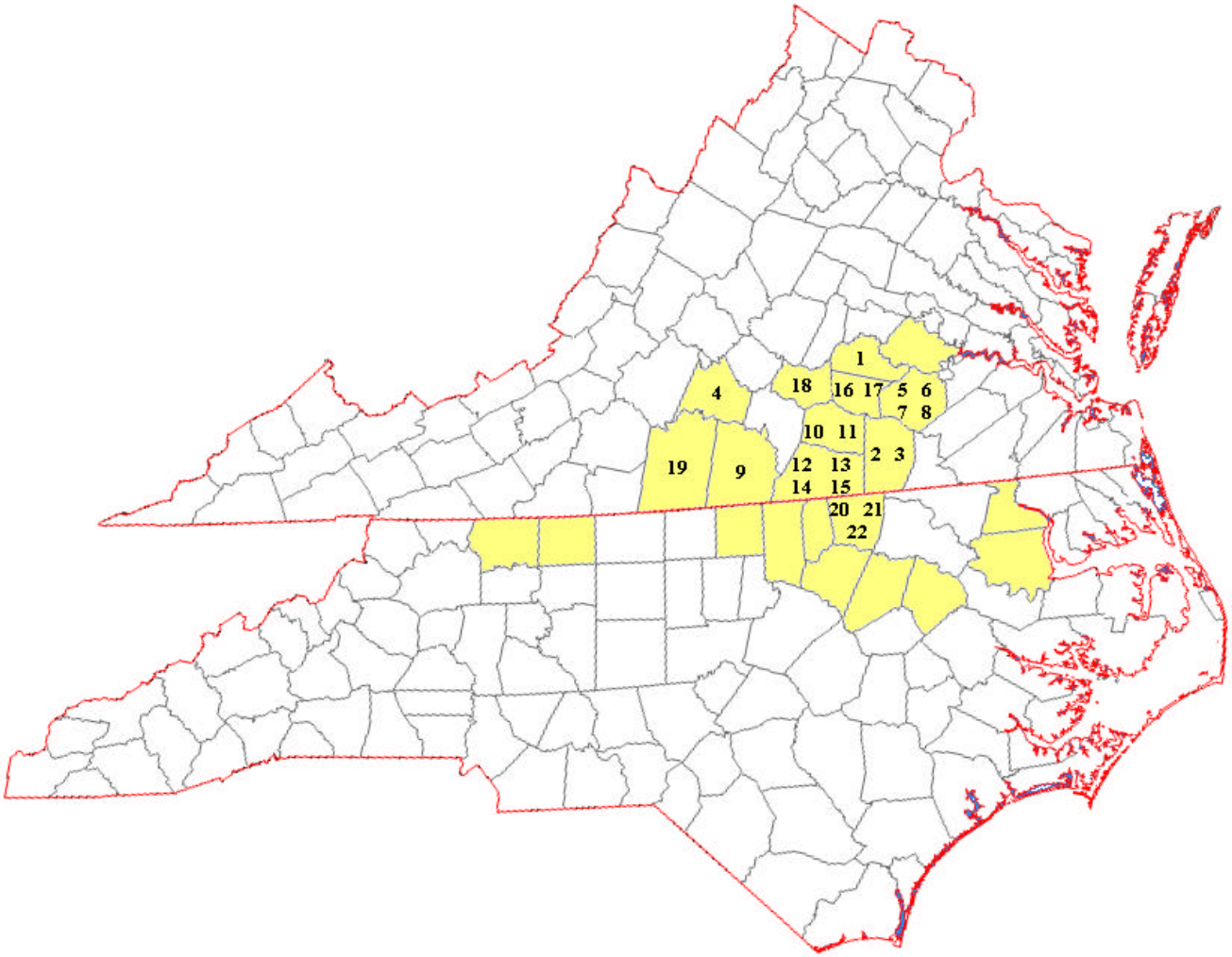
[1] Abbreviations for isolates consist of the first letter of genus and species (Gts = *Globodera tabacum solanacearum*; Gtt = *Globodera tabacum tabacum*; Gtv = *Globodera tabacum virginiae*; Hc = *Heterodera carotae*; Hg = *Heterodera glycines*; Hs = *Heterodera schachtii*; Ht = *Heterodera trifoli*; Hr = *Heterodera rumicis*; Gm = *Globodera "mexicana"*), first two letters of county name (Am = Amelia; Br = Brunswick; Ca = Campell; Di = Dinwiddie; Ha = Halifax; Lu = Lunenburg; Me = Mecklenburg; No = Nottoway; Pr = Prince Edward; Pi = Pittsylvania; Wa = Warren), and first letter of source (grower name or culture collection).

Table 2.6: Jaccard's similarity matrix of four outlier isolates[1].

	1: Gts-AmH	7: Gts-DiB	14: Gts-MeH2	19: Gts-PiO
1	1.000			
7	0.311	1.000		
14	0.214	0.226	1.000	
19	0.239	0.264	0.274	1.000

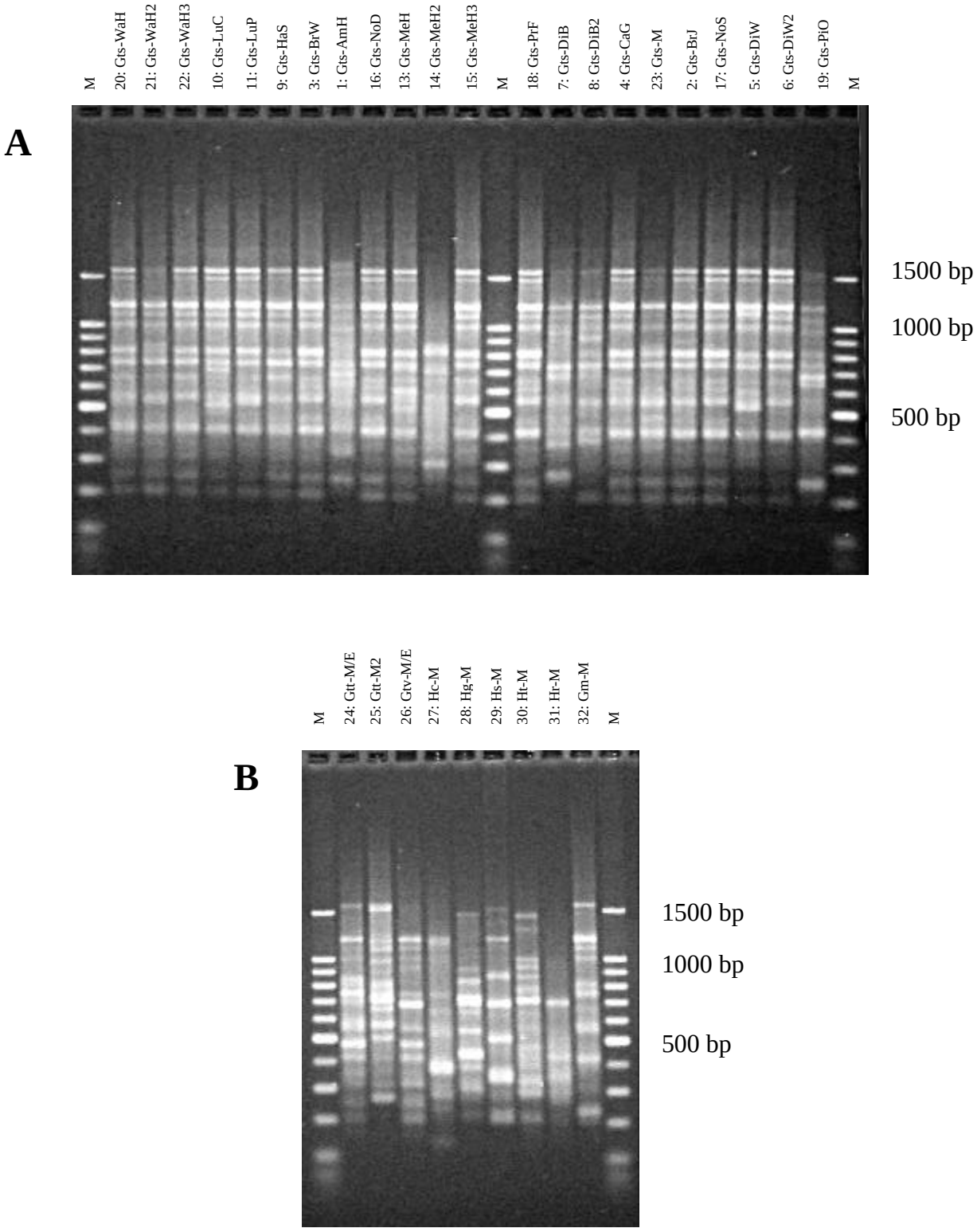
[1] Abbreviations for isolates consist of the first letter of genus and species (Gts = *Globodera tabacum solanacearum*), first two letters of county name (Am = Amelia; Di = Dinwiddie; Me = Mecklenburg; Pi = Pittsylvania), and first letter of source (grower name).

Figure 2.1: Locations of *Globodera tabacum solanacearum* isolates obtained from counties in the southern piedmont of Virginia and northern piedmont of North Carolina. *G. t. solanacearum* has been reported in all highlighted counties.



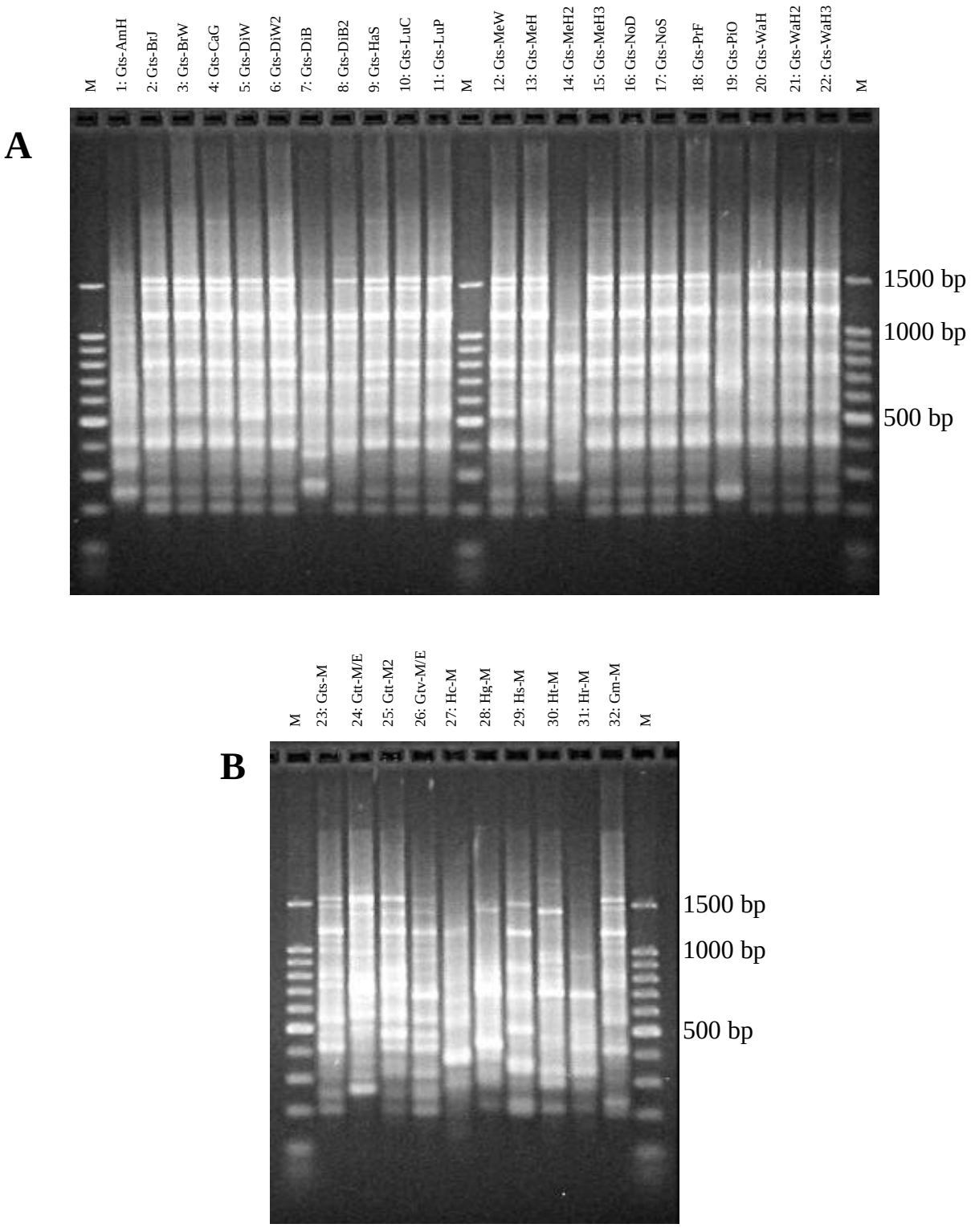
1 = Amelia County, 2 and 3 = Brunswick County, 4 = Campbell County, 5, 6, 7, and 8 = Dinwiddie County, 9 = Halifax County, 10 and 11 = Lunenburg County, 12, 13, 14, and 15 = Mecklenburg County, 16 and 17 = Nottoway County, 18 = Prince Edward County, 19 = Pittsylvania County, 20, 21, and 22 = Warren County.

Figure 2.2: Rep 1 using primer 1. RAPD profiles of 22 isolates of *Globodera tabacum solanacearum* (A), and outgroup (B). This rep does not include isolate 12.



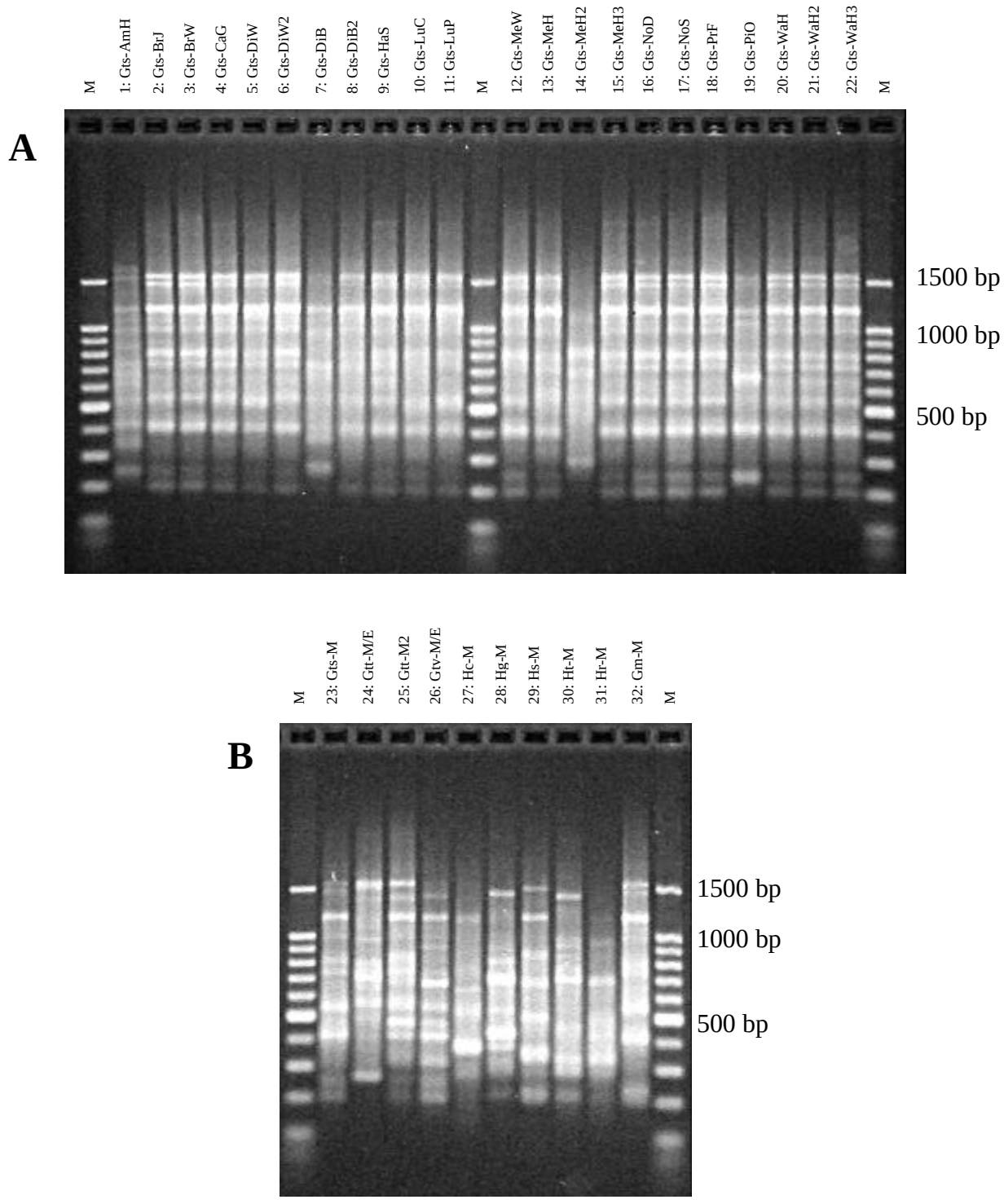
[1] Abbreviations for isolates consist of the first letter of genus and species (Gts = *Globodera tabacum solanacearum*; Gtt = *Globodera tabacum tabacum*; Gtv = *Globodera tabacum virginiae*; Hc = *Heterodera carotae*; Hg = *Heterodera glycines*; Hs = *Heterodera schachtii*; Ht = *Heterodera trifoli*; Hr = *Heterodera rumicis*; Gm = *Globodera "mexicana"*), first two letters of county name (Am = Amelia; Br = Brunswick; Ca = Campell; Di = Dinwiddie; Ha = Halifax; Lu = Lunenburg; Me = Mecklenburg; No = Nottoway; Pr = Prince Edward; Pi = Pittsylvania; Wa = Warren), and first letter of source (grower name or culture collection). M = 100 bp molecular weight marker (Promega)

Figure 2.3: Rep 2 using primer 1. RAPD profiles of 22 isolates of *Globodera tabacum solanacearum* (A), and outgroup (B).



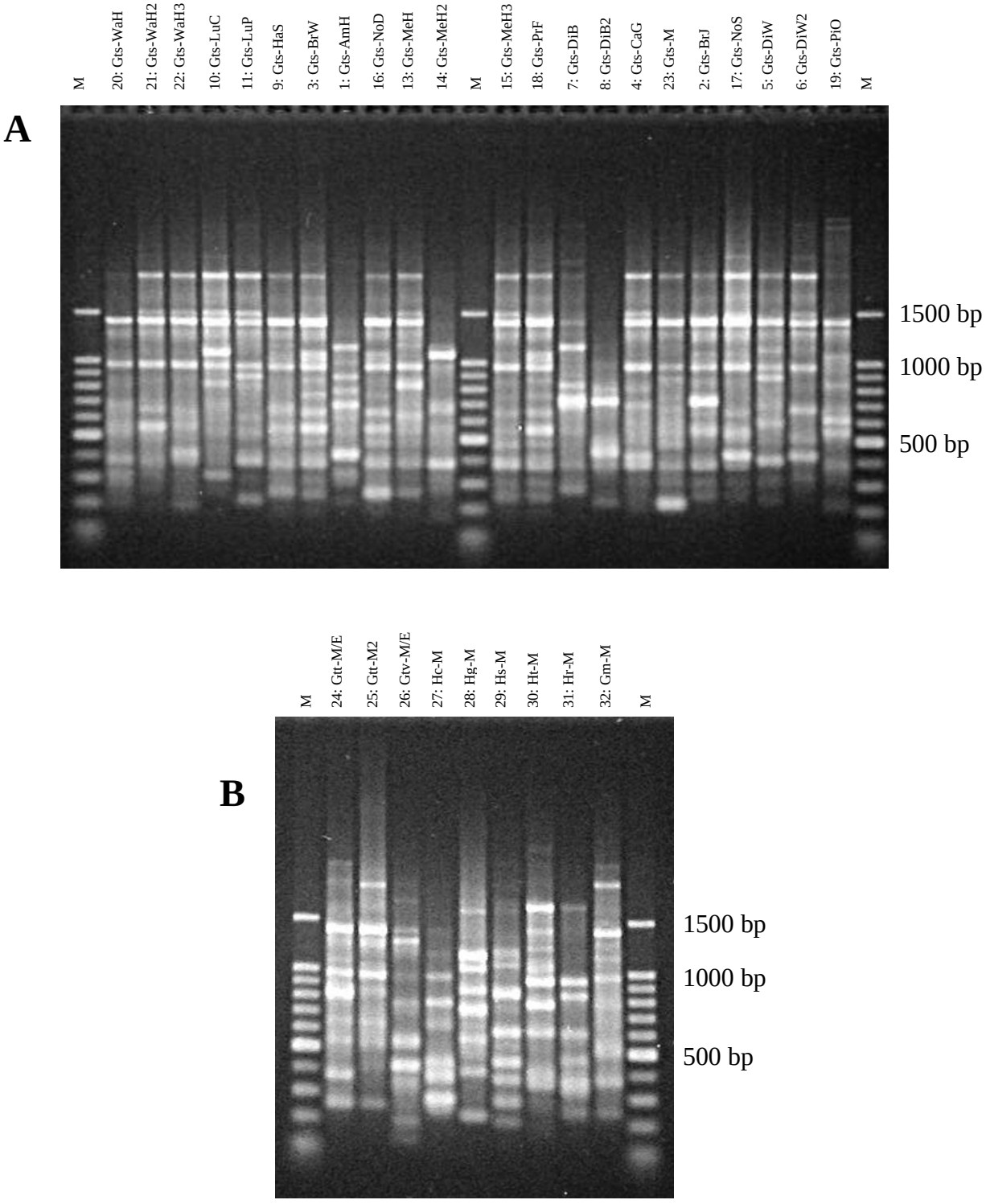
[1] Abbreviations for isolates consist of the first letter of genus and species (Gts = *Globodera tabacum solanacearum*; Gtt = *Globodera tabacum tabacum*; Gtv = *Globodera tabacum virginiae*; Hc = *Heterodera carotae*; Hg = *Heterodera glycines*; Hs = *Heterodera schachtii*; Ht = *Heterodera trifoli*; Hr = *Heterodera rumicis*; Gm = *Globodera "mexicana"*), first two letters of county name (Am = Amelia; Br = Brunswick; Ca = Campell; Di = Dinwiddie; Ha = Halifax; Lu = Lunenburg; Me = Mecklenburg; No = Nottoway; Pr = Prince Edward; Pi = Pittsylvania; Wa = Warren), and first letter of source (grower name or culture collection). M = 100 bp molecular weight marker (Promega).

Figure 2.4: Rep 3 using primer 1. RAPD profiles of 22 isolates of *Globodera tabacum solanacearum* (A), and outgroup (B).



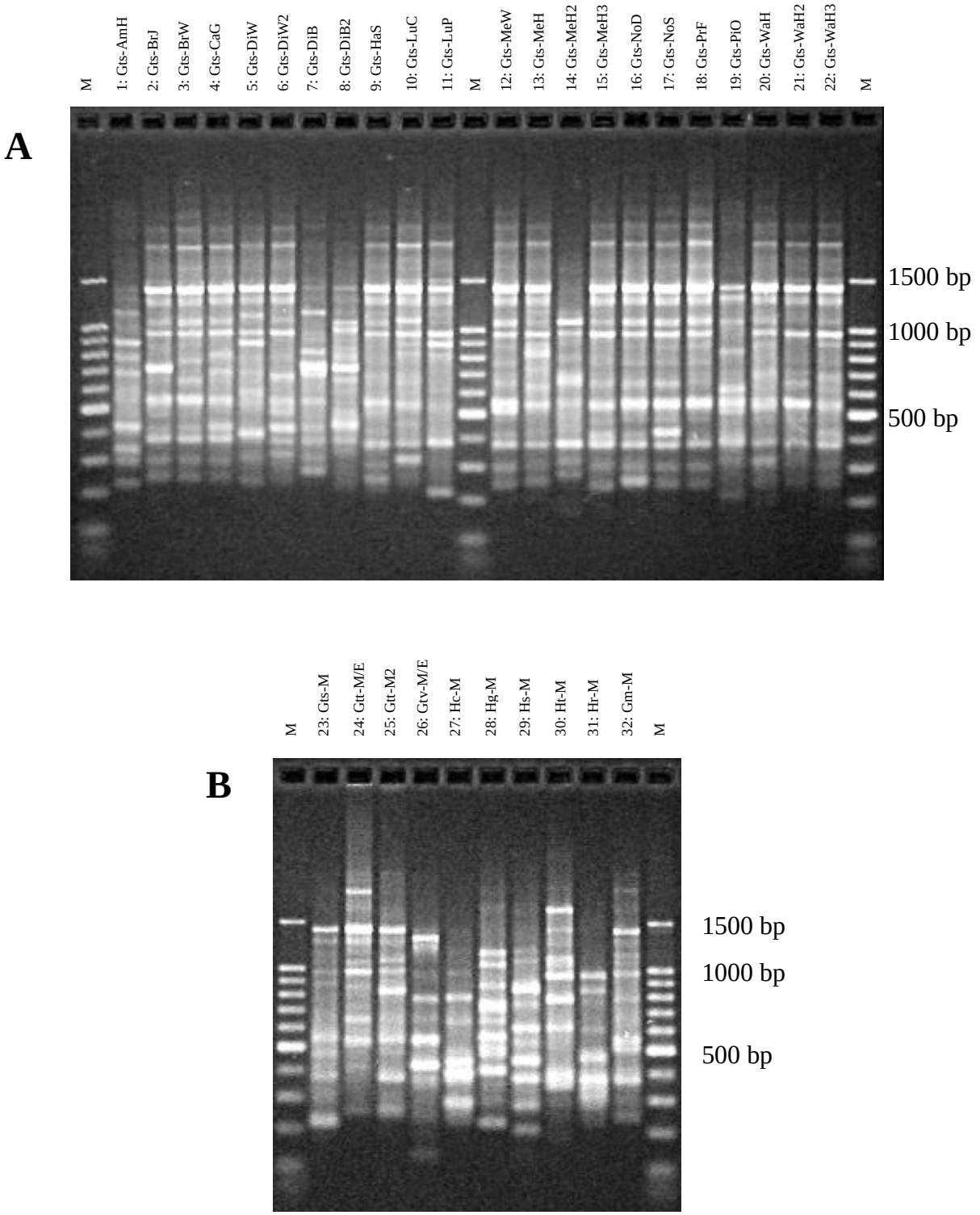
[1] Abbreviations for isolates consist of the first letter of genus and species (Gts = *Globodera tabacum solanacearum*; Gtt = *Globodera tabacum tabacum*; Gtv = *Globodera tabacum virginiae*; Hc = *Heterodera carotae*; Hg = *Heterodera glycines*; Hs = *Heterodera schachtii*; Ht = *Heterodera trifoli*; Hr = *Heterodera rumicis*; Gm = *Globodera "mexicana"*), first two letters of county name (Am = Amelia; Br = Brunswick; Ca = Campell; Di = Dinwiddie; Ha = Halifax; Lu = Lunenburg; Me = Mecklenburg; No = Nottoway; Pr = Prince Edward; Pi = Pittsylvania; Wa = Warren), and first letter of source (grower name or culture collection). M = 100 bp molecular weight marker (Promega)

Figure 2.5: Rep 1 using primer 2. RAPD profiles of 22 isolates of *Globodera tabacum solanacearum* (A), and outgroup (B). This rep does not include isolate 12.



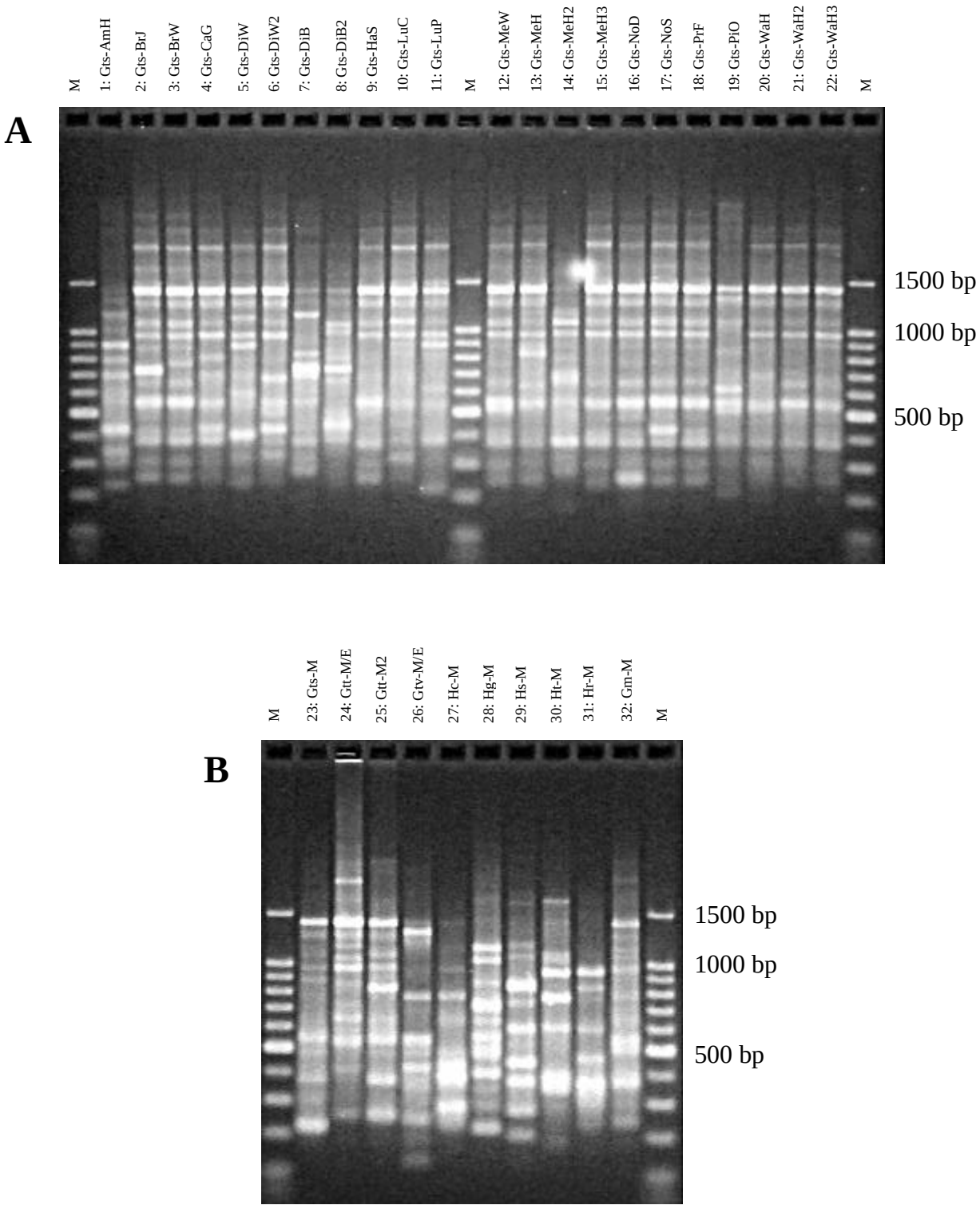
[1] Abbreviations for isolates consist of the first letter of genus and species (Gts = *Globodera tabacum solanacearum*; Gtt = *Globodera tabacum tabacum*; Gtv = *Globodera tabacum virginiae*; Hc = *Heterodera carotae*; Hg = *Heterodera glycines*; Hs = *Heterodera schachtii*; Ht = *Heterodera trifoli*; Hr = *Heterodera rumicis*; Gm = *Globodera "mexicana"*), first two letters of county name (Am = Amelia; Br = Brunswick; Ca = Campell; Di = Dinwiddie; Ha = Halifax; Lu = Lunenburg; Me = Mecklenburg; No = Nottoway; Pr = Prince Edward; Pi = Pittsylvania; Wa = Warren), and first letter of source (grower name or culture collection). M = 100 bp molecular weight marker (Promega)

Figure 2.6: Rep 2 using primer 2. RAPD profiles of 22 isolates of *Globodera tabacum solanacearum* (A), and outgroup (B).



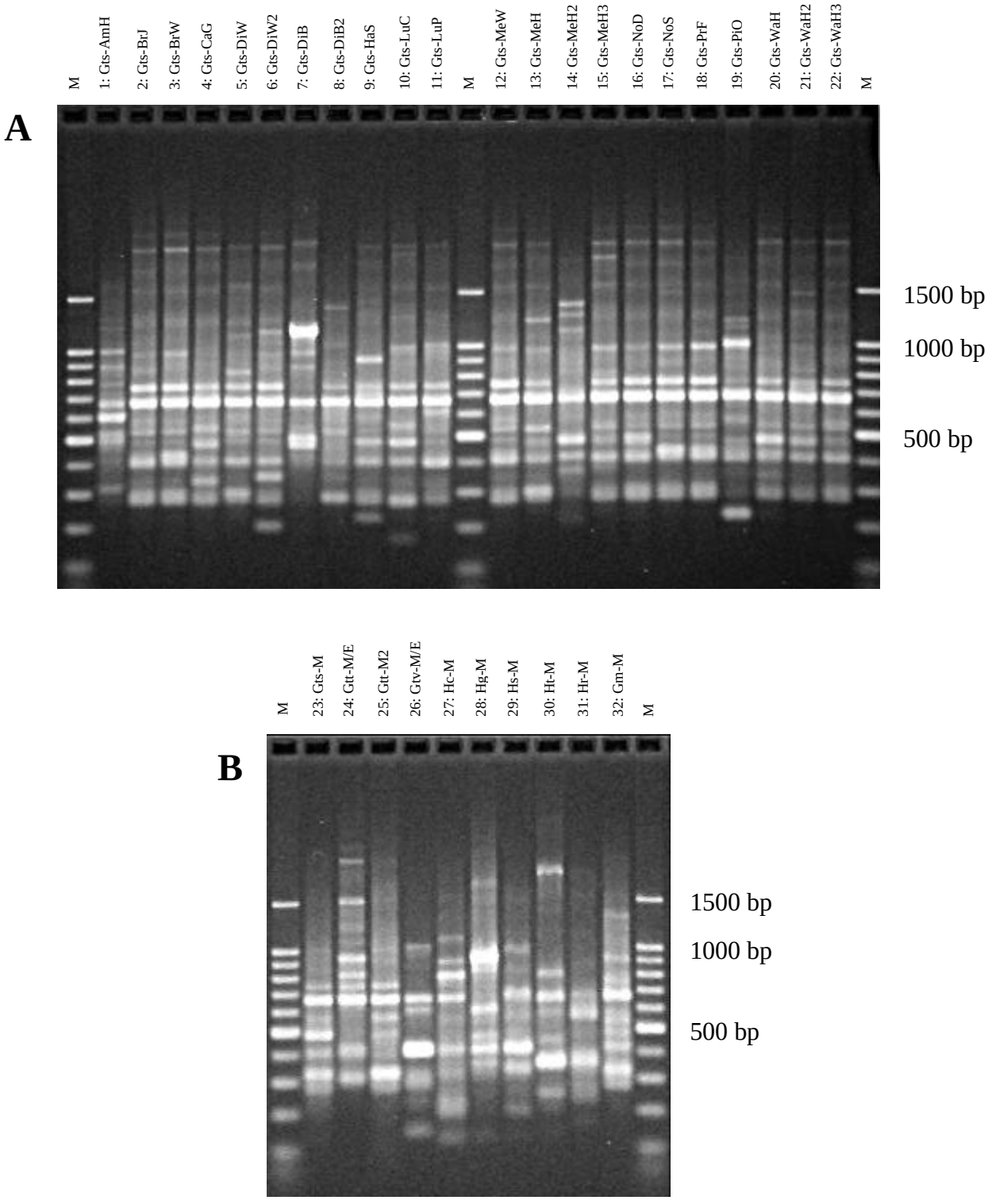
[1] Abbreviations for isolates consist of the first letter of genus and species (Gts = *Globodera tabacum solanacearum*; Gtt = *Globodera tabacum tabacum*; Gtv = *Globodera tabacum virginiae*; Hc = *Heterodera carotae*; Hg = *Heterodera glycines*; Hs = *Heterodera schachtii*; Ht = *Heterodera trifoli*; Hr = *Heterodera rumicis*; Gm = *Globodera "mexicana"*), first two letters of county name (Am = Amelia; Br = Brunswick; Ca = Campell; Di = Dinwiddie; Ha = Halifax; Lu = Lunenburg; Me = Mecklenburg; No = Nottoway; Pr = Prince Edward; Pi = Pittsylvania; Wa = Warren), and first letter of source (grower name or culture collection). M = 100 bp molecular weight marker (Promega)

Figure 2.7: Rep 3 using primer 2. RAPD profiles of 22 isolates of *Globodera tabacum solanacearum* (A), and outgroup (B).



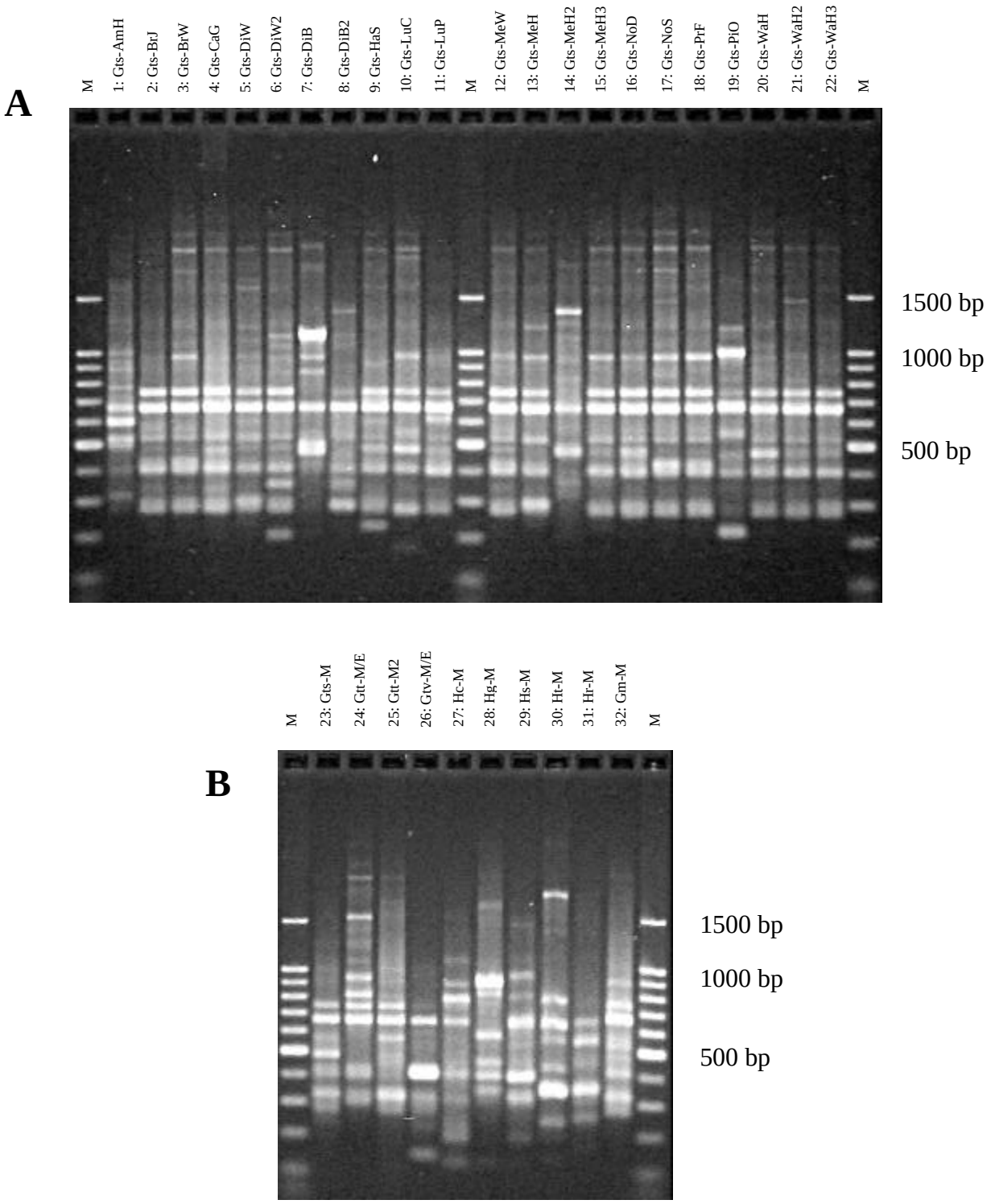
[1] Abbreviations for isolates consist of the first letter of genus and species (Gts = *Globodera tabacum solanacearum*; Gtt = *Globodera tabacum tabacum*; Gtv = *Globodera tabacum virginiae*; Hc = *Heterodera carotae*; Hg = *Heterodera glycines*; Hs = *Heterodera schachtii*; Ht = *Heterodera trifoli*; Hr = *Heterodera rumicis*; Gm = *Globodera "mexicana"*), first two letters of county name (Am = Amelia; Br = Brunswick; Ca = Campell; Di = Dinwiddie; Ha = Halifax; Lu = Lunenburg; Me = Mecklenburg; No = Nottoway; Pr = Prince Edward; Pi = Pittsylvania; Wa = Warren), and first letter of source (grower name or culture collection). M = 100 bp molecular weight marker (Promega)

Figure 2.8: Rep 1 using primer 3. RAPD profiles of 22 isolates of *Globodera tabacum solanacearum* (A), and outgroup (B).



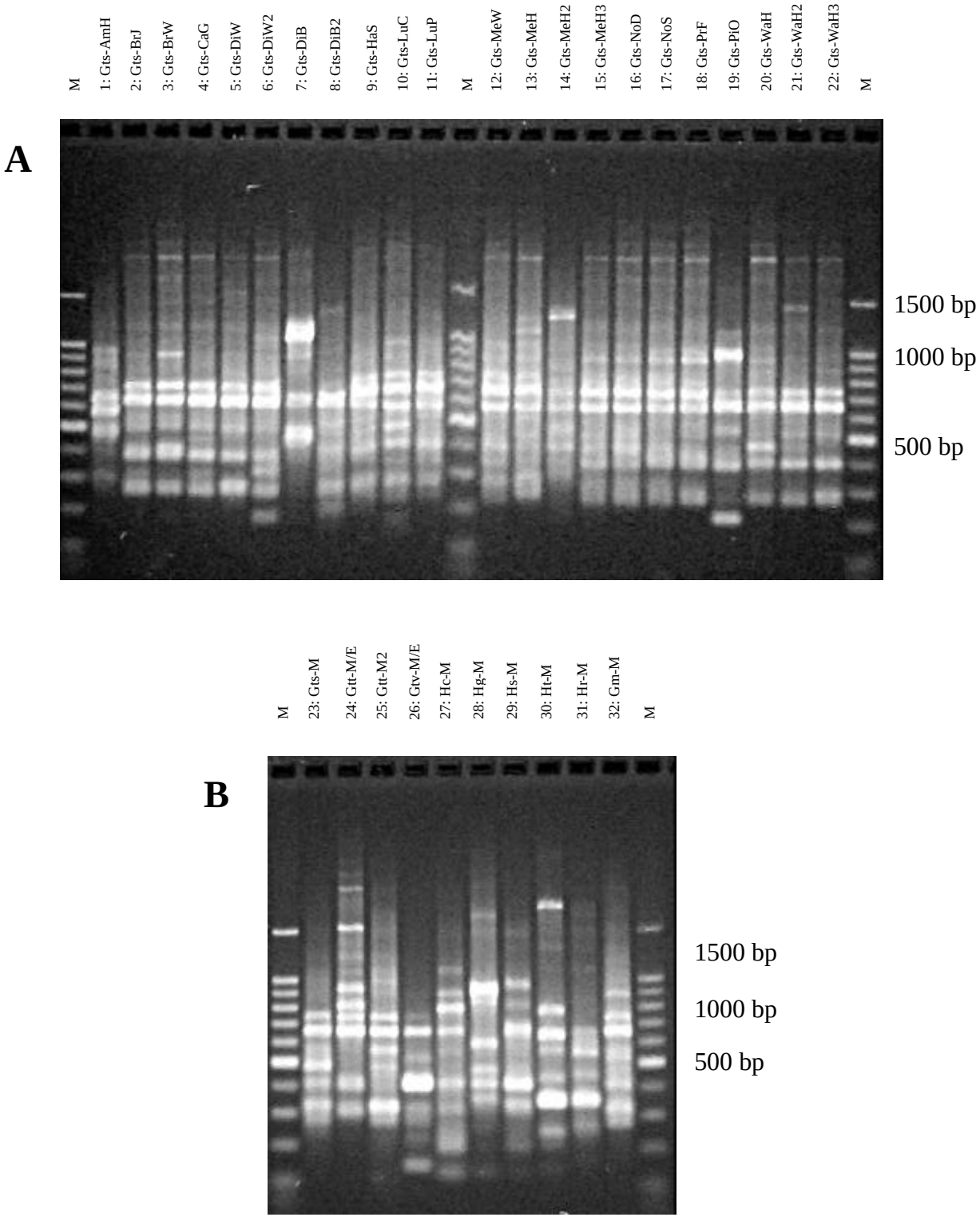
[1] Abbreviations for isolates consist of the first letter of genus and species (Gts = *Globodera tabacum solanacearum*; Gtt = *Globodera tabacum tabacum*; Gtv = *Globodera tabacum virginiae*; Hc = *Heterodera carotae*; Hg = *Heterodera glycines*; Hs = *Heterodera schachtii*; Ht = *Heterodera trifoli*; Hr = *Heterodera rumicis*; Gm = *Globodera "mexicana"*), first two letters of county name (Am = Amelia; Br = Brunswick; Ca = Campell; Di = Dinwiddie; Ha = Halifax; Lu = Lunenburg; Me = Mecklenburg; No = Nottoway; Pr = Prince Edward; Pi = Pittsylvania; Wa = Warren), and first letter of source (grower name or culture collection). M = 100 bp molecular weight marker (Promega)

Figure 2.9: Rep 2 using primer 3. RAPD profiles of 22 isolates of *Globodera tabacum solanacearum* (A), and outgroup (B).



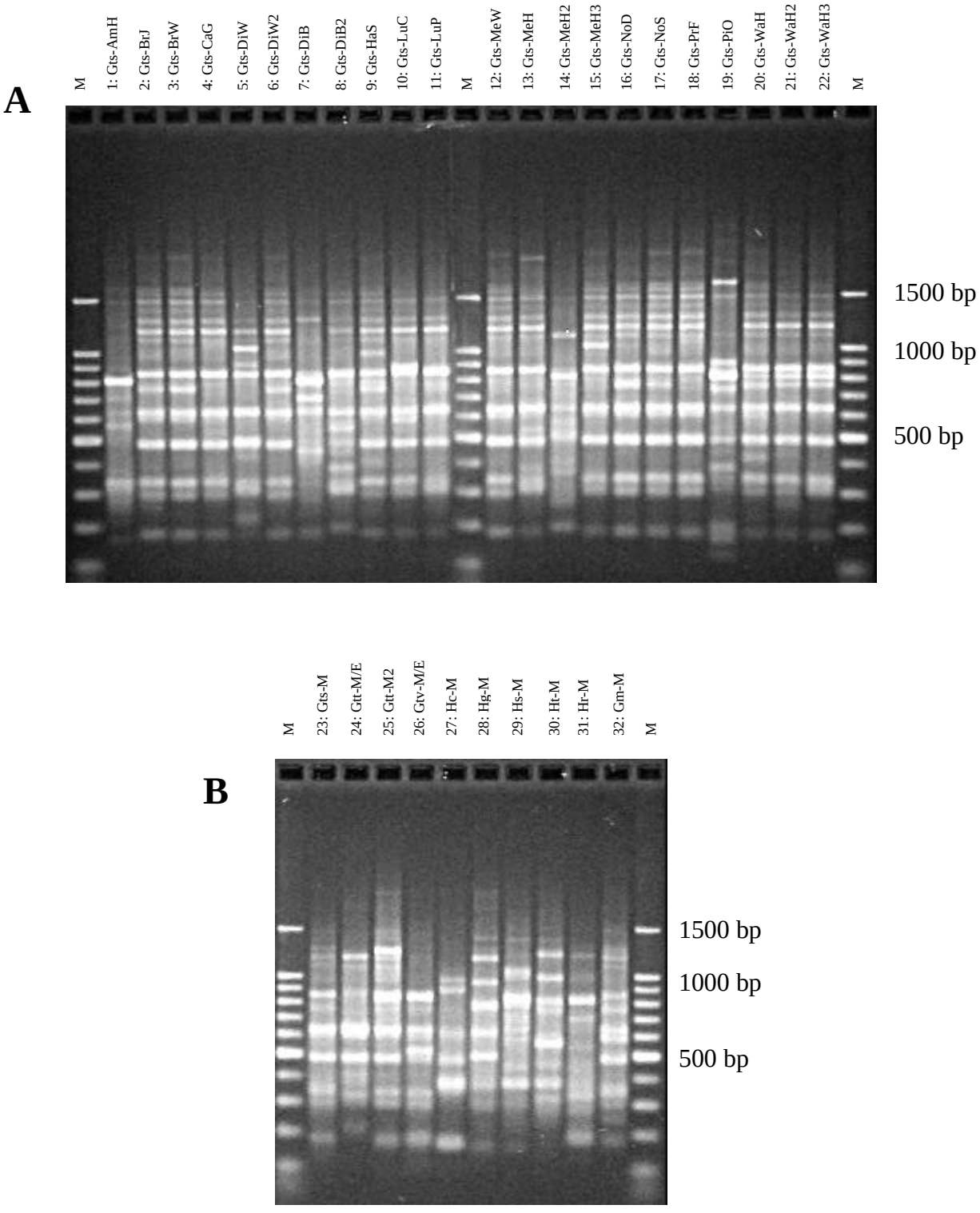
[1] Abbreviations for isolates consist of the first letter of genus and species (Gts = *Globodera tabacum solanacearum*; Gtt = *Globodera tabacum tabacum*; Gtv = *Globodera tabacum virginiae*; Hc = *Heterodera carotae*; Hg = *Heterodera glycines*; Hs = *Heterodera schachtii*; Ht = *Heterodera trifoli*; Hr = *Heterodera rumicis*; Gm = *Globodera "mexicana"*), first two letters of county name (Am = Amelia; Br = Brunswick; Ca = Campell; Di = Dinwiddie; Ha = Halifax; Lu = Lunenburg; Me = Mecklenburg; No = Nottoway; Pr = Prince Edward; Pi = Pittsylvania; Wa = Warren), and first letter of source (grower name or culture collection). M = 100 bp molecular weight marker (Promega)

Figure 2.10: Rep 3 using primer 3. RAPD profiles of 22 isolates of *Globodera tabacum solanacearum* (A), and outgroup (B).



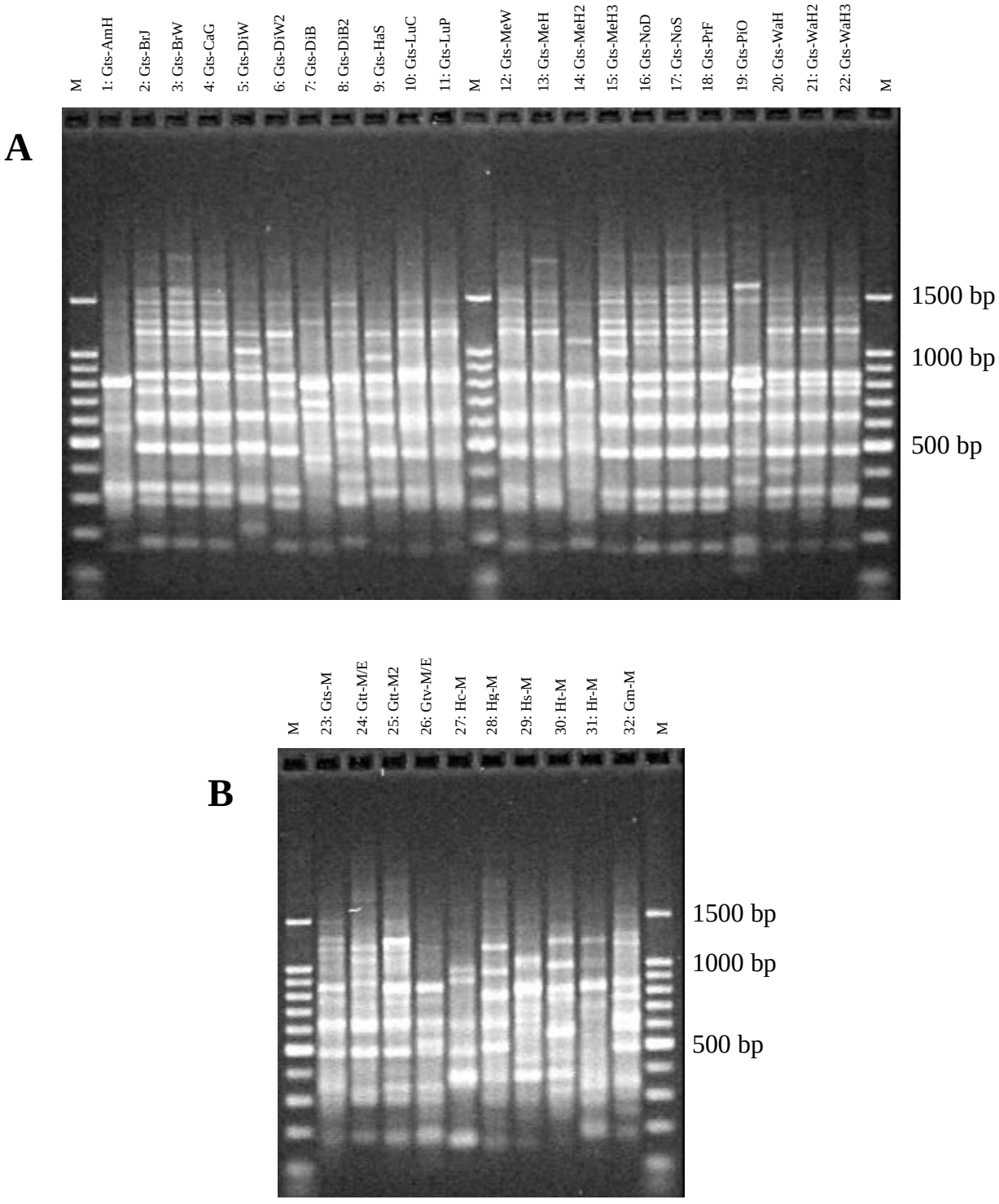
[1] Abbreviations for isolates consist of the first letter of genus and species (Gts = *Globodera tabacum solanacearum*; Gtt = *Globodera tabacum tabacum*; Gtv = *Globodera tabacum virginiae*; Hc = *Heterodera carotae*; Hg = *Heterodera glycines*; Hs = *Heterodera schachtii*; Ht = *Heterodera trifoli*; Hr = *Heterodera rumicis*; Gm = *Globodera "mexicana"*), first two letters of county name (Am = Amelia; Br = Brunswick; Ca = Campell; Di = Dinwiddie; Ha = Halifax; Lu = Lunenburg; Me = Mecklenburg; No = Nottoway; Pr = Prince Edward; Pi = Pittsylvania; Wa = Warren), and first letter of source (grower name or culture collection). M = 100 bp molecular weight marker (Promega)

Figure 2.11: Rep 1 using primer 4. RAPD profiles of 22 isolates of *Globodera tabacum solanacearum* (A), and outgroup (B).



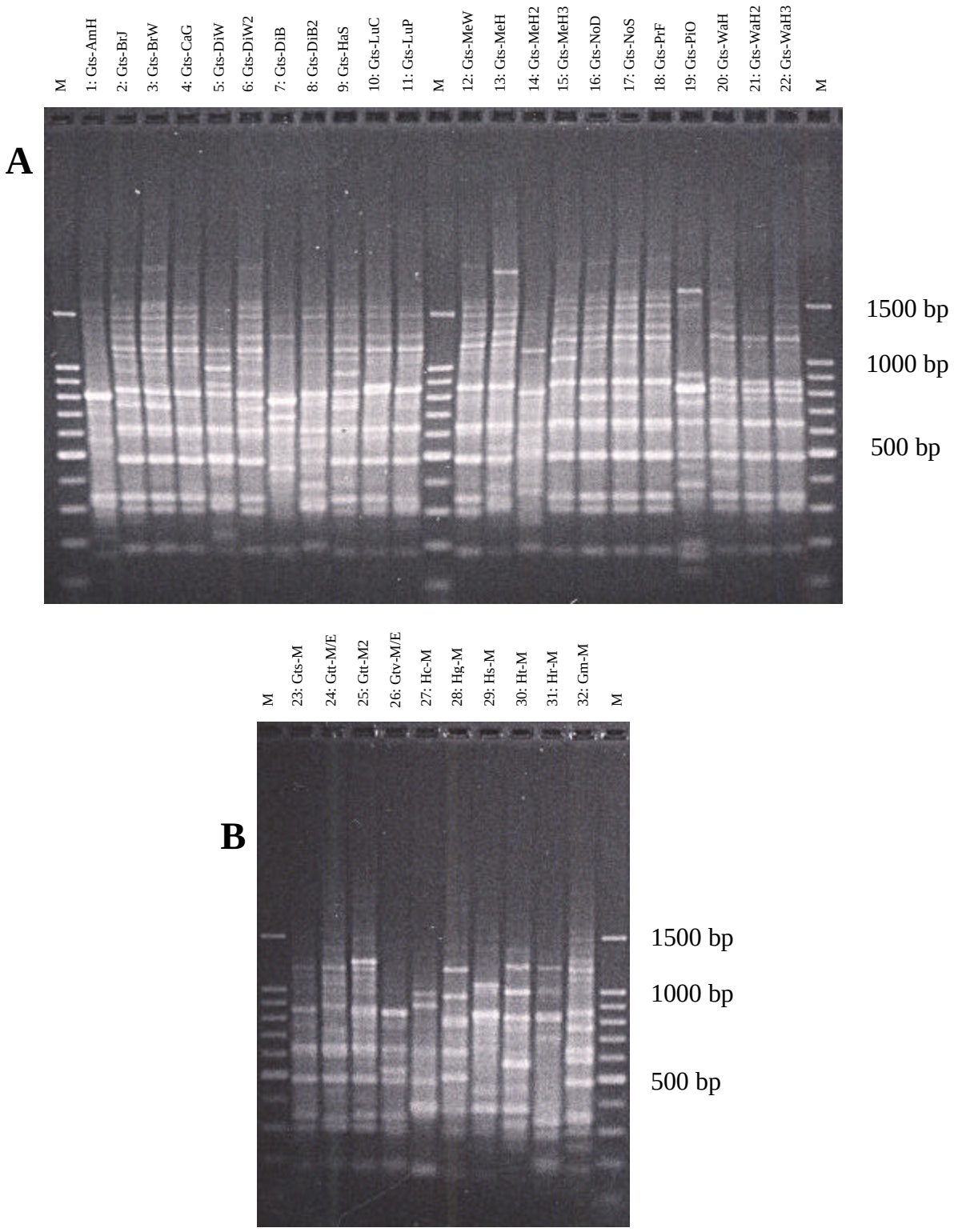
[1] Abbreviations for isolates consist of the first letter of genus and species (Gts = *Globodera tabacum solanacearum*; Gtt = *Globodera tabacum tabacum*; Gtv = *Globodera tabacum virginiae*; Hc = *Heterodera carotae*; Hg = *Heterodera glycines*; Hs = *Heterodera schachtii*; Ht = *Heterodera trifoli*; Hr = *Heterodera rumicis*; Gm = *Globodera "mexicana"*), first two letters of county name (Am = Amelia; Br = Brunswick; Ca = Campell; Di = Dinwiddie; Ha = Halifax; Lu = Lunenburg; Me = Mecklenburg; No = Nottoway; Pr = Prince Edward; Pi = Pittsylvania; Wa = Warren), and first letter of source (grower name or culture collection). M = 100 bp molecular weight marker (Promega)

Figure 2.12: Rep 2 using primer 4. RAPD profiles of 22 isolates of *Globodera tabacum solanacearum* (A), and outgroup (B).



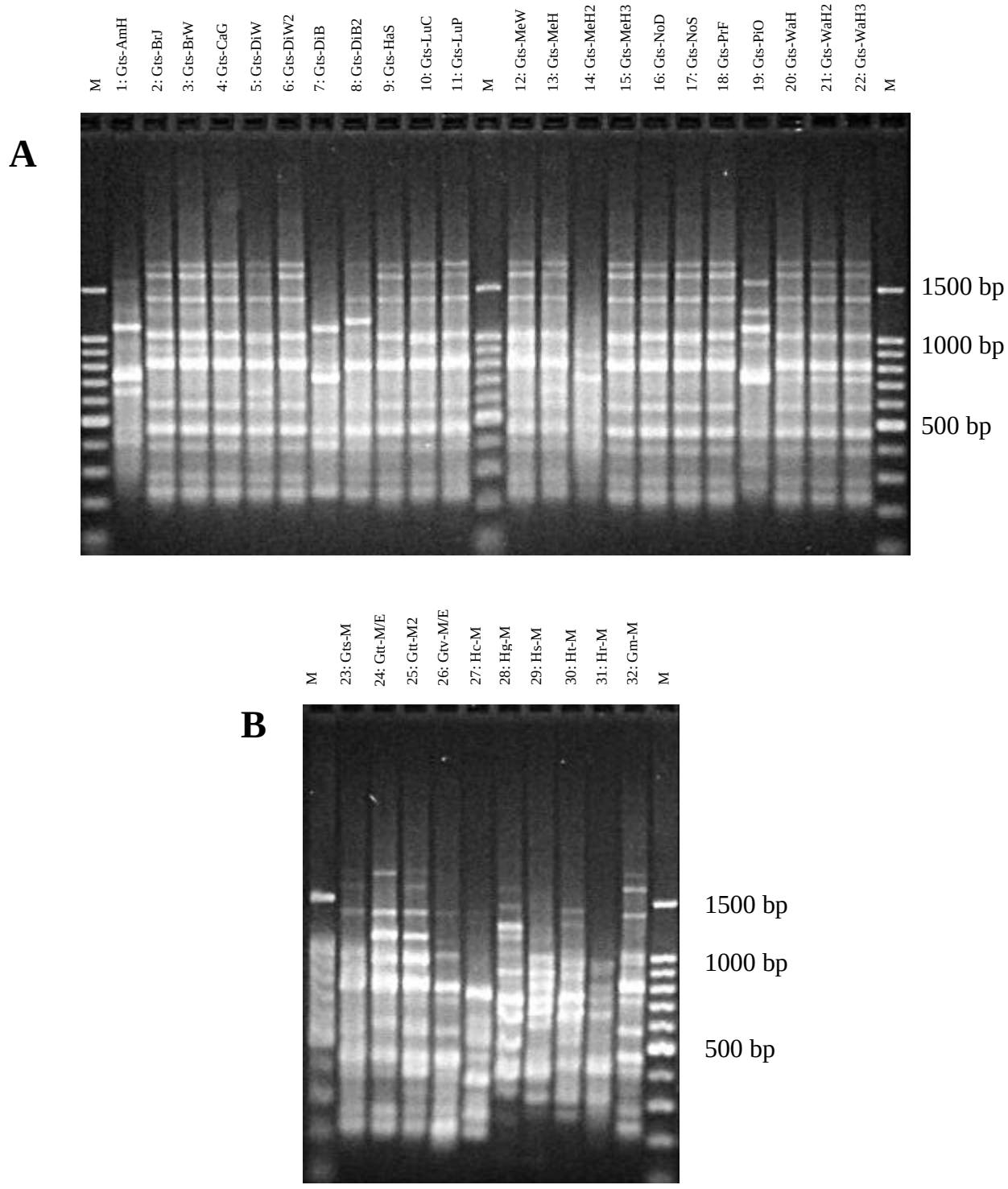
[1] Abbreviations for isolates consist of the first letter of genus and species (Gts = *Globodera tabacum solanacearum*; Gtt = *Globodera tabacum tabacum*; Gtv = *Globodera tabacum virginiae*; Hc = *Heterodera carotae*; Hg = *Heterodera glycines*; Hs = *Heterodera schachtii*; Ht = *Heterodera trifoli*; Hr = *Heterodera rumicis*; Gm = *Globodera "mexicana"*), first two letters of county name (Am = Amelia; Br = Brunswick; Ca = Campell; Di = Dinwiddie; Ha = Halifax; Lu = Lunenburg; Me = Mecklenburg; No = Nottoway; Pr = Prince Edward; Pi = Pittsylvania; Wa = Warren), and first letter of source (grower name or culture collection). M = 100 bp molecular weight marker (Promega)

Figure 2.13: Rep 3 using primer 4. RAPD profiles of 22 isolates of *Globodera tabacum solanacearum* (A), and outgroup (B).



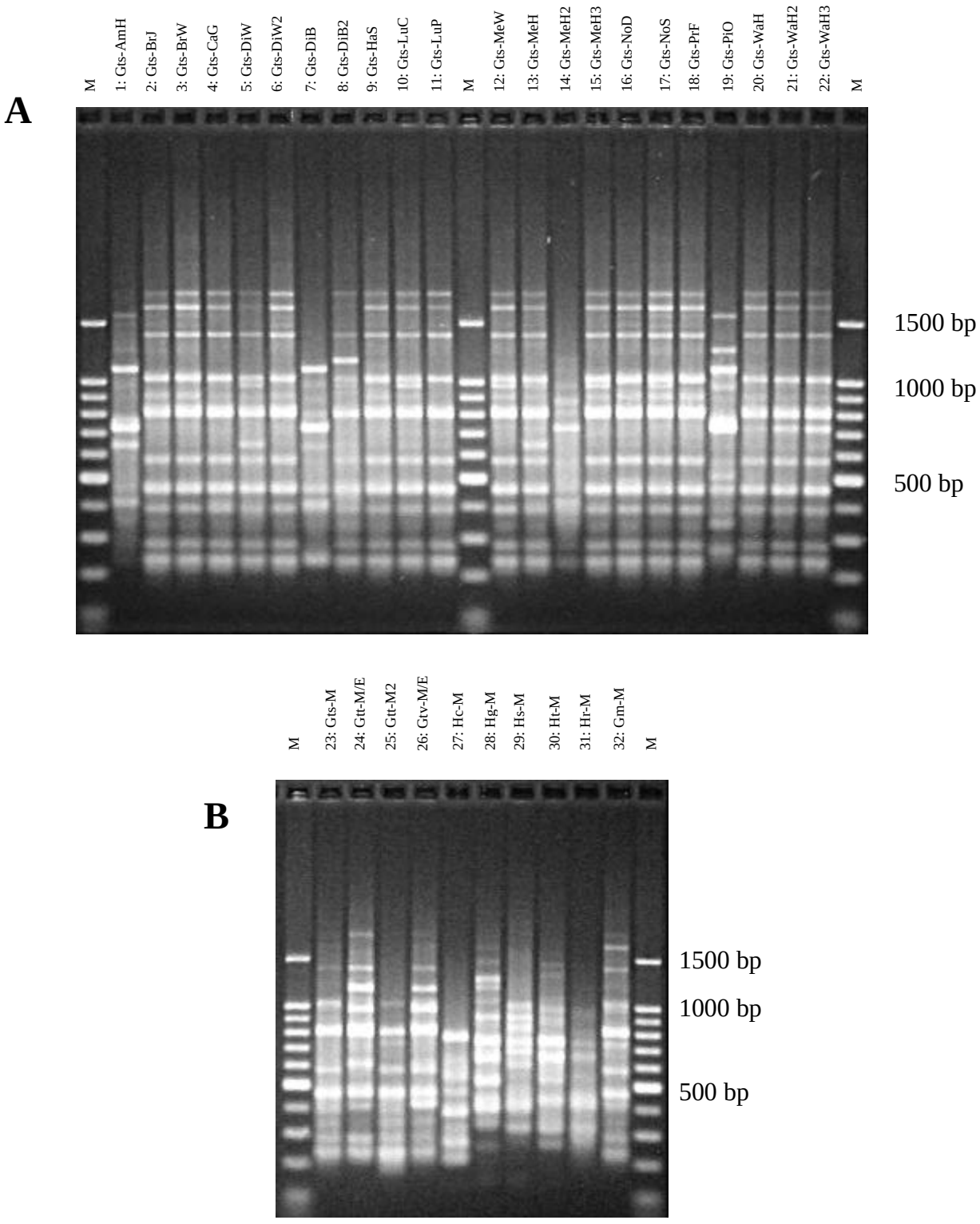
[1] Abbreviations for isolates consist of the first letter of genus and species (Gts = *Globodera tabacum solanacearum*; Gtt = *Globodera tabacum tabacum*; Gtv = *Globodera tabacum virginiae*; Hc = *Heterodera carotae*; Hg = *Heterodera glycines*; Hs = *Heterodera schachtii*; Ht = *Heterodera trifoli*; Hr = *Heterodera rumicis*; Gm = *Globodera "mexicana"*), first two letters of county name (Am = Amelia; Br = Brunswick; Ca = Campell; Di = Dinwiddie; Ha = Halifax; Lu = Lunenburg; Me = Mecklenburg; No = Nottoway; Pr = Prince Edward; Pi = Pittsylvania; Wa = Warren), and first letter of source (grower name or culture collection). M = 100 bp molecular weight marker (Promega)

Figure 2.14: Rep 1 using primer 5. RAPD profiles of 22 isolates of *Globodera tabacum solanacearum* (A), and outgroup (B).



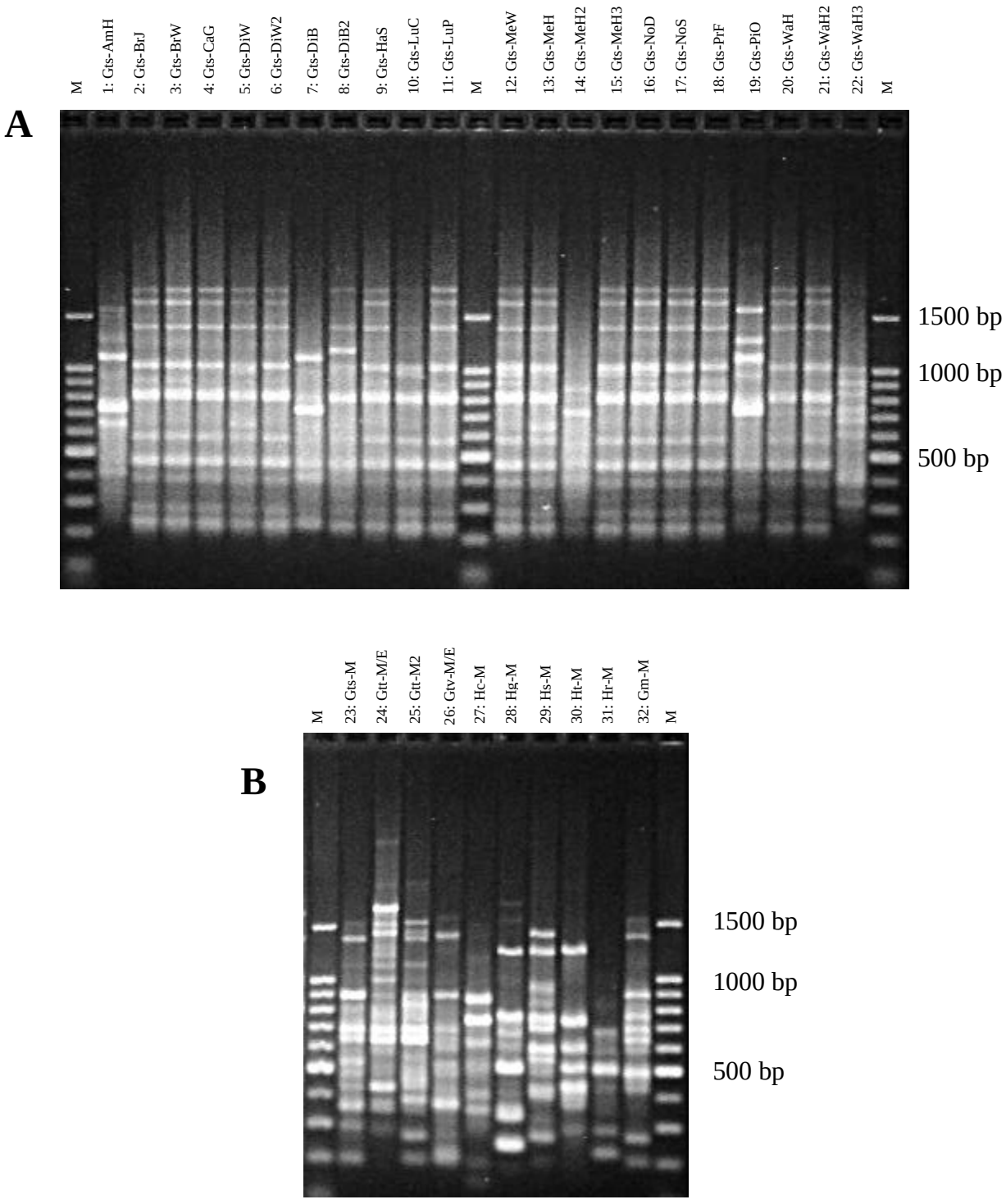
[1] Abbreviations for isolates consist of the first letter of genus and species (Gts = *Globodera tabacum solanacearum*; Gtt = *Globodera tabacum tabacum*; Gtv = *Globodera tabacum virginiae*; Hc = *Heterodera carotae*; Hg = *Heterodera glycines*; Hs = *Heterodera schachtii*; Ht = *Heterodera trifoli*; Hr = *Heterodera rumicis*; Gm = *Globodera "mexicana"*), first two letters of county name (Am = Amelia; Br = Brunswick; Ca = Campell; Di = Dinwiddie; Ha = Halifax; Lu = Lunenburg; Me = Mecklenburg; No = Nottoway; Pr = Prince Edward; Pi = Pittsylvania; Wa = Warren), and first letter of source (grower name or culture collection). M = 100 bp molecular weight marker (Promega)

Figure 2.15: Rep 2 using primer 5. RAPD profiles of 22 isolates of *Globodera tabacum solanacearum* (A), and outgroup (B).



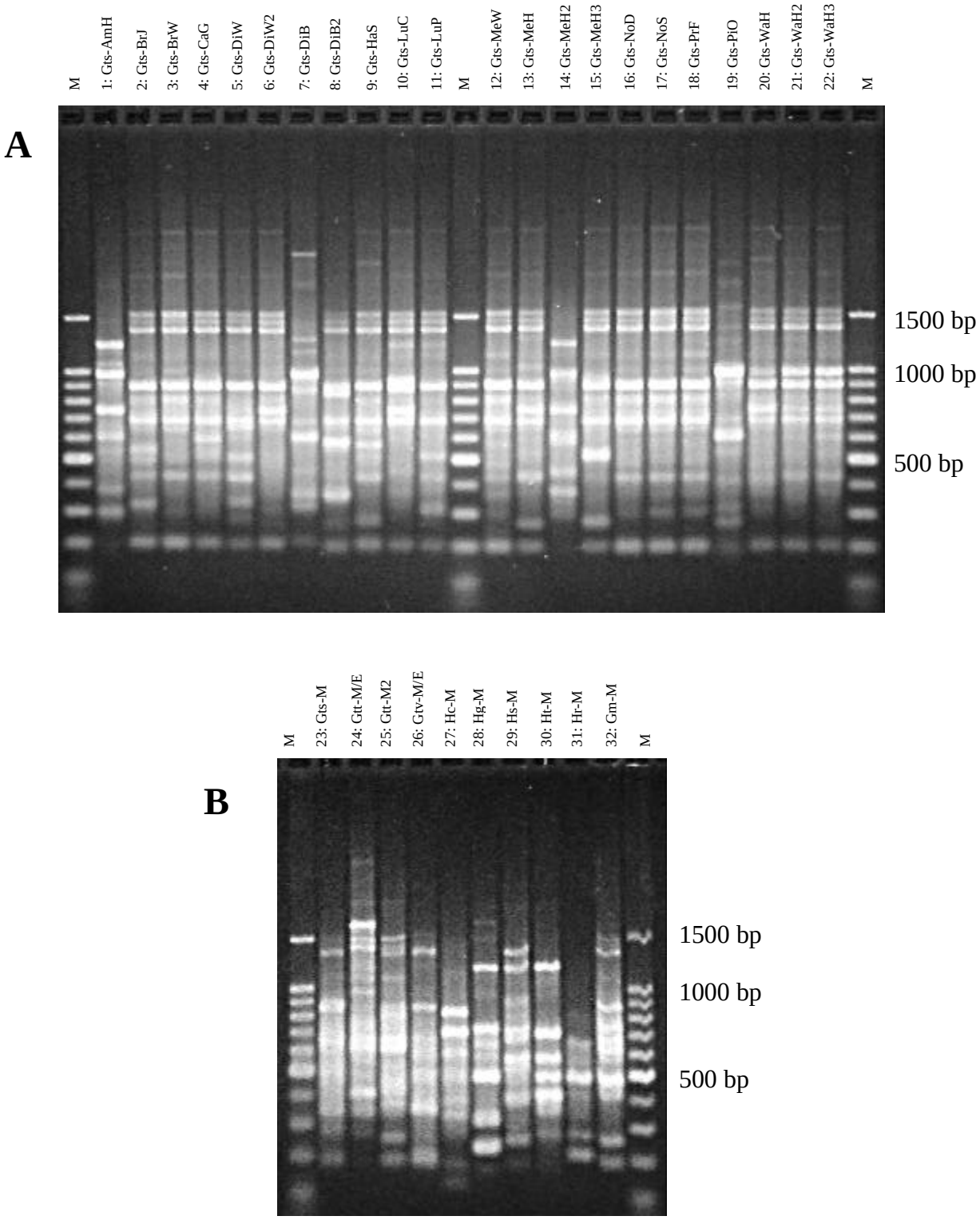
[1] Abbreviations for isolates consist of the first letter of genus and species (Gts = *Globodera tabacum solanacearum*; Gtt = *Globodera tabacum tabacum*; Gtv = *Globodera tabacum virginiae*; Hc = *Heterodera carotae*; Hg = *Heterodera glycines*; Hs = *Heterodera schachtii*; Ht = *Heterodera trifoli*; Hr = *Heterodera rumicis*; Gm = *Globodera "mexicana"*), first two letters of county name (Am = Amelia; Br = Brunswick; Ca = Campell; Di = Dinwiddie; Ha = Halifax; Lu = Lunenburg; Me = Mecklenburg; No = Nottoway; Pr = Prince Edward; Pi = Pittsylvania; Wa = Warren), and first letter of source (grower name or culture collection). M = 100 bp molecular weight marker (Promega)

Figure 2.16: Rep 3 using primer 5. RAPD profiles of 22 isolates of *Globodera tabacum solanacearum* (A), and outgroup (B).



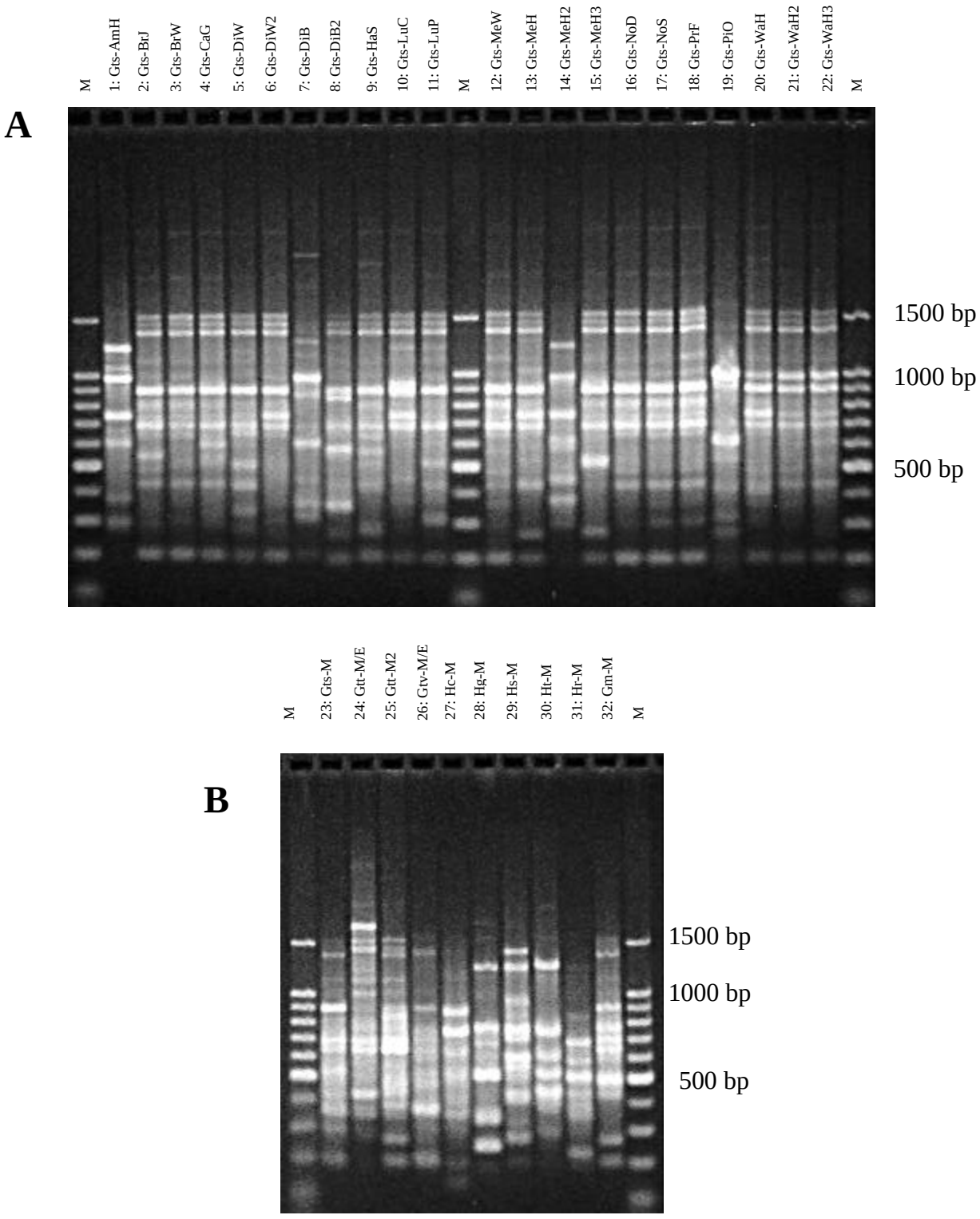
[1] Abbreviations for isolates consist of the first letter of genus and species (Gts = *Globodera tabacum solanacearum*; Gtt = *Globodera tabacum tabacum*; Gtv = *Globodera tabacum virginiae*; Hc = *Heterodera carotae*; Hg = *Heterodera glycines*; Hs = *Heterodera schachtii*; Ht = *Heterodera trifoli*; Hr = *Heterodera rumicis*; Gm = *Globodera "mexicana"*), first two letters of county name (Am = Amelia; Br = Brunswick; Ca = Campell; Di = Dinwiddie; Ha = Halifax; Lu = Lunenburg; Me = Mecklenburg; No = Nottoway; Pr = Prince Edward; Pi = Pittsylvania; Wa = Warren), and first letter of source (grower name or culture collection). M = 100 bp molecular weight marker (Promega)

Figure 2.17: Rep 1 using primer 6. RAPD profiles of 22 isolates of *Globodera tabacum solanacearum* (A), and outgroup (B).



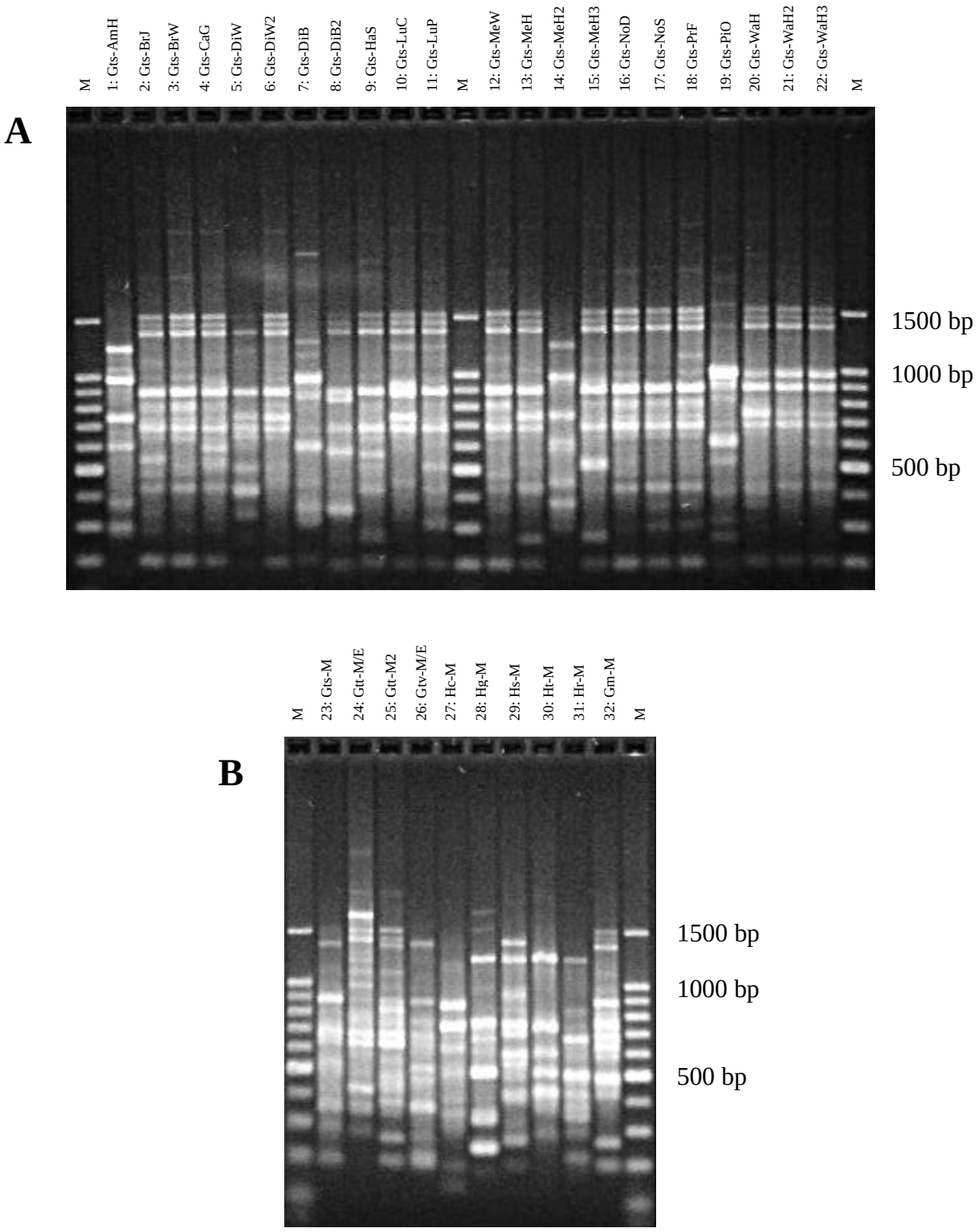
[1] Abbreviations for isolates consist of the first letter of genus and species (Gts = *Globodera tabacum solanacearum*; Gtt = *Globodera tabacum tabacum*; Gtv = *Globodera tabacum virginiae*; Hc = *Heterodera carotae*; Hg = *Heterodera glycines*; Hs = *Heterodera schachtii*; Ht = *Heterodera trifoli*; Hr = *Heterodera rumicis*; Gm = *Globodera "mexicana"*), first two letters of county name (Am = Amelia; Br = Brunswick; Ca = Campell; Di = Dinwiddie; Ha = Halifax; Lu = Lunenburg; Me = Mecklenburg; No = Nottoway; Pr = Prince Edward; Pi = Pittsylvania; Wa = Warren), and first letter of source (grower name or culture collection). M = 100 bp molecular weight marker (Promega)

Figure 2.18: Rep 2 using primer 6. RAPD profiles of 22 isolates of *Globodera tabacum solanacearum* (A), and outgroup (B).



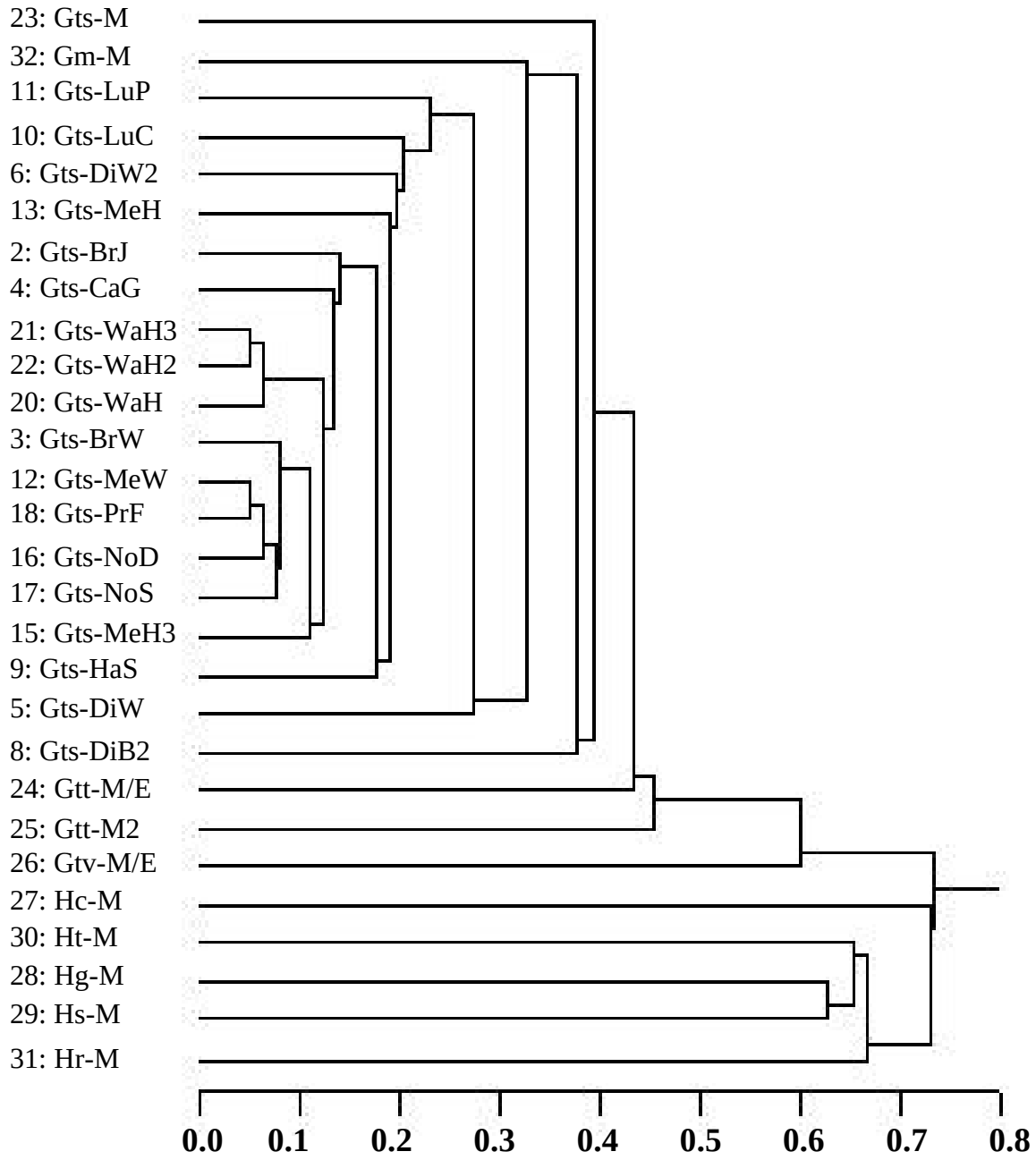
[1] Abbreviations for isolates consist of the first letter of genus and species (Gts = *Globodera tabacum solanacearum*; Gtt = *Globodera tabacum tabacum*; Gtv = *Globodera tabacum virginiae*; Hc = *Heterodera carotae*; Hg = *Heterodera glycines*; Hs = *Heterodera schachtii*; Ht = *Heterodera trifoli*; Hr = *Heterodera rumicis*; Gm = *Globodera "mexicana"*), first two letters of county name (Am = Amelia; Br = Brunswick; Ca = Campell; Di = Dinwiddie; Ha = Halifax; Lu = Lunenburg; Me = Mecklenburg; No = Nottoway; Pr = Prince Edward; Pi = Pittsylvania; Wa = Warren), and first letter of source (grower name or culture collection). M = 100 bp molecular weight marker (Promega)

Figure 2.19: Rep 3 using primer 6. RAPD profiles of 22 isolates of *Globodera tabacum solanacearum* (A), and outgroup (B).



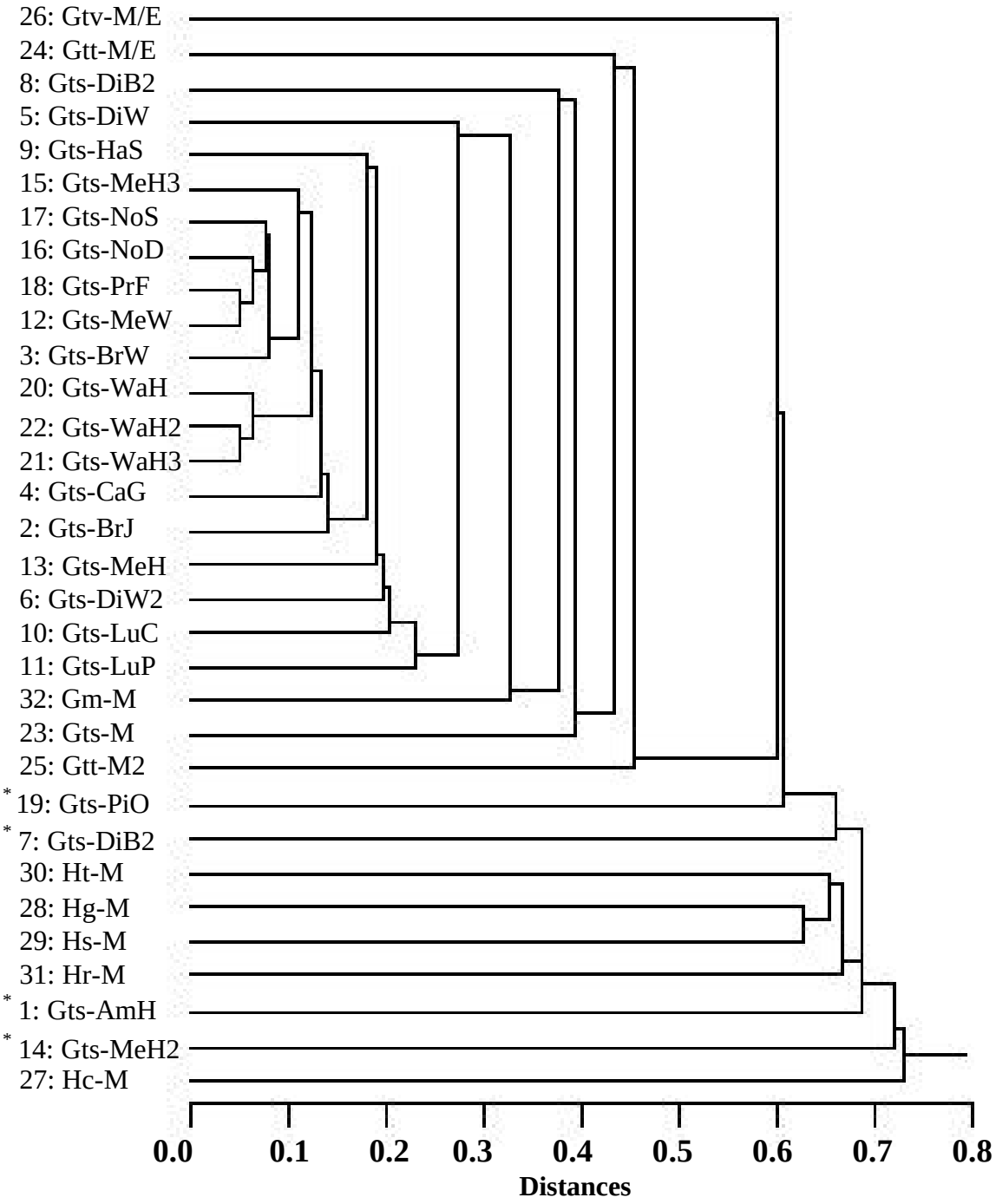
[1] Abbreviations for isolates consist of the first letter of genus and species (Gts = *Globodera tabacum solanacearum*; Gtt = *Globodera tabacum tabacum*; Gtv = *Globodera tabacum virginiae*; Hc = *Heterodera carotae*; Hg = *Heterodera glycines*; Hs = *Heterodera schachtii*; Ht = *Heterodera trifoli*; Hr = *Heterodera rumicis*; Gm = *Globodera "mexicana"*), first two letters of county name (Am = Amelia; Br = Brunswick; Ca = Campell; Di = Dinwiddie; Ha = Halifax; Lu = Lunenburg; Me = Mecklenburg; No = Nottoway; Pr = Prince Edward; Pi = Pittsylvania; Wa = Warren), and first letter of source (grower name or culture collection). M = 100 bp molecular weight marker (Promega)

Figure 2.20: Nearest neighbor clustering for 23 isolates[1] within the *G. t. tabacum* species complex and 5 species within the genus *Heterodera*. Clustering was based on Jaccard's similarity coefficient as calculated with 140 RAPD markers generated from six different random primers.



[1] Abbreviations for isolates consist of the first letter of genus and species (Gts = *Globodera tabacum solanacearum*; Gtt = *Globodera tabacum tabacum*; Gtv = *Globodera tabacum virginiae*; Hc = *Heterodera carotae*; Hg = *Heterodera glycines*; Hs = *Heterodera schachtii*; Ht = *Heterodera trifoli*; Hr = *Heterodera rumicis*; Gm = *Globodera "mexicana"*), first two letters of county name (Am = Amelia; Br = Brunswick; Ca = Campell; Di = Dinwiddie; Ha = Halifax; Lu = Lunenburg; Me = Mecklenburg; No = Nottoway; Pr = Prince Edward; Pi = Pittsylvania; Wa = Warren), and first letter of source (grower name or culture collection).

Figure 2.21: Nearest neighbor clustering for 27 isolates[1] within the *G. tabacum* species complex and 5 species within the genera *Heterodera*. Clustering was based on Jaccard's similarity coefficient as calculated with 140 RAPD markers generated from six different random primers.



[1] Abbreviations for isolates consist of the first letter of genus and species (Gts = *Globodera tabacum solanacearum*; Gtt = *Globodera tabacum tabacum*; Gtv = *Globodera tabacum virginiae*; Hc = *Heterodera carotae*; Hg = *Heterodera glycines*; Hs = *Heterodera schachtii*; Ht = *Heterodera trifoli*; Hr = *Heterodera rumicis*; Gm = *Globodera "mexicana"*), first two letters of county name (Am = Amelia; Br = Brunswick; Ca = Campell; Di = Dinwiddie; Ha = Halifax; Lu = Lunenburg; Me = Mecklenburg; No = Nottoway; Pr = Prince Edward; Pi = Pittsylvania; Wa = Warren), and first letter of source (grower name or culture collection).

* Isolates removed from analysis.

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Chapter 3: Selection of *Globodera tabacum solanacearum* for virulence against a resistant flue-cured tobacco cultivar.

Abstract: A single isolate of *Globodera tabacum solanacearum* was subjected to five generations of selection pressure from the resistant flue-cured tobacco cultivar NC567. The susceptible flue-cured tobacco cultivar K326 was used as a comparison to compute percent reproduction. Variable reproduction of *G. t. solanacearum* was observed on both cultivars over all generations. This variation may be attributed to differences among generations in the time interval between inoculation and cyst extraction, temperature, possible diapause effects, and/or daylength. Ninety-eight cysts were produced in the fifth and final generation compared to the 14 to 50 cysts produced during each of the previous four generations. An increase in reproduction on the resistant versus the susceptible host across the five generations could be due to selection of specific virulence genes. Research involving continuous reproduction on the resistant host for additional generations would be necessary in order to conclude whether or not tobacco cyst nematode (TCN) virulence is being selected.

Key Words: Tobacco cyst nematodes, *Globodera tabacum solanacearum*, Virulence, Resistance, Pathotype

Introduction

The tobacco cyst nematode [*Globodera tabacum solanacearum* (Miller and Gray, 1972) Behrens, 1975] Stone 1983 (TCN) is an economically important soilborne pathogen of flue-cured tobacco (*Nicotiana tabacum* L.) in Southside Virginia, and is present and spreading within North Carolina (Johnson, 1998). Approximately 30% of Virginia's total flue-cured tobacco acreage is infested, with average yield reductions ranging from 15% to 100% (Virginia Impacts, 2001; Komm *et al*., 1983). The estimated annual crop loss and pesticide expense to farmers is $3 million (Rideout, 2000). Current control measures for TCN include crop rotation, sanitation, and use of nematicides (Reed *et al*., 2001). Crop rotation and resistant cultivars can reduce nematicide use by more than 50%, which would save producers over $1 million annually, in addition to the improvement to environmental quality in TCN-infested areas (Virginia Impacts, 2001).

In the past, resistant cultivars performed poorly in terms of yield and quality when compared with susceptible cultivars planted in nematicide-treated soil (Johnson, 1990; Johnson *et al*., 1989). Agronomically desirable cultivars that suppress TCN reproduction have been released recently and are now widely grown throughout Southside Virginia and North Carolina (Johnson, 2002, personal communication). Widespread use of resistance to plant parasitic nematodes creates selection pressure that commonly leads to development of new nematode biotypes (Elliott *et al*., 1986; Triantaphyllou, 1987; Young, 1992). Resistance-breaking biotypes have been reported for potato cyst nematodes (Triantaphyllou, 1975; Kort *et al*., 1977) and soybean cyst nematodes (Caviness, 1992), but have not yet been reported for TCN. Elliot *et al*. (1986) found TCN resistance to be durable over a period of three years, but this study involved only one isolate of the pathogen at one location over a relatively short

period of time. Rideout *et al.* (2000) looked for pathotype differences among 15 geographic isolates of *G. t. solanacearum* from Virginia, Maryland, and North Carolina. No differences were observed among these isolates in nematode development and reproduction on a resistant and a susceptible flue-cured tobacco cultivar, suggesting that different *G. t. solanacearum* biotypes do not exist or exist at extremely low frequencies. This study was also conducted over only one or two generations, and concluded that a longer-term study would more fully document the long-term effectiveness of resistance to *G. t. solanacearum* (Rideout *et al*., 2000). In an eleven generation study, Turner (1990) found the efficacy of resistance to *G. pallida* declined gradually after four to five generations of reproduction on resistant potato hybrids of *Solanum vernei*. In this research, we examined the reproduction of one *G. t. solanacearum* isolate over five generations on a resistant (NC567) and a susceptible (K326) cultivar of flue-cured tobacco.

Materials and Methods

Obtaining a TCN isolate - Soil from a TCN-infested field at the Virginia Tech Southern Piedmont Agricultural Research and Extension Center (SPAREC) in Blackstone, Virginia was sampled using a standard 2 cm diameter soil probe to a maximum depth of 20 cm. To obtain an adequate representation of the nematode population in the entire field, sampling was conducted over the entire area of the field in a criss-cross pattern (Barker *et al*., 1984). The field had been used as a nematicide research plot and had been in tobacco production for the previous two years. Soil was air dried and cysts were extracted using a modified Fenwick can (Caswell *et al*., 1985).

Seedling Preparation - Seeds were germinated in vermiculite and grown in 11 cm-diameter clay pots for approximately four weeks. Individual tobacco seedlings were

transplanted into 11 cm-diameter clay pots containing approximately 300 cm^3 of a 1:1 Profile:field soil (steam-sterilized sandy loam) mix. Profile, made by Schultz®, is a clay soil conditioner designed to improve drainage and aeration in heavy, compacted clay soils, and helps improve water and nutrient retention in dry sandy soils. This soil mix was utilized to improve soil aeration and water drainage for optimal nematode reproduction. Seedlings were allowed to grow for two weeks prior to inoculation for adequate root establishment.

Inoculation - TCN eggs were obtained by crushing cysts in a household blender (Oster Osterizer, Sunbeam® Products, Boca Raton, FL) at high speed for 60 seconds. Inoculation was performed by placing a tap water suspension of approximately 6,000 eggs into a trench around each seedling. The trench was covered with 100 cm^3 of a 1:1 Profile:soil mix to cover exposed eggs and encourage additional root growth (Hayes *et al*., 1997). Plants were lightly hand-watered for the first week after inoculation to ensure that unhatched nematode eggs were not lost through the bottom of pots; thereafter, plants were watered and fertilized using an automated system. Low nematode reproduction on the resistant host during some generations did not allow maintenance of a constant initial nematode inoculum level across all generations. Nematode reproduction during the first generation limited inoculum densities to 5,625 eggs/pot for 10 pots each of the susceptible and resistant cultivar for the second generation. Due to the extremely low reproduction on the resistant host at the second generation, the third generation consisted of a single pot of NC567 infested with 75 cysts and a single pot of K326 infested with 3,600 eggs. Three pots of K326 were also each inoculated with 6,000 nematode eggs obtained from the second generation from K326. Low nematode reproduction in the third generation on NC567 limited inoculum for the fourth generation to 2,340 eggs applied to a single pot of NC567. Nine pots of NC567 were also infested with

6,000 eggs/pot using eggs extracted from cysts that had developed on K326 after two generations on NC567. Inoculum for a fifth consecutive generation on the resistant host NC567 consisted of all of the eggs from all of the cysts that had developed on the single plant of NC567 from the fourth generation. Cysts exposed to resistance in generations 1-2, the susceptible host in generation 3, followed by exposure to resistance in generation 4 were also again crushed and inoculated onto K326 for a fifth generation. Two plants of K326 were inoculated with 6,000 eggs/pot for a fifth consecutive generation of reproduction on a susceptible host.

Experimental Design - Ten plants of each variety were inoculated for the first generation. The number of replicates for each subsequent generation depended on the quantity of cysts obtained from the previous generation. For example, if only 4,000 eggs were obtained from all 10 plants of NC567, then only 1 pot would be inoculated with 4,000 eggs in the next generation. Because some generations consisted of only one culture and were not replicated, a formal statistical analysis was not possible. Correlations were observed between generations and non-statistical conclusions were drawn.

Data Collection - Approximately seventy days after inoculation for each of five generations, watering was discontinued and the soil was allowed to dry. The pots were emptied and roots and soil were separated. For both cultivars, cysts were extracted from soil, air-dried, and counted under a dissecting microscope. For the susceptible control (K326), one gram of feeder roots was sampled, and for the resistant cultivar (NC567), two grams of feeder roots were sampled for a better representation of the low occurrence of nematode penetration and feeding. Root samples were washed and stained with acid fuchsin, according to Byrd *et al*. (1983). The number of vermiform (juveniles that had successfully penetrated roots

without obvious feeding), swollen (nematodes with a distinct sausage shape), pyriform (flask-shaped nematodes), and adult (saccate nematodes bearing eggs) nematodes were distinguished (Rideout *et al*., 2000; Wang *et al*., 1999).

Results

Average reproduction on the resistant cultivar NC567 in generation 1 was 7.35% of the susceptible control K326 (Table 3.1). Fewer cysts were obtained from each cultivar in generation 2 than in generation 1. However, the percent reproduction (38.6%) on the resistant versus the susceptible variety in generation 2 was higher than in generation 1. Reproduction on the resistant variety was 7.08% that of the susceptible control in generation 3, but inoculum for the two cultivars had not been standardized. Four hundred and twenty cysts were recovered from the susceptible variety inoculated with 3,600 eggs from NC567, enough for more replications on NC567 in generation 4. Reproduction was very low in generation 4, with an average of 3.22 cysts recovered from NC567, which was only 3% that of the susceptible control. The nematodes from cysts exposed only to NC567 reproduced and yielded 98 cysts in generation 5, which was 28.57% of the reproduction on the susceptible control.

Discussion

Reproduction was greatest on the susceptible cultivar during the first generation, perhaps due to the phenomenon of diapause, an extreme form of dormancy. Potato cyst nematode eggs can not be stimulated to hatch until diapause has ended, irrespective of environmental conditions (Turner and Evans, 1998). Potato cyst nematodes undergo only one generation per growing season (Bakker *et al*., 1993), whereas TCN can undergo 3 to 5 generations per season (Johnson, 1998; Adams *et al*., 1982), suggesting that diapause may not

be essential for TCN eggs to begin hatching. However, LaMondia (1995) found increased egg hatch from *G. t. tabacum* cysts of variable age recovered from field soil compared to newly produced cysts from plants in growth chambers. The *G. t. solanacearum* eggs used as inoculum for the first generation were extracted from a soil sample taken in March, so the nematodes could have just completed a winter diapause. The lower cyst numbers recovered in subsequent generations could have resulted from decreased egg hatch due to diapause effects. If the lowered hatching observed in subsequent generations was diapause-related, it might have been possible to prevent this problem by chilling cysts (Fisher *et al*., 1981) or by keeping cysts moist between generations. Janssen *et al*. (1987) found that keeping cysts moist circumvented diapause in *G. rostochiensis,* which indicates that desiccation may be a trigger for the induction of diapause.

Although no research has been conducted on TCN diapause, temperature is a proven factor in hatching of TCN (Wang *et al*., 2000; LaMondia, 1995). Because this experiment was conducted throughout the year, generations 1 and 5 occurred in the spring, generation 2 in late summer and early fall, generation 3 in late fall and winter, and generation 4 in winter and early spring. Wang *et al*. (1997) found higher temperatures to be correlated with increased hatching of TCN, but in our research, generation 1 was inoculated in March, when the ambient temperature of the greenhouse was relatively low.

Several yellow and white cysts were observed in generation 2, suggesting that the 52 day period between infestation and extraction was too short. In addition, the average number of cysts produced on the susceptible variety in generation 2 was only approximately 10% that from generation 1. The shorter daylengths of September and October may have slowed nematode reproduction, increasing the time interval necessary to fully capture one full

nematode generation. Franco and Evans (1979) observed increased hatching of PCN when daylength increased from 12 to 16 hours. Salazar and Ritter (1993) also observed high hatching rates under long-day conditions and lower hatching rates under short-day regimes. Hominick (1986) found that the amount and/or intensity of light on plants impacted hatching response of *G. rostochiensis*. The additional time allowed between infestation and extraction in generation 3 (93 days) resulted in increased reproduction.

In a similar experiment using *G. pallida*, Turner *et al*. (1983) observed a general trend of very low initial reproduction followed by increased reproduction. This trend in our results suggests that virulence in TCN may be selected, and higher reproduction may be observed in the next generations. In a study examining reproduction after five consecutive generations on a resistant versus susceptible host, Turner (1990) observed selection to stabilize between generations 9 and 11, in that reproductive potential was indistinguishable on resistant and susceptible test clones. The virulent population did not revert back to a predominantly avirulent population after four generations on a susceptible cultivar, but remained genetically distinct from their original unselected field populations (Turner, 1990).

A complex of major and minor genes mediates resistance in *Solanum vernei*, the parent of the resistant clones used by Turner (1990). In general, multigenic resistance is considered more durable than single-gene resistance, yet it was overcome by a *G. pallida* isolate selected for virulence. Crowder *et al*. (2000) found there to be both a multigenic and a single dominant gene for resistance to *G. t solanacearum* in flue-cured tobacco, depending on the source. Coker 371 Gold, which possesses a single dominant resistance gene, is the parent of many commonly used, recently introduced resistant hybrids. The source of resistance in NC567 is unknown, but if the multigenic resistance of *Solanum vernei* cultivars begins to

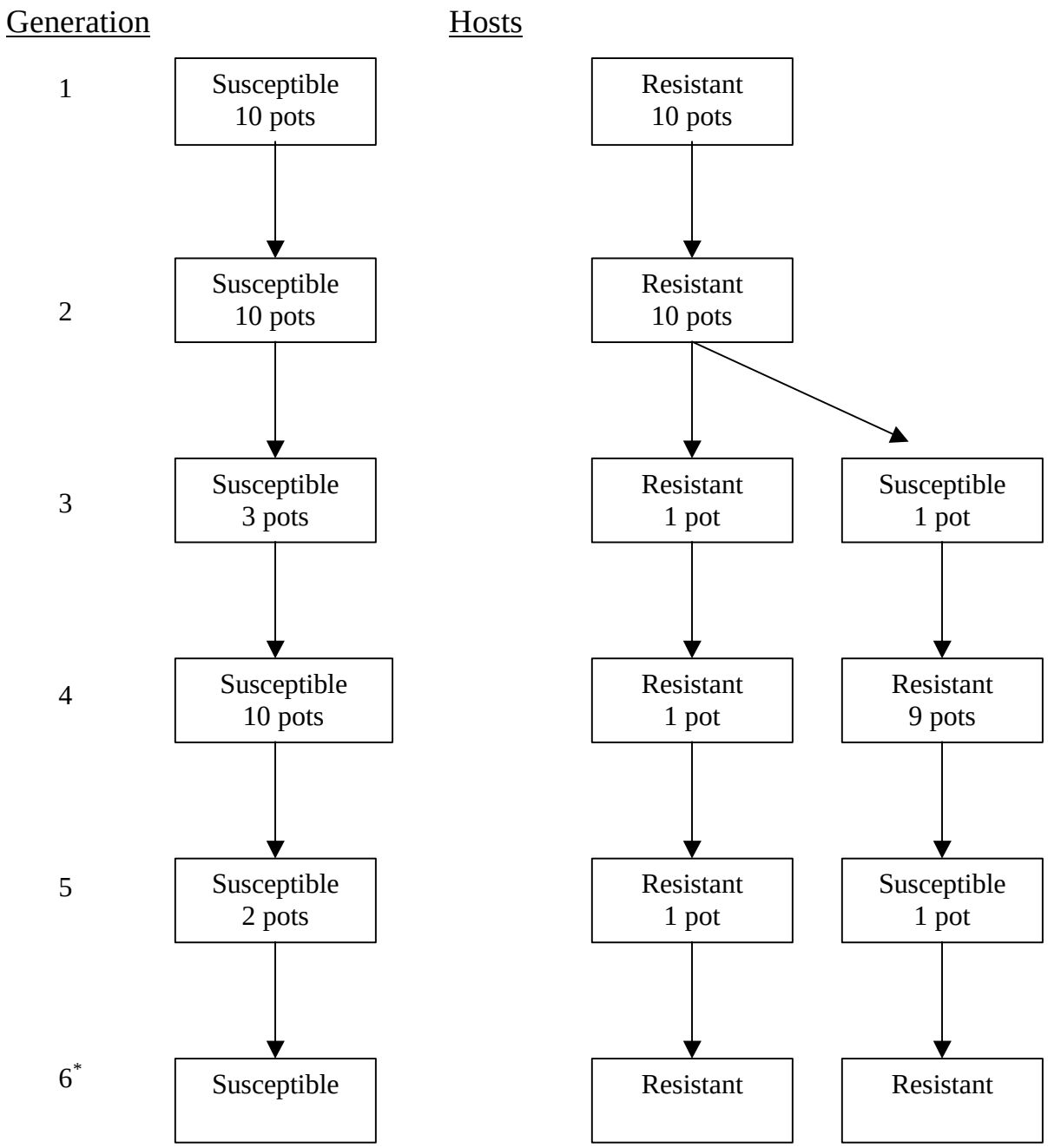
break in only four generations, and resistance appears to be declining in NC567 after five generations, the monogenic resistance of commonly grown flue-cured cultivars may be in jeopardy. Under field conditions, extended rotations should slow the rate of selection, but because TCN undergoes more generations in a growing season, selection for virulence might be faster than observed in PCN. At this point, no definitive conclusions can be made about selection for virulence in TCN. In order to truly evaluate the effects of prolonged exposure of TCN to resistance, this experiment needs to be carried out for at least five more generations.

Table 3.1: *Globodera tabacum solanacearum* development and reproduction on roots and in soil of a resistant (NC567) and susceptible (K326) flue-cured tobacco cultivar.

					Roots					Percent
Generation	Cultivar	Dates	Time	Reps	Vermiform	Swollen	Pyriform	Adult	Cysts	Reproduction
1	K326	Mar 8. - May 16. '01	69 days	10	157.8	2.5	0.8	1.2	685.9	7.35%
	NC567			10	19.7	0.8	0.2	2.1	50.4	
2	K326	Aug. 14 - Oct. 5 '01	52 days	10	18.4	11.8	1.8	0.1	56.8	38.56%
	NC567			10	32.3	18.1	4.2	0.4	21.9	
3	K326	Oct. 6 - Jan. 7 '01-'02	93 days	3	23.47	10.23	2.07	0.87	621.67	7.08%
	K326 *			1	160	68	48	104	420	
	NC567			1	31	3	1	0	44	
4	K326	Feb. 8 - Apr. 26 '02	77 days	10	8.1	1.7	0.4	1.2	107.7	13.00%
	NC567 *			9	0	0	0	0	3.22	
	NC567			1	0	0	0	0	14	
5	K326	Apr. 27 - Jun. 29 '02	63 days	2	154	6	0.5	4	343	28.57%
	K326 *			1	119	3	1	0	360	
	NC567			1	5	1	0	0	98	

* Inoculated with cysts from the opposite cultivar in the previous generation.

Figure 3.1: Flowchart presenting experimental plan for serial inoculation of resistant (NC567) and susceptible (K326) cultivars of flue-cured tobacco.



* Generation 6 is a depiction of the future continuation of this project.

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VITA

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