DNA POLYMORPHISM IN THE DUTCH ELM DISEASE FUNGUS OPHIOSTOMA ULMI

BY

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ABSTRACT

The current devastating epidemics of Dutch elm disease in North America, Europe and south-west Asia are caused by the emergence and spread of a highly pathogenic aggressive subgroup of the fungus Ophiostoma (Ceratocystis) ulmi, which is rapidly replacing the 'old' non-aggressive subgroup, believed to be responsible for milder disease epidemics earlier in the century. The aggressive subgroup had previously been divided into the Eurasian (EAN) and North American (NAN) races on the basis of a range of biological characteristics.

Analysis of both nuclear and mitochondrial DNA using restriction fragment length polymorphisms (RFLPs) distinguished EAN and NAN isolates and allowed an assessment of their genetic variability. It was demonstrated that there is significantly greater variation in the mitochondrial DNA within the NAN race than within the EAN race or non-aggressive sub-group. Both the nuclear and mitochondrial data support a single origin for the two aggressive races. Nuclear RFLPs in conjunction with vegetative compatibility group and epidemiological data are consistent with the view that the NAN race may well have evolved in North America from isolates of the EAN race imported from Romania or the Soviet Union.

It has also been demonstrated that within the NAN race, large vegetative compatibility groups remain homogeneous with respect to nuclear DNA RFLPs through considerable periods of time. Indeed, they appear to remain clonal from epidemic front regions through to post epidemic regions, apparently as a result of selective pressures. Finally, it was shown that in epidemic front and relatively recent post epidemic regions occasional, otherwise typically aggressive isolates, exhibited some non-aggressive nuclear or mitochondrial DNA polymorphisms. This would appear to suggest that in areas where the non-aggressive sub-group is endemic and the aggressive races invade there is at least a limited possibility of hybridisation and recombination, or at least hyphal anastomosis followed by cytoplasmic DNA transfer, between the two sub-groups.

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INTRODUCTION

The spread of Dutch elm disease is one of the theories postulated to explain the well documented 'Elm Decline' in Britain and continental Europe around 3000 BC (Watts, 1961; Hove, 1968; Smith and Pilcher, 1973). Though it is unlikely that such a historical scenario will ever be proven, it is beyond doubt that Dutch elm disease has been responsible for the death of millions of elms in Europe, North America and south-west Asia during the present century (Gibbs, 1978).

Dutch elm disease appeared in Europe around 1920 (Guyot, 1921) and in North America in the late 1920s (Clinton and McCormick, 1936; Peace, 1960; Gibbs, 1978). During the following two decades it became epidemic in both regions. Though damaging however, this epidemic was considerably less serious than that which has occurred during the 1970's and 1980's.

Due to the devastating effect of the disease, the causal fungus Ophiostoma ulmi, has been the subject of intensive mycological, pathological and population studies, particularly during the last twenty years.

It was the intention of this study to investigate the population genetics, epidemiology and evolution of the disease by making use of DNA restriction fragment length polymorphisms (RFLPs). The execution of this project has been greatly facilitated by the availability of the Forestry Commission's international stock collection of more than 4,000 isolates along with accumulated data regarding their subgroup, mating type and vegetative compatibility interactions.

N.B. Where the word 'polymorphism' refers to a single electrophoretic type, this word should read 'morph' or 'morphism'.

1.1 Taxonomy of O. ulmi

The taxonomic name of the causal fungus of Dutch elm disease is now generally accepted as Ophiostoma ulmi (Buisman) Nannfeldt. It is a member of the Ascomycotina, subclass Pyrenomycetes, and a member of the family Ophiostomataceae. The members of the Ophiostomataceae are distinguished by differences in the morphology of their ascospores and perithecia.

Ophiostoma ulmi has a synnematal imperfect stage which was originally named Graphium ulmi, now Pesotum ulmi (Schwartz) (Crane and Schoknecht, 1973), and a perfect stage originally called Ceratostomella ulmi (Buisman) which was renamed Ceratocystis ulmi (Moreau, 1952). Recently however it has been renamed Ophiostoma ulmi on the basis of fungicide tolerances and cell wall composition. It was first noted by Fergus (1956) that resistance to the fungicide actidione (cycloheximide) was possessed by several members of the Ceratocystis genus. Rosinski and Campana (1964) demonstrated the presence of both chitin and cellulose in the cell walls of Ceratocystis ulmi and Jewell (1974) extended this work to include many members of the Ceratocystis and Europhium genera. As a result he proposed that cell wall composition be used as a taxomnomic character. After comparing the distribution of tolerance to cycloheximide with the Ceratocystis classification based on morphology, Harrington (1981) found a good correlation both to morphological characters and presence of cellulose in the cell walls. De Hoog and Scheffer (1984) reappraised the situation including data from anamorph characters and concluded that a distinction between Ceratocystis sensu strictu and Ophiostoma was necessary. Elliott Horner et al., (1986) have also concluded that Ophiostoma should include the majority of species, previously ascribed to Ceratocystis, which have the unusual characteristics of cycloheximide tolerance and containing cellulose and rhamnose as well as chitin in their cell walls.

Attempts have been made to classify the Ceratocystis group using serological techniques (Amos and Burrell, 1966), and the use of

monoclonal antibodies for differentiating between subgroups of Ophiostoma ulmi is being investigated (Dewey et al., 1989).

1.2 Biology of Ophiostoma ulmi

Ophiostoma ulmi has a sexual and an asexual stage, a yeast-mycelial dimorphism, and a mycelial-mycelial dimorphism (see later). In addition there are four recognised spore stages, whose inter-relationships are illustrated in Fig. 1.1 and which provide great scope for work in developmental genetics (Brasier, 1988a).

The asexual spores (conidia) are borne either on the vegetative mycelium or form on a synnema. Synnematal spores, often referred to as the Pesotum or Graphium state, are borne on a sticky, creamy white spore drop at the tips of black synnematal stalks about 1-2 mm high. The spores are hyaline, single celled and oval, measuring 2-5 x 1-3 um, the synnemata being attached to the substrate by dark basal rhizoidal hyphae. Mycelial spores were originally termed the Cephalosporium state though the term Sporothrix state is now preferred. In this state clusters of conidia form embedded in a mucillagenous drop on short side branches or conidiophores. These conidia are also hyaline and single celled, but more elongated, often with one side curved convexly and commonly 4-6 x 2-3 um.

In Ophiostoma ulmi the 'yeastlike' stage is produced by the budding off of single cells. The choice between yeast-like growth or mycelial propagation may be influenced by nutritional factors (Kulkarni and Nickerson, 1981) such as the availability of proline, though these results have not been confirmed. In addition Muthukumar and Nickerson (1984) established that the induction of the yeast phase in proline-containing medium depends on the absence of calcium ions and they suggested dimorphism might be controlled by a calcium-calmodulin system (Muthukumar et al, 1985). However they were unable to find differential levels of calmodulin in the two types of growth. Smalley (1962) has shown that water availability is also important, and when grown on solid complete medium growth is mainly mycelial, whereas in shaken liquid cultures yeast-like growth predominates.

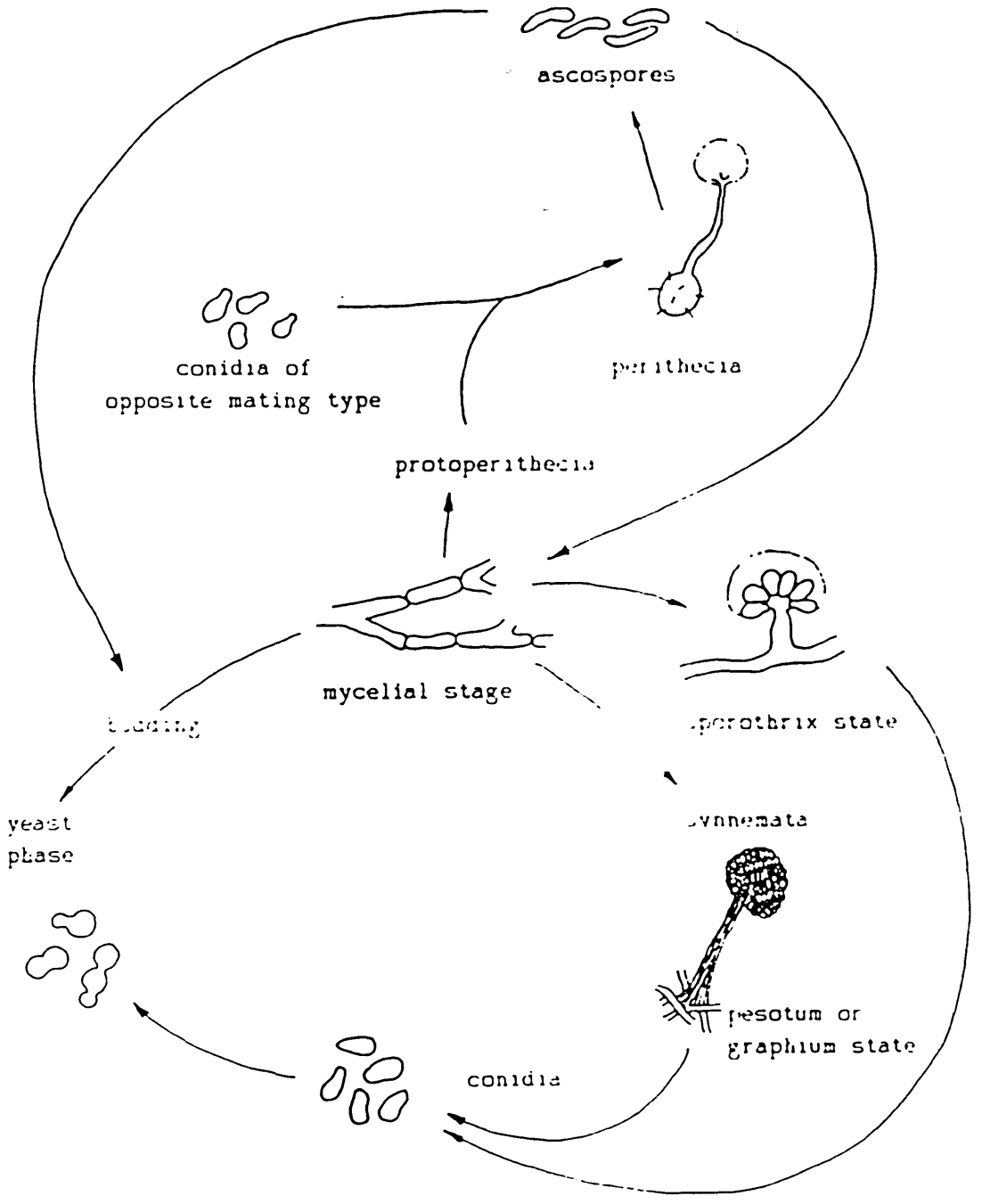

Fig. 1.1. Spore stages of Ophiostoma ulmi

The sexual stage is referred to as the Ophiostoma stage (previously Ceratocystis). The fungus is heterothallic with two mating types, known as A and B (Buisman, 1932; Swingle, 1936; Shafer and Liming, 1950). Both produce protoperithecia which develop into perithecia after fertilization by spores from an individual of the opposite mating type. The perithecia are black with a flask shaped base commonly 100-150 um wide and a long neck up to 350 um long. The sexual spores (ascospores) which are hyaline, single celled and slightly reniform, are produced within evanescent asci and are about 4.5 x 1.5 um. The ascospores of O. ulmi, in common with other members of the Ophiostomataceae, are embedded in a gummy mass which absorbs water and swells to emerge from the ostiole at the tip of the perithecium. There they form a sticky spore droplet supported by a ring of ostiolar hyphae. Perithecia are attached to the substrate by dark rhizoidal hyphae.

1.3 Disease Cycle of Ophiostoma ulmi

Dutch elm disease is a vascular wilt disease in which initial wilting of the foliage on affected branches is followed by yellowing and browning of the leaves and ultimately dieback of the twigs and branches. The cycle of the disease is illustrated in Fig. 1.2.

In streetsides and hedgerows transmission of the fungus may sometimes occur via root grafts, but in most cases it is facilitated by beetle vectors. In Europe three species of the Scolytus beetles have been demonstrated to initiate new disease in healthy trees, namely Scolytus scolytus, S. laevis and S. multistriatus. In North America S. multistriatus, introduced from Europe around 1910 (Chapman, 1910), and the indigenous Hylogropinus rufipes are the principle transmitters.

During the summer and autumn months adult females bore into the bark of diseased elms to form breeding galleries along the length of which they lay their eggs at intervals. The larvae make secondary tunnels approximately at right angles to the mother gallery whichform a characteristic pattern (Fig. 1.2). The mycelium of the fungus grows

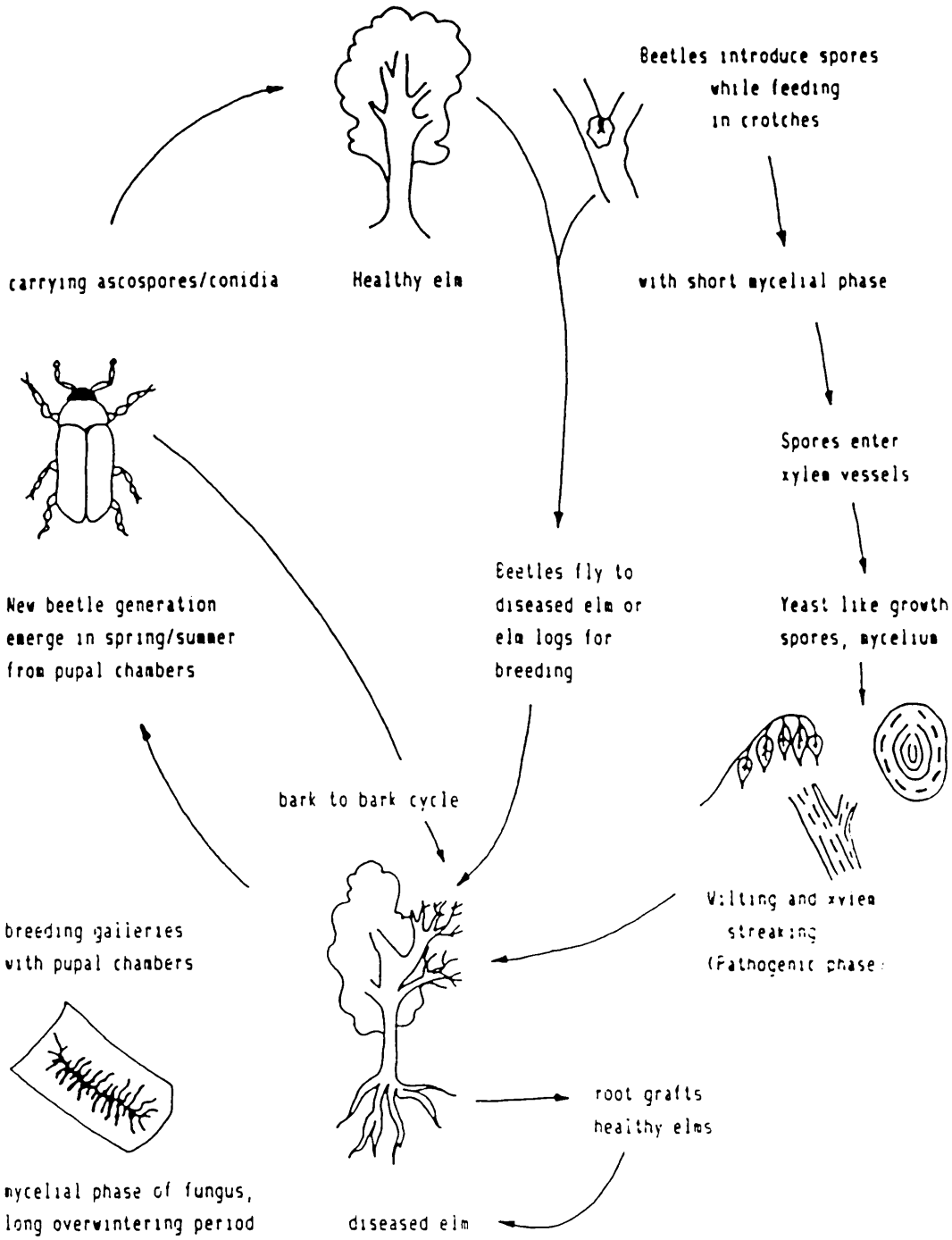

Fig. 1.2. Diseae cycle of Ophiostoma ulmi

within these breeding galleries and the surrounding bark producing asexual and sexual spores.

There is a succession of three main spore stages during an overwintering period within the breeding galleries. Synnemata decline during this period while perithecia are at a maximum in December and January. Mycelial conidia are at a high level at around the time the beetles emerge between May and October (Lea and Brasier, 1983).

The beetles emerge from the breeding galleries carrying the fungal inoculum from the pupal chamber (Webber and Brasier, 1984), the inoculum consisting mainly of conidia, ascospores and mycelial fragments, and then fly to healthy elms which they may infect through the feeding grooves they cut into twig crotches. It would appear however that though these grooves are the source of new infection in healthy trees, beetles may also initiate a purely saprophytic as opposed to pathogenic bark to bark cycle by flying directly to new breeding material (Webber and Brasier, 1984) where they then excavate new breeding galleries without intermediate crotch feeding.

Through twig crotch feeding the fungus enters the xylem where it exists in a yeast-like pathogenic phase. This yeast phase facilitates the propagation of the disease through the whole tree via the vascular system, and results in the xylem streaking and wilting which is characteristic of the disease. Initial growth of the pathogen may be supported by available xylem sap nutrients though further nourishment can be obtained through the action of cell wall degrading enzymes produced by the pathogen Elgersma (1983). A variety of work has shown that enzyme preparations from aggressive isolates release more arabinose and rhamnose from elm material than those from non-aggressive isolates strongly suggesting that cell wall degrading enzymes are involved in pathogenesis. It has been suggested that these enzymes in concert with other host and pathogen derived growth substances may induce tyloses which may play a role in the blocking of water transport. However, gelation of primary cell walls by acidification may be partly responsible for impeding water flow (Elgersma, 1983).

Research on the production of toxins by O. ulmi has resulted in the isolation of four groups of phytotoxic compounds; namely

phenolics, polysaccharides, a protein named cerato-ulmin and a glycopeptide. The former two compounds are thought to have little or no effect in this case, although cerato-ulmin production in shake cultures has been reported to be correlated with aggressiveness and when administered to elm cuttings it induces wilting, chlorosis and necrosis. In addition phytotoxicity of a partially purified glycopeptide fraction in elm shoots has been reported, and is thought to act by physically plugging pit membranes (Scheffer, 1983).

The trees weakened by the disease become in turn ideal breeding sites for the beetles. Regarding the fungal cycle in the disease it has been hypothesised that a continuous saprophytic bark to bark phase, as described above, may mean that individuals of O. ulmi may become trapped within the pathogenic phase and thus make no further contribution to the saprophytic phase and thus to beetle inoculum, even after the death of the tree. Such a sequence has been questioned on evolutionary grounds (Webber and Brasier, 1984). It had been shown by Brasier (1982a; b) that there is significant pathogenic variation within the O. ulmi subgroups, and by Lea (1977) that host selection may favour more pathogenic genotypes. Unless the pathogenic phase contributes to the beetle inoculum and is consequently fed back into the fungal gene pool pathogenic fitness is unlikely to be maintained within the population since there would be little opportunity for host mediated selection to occur.

Experiments using genetically marked isolates carried out by Webber and Brasier (1984) clearly demonstrated the existence of two distinct disease cycles; the first involving the pathogenic xylem phase and the second a solely saprophytic bark to bark cycle. They also showed that when O. ulmi from the pathogenic phase is fed back into the saprophytic gene pool, genetic recombination occurs prior to the emergence of the next generation of beetles (Webber et al., 1989). It was suggested that the majority of this recombination probably occurs during the flush of perithecial formation in the bark during the winter months. They concluded that on emergence the beetles would carry a significant proportion of inoculum derived directly or via recombination from the fungus which had killed the tree.

1.4 Elm Susceptibility

Ever since the first outbreaks of Dutch elm disease early this century, a great deal of effort has been made to assess the resistance of different elm species and breed resistant varieties. There are at least 32 species of elm within the northern hemisphere, of which Europe and Central Asia has five, North America eight and the Himalayas and Eastern Asia 23. The highest levels of resistance are found among Asian elms such as Ulmus parvifolia (chinese elm), U. pumila(siberian elm) and U. wilsoniana though several other Asian elms show resistance. By contrast only one species of European elm U. carpinifolia, and in North America only the relatively unimportant U. crassifolia have some resistance (Heybroek, 1981; Ozolin and Kryukova, 1980).

Gibbs et al. (1975) assessed the percentage of the crown showing leaf symptoms and the percentage of main stem dieback after 8 and 12 weeks respectively among a range of elm species following inoculation with a number of aggressive and non-aggressive isolates. Of particular interest is that relative to the major British and Mediterranean species U. procera, the disease develops much more rapidly in the predominant Eastern and Central North American species U. americana. Furthermore U. americana is highly susceptible to the non-aggressive subgroup, whereas U. procera is relatively very resistant. The higher susceptibility of the American elm population may well account for some of the differences between the O. ulmi population structure in North America and Europe, although in North America where there is lower resistance to the non-aggressive by the indigenous elm population, the non-aggressive would appear to be declining as rapidly as in Europe (Brasier, 1987b; Gibbs, 1978; Houston, 1985).

1.5 The Origins of Dutch Elm Disease

Brasier (1990a) has recently reassessed the evidence concerning the origin of Dutch elm disease and in particular the two major epidemics of this century. Following the dramatic appearance of the disease in Europe at the beginning of the present century it became widely believed that O. ulmi had been imported from China. Brasier (1990a) concluded however that this hypothesis was only based on two lines of circumstantial evidence. Firstly, the resistance of eastern Asiatic elm species, and secondly, the great diversity of elm species in China.

Both these facts led to earlier speculation that both the elm family and its attendant pathogens may have evolved together in this region. Brasier (1990a) however, presents evidence which calls such a hypothesis into question. He points out that some forms of the resistant elm U. pumila, which is phenotypically highly variable and very common in China, are known to be highly susceptible to O. ulmi, and several other Chinese elm species are also reported to be susceptible. Furthermore, the disease appears to be absent from Japan, which has long had close links with China, although the Japanese elm flora contains two relatively susceptible species. In the light of all the available evidence Brasier (1990a) favours a European and/or Himalayan origin for the disease.

1.5.1 The First Epidemic of the 20th Century

The first known record of Dutch elm disease, is its discovery in Picardy, France in 1918 (Guyot, 1921). In 1920 the fungus, later named Graphium ulmi, was first isolated from a diseased elm by Schwartz in the Netherlands (Schwartz, 1922), and the first confirmative inoculation experiments were carried out in Germany in 1927 (Wollenweber, 1927). By 1931 the role of the bark beetles in disease transmission had been established (Fransen, 1931) and in the following year the perfect form of the fungus, Ceratostomella ulmi, was reported and its heterothallism demonstrated (Buisman, 1932).

The first symptoms of the disease were recorded in Britain

by 1927 (Peace, 1960) and in North America by 1930, where it appears to have been introduced by a shipment of elm logs from Europe (Clinton and McCormick, 1936; Gibbs, 1978). It is difficult to determine however, whether these reports reflect the rapid spread of the disease, or merely the first observations, since annual rings of felled trees in Britain show evidence of the disease as early as 1912 (Peace, 1960). Contemporary records as interpreted by Peace (1960), Gibbs (1978) and Brasier (1990a) suggest that the first epidemic originated in France, Belgium and Germany, progressed steadily eastwards across Europe and southwest Asia, and was imported into Great Britain and North America to the West (Fig. 1.3).

The first epidemic appears to have peaked in Britain around the mid 1930's and to have gradually declined thereafter (Peace, 1960). In fact, despite the widespread nature of this early epidemic, the damage to the elm population was fairly limited in comparison with the recent epidemic, killing only 10-20 % of the British elm population during the period 1927-60 (Peace, 1960; Gibbs, 1981). In North America there was no remission of the disease in the same way as in Europe, as the American elm population is much more highly susceptible to the non-aggressive subgroup (see Section 1.4).

1.5.2 The Second Epidemic of the 20th Century

In the latter part of this century major changes have occurred in the structure of the O. ulmi population. It was first noted in the late 1960's that there were new outbreaks of the disease in Great Britain and it was soon realised that this marked the onset of a new and far more serious epidemic in which elm mortality rates were almost 100 % among mature trees.

Early studies of this new outbreak revealed that a new more aggressive form of O. ulmi was responsible (Gibbs et al., 1972). Furthermore, it was soon shown that the pathogen existed as two different subgroups, termed aggressive and non-aggressive (Gibbs and Brasier, 1973; Brasier and Gibbs, 1973). The two subgroups have since been shown to have substantial differences in cultural morphology, pathogenicity, toxin and enzyme production, mating type frequency,

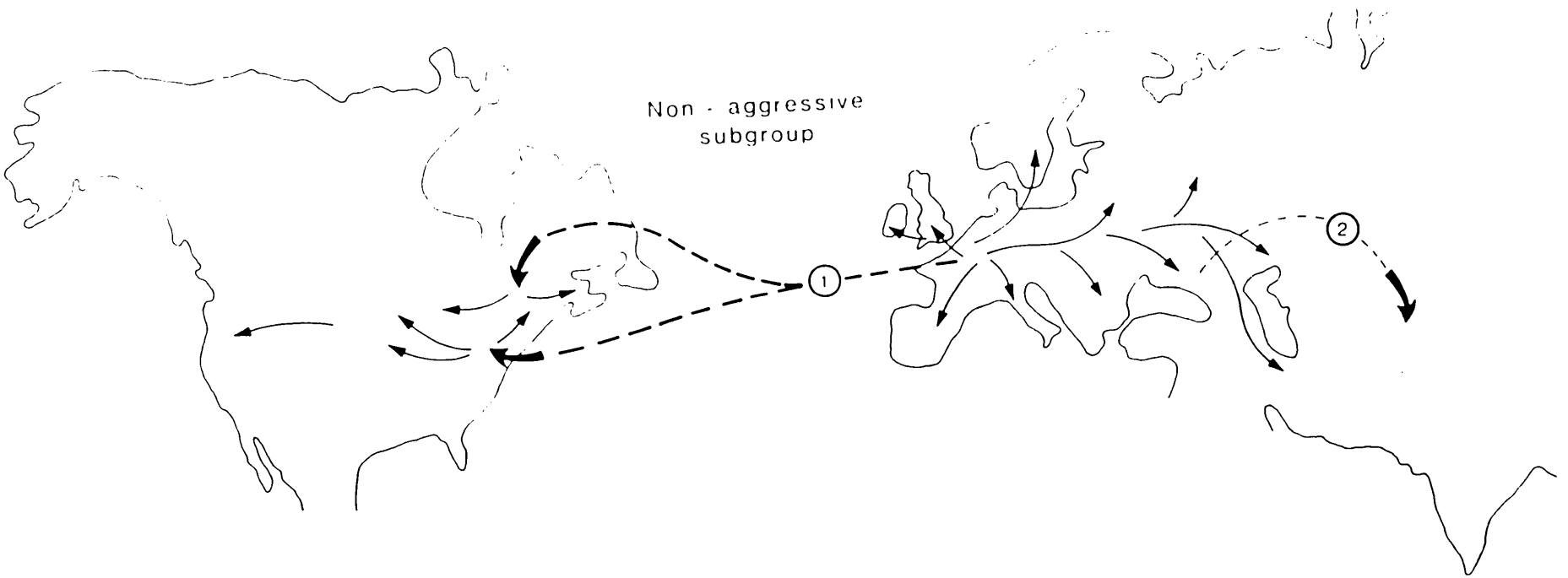

Fig. 1.3. Suggested pattern of spread of the non-aggessive subgroup of O. ulmi during the first epidemic of Dutch elm disease. Small arrows, overland spread. Large arrows, major introductory events as follows: 1, introduction of non-aggressive from north-west Europe to North America, c. 1920's; 2, introduction of non-aggressive from Krasnodar to Tashkent, c. late 1930's. Figure courtesy of Dr. C.M. Brasier (Brasier, 1990a)

growth rate, and temperature optima for growth (Table 1.1). No isolates have been found with truly intermediate phenotypes (Brasier, 1982b).

Brasier and Gibbs (1973) concluded that the non-aggressive subgroup, being apparently endemic to Britain, was responsible for the outbreaks earlier in the century, whereas the aggressive subgroup appeared to have been recently introduced into Britain from Canada through the importation of Rock elm (Ulmus thomasii) timber.

The apparently long standing presence of the aggressive subgroup in Italy, and its discovery in Iran (Brasier and Afsharpour, 1979), suggested a possible division within the aggressive subgroup. Soon after the aggressive subgroup was found to exist as two distinct races (Brasier, 1979; Brasier, 1981; Brasier, 1982b) which were designated the North American (NAN) and the Eurasian (EAN). Moreover it became apparent that that there were two distinct epidemic fronts of the aggressive subgroup in Europe, one spreading from the East (the EAN) and the other from the West (the NAN) - Fig. 1.4. It is these two new aggressive races that have been responsible for the highly destructive epidemic of the last 20 years and which have decimated elm populations across North America, Europe and south-west Asia. Both races are rapidly replacing the 'old' weakly pathogenic non-aggressive subgroup throughout it's range (Brasier, 1983a; 1986a; 1987b).

As regards the origin of the two aggressive subgroups, a presumptive pattern has been suggested by Brasier (1990a) following extensive surveys of the aggressive populations throughout North America, Europe and southeast Asia (Fig. 1.4). He proposes that the NAN may have first appeared in North America during the 1940's, the initial outbreaks occuring in the mid-western United States. He goes on to suggest that this outbreak may have resulted from the introduction of the EAN aggressive via imported elm material and that the NAN then evolved from the EAN as a result of adaptation to the large and highly susceptible American elm population. The subsequent spread of the NAN has recently been reviewed by Brasier (1990a). The NAN spread steadily through the United States and Canada and was then imported into northwest Europe, probably via Britain, in the 1960's

		The Aggressive Strain	
	The Non-Aggressive Strain	North American Race (NAN)	Eurasian Race (EAN)
†Growth rate (mm/day)			
20°C	(1.5) - 2.0-3.1 - (3.5)	3.2-4.8 (-5.5)	3.1-4.4 (-4.8)
33°C	1.1-2.8	0.1-0.5	0 (-0.1)
Optimum temperature for growth °C	30°	20-22°	20-22°
Upper limit for growth °C	35°	33°	32°
Colony morphology (Figs. 2, 3)	from a smooth surface to a lawn of moderately dense relatively undifferentiated aerial mycelium. Weak diurnal zonation.	Fibrous-striate petaloid. Strong diurnal zonation.	Less striate and petaloid, often lobed and uneven. Strong diurnal zonation.
Mating type frequency	A and B types equal	B type predominant	B type predominant
Fertility reaction (when fertilised by other strains/races)	Universal acceptor - accepts non-aggressive and both races of aggressive strain.	Accepts both races of the aggressive strain equally. Strongly rejects the non-aggressive strain.	Partially rejects NAN race. Accepts EAN race. Strongly rejects the non-aggressive strain.
†Pathogenicity (% defoliation on clonal *Ulmus procera*)	10-35% (-40%) Typically recovers	80-100% No recovery	60-100% Recovery occasional
†Cerato-ulmin production	Low	High	ND

*Based on tests carried out under the conditions described in Brasier (1981).

†Data given are common ranges with extremes in parenthesis.

Table 1.1 Characteristics of the three subgroups of O. ulmi. Courtesy of Dr. C.M. Brasier (Brasier, 1982b).

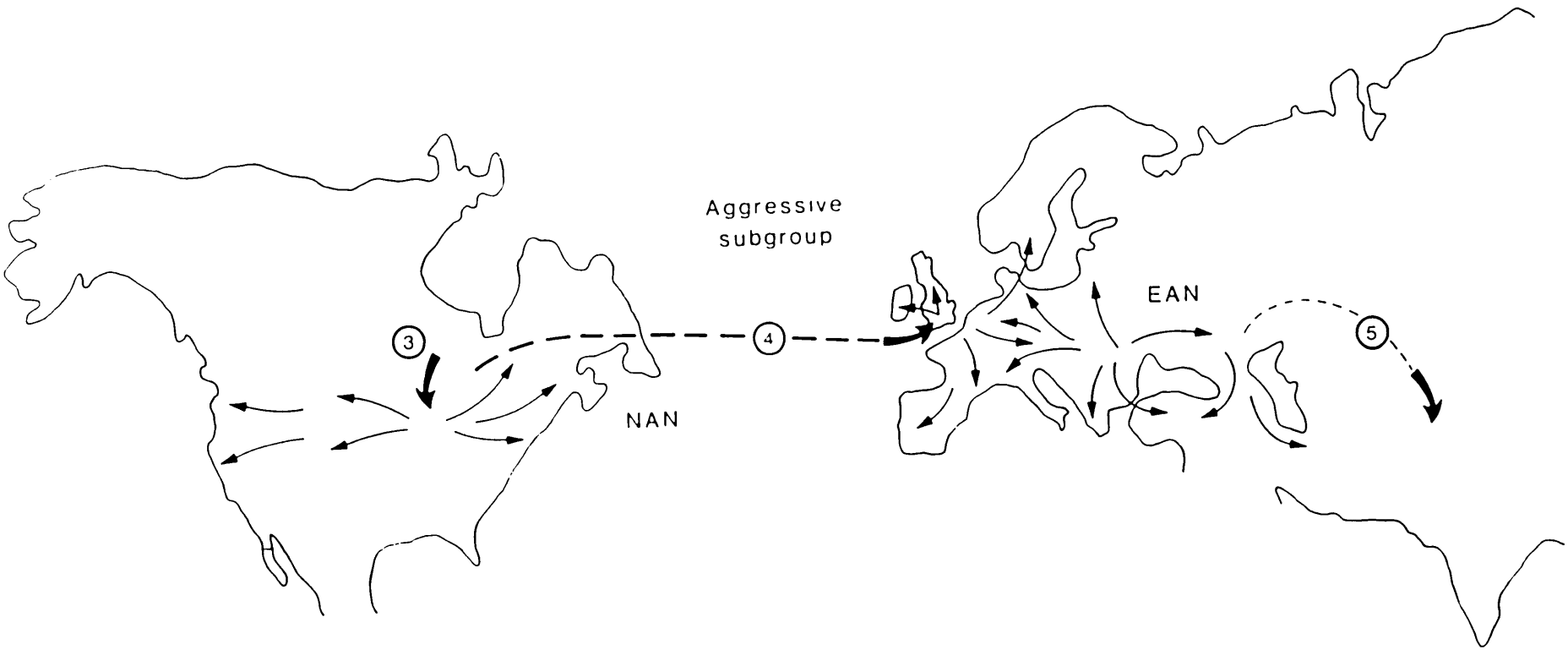

Fig. 1.4. Suggested pattern of spread of the EAN and NAN aggessive subgroups of*O. ulmi* during the second epidemic of Dutch elm disease. Small arrows, overland spread. Large arrows, major introductory events as follows: 3, introduction of a form close to the EAN aggressive into North America (Illinois area), c. 1940's, and its subsequent evolution into the NAN; 4, introduction of the NAN from the Toronto area into Britain, c. 1960; 5, introduction of EAN into the Tashkent area, c. mid 1970's. Figure courtesy of Dr. C.M. Brasier (Brasier, 1990a)

and 1970's. In recent years the NAN epidemic has spread northeast into Scandanavia and south into the Iberian peninsula where epidemic front sites may still be found (Fig. 1.4).

The first evidence of the second epidemic, due to the EAN aggressive may be traced to the 1950's or possibly earlier, in northeast Romania. The EAN is then believed to have spread westward across the Ukraine and also accross Yugoslavia to northern Italy during the 1960's (Brasier, 1990a). Brasier then goes on to record the progress of the EAN further west to Czechoslovakia and Poland in the mid-1970's and to Denmark, East Germany and Southern Sweden in the mid - late 1970's. It had apparently also reached Volgograd to the east by the late 1960's, northern Iran in the 1970's and has spread southeast to Turkey and Greece during the 1970's and 80's (Fig. 1.4).

The first documented appearance of the disease in Central Asia was around 1938 in Tashkent, Uzbekistan, and an isolate collected there in 1969 was found to be less pathogenic than samples from Volgograd (Kryukova, 1972). This suggested that the current Volgograd outbreak is caused by the aggressive while the earlier Tashkent outbreak was probably the non-aggressive. Brasier (1980) sampled the Volgograd area and confirmed that the EAN was the current form present, while a survey at Tashkent in 1986 (Brasier, 1987b) revealed that the EAN is now numerically dominant over the non-aggressive.

Several parts of China, which is geographically separated from Soviet Central Asia by three mountain ranges, were extensively sampled by Brasier in 1987. However, despite the presence of susceptible elm varieties, frequent stressed trees and actively breeding scolytid beetles, no evidence whatsoever of the disease was found, and led Brasier to conclude that Dutch elm disease is most probably absent in China (Brasier, 1990a) though its possible occurrence there cannot be altogether eliminated.

1.6 The Two Races of the Aggressive Subgroup

It is possible to distinguish between isolates of the EAN and NAN races on the basis of a range of characteristics, including colony morphology, growth rate and pathogenicity. and occurrence of a dimorphism in the EAN (Brasier, 1982; 1979; 1986b) - see Table 1.1. This dimorphism is perhaps the most striking difference between the two races. It consists of the ability to alternate between a wild type and a distinctly different uniform powdery mutant ('up-mut') form. It would appear to be a fundamental characteristic of the EAN race, though it is remarkably unpredicatable and is probably responsible for the greater instability and slower growth of EAN wild type isolates in comparison with NAN isolates (Brasier, 1986b).

Also important as a means of distinguishing between the three subgroups however is the presence of unidirectional reproductive barriers which have been described in some detail by Brasier (1979; 1981; 1984). Both races of the aggressive strain as recipients (female) strongly reject the non-aggressive strain as a male mating partner whereas the non-aggressive strain acts as a universal receptor, accepting non-aggressive, NAN or EAN mating partners equally. The barrier between the two races of the aggressive strain is also unidirectional in that the EAN, as recipient, partially rejects the NAN while the NAN accepts the EAN equally with itself. An additional feature of the unidirectional barrier between the non-aggressive and aggressive subgroups is that even when perithecia are produced they are often poorly developed or abnormal. Brasier (1987a) has concluded that the non-aggressive and aggressive subgroups are strongly reproductively isolated and that they should be considered at least subspecies of O. ulmi and possibly sibling species (Brasier, 1990a).

Of particular importance ecologically is to what extent the above mating barriers also operate under natural conditions of mycelial interaction. Using mites, which are thought to be a natural aid to mating in O. ulmi, it was shown that under conditions more

closely resembling those found within the elm bark, the agrressive to non-aggressive mating barrier is virtually total (Brasier, 1978).

1.7 Inter-mycelial Recognition Systems in Ophiostoma ulmi

During the annual disease cycle of Dutch elm disease there are two points where mycelial interaction between the various genotypes may occur. The first is in the feeding grooves, which are cut by the vector scolytid beetles in the elm's twig crotches, and are where a short mycelial phase may be established before the fungus enters the xylem. By far the most important opportunity for mycelial interaction is probably in the essentially saprophytic stage, during the long bark or overwintering phase which may last up to 8-10 months (see Section 1.3). Furthermore it is during this phase that most is known about the structure of the O. ulmi population. Lea (1977) has demonstrated that during the bark phase a three-dimensional mosaic of phenotypic variants exists. Variability between isolates was observed in terms of morphological characteristics, growth-rate and mating type reflecting a dynamic patchwork of genetic variation which is presumably under a host of selective pressures, and also regulated by the inter-mycelial recognition systems of the fungus.

It is the inter-mycelial recognition systems (or compatibility/ incompatibility systems) which are the ultimate determinants of genetic isolation. Consequently these physiological mechanisms promoting 'self-non-self' discrimination could play a major role as a mechanism of evolutionary divergence. In addition these systems are intriguing because of their probable regulatory role in pathogenicity and population structure, quite apart from their intrinsic interest as mycological phenomena.

Brasier (1984, 1988b) describes three known inter-mycelial recognition systems in O. ulmi; namely vegetative-compatibility (vc) interactions, mating type interactions and subgroup incompatibility.

1.7.1 Vegetative Incompatibility

Vegetative (or somatic) incompatibility occurs between individual mycelia, and may be observed in culture by the pairing of O. ulmi isolates on elm sapwood agar. It is a 'self-nonself' recognition system, in many ways analogous to tissue incompatibility

systems in animals, and promotes the breakdown of hyphal fusions between adjacent mycelia. In all, five different reactions are possible which are visually distinguishable. Their features are summarised below:

'w' or 'wide' reaction	Fully incompatible reaction, diffuse white mycelial barrage with synnematal lines of varying intensity parallel to barrage.
'n' or 'narrow' reaction	Narrow denser white mycelial barrage with some synnematal lines close to barrage.
'l' or 'line' reaction	Thin white mycelial barrage line, no synnemata associated with barrage.
'lg' or 'line-gap'	A thin gap or barrage between the two colonies, no synnemata.
'c' or 'compatible'	Either no reaction or slight diffuse mycelial thickening at the junction line of the two colonies.

Isolates of the same vegetative compatibility (vc) group will give a c reaction, and those of different vc groups w, n, l or lg reactions. By far the commonest reaction observed when wild isolates are paired against each other is the w reaction (often in greater than 95% of cases), indicating that the total number of different vc groups is likely to be extremely large.

The vc group of individual isolates is assessed by pairing isolates together on elm sapwood agar. Isolates are inoculated 2cm apart either against a known tester or in all possible combinations, incubated at 20°C (aggressive subgroup) or 30°C (non-aggressive subgroup) for 7-10 days, left in diffuse light for 3-5 weeks and scored. Vegetative incompatibility in aggressive isolates was determined on the basis of a clear w or n reaction. Owing to the poor definition of w and n reactions in pairings of non-aggressive isolates, incompatibility was determined on the basis of a strong mycelial barrage equivalent at least to an n reaction (Mitchell, 1988).

The large number of vc groups found in Ophiostoma ulmi suggests

that vegetative incompatibility is controlled by a bi or multi-allelic and/or polygenic system. This has been demonstrated by the results of back-crossing experiments in which two NAN isolates giving a fully incompatible w reaction were crossed and 20 of the resulting F1 progeny were scored by pairing them against each parent (Brasier, 1984). Two isolates giving the weakest vc reactions were then successively backcrossed to the original parents until all incompatibility was eliminated. The fact that it took four back-crosses before all compatible reactions were achieved suggests polygenic control by at least three or four loci. In addition there is a 1:1 segregation ratio of n to w reactions in the F1 generation and F1 isolates giving n reactions to one parent gave a w reaction against the other and vice versa. This indicates that the n reaction must differ from the w reaction by a single 'w' locus. That a w reaction occurs when two isolates possess different alleles at a 'w' locus and an n reaction occurs when they possess the same w allele is also suggested by the 1:1 segregation ratio. Furthermore, the far greater prevalence of w reactions than n reactions, in random pairings of wild isolates, lends weight to the idea of multiallelism or multiple loci at the 'w' locus.

Such results appear to support a polygenic and multiallelic explanation of the vegetative incompatibility system. However, its final expression is likely to be complicated by differences of a qualitative nature in the interaction of different vc alleles and loci, and the likely influence of modifiers and background genotype. This is exemplified by differences in the morphology of w, n, l and lg reactions in different isolate combinations even when, in the case of lg reaction type, any two isolates appear only to differ by only a single vc locus.

As stated above, the w reaction accounts for around 95% of vegetative incompatibility reactions between pairs of O. ulmi isolates, and in most cases produces lines of synnemata along either side of the diffuse white mycelial zone and into each culture as the w reaction develops. This phenomenon, which may take up to 40 days or more to develop, occurs to a lesser extent in n reactions but is absent in l, lg and c reactions. It has been termed the 'Penetration

effect' by Brasier (1984)

By the use of genetic markers the origin of the synnemata was shown to be that of the opposing isolate, demonstrating penetration of one mycelium by another. Penetration may be entirely uni-directional, equally bi-directional or unequally bi-directional, as well as varying in extent. When cultures of opposing mating type were paired against each other fewer synnemata were produced but the extent of penetration could be gauged by the formation of perithecia either side of the barrage zone (Brasier, 1984).

A number of pairing experiments (Brasier, 1984) revealed that penetrating ability is strongly hierarchical and seems to vary between members of the same vc group. The latter result seems to indicate that penetrating ability is not largely regulated by vc genes. Though mycelial vigour may be involved the nature and origin of any genetic control remains unknown. Other experiments have confirmed that penetration is a result of mycelial introgression and not nuclear migration (Brasier, 1988; pers. comm.).

Where paired isolates are of opposite mating type there are considerable differences in the extent of perithecial production between different vegetative incompatibility reaction types. The production of perithecia appears to mirror that of mycelial penetration in that the average extent of perithecial formation in w and n reactions is comparable to the average extent of synnematal formation in the same reactions. Whereas w reactions produce perithecia to a considerable depth either side of the junction line, n, l, lg and c reactions produce progressively reduced levels of perithecia. Given that the number of ascogonia per unit area available for fertilisation is constant throughout a culture then it was estimated that potential perithecial formation in w reactions would be nearly twice that in n reactions and up to ten times that in c reactions. In other words there is an increase in sexual reproduction potential from c to w reaction type.

1.7.2 Mating Type.

Ophiostoma ulmi was shown to be heterothallic by Buisman (1932), and its two mating types were designated as A and B by Shafer and Liming (1950). It has also been demonstrated that both the wild type mating types are bisexual since both A and B mating type isolates may become female sterile in culture (Holmes, 1977). This second system of inter-mycelial recognition also determines whether or not perithecia develop from ascogonial initials in a vegetative mycelium.

The back-crossing experiments described above to investigate vegetative incompatibility also indicated that the vc loci are genetically unlinked to the mating type locus and thus functionally independent. Mating type was shown to segregate in a normal 1:1 ratio in all four back-cross generations. In fact in most crosses, even between sub-groups, the ratio of A:B types in the progeny is reasonably close to 1:1, with a consistent but slight bias towards B types being thought to be due to a pleiotropic effect of the A mating type in retarding germling growth. All these results suggest that control of mating type is exerted by the presence of two alleles, A and B, at the mating type or mt locus (Brasier, 1984).

1.7.3 Subgroup Interactions.

The characteristics of this form of intermycelial recognition have been described in section 1.5 above. Though this system operates via the mating system it is independent of mating type.

In O. ulmi genetic control of subgroup fertility barriers is little understood as is the case for the majority of fungi. However some research has been carried out by Brasier (1984) on the heritability of the NAN to EAN barrier. The progeny of reciprocal crosses between the EAN and NAN races show a bias towards the NAN fertility phenotype suggesting polygenic nuclear control of hybridization barriers with some non-additive interaction. More recently a possible two locus two allele system has been suggested for the non-aggressive to aggressive barrier (Kile and Brasier, 1990).

1.8 The Role of Inter-mycelial Recognition Systems in *O. ulmi*

1.8.1 Vegetative Incompatibility.

That vegetative or heterokaryon incompatibility systems are widespread within the fungi, particularly in the Ascomycotina and Basidiomycotina, is now apparent (Burnett, 1976, Jennings and Rayner, 1984). However their role and importance within fungal biology remains a topic of much research and discussion. For many years it was was believed that populations of fungi were essentially unit mycelia comprised of physiological mosaics of genotypes subject to considerable levels of nuclear migration and heterokaryosis (Burnett and Partington, 1957; Raper, 1966; Raper and Flexer, 1970). However belief in their existence as 'a single physiological and ecological unit, although genetically a mosaic' (Burnett, 1976) has fallen from grace. Grindle (1963) observed cultural and morphological uniformity within heterokaryon compatibility groups of *Aspergillus nidulans* and Caten and Jinks (1966) suggested that the formation of heterokaryons in the presence of a vegetative compatibility system is likely to be restricted to pairs of isolates which are more or less genotypically identical.

In *O. ulmi* it has been shown that the majority of mycelia in elm bark samples comprise mosaics of different vc groups likely to produce fully vegetatively incompatible w reactions against each other so suppressing the opportunity for heterokaryosis to occur. Furthermore, even when paired isolates are of the same vc group, but have otherwise differing genetic backgrounds, nuclear transfer does not seem to occur (Brasier, 1984). Rather, vegetative incompatibility systems have been proposed as more understandable when considered in terms of territorial competition on the part of the different genotypes with the mosaic being a consequence of the system itself (Rayner *et al*, 1984).

Brasier (1984) has suggested that in the 'penetration effect' in *O. ulmi* (see above), where hyphae of one mycelium apparently penetrate the mycelium of another it may be that this results in the eventual replacement of one genotype by the other. Thus the genetic

mosaic described above may result not only from initial colonization events, but also from subsequent invasions and replacements. Such a situation would extend the concept of territorial defence and competition and has considerable implications for understanding the population dynamics and ecology of O. ulmi and plant pathogens in general.

A further consequence of vegetative incompatibility via the penetration effect may be the restriction of inbreeding. Brasier (1984) has shown that if two isolates are of different mating types penetration serves to promote the fertility of vegetatively incompatible pairings by extending the area within which sexual mating may occur. As mentioned above w reactions are estimated to produce around ten times as many perithecia as c reactions. Since there is likely to be a free recombination of vc loci within a polygenic - heterogenic vegetative incompatibility system, then within a local population fully vegetatively compatible isolates are likely to be the most closely related and fully incompatible isolates the least closely related as regards other loci in their genome. As a result mating between more closely related genotypes will tend to be restricted such that the penetration effect may promote outbreeding in local populations.

Observations of the restriction of cytoplasmic infection by heterokaryon incompatibility in Aspergillus amstelodami (Caten, 1972) and Endothia parasitica (Grente and Sauret, 1969) led to the suggestion that incompatibility systems may serve to prevent cytoplasmic transfer (Day, 1970; Caten; 1972). Caten showed that a reduction in cytoplasmic transfer was positively correlated with increasing heterokaryon incompatibility, and in O. ulmi less than 4 % cytoplasmic d-factor (mycovirus) transfer has been demonstrated between fully vegetatively incompatible isolates compared with 100 % transfer between fully compatible isolates (Brasier, 1984). Given the severely detrimental effect of this cytoplasmic infection in O. ulmi the vegetative incompatibility system may secure the continuing success of the fungus in the face of such a potentially deleterious agency, especially given the widespread opportunities for d-factor spread during mycelial interaction in the mosaics of O. ulmi in elm

bark.

The relative importance of vc in restricting cytoplasmic infection, in promoting outbreeding and in territorial defence, and the extent of their interaction, are difficult to gauge. Indeed their influence is likely to vary at different stages of an epidemic according to the potential for mycelial interaction to occur and possibly mycovirus pressure.

1.8.2 Mating Type.

As in other many other Ascomycetes the two allele single locus mating recognition system of O. ulmi serves to prevent selfing and thus promote obligatory outcrossing, though the regulation of mating competence appears to differ between subgroups. While there is an approximate 1:1 ratio of mating type alleles in the non-aggressive subgroup the A type accounts for only about 15 % in either aggressive subgroup (Brasier, 1984). Although this might be expected to reduce the mating competence of the aggressive population, in practice the low frequency of the A-type may be largely offset by its higher rate of protoperithecial formation. Where at epidemic fronts the scarcer A-type is initially often absent, it may be that pseudoselfing (Brasier and Gibbs, 1975) leads to the initiation of sexual reproduction, such that recombination of background genetic variation occurs.

Perithecial formation and ascospore production are initiated by mating type recognition at various stages of the O. ulmi life cycle and there is evidence that where parental isolates are d-infected, d-factors are not transmitted to the ascospores (Brasier, 1984; 1986b; Rogers et al., 1986b). Thus, sexual reproduction may serve to maintain population fitness by preserving a proportion of the population free from the cytoplasmic infection. Indeed the same phenomena has been demonstrated in other fungi such as Endothia parasitica (Anagnostakis, 1984) and Geaumannomyces graminis (McFadden et al, 1983).

The complex mycelial mosaics of O. ulmi populations in elm bark with the consequent availability of a variety of potential adjacent

mating partners together with the additional opportunity for cross fertilisation provided by the mediation of the microfauna also enhances out-crossing potential. The resulting genetic recombination provides a sufficient reservoir of variation for the maintenance of populations via natural selection (Brasier, 1984).

1.8.3 Subgroup Interactions.

Inter and intra specific fertility barriers are common throughout the fungi and one of their primary effects appears to be the maintenance of genetic isolation which is an aspect of evolutionary divergence. Such divergence may occur either allopatrically as a result of chance genetic drift eg. through geographical isolation, or sympatrically through the more active promotion and maintenance of isolation in the same geographical environment (Burnett; 1975; Burnett, 1983; Brasier; 1987). In O. ulmi the aggressive and non-aggressive subgroups have a whole range of significantly differing characteristics (Table 1.1) and the progeny of forced crosses demonstrate both remarkable morphological variation and negative interaction of continuous characteristics indicating genomic divergence and strong genomic incongruity (Brasier, 1977). Possible rare hybrid types have been found among wild isolates at epidemic front sites and these may be the result of introgression through rare crossing events, though the possibility of somatic recombination cannot be entirely ruled out. Their apparent absence from immediate post epidemic sites suggests that they do not survive, and Brasier (1984) has suggested that this points to a considerable degree of evolutionary divergence between the aggressive and non-aggressive subgroups maintained by a combination of the fertility barriers and natural selection.

Whether the same may apply to the barriers between the NAN and EAN is unclear although Brasier (1984) is of the opinion that the ability of even small reductions in gene flow between sub-populations to promote genetic isolation is often underestimated.

The extent to which the barriers are a consequence of, or a contributing factor to the evolutionary divergence of the subgroups

also remains in question, however Brasier (1984) has suggested that the aggressive / non-aggressive barrier most likely results from ecological specialisation within the same region, whereas the EAN / NAN barrier results from geographical isolation followed by genetic drift.

1.9 Vegetative Compatibility and Population Structure.

The multi-allelic and/or polygenic nature of the vegetative compatibility system in O. ulmi has been described above (see Section 1.6). As a normally outcrossing species, with its two mating types A and B, it is capable of generating large numbers of vc groups. These may be employed as indicators of population heterogeneity.

Initial analysis of a world wide sample of the three subgroups suggested that each of the two aggressive races contained a single dominant vc group, which were termed the NAN and EAN vc 'supergroups' (Brasier, 1984). These groups were found to account for 40 % and 60 % respectively of NAN and EAN isolates, though the vc group was different in each case. In the last few years a different NAN vc supergroup has been identified as the largest single vc group in North America and this has been termed the American NAN vc supergroup (Brasier and Kirk, 1990). The original NAN vc supergroup is dominant in Europe and has been termed the European NAN vc supergroup. In contrast most aggressive non supergroup isolates, tend to be of different unique vc groups and the non aggressive in Europe is now almost totally heterogeneous.

A comparison of a recent epidemic site in Poland and a post epidemic site in Romania revealed that the EAN vc supergroup accounted for about 50 % and 20 % of the samples respectively (Table 1.2). The latter percentage is similar to that found for the NAN vc supergroup in several NAN post-epidemic sites in Britain. The remaining heterogeneous components at both sites consisted almost entirely of different vc groups, though quite a high proportion also carried the same 'w' allele as the supergroup. Furthermore, the

Site and status	Sample size	Uniform or supergroup component			Second vc group		Heterogeneous component			
								vc group frequency		
		% of sample	% A type	% d-infected	% of sample	% A type	% of sample	No. of groups/no. of isolates	Ratio isolates/groups	% A type
a, EAN aggressive sites										
Poland (1980) recent epidemic	52	49	0	31	8	0	43	19/20	1.05	39
Romania (1980) post-epidemic	71	20	0	7	7	0	73	22/26	1.18	12
b, NAN aggressive post-epidemic sites, southern Britain										
Chichester (1982)	40	15	0		—	—	85	12/13	1.08	15
Southampton area	72	19	0	0			82			22
Orsett (1983)	86	13	0	10	—	—	87	14/14	1.00	16
South Essex (1983)	71	10	0	0			90			9
Tewkesbury (1983)	67	16	0	18	16	0	67	9/10	1.11	13
Gloucestershire (1983)	83	7	0	0	10	0	83			7
Total or mean	419	13	0	6			82			14
c, NAN aggressive epidemic front sites, Spain										
Avila area (1985)	74	88	40	3	—	—	12	5/9	(1.80)	(44)
Guadalajara area (1985)	39	95	0	49	—	—	5	2/2	(1.00)	(50)

Table 1.2 Structure of epidemic and post-epidemic population samples. Coutesy of Dr. C.M. Brasier (Brasier, 1988a).

frequency of A mating type isolates was approximately 40 % and 10 % respectively in the heterogeneous components in both the EAN and NAN samples, whereas it was absent in the supergroup components.

The apparently very low level of the A mating type within the supergroup component of post-epidemic aggressive populations led to the suggestion that this portion of the population might be largely asexually propagated, with the heterogeneous component being largely maintained by sexual recombination (Brasier, 1988; Mitchell and Brasier, in preparation). One possible explanation of the presence of the supergroups in the EAN and NAN aggressives as opposed to the non-aggressive is that they are the product of founder effects during epidemic spread. However, their continued presence after many years, despite numerous cycles of sexual outcrossing, indicated that they were perhaps also favoured through some kind of selective advantage (Brasier, 1988; Mitchell and Brasier, in preparation).

The evidence of a higher proportion of supergroup isolates in Poland (recent epidemic) compared with Romania (post epidemic), also suggested that the supergroups could be a characteristic feature of epidemic fronts. To investigate this further Brasier studied a recent NAN outbreak in and around Lisbon (Brasier, 1988; Mitchell and Brasier, in preparation). In 1985 sampling at epidemic front sites outside the Lisbon area revealed only NAN supergroup isolates, all of which were B types. In contrast, at older epidemic sites close to the outbreak centre in Lisbon itself the supergroup component had fallen to around 60 %. A heterogeneous component comprising 25 different vc groups among 40 isolates was also present, and 15 % of these were A types. Not only did this appear to confirm the occurrence of population uniformity at epidemic fronts but suggested the existence of an intermediate stage between a near clonal frontal population and a largely heterogeneous post epidemic population.

The following year in October 1986 an epidemic front site at Tomar and a somewhat older site at Mafra, nearer to Lisbon, were resampled. A number of pathogenic phase (twig) and saprophytic phase (bark) samples were taken from each site. At Tomar both the pathogenic and saprophytic phases now contained a heterogeneous component though this was considerably smaller than that found at the

older Mafra site (Table 1.3). Furthermore, the ratio of isolates to vegetative compatibility groups within the heterogeneous component was over three times greater at Tomar than at Mafra, and the heterogeneous component was significantly larger in the saprophytic phase compared with the pathogenic phase at both sites. It was also observed that around 90 % of the heterogeneous component at the epidemic front site was of the normally rare A mating type, compared with only 20-40 % at the older post epidemic site (Table 1.3).

These results showed that heterogeneity may be generated very quickly, particularly in the saprophytic phase. Though the origin of the new vc groups is still open to speculation, their emergence appears to coincide with a dramatic increase in the A mating type, which being particularly fertile may cause a burst of sexual reproduction. Eventually the population structure appears to stabilise to a situation similar to that found in Romania and at other post epidemic sites; complete displacement of the non-aggressive, a minority purely B type supergroup component and a majority highly heterogeneous component containing a low percentage of A types. It would seem that the Mafra population may already be nearing such a structure.

1.10 D-factors and Other Determinants of Population Structure.

When considering the population structure of Ophiostoma ulmi one must also take into account the influence of the 'd-factor' (Brasier, 1983b; 1986c). This is a cytoplasmically transmissible disease which in one case has been shown to be associated with a virus-like dsRNA fragment (Rogers et al., 1986a; 1986b; 1987; 1988) and which can seriously debilitate the fungus (Brasier, 1986c). It can adversely affect growth, fitness, conidial viability and infection potential towards healthy elms, and is readily transferred via hyphal fusions between individuals of the same vc group. It may also be passed into asexual conidiospores, but is not transmitted into sexually produced ascospores, and is strongly restricted by vegetative incompatibility reactions (Brasier, 1984; 1986c; 1990b).

As a result dominant vc groups such as the European NAN vc supergroup, the Americn NAN vc supergroup and the EAN vc supergroup would seem likely to be at great risk from the spread of these d-factors, especially in the bark phase where there is the greatest chance of hyphal interaction between individuals.

In fact the supergroup components of epidemic front populations at Poland and Tomar, mentioned above, do indeed exhibit high d-infection levels. In the polish sample a minimum 31 % of isolates were d-infected while at Tomar the level was as high as 44 % in the bark phase (Table 1.3). In the pathogenic twig phase at Tomar however there was only 13 % d-infection suggesting strong selection against d-infected isolates in the pathogenic phase. D-infection levels in the supergroup component at post-epidemic sites such as Romania and UK were also considerably lower, at around 7 %.

At Tomar and Mafra there were also significant differences in the level of d-infection between the supergroup and heterogeneous components, and between the pathogenic and saprophytic stages. High levels of infection were not found in the pathogenic phase at either site but there were considerable levels of infection in the saprophytic stage of both the supergroup and heterogeneous components. Furthermore, the infection level among the bark stage heterogeneous component at the older Mafra site was around half that at the more recent frontal site of Tomar. In contrast the infection level in the supergroup component increased significantly from 44 % to 67 % from Tomar to Mafra (Table 1.3).

This led Brasier (1988a) to suggest that the explosion of variability in vc groups at epidemic fronts might be driven, at least in part by d-factor spread in the pathogen clones. As a result it was proposed that the ability of the aggressive subgroups to maintain an intensive epidemic may be partly because there is a sufficiently rapid genetic diversification of the pathogen population to ameliorate the effects of high d-infection levels in the clones.

Similarly, as the generation of new vc groups and the production of d-factor free ascospores through sexual outcrossing would tend to restrict the spread of d-factors, Mitchell and Brasier (in preparation) proposed that debilitation of the dominant frontal

Site	Status	NAN sample size	Uniform or supergroup component			Heterogeneous component				
							vc group frequency			
			% of sample	% A type	% d-infected	% of sample	No. of groups/no. of isolates	Ratio isolates/groups	% A type	% d-infected
Tomar	Current epidemic front									
	Pathogenic phase	59	93	4	13	7	(3/4)	(1.33)	(75)	(0)
	Saprophytic phase	150	64	5	44	36	9/45	5.0	91	38
Mafra	Recent epidemic front									
	Pathogenic phase	57	37	0	0	67	21/29	1 38	19	6
	Saprophytic phase	102	9	33	67	91	52/83	1.60	46	21

Table 1.3 Comparison of current epidemic and recent epidemic NAN aggressive samples from Portugal, 1986. Courtesy of Dr. C.M. Brasier (Brasier, 1988a).

vc clones by high levels of cytoplasmic infection might be a major cause of the rapid change in population structure. Thus, a balance between the fitness advantage of the vc supergroup and the disadvantage of high levels of d-infection may partly determine its frequency within a particular population.

Other results from Tomar and Mafra also provide evidence in support of the idea that the supergroups are the products of selection maintained by asexual reproduction. Pathogenicity of Tomar supergroup isolates compared with heterogeneous component isolates was significantly higher, and the supergroup component is phenotypically uniform whereas the heterogeneous component exhibits considerable variability. In addition there are a number of indications that there is strong selection between the saprophytic and pathogenic phases: There is a smaller heterogeneous component in the pathogenic phase at both sites, a lower frequency in both phases of less pathogenic A types at Mafra, a lower frequency of the weakly pathogenic non-aggressive subgroup in the pathogenic phase at Tomar, and a lower frequency of diseased or d-infected isolates in the pathogenic phase at both sites (Table 1.3).

As a result Brasier (1988a) proposed that at least during the early stages of an outbreak in the pathogenic phase the B mating type supergroup isolates may possess a selective advantage over the range of heterogeneous component genotypes. He suggested that this might aid the perpetuation of the supergroup genotype by ensuring a reasonably high proportion of supergroup isolates during the subsequent saprophytic phase, so promoting its dispersal by the next generation of vector beetles. Finally, a number of environmental changes were proposed which might act as a selection for diversity in the pathogen population and thus result in the decline of the supergroup component. The progression of the epidemic outbreak would result in a general increase in the heterogeneity of the environment, large numbers of small elms, and the eventual collapse and change in structure of the beetle vector population (Brasier, 1988a; Brasier, 1990b).

1.11 Phylogenetic Studies.

Within the fungi, a whole range of characters have been employed in the elucidation of evolutionary relationships. Pre-Darwinian phenetic classifications took into account as many characters as possible and in contrast to phylogenetic classifications did not attach more weight to characters that are assumed to be the result of evolutionary relationships. Traditionally, morphological and cultural characteristics have been the most frequently used characters, and still remain the most important in constructing phylogenies of higher fungal taxa.

In recent years there has been enormous interest in the use of genetic markers. This is especially true when considering the phylogeny of genera, species and subspecies, where morphological differences are often slight or ambiguous. It remains to be seen however, whether the intraspecific variation exhibited by many genetic markers will preclude their widespread utilization in the phylogeny of higher taxonomic groupings. For taxonomic and phylogenetic analyses it is vital that the characters under study exhibit a measure of both diversity and conservation. It is the balance between them which determines the taxonomic level at which any particular marker will be of use (Taylor, 1986).

1.11.1 Protein Polymorphism.

Heritable variation of characteristics has long been employed in phylogenetic studies despite the lack of an understanding of their underlying cause. Although their fundamental determinant is now known to be the DNA sequence of an organism's genome, it is through the mediation of enzymes or other proteins that such variation is generally observed. The advent of molecular techniques has allowed the detection of a variety of enzyme polymorphisms or isozymes - different molecular forms of a protein arising from any cause whether genetic or nongenetic; and in particular allozymes - unique electrophoretic forms of an enzyme or protein determined by a specific allele.

At higher taxonomic levels gel electrophoresis of total soluble proteins has been widely employed, and the introduction of simplified techniques of isoelectric focusing has allowed the higher resolution of arrays of proteins at minimal costs (Hoyle, 1979). This method employs horizontal, thin layer polyacrylamide gel electrophoresis of arrays of buffer-soluble proteins. Adaskaveg et al. (1988) for example, have used isoelectric focusing to look at the relationship of a range of Pythium species. Using Coomassie staining they were able to reliably differentiate between six closely related morphological species, though there was little variation between isolates within a species. Similarly, Erselius and Vallavieille (1984) using the same system, were able to distinguish five species of Phytophthora but found little variation between protein patterns among isolates of each species and isozyme uniformity among worldwide isolates of Erysiphe graminis f. sp. hordei has also been recorded (Clark et al., 1989).

Stasz et al. (1989) studied five morphological species of Trichoderma using allozymes and they found that most alleles were widely distributed among most species rather than being species specific, suggesting that extensive genetic exchange occurred among the taxa. In studies of isozyme variation within and among the host specialised varieties of Leptographium wageneri Zambino and Harrington (1989) found one electrophoretic type was abundant and broadly distributed within each variety. They concluded that in this case the current designation of the three host-specialised varieties was justified and suggested that genetic recombination was rare or absent in nature.

Isozyme analysis by starch gel electrophoresis can be a particularly powerful method for taxonomic, genetic and population studies and its applicability to taxonomic classification has been shown by Newton et al. (1985) who were able to consistently assign isolates of the yellow rust Puccinia striiformis to one of two formae speciales based on allozyme variation though they found no variability within either biotype. A similar lack of variation was found within the rice blast fungus Pyricularia oryzae (Leong and Williams, 1986) even though considerable variation in pathogenicity

had been recorded for many rust and rice blast populations (Burdon and Roelfs, 1985a).

Studying a wild population of Agaricus bisporus, Kerrigan and Ross (1989) found considerable allozyme polymorphism in eighteen field collected isolates from coastal central California. The isolates were genotypically scored at six polymorphic loci and with one exception each isolate was genetically unique within the field sample. In addition, by analysis of Puccinia graminis f. sp. tritici, Burdon and Roelf (1985b) demonstrated the presence of nine different isozyme phenotypes in populations where sexual reproduction was known to occur. This led Clark et al. (1989) to suggest that in Erysiphe uniformity may reflect clonal populations.

That it is possible to distinguish the aggressive and non-aggressive subgroups of O. ulmi using a range of cultural characteristics has already been described. In addition however the two groups have been distinguished by examining their electrophoretic patterns of soluble proteins and specific isozymes (Bernier et al, 1983; Jeng, 1986; Jeng and Hubbes, 1983). This is perhaps relatively unsurprising given the fact that the two subgroups are strongly reproductively isolated from each other. The EAN and NAN subgroups are considerably more closely related than the aggressive and non-aggressive as evidenced by data concerning a whole a range of cultural characteristics (Table 1.1). One might therefore expect there to be more limited differences in their electrophoretic characteristics and indeed this is the case. Jeng et al. (1988) examined a total of seven isozymes and were able to differentiate the EAN and NAN using peroxidase and tetrazolium oxidase. Analysis with of the enzymes alcohol dehydrogenase, esterase, malic dehydrogenase, hexokinase, and phosphoglucomutase however revealed no major differences between the two aggressive races.

1.11.2 Reassociation Kinetics.

The kinetics and stability of DNA:DNA reassociations in solution hybridisation has also been used to estimate evolutionary relatedness although problems of comparison between different studies

may arise as a result of differences in stringency levels. Weber et al., (1986) compared the mitochondrial and nuclear genomes of two Coprinus species. In both cases the majority of the genome did not cross-hybridise indicating a considerable level of sequence divergence between members of the same genus. Nuclear DNA reassociation between five closely related species of the Saccharomyces genus also revealed large sequence divergences supporting the separation of the species into distinct taxa (Martini and Kurtzman (1988). In contrast, within the Ascomycete genus Neurospora, greater than 80 % cross-hybridisation has been demonstrated (Dutta and Ojha, 1972).

1.11.3 Restriction Fragment Length Polymorphisms.

The advent of recombinant DNA technology, and in particular the discovery of type II restriction endonucleases, has allowed the investigation of the most fundamental level of genetic variability; namely variation in the sequence of DNA itself. Type II restriction endonucleases recognise specific nucleotide sequences, cleaving double stranded DNA into a range of different sized restriction fragments. DNA sequence variation between individual organisms consisting of point mutations, large or small scale insertions or deletions, or gross chromosomal rearrangements, may result in differences in the pattern of restriction fragments observed following their electrophoretic separation. Such variations are known as restriction fragment length polymorphisms (RFLPs) and have been increasingly employed for a wide range of genetic analyses.

One advantage of RFLPs over protein polymorphisms is a result of the redundancy of the genetic code. 'Silent' DNA modifications may occur as a result of base substitution such that their amino acid sequence remains unaltered, and is thus undetectable by protein analysis. In addition, variation in the unexpressed regions of DNA such as regulatory sequences, pseudogenes, flanking sequences, introns, and repetitive and satellite DNA may also be examined. Indeed, since such sequences are likely to be less restricted by the functional constraints of coding DNA, the level of polymorphism they

exhibit might be expected to be higher.

Perhaps the most important use of RFLPs is that they provide an enormous pool of genomic markers for genetic analyses. The population and genetic studies of many organisms, including O. ulmi, has been hindered by the lack of easily scorable inherited traits. Furthermore, particularly in the fungi, variation in morphology is often unstable and influenced by external environmental factors. Such instability may also be exhibited by commonly used markers such as fungicide resistance, virulence and mating type. Isozymes, which have been discussed in Section 1.10.1, are valuable in that they demonstrate Mendelian inheritance, are codominant, and are generally uninfluenced by environmental factors. Compared with RFLPs however, the level of polymorphism they reveal is severely restricted.

1.11.4 Mitochondrial DNA Polymorphisms.

Restriction fragment length polymorphisms of mt DNA have been a particularly fruitful source of information for evolutionary and population studies for a variety of reasons. The size of fungal mt DNA varies considerably between and even within closely related species. However, it is still sufficiently small to allow the resolution of all its restriction fragments following gel electrophoresis. Inheritance of mt DNA is usually uniparental, each organism containing only a single mt DNA species. Consequently mitochondrial genomes are essentially haploid making analysis simpler than for the nuclear DNA of diploid organisms. Most important perhaps is the fact that mt DNA appears to evolve at faster rate than that of n DNA (Brown et al., 1979), though it remains unclear whether this is due to a high rate of mutation, to a high rate of fixation, or to both. For these reasons mt DNA is a potentially extremely useful molecule for evolutionary biologists in assessing the relationships among species and populations which have only recently diverged.

The theoretical basis of research in this field has been the molecular clock hypothesis (Kimura, 1969; Wilson et al., 1977) which maintains that fixed mutations accumulate at a constant rate such that differences between the genomes of any two organisms are

proportional to the time that the two genomes have been isolated. The hypothesis has been applied in the study of a range of animal species, from Tetrahymena (Morin and Cech, 1988), to Drosophila (Latorre et al., 1986), pocket gophers (Avise et al., 1979), primates (Ferris et al., 1981) and humans (Cann et al., 1987). In these species the vast majority of mutations are point mutations or nucleotide sustitutions making the interpretation of the polymorphic data at least 'relatively' simple.

In the fungi on the other hand, it has been demonstrated that even within a single species mt DNA may exhibit length mutations (eg. Saunders et al., 1977). This complicates evolutionary analysis since the different types of mutations, such as nucleotide substitutions, length mutations and rearrangements, must be compared independently (Taylor, 1986). In order to differentiate the different mutation types the genome must first be mapped.

Another requirement for the use of mt DNA data in evolutionary studies is that the mode of inheritance is known. Although uniparental inheritance has been demonstrated in several fungi, it is not the rule, and recombination of mt DNA has been demonstrated in several species (Taylor, 1986).

RFLPs in mt DNA have been employed in taxonomic studies as high as the generic level, though in general r RNA homology has been employed above the species level due to highly conserved nature of these gene sequences. Hoeben and Clark-Walker (1986) have utilized gene order and restriction endonuclease sites to assess the relationship of a number of yeast genera, while several groups have investigated sub-generic fungal relationships. Working with several Boletaceae species Bruns and Palmer (1986) concluded that while restriction site changes and length mutations were essential for comparison of closely related species, gene order differences were most useful among more distantly related taxa (Taylor, 1986). Similarly, length mutations had been found to be the most prevalent form of variability among Neurospora species (Collins and Lambowitz, 1983). Weber et al. (1986) demonstrated a two fold mt DNA size difference between two species of Coprinus, along with gene order differences and an apparently complete lack of common restriction

sites, thus lending considerable weight to their assignment in different sections of the genus. Smith and Anderson (1989) were able to distinguish between eight biological species of Armillaria, and Croft (1987) demonstrated distinct mt DNA RFLP maps in five different taxonomic species of the 'nidulans' group of Aspergillus. Of the five, three species, A. nidulans, A. nidulans var. echinulatus and A. quadrilineatus were particularly closely related, exhibiting very few polymorphisms, though their circular genomes were shown to differ in size due to the presence or absence of several optional introns. In Phytophthora megasperma and P. parasitica, members of the oomycetes, Foerster et al. (1987) demonstrated that, despite a common gene order, there were size differences and extensive restriction site polymorphism between these two morphological species, and between two morphologiclly similar formae speciales of P. megasperma.

Below the species level mt DNA polymorphisms have also been widely employed. Garber and Yoder (1984) detected few differences in the mt DNA of geographically diverse isolates of C. heterostrophus, and similar results have been reported for P. infestans (Klimczak and Prell, 1985; Carter et al., 1990) and Bremia lactucae (Hulbert and Michelmore, 1988), while no intraspecific variation at all was found within Aspergillus nidulans or Aspergillus nidulans var. echinulatus (Croft, 1987). In contrast, Specht et al (1983) were able to distinguish between four geographically widely spaced strains of Schizophyllum commune and work with Neurospora has revealed considerable variation between amd within a number of species. Length mutations, nucleotide sustitutions and even size variation are common in Neurospora, suggesting that divergent isolates represent geographically distinct populations (Taylor et al., 1986; Taylor, 1986). This work has also been extended to investigate the phylogeny of heterothallic and pseudohomothallic Neurospora species (Taylor, 1989)

1.11.5 Nuclear DNA Polymorphisms.

Nuclear DNA polymorphisms have been extensively employed in a whole range of genetic studies. Single copy clones of random n DNA fragments are particularly useful for genetic mapping. They may be radiolabelled and used to probe Southern blots of DNA digests separated by agarose gel electrophoresis. The polymorphisms they detect then allow the assignment of the fragments to linkage groups in the same way as other variable genetic markers. One may exclude non-coding DNA by employing clones from a c DNA library and in fact most of the early work on n DNA polymorphisms was on genes of known functions, particularly those responsible for congenital disease in humans.

Closely linked n DNA polymorphisms (haplotypes) have frequently been employed in evolutionary analyses. Wainscoat et al. (1986) for example, studied haplotypes in the human beta-globin gene cluster among individuals from eight diverse populations and observed a major division of between African and Eurasian groups.

Repetitive n DNA sequences are particularly useful in population and epidemiological studies, where large amounts of information may be gleaned from the use of a few clones. Where the frequency of polymorphisms is extremely high, as in the case of hypervariable minisatellite DNA sequences in humans and other organisms, individuals may be 'fingerprinted' - a technique which has widespread implications in forensic medicine and paternity testing as well as biological and ecological research (Jeffreys, 1985). These human hypervariable sequences have successfully detected polymorphisms in dogs, cats, mice, birds, and plants, and recently Braithwaite and Manners (1989) managed to detect DNA polymorphisms in the fungus Colletrichum gloeosporioides. They were able to distinguish between two genetically distinct pathotype populations in Australia, though no polymorphisms within each of the pathotypes was observed.

Repetitive, generally non-coding DNA tends to be much less conserved than fragments of coding DNA and thus for population and taxonomic work probes should be selected according to the genetic

variability which one might expect among the sample under study. Repetitive polymorphic clones have been utilized by O'Dell et al. (1989) in population studies of Erisyphe graminis on barley, oats and rye, while Carter et al., (1990) used random, anonymous single copy clones to investigate relationships among a world wide sample of 24 Phytophthora infestans isolates. They detected sufficient polymorphisms to distinguish between most of the isolates. These probes also detected different ploidy levels and allowed an examination of relative homozygosity and heterozygosity frequencies.

Croft et al. (1987) employed random n DNA clones to investigate the phylogenetic relationships of the of the 'nidulans' group of Aspergillus. They found that such probes were sufficiently polymorphic to efficiently distinguish between different species, but revealed almost no polymorphisms within individual species. Even when variability below the species level was observed RFLPs were only found between and never within heterokaryon compatibility groups.

In a survey of 16 isolates of Armillaria mellea representing 8 biological species, Anderson et al. (1987) utilized ten random n DNA clones to investigate genetic relatedness. They concluded that the polymorphism they encountered essentially confirmed previous species designations based on mating behaviour, there being clear distinctions between species but little or no variation within them. This was taken to be evidence to support the idea of reproductive isolation and genetic divergence between groups in this genera.

Hulbert and Michelmore (1988) have also used random n DNA clones to investigate genetic variation in a diverse geographical sample of 25 Bremia lactucae isolates. The greatest variability was found among European isolates, the region thought to be the centre of diversity of the fungus, and is consistent with the high levels of sexual reproduction noted in this area. They also found evidence of polyploidy or stable heterokaryons an indications of somatic fusion.

METHODS AND MATERIALS

2.1 Abbreviations

bp	base pair
BSA	bovine serum albumin
dist. water	distilled water
DMSO	dimethylsulphoxide
DNase	deoxyribonuclease
ds	double stranded
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid disodium salt
hrs	hour(s)
kbp	kilobase pair
min	minutes
PAGE	polyacrylamide gel electrophoresis
p.s.i.	pounds per square inch
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sec	second(s)
Sevag	chloroform: isoamyl alcohol 24:1 (v/v)
st. dist. water	sterile distilled water
tris	tris(hydroxymethylaminomethane)
U	unit
UV	ultraviolet
v-c	vegetative compatibility
X-gal	5-bromo-4-chloro-3-indole-B-D-galactopyranoside

2.2 Names and Addresses of Commercial Companies

Amersham International: Lincoln Place, Green End, Aylesbury, Bucks HP20 2TP.

BCL Cellophane: Bath Rd., Bridgewater, Somerset TA6 4PA.

BDH: Freshwater Rd., Dagenham, Essex RM8 1RZ.

Beckman: Progress Rd., Sands Industrial Estate, High Wycomb, Bucks HP12 4SL.

Biorad Labs Ltd: Greenhill Crescent, Holywell Industrial Estate, Watford, Hertfordshire, WD1 8RD.

Difco Laboratories: PO Box 14B, Central Ave., East Molesey, Surrey KT8 0SE

Dupont (UK) Ltd: Agricultural Chemicals Department, Wedgwood Way, Stevenage, Herts SG1 4QN.

Gallenkamp: Belton Rd. West, Loughborough, Leicestershire LE11 0TR.

Genetic Research Instrumentation: Gene House, Dunmow Rd., Felsted Nr. Dunmow, Essex CM6 3LD.

Ilford Ltd: Christopher Martin Rd., Basildon, Essex SS14 3ET.

May and Baker: Rainham Rd., South, Daghenham, Essex RM10 7XS

Mini-Instruments Ltd: 8 Station Industrial Estate, Burnham-on-Crouch, Essex CM0 8RN.

MSE (Fisons Scientific Equipment): 41/45 Gatwick Rd., Crawley, Sussex RH10 2UL.

Northumbria Biologicals Ltd: South Nelson Industrial Estate, Cramlington NE23 9HL.

NEN Research Products: Du Pont (UK) Ltd., Biotechnology Systems Division (see above).

Novo Biolabs Ltd: DK 2880, Bagsvaerd, Denmark.

Oxoid Ltd: Wade Rd., Basingstoke, Hampshire RG24 0PW.

Pharmacia: Pharmacia House, Midsummer Blvd., Milton Keynes MK9 3HP.

Rose Chemicals Ltd: 83 Darenth Rd., London N6 6EB.

Sigma: Fancy Rd., Poole, Dorset, BH7 7NH.

Sterilin Ltd: Sterilin House, Clockhouse Lane, Feltham, Middlesex TW14 8QS.

Stratech Scientific Ltd:

	37 Mildmay Grove, London N1.
Whatman Labsales Ltd:	Coldred Rd., Parkwood, Maidstone, Kent ME15 9XN

2.3 Safety Considerations

A laboratory coat was worn at all times and disposable gloves worn when handling dangerous chemicals, radioactive isotopes and carrying out recombinant DNA work. Safety glasses were worn when working with radioactive isotopes and these experiments carried out behind a perspex sceen. A Geiger monitor was used to check potential radioactive contamination. All radioactive waste was disposed of in accordance with the Imperial College Safety Regulations.

An Envair class II microbiological safety cabinet was used for all work involving recombinant bacteria and O. ulmi isolates, and all waste materials and media were autoclaved prior to disposal.

Registration with the Advisory Committee on Genetic Manipulation (ACGM) and the required medical examination were completed for work with recombinant DNA.

2.4 Miscellaneous General Methods

Most laboratory reagents were supplied by Sigma or BDH and were of analytical grade.

All plasticware, glassware and solutions used for recombinant DNA work were sterilized by autoclaving at 15 p.s.i., 121^{o}C for 20 min. Any solutions which could not be autoclaved were filter sterilized through Millipore 0.2 um filters.

Phenol (Rose Chemicals Ltd.) was redistilled and stored in aliquots at -20^{o}C. It was saturated with TE (pH 8.0) before use and stored at 4^{o}C. On occasions 8-hydroxyquinoline was added to a final concentration of 0.1 % (w/v).

Phenol extraction involved mixing one volume of TE-saturated phenol to a sample, vortexing until an emulsion was formed and then

separating the two phases by microcentrifugation for three minutes. The upper aqueous layer was removed into a clean Eppendorf tube and was then usually re-extracted with a 1:1 (v/v) mixture of phenol and Sevag (24:1 (v/v) mixture of chloroform and isoamyl alcohol) and finally with Sevag alone to remove traces of phenol.

Ethanol precipitation involved adding two volumes of cold absolute alcohol to a nucleic acid solution and incubating either on cardice for 20 min, at -70°C for 2 hrs or at -20°C overnight. Solutions of low ionic strength were made up to 0.3 M in sodium acetate prior to addition of ethanol. The nucleic acid was collected by microcentrifugation for 20 min and the pellet washed with 70% (v/v) ethanol and dried under vacuum.

Centrifugations were performed in MSE HS18 or Beckman J2-21 centrifuges for low speed spins and in Beckman L8-55 or L8-70 ultracentrifuges for high speed spins. A Hettich microfuge was used for microcentrifugation of Eppendorf tubes.

The concentrations of DNA samples were determined by comparing measured aliquots of the DNA sample solution against known concentrations of DNA standards in ethidium bromide-stained agarose gels.

2.5 Commonly Used Solutions and Buffers

Denhardt's solution (100x): 2 % (w/v) BSA, 2 % (w/v) Ficoll (Pharmacia), 2 % (w/v) PVP (polyvinylpyrolidone).

Oligolabelling buffer: prepared by mixing solutions A:B:C in the ratio of 100:250:150 by volume.

Solution O: 1.25 M Tris-Cl, 0.125 M $MgCl_2$ at pH 8.0. Stored at 4°C.

Solution A: 1 ml of solution O, 18 ul mercaptoethanol, 5 ul dATP, 5 ul dTTP, 5 ul dGTP (each triphosphate previously dissolved in TE pH 7.0. Stored at -20°C.

Solution B: 2 M Hepes pH 6.6. Stored at 4°C.

Solution C: Hexadeoxyribonucleotides (Pharmacia) evenly suspended in TE at 90 OD units/ml. Stored at -20°C.

TAE: 40 mM Tris, 1 mM EDTA, Adjusted to pH 8.2 with acetic acid.

TBE: 0.09 M Tris, 0.09 M boric acid, 2.5 mM EDTA, pH 8.2

TFB: 100 mM KCl, 45 mM $MnCl_2$, 10mM $CaCl_2$, 3 mM hexamine cobalt (III) trichloride (Sigma), 10mM 2(N-morpholino)ethane suphonic acid pH 6.3 (Sigma), filter sterilized and stored at 4°C.

SSC: 0.15 M NaCl, 0.015 M sodium citrate pH 7.0.

SSPE: 0.18 M NaCl, 0.01 M sodium phosphate, 0.001 M EDTA pH 7.7.

Buffers were adjusted to the correct pH with concentrated HCl or NaOH as appropriate unless otherwise stated. Many of these buffers were made up as 10x or 20x concentrations and diluted appropriately.

2.6 Growth Media and Antibiotics

Malt Extract Agar (MEA): 33 g Oxoid malt extract agar and 10 g Oxoid technical agar per litre of distilled water.

Actidione and Streptomycin Agar (A+S): MEA, as above, containing 0.1 g actidione (cycloheximide) (Sigma) added as a 5 g per litre solution before autoclaving, and 10 ml of streptomycin solution (1 g in 75 ml of st. dist. water) added after autoclaving to the cooled agar.

Minimal Agar: 500 ml 3 % Bacto Agar (Difco), 500 ml M9 salts (6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, dist water to 1 litre), 1 ml 0.1 M CaCl2, 1 ml 1.0 M MgSO4, 0.5 ml 1 % (w/v) vitamin B1 (BDH) and 1 ml 20 % (w/v) glucose.

2xTY Broth: 20 g Bacto Tryptone, 10 g Yeast Extract and 6 g NaCl per litre of distilled water.

2xTY Agar: 2xTY broth containing 16g Bacto Agar (Difco).

Ampicillin (Sigma) sodium salt was prepared as a 20 mg/ml stock in dist. water and stored at -20°C. It was then added to broth and plates to a concentration of 50-100 ug/ml.

X-gal (NBL) was prepared as a 20 mg/ml stock in dimethylformamide and stored at 4°C.

SOB medium:

2.7 Culturing of Isolates

2.7.1 Inoculation of Media

All media were inoculated within a laminar air flow cabinet using a flamed needle.

2.7.2 Stock Culture Maintenance

Short term stock cultures were maintained in MEA Petri dishes, in darkness for up to four weeks or at 20°C or up to eight weeks at 4°C. Long term stock cultures were stored on slopes in bijou bottles at -20°C.

2.7.3 Treatment of Cellophane

10cm diameter discs were cut from Cellophane 525 PU (Donation from BCL Cellophane), boiled for 10 min in EDTA (1 g/litre), washed 3 times in dist. water and then autoclaved for 20 min at 121°C and 15 p.s.i. They were overlaid onto the agar using flamed forceps in a

laminar air flow cabinet and then allowed to dry overnight before inoculating. This appeared to reduce yeast-like growth.

2.7.4 Cultures for DNA Extraction

9 cm Petri dishes containing MEA overlain with cellophane were inoculated with 5 evenly spaced mycelial plugs from a stock plate and incubated at 20°C in darkness. The mycelium was harvested after 14-18 days and either used immediately for DNA extraction or frozen in liquid nitrogen and stored at -70°C.

2.8 DNA Preparation

2.8.1 Large Scale Fungal DNA Preparation

Fungal DNA was prepared by the method of Garber and Yoder (1983) with a few modifications. Around 40-60 g of fungal mycelium was ground using a pre-cooled pestle and mortar with liquid nitrogen to the consistency of a fine powder. The powder was suspended in 250 ml of ice cold STE buffer (0.35 M Sucrose, 50 mM EDTA, 10 mM Tris, pH 7.4) containing 20 ug/ml proteinase K (Sigma). The cell homogenate was centrifuged at 3500 rpm for 10 min at 4°C in an HS18 6 x 250 ml rotor to pellet the cell walls and the supernatant containing the organelles was poured through 2 layers of cheesecloth into a beaker and the pellet discarded. The supernatant was centrifuged at 25,000 rpm in a Beckman SW50 rotor for 48 min at 4°C and the pellets were resuspended gently in 2.5 ml of ice cold lysis buffer (250 mM NaCl, 50 mM EDTA, 10 mM Tris, pH 7.4) containing 20 ug/ml proteinase K. One quarter volume of 10 % (w/v) SDS was then added to yield a final concentration 2 % (w/v) SDS. Lysis of the organelles was promoted by incubating the suspension at 65°C for 40 min. The tubes were left on ice for 5-10 min and then centrifuged at 7,000 rpm in an HS18 8 x 50 rotor for 10 min at 4°C to pellet any non-lysed material. The supernatant was transferred to Eppendorf tubes

microcentrifuged for 15 min at 4°C and then the clear supernatant was decanted and extracted with phenol/Sevag.

2.8.2 Separation of Nuclear (n) and Mitochondrial (mt) DNA

Caesium chloride and the fluorescent dye Bisbenzimide (Sigma) were added to the extracted DNA solution to an adjusted density of 1.6 g/ml and a Bisbenzimide concentration of 120 ug/ml. Polyallomar 13.5 ml tubes were filled with this solution, loaded in a Type 70.1 Ti fixed angle rotor and centrifuged for 16 h at 55,000 rpm. The Bisbenzimide binds preferentially to A-T rich sequences of DNA, which are more common in mt DNA, so further reducing its density relative to n DNA and causing it to band higher up the tube following centrifugation. The DNA bands appeared blue-white under long wavelength uv light and the n and mt DNA bands were removed separately using a syringe. The mt and n DNA were then recentrifuged separately to reduce any cross-contamination and all traces of Bisbenzimide were removed by extracting several times with isopropanol. An equal volume of isopropanol was added to the DNA containing solutions and the tubes rocked until mixing was complete. The upper layer containing solvent and dye was drawn off and discarded and the process repeated a further four times. The DNA was diluted with 3 volumes of 1xTE and precipitated at -20°C overnight following addition of 0.05 volume of 8 M LiCl and 2 volumes of absolute ethanol.

The DNA was pelleted by microcentrifugation at 4°C for 10 min. The supernatant was discarded and 40 ul and 400 ul of 1xTE was added to the mt and n DNA pellets respectively. The DNA was allowed to resuspend at 4°C overnight and then finally gently mixed to homogeneity. The DNA was then ready for immediate use or stored at -20°C until required.

2.8.3 Small Scale Fungal DNA Preparation

Small amounts of fungal DNA were prepared by the method of Raeder and Broda et al. (1985) with a few modifications. Around 1-2 g

of fungal mycelium was ground with liquid nitrogen, using a pre-cooled pestle and mortar, to the consistency of a fine powder. Around 250 ug of this powder was transferred to each of a number of 1.5 ml Eppendorf tubes, resuspended in 500 ul of extraction buffer (200 mM Tris-HCl pH 8.5, 25 mM EDTA, 250 mM NaCl, 0.5 % SDS) and then mixed to homogeneity with 350 ml phenol and 150 ml Sevag. Following microcentrifugation for 1 hr at 4°C, the upper aqueous phase was drawn off and transferred to a new 1.5 ml Eppendorf tube containing 25 ul of 10 mg/ml RNase A solution (Sigma). The solution was mixed by shaking, incubated for 10 min at 37°C, then extracted with 1 volume of Sevag. The upper phase was transferred to a new 1.5 ml Eppendorf tube mixed with 0.54 volumes of isopropanol and the immediately visible DNA precipitate was allowed to settle. As much liquid as possible was then drawn off, care being taken not to disturb the precipitate, and the tube was then microcentrifuged for about 5 sec before any remaining liquid was removed. The DNA pellet was washed with 1 ml 70 % (v/v) ethanol and recentrifuged. After removal of the ethanol the DNA was vacuum dried for around 10 min and then 400 ul 1x TE was added to each tube. The DNA was allowed to resuspend at 4°C overnight and then finally mixed gently to homogeneity. The DNA was then ready for immediate use or stored at -20°C until required.

2.8.4 Isolation of DNA from Agarose

DNA was isolated using the 'GENECLEAN' kit (Stratech Scientific Ltd.) according to the manufacturers recomendations. The bands of interest were excised from an ultrapure, low melting point agarose gel, added to 2-3 volumes of a saturated NaI stock solution and incubated at 55°C for 5 min to dissolve the agarose. 'GLASSMILK' suspension was added (5 ul to solutions containing 5 ug or less of DNA and an additional 1 ul for each 0.5 ug of DNA above 5 ug) and then the solution was incubated for a further 5 min. The GLASSMILK/DNA complex was pelleted by microcentrifugation for 5 sec and the supernatant was discarded. The pellet was washed three times with 'NEW' solution, recentrifuging and discarding the supernatant

each time. Finally, the DNA was eluted into 1x TE buffer and stored at -20°C until required.

2.8.5 Small Scale Plasmid DNA Preparation

Single colonies were toothpicked into 10 ml Sterilin tubes containing 1.5 ml of 2xTY broth plus ampicillin, and grown at 37°C overnight with shaking. The cultures were transferred to 1.5 ml Eppendorf tubes and the cells harvested by microcentrifugation for 1 min. The supernatants were discarded and the pelleted cells resuspended by vortexing in 100 ul ice cold 1x TE pH 8.0. Two hundred microlitres 0.2 N NaOH, 1 % (w/v) SDS were added, the contents mixed by inversion and the cells were then incubated on ice for 5 min to lyse the cells. One hundred and fifty microlitres of ice cold 3 M potassium acetate (pH 4.8) was added and the mixture was vortexed and held on ice for a further 5 min. The cell debris was pelleted by microcentrifugation for 5 min and the supernatants were transferred to clean Eppendorf tubes and then extracted with phenol/Sevag. The aqueous phase was recovered and the plasmid DNA precipitated by adding 1 ml of ethanol and incubating at room temperature for 2 min. The DNA was collected by microcentrifugation for 5 min, washed with 70 % (v/v) ethanol, dried under vacuum and then resuspended in 50 ul TE (pH 8.0) containing 20 ug/ml RNase A. The RNA was digested by incubating at 37°C for 30-60 min. The plasmid DNA could then be analysed by restriction enzyme digestion and agarose gel electrophoresis, or stored frozen at -20°C until required.

2.9 Manipulation of DNA

2.9.1 Restriction Enzyme Digestion

Restriction endonucleases were purchased from NBL, Amersham and Pharmacia and stored at -20oC. Reactions were performed in one of four buffers (see below) which were made as 10x stocks and stored at -20°C.

Generally, reactions containing 0.1-1.0 ug plasmid DNA in a 10-20 ul volume of 1x restriction buffer were digested with 2-10 units of restriction enzyme at 37°C for 1-2 h. Large scale digestions of mt DNA, n DNA or total DNA of fungi were usually digested in reactions containing approximately 0.5, 4.0 and 4.0 ug DNA respectively. They were digested with 2 units of restriction enzyme per 1 ug DNA at 37°C overnight.

2.9.2 Agarose Gel Electrophoresis

Biorad electrophoresis equipment was used for analysing DNA samples. Horizontally submerged slab gels were cast in one of four sizes; 'medium' 15 x 15 x 0.5 cm, 'large' 15 x 25 x 0.5 cm or 'mini-gels' 10 x 6.5 x 0.5 cm.

Agarose (from Sigma) was prepared at various concentrations, most commonly between 0.6 and 1 % by boiling in TAE or TBE buffer. It was allowed to cool to about 60°C and then ethidium bromide (10 mg/ml stock stored at 4°C in the dark) added to a final concentration of 0.5 mg/ml. Gels were cast and allowed to set for at least 30 min before being used. Samples (usually 5-10 ul) were mixed with 0.1 vol loading buffer (0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol, 30 % (w/v) glycerol) prior to loading into the gel slots. Electrophoresis in 1x TAE or TBE was at 20 V overnight for 'large' gels, 70-100 V for 3-5 hrs for 'medium' gels and 70-100 V for 30-60 min for 'mini-gels'.

Following electrophoresis, DNA bands were visualised using a short-wave ultra violet transilluminator. Photographs were taken using Pentax MX camera fitted with two Y(K2) yellow filters (Hoya), one UV(0) filter (Hoya) and loaded with Ilford Pan F 35 mm film.

2.9.3 Southern Blotting

DNA fragments separated by agarose gel electrophoresis were transferred to Hybond-N+ (Amersham) membrane, essentially according to maufacturers instructions. Complete digestion of the DNA was confirmed by viewing the ethidium bromide stained gels under UV

light. The DNA was depurinated by incubating the agarose gel in 0.25 M HCl for 15 min with occasional shaking. After rinsing the gel in distilled water it was placed in denaturing solution (1.5 M NaCl, 0.5 M NaOH) so as to completely cover the gel and shaken at room temperature for 30 min. The gel was then rinsed in distilled water, placed in neutralizing buffer (1.5 M NaCL, 0.5 M Tris-HCl pH 7.2, 0.001 % EDTA) and shaken at room temperature for a further 15 min.

A glass dish was filled with blotting buffer (20x SSPE) and a raised glass platform was made and covered with a wick made from three sheets of Whatman 3MM filter paper and saturated with blotting buffer. The gel was placed on the wick, care being taken to avoid trapping air bubbles beneath it, and it was surrounded with cling film to prevent the blotting buffer being absorbed directly into the paper towels above. A sheet of Hybond-N+ membrane was cut to the exact size of the gel and placed on top of the gel, once again taking care to avoid trapping air bubbles. Three sheets of Whatman 3MM paper cut to size and wetted with blotting buffer were placed on the Hybond-N+ membrane, and on top of them were placed a stack of absorbent paper towels about 5 cm in height. A heavy glass plate was placed on top of this to keep the paper towels in position and transfer was allowed to proceed overnight. The glass tray was covered with cling film to prevent evaporation of the blotting buffer.

After blotting was complete the apparatus was carefully dismantled and the membrane was marked with pencil to allow later identification of tracks. To fix the DNA the membrane was removed and placed on a pad of absorbant Whatman 3MM filter paper 2-3 pieces thick soaked in 0.4 M NaOH. After leaving to soak for around 20 min the membrane was rinsed briefly by immersion in 5x SSPE with gentle agitation. The membrane was then immediately used for hybridization or wrapped in cling film and stored at $4^{o}C$ until required.

2.9.4 Dot Blotting

A square grid of pencil dots, 1 cm apart, were marked onto an appropriately sized piece of Hybond-N+ hybridization membrane. Using a pipette 1 ug of each DNA sample was spotted onto the membrane at

the position of the pencil marks in approximately 2 ul aliquots. The membrane was allowed to dry between each aliquot. The DNA was denatured by placing the membrane, DNA side up on a filter paper pad soaked in denaturing solution and left for five minutes. The membrane was then transferred to a second filter paper wad soaked in neutralizing solution and left for 1 min before fixing as described for Southern blotting in section 2.9.2.

2.9.5 Hybridisation

The membrane was sealed in a bag with prehybridization buffer (5x SSPE, 5x Denhardt's solution, 0.5 % SDS) - usually 10 ml for a 15 x 15 cm 'medium' sized membrane - containing a final concentration of 100 ug/ml sheared and denatured salmon sperm DNA. The bag was then incubated for a minimum of 30 min at 65°C with constant agitation.

The oligo-labelled probe (see section 2.10.6 for preparation) was denatured by boiling for 10 min, cooled briefly on ice and added to the bag containing the membrane, resealed and incubated overnight at 65°C with constant agitation.

After hybridization the solution was poured from the bag, the membrane removed and washed by agitation first in 2x SSPE, 0.1 % SDS for 15 min, and then in 1x SSPE, 0.1 % SDS, for 10 min, both at 65°C. The second wash was repeated a second time and if 'very high stringency hybridization' only was required a third wash with 0.1x SSPE, 0.1 % SDS for 10 min at 65°C was carried out.

While still wet the membrane was sealed in a plastic bag and exposed to Kodak X-Omat S film in a light proof cassette with two intensifying sceens. The cassettes were left for as long as necessary at -70°C and the films were then developed using a Kodak X-Omat automatic processor.

2.9.6 Rehybridization

To facilitate the use of the same blot with several new probes, the membranes were kept wet until the previously used probes were removed by immersion in boiling 0.5 % SDS. After the SDS had cooled

to room temperature the membranes were tested for remaining probe by exposing them to X-ray film. Once all the probe had been removed the membranes could then be rehybridized immediately to a different probe, or stored at 4°C until required.

2.9.7 Oligonucleotide Labelling of DNA Probes

All radiolabelled probes were prepared by the methods of Feinberg and Vogelstein (1983, 1984). When the labelling of cloned plasmid inserts was required, around 1-2 ug of plasmid DNA was first cleaved with an appropriate restiction endonuclease and the fragments electrophoretically separated in a 1.5 % low melting point agarose gel (Sigma). The desired insert band was excised cleanly and put into a preweighed 1.5 ml Eppendorf tube and sterile distilled water was added at a ratio of 3 ml water/g of gel. The contents of the tube were then boiled for 7 min to dissolve the gel and denature the DNA. The insert was stored at -20°C until required for labelling, when the gel was reboiled for 3 min and stored for 10-60 min in a 37°C water bath until the labelling reaction was initiated.

The labelling reaction was carried out at 37°C after the sequential addition of the following reagents: Sterile distilled water (to a volume of 50 ul) was added to 5 ul 10x oligolabelling buffer, 2 ul of 10 mg/ml BSA and around 50 ng of DNA in agarose (up to 37.5 ul. Two microlitres of (32P) dCTP, 10 uCi/ul (Amersham) and 2 U of E. coli DNA polymerase I (Klenow fragment) were then added, thoroughly mixed and the incubation begun. The reaction was terminated after around 2 hrs by the addition of 200 ul of stop buffer (20 mM NaCl, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.25 % SDS). The purification of the precursor nucleotide triphosphate from the labelled product prior to hybridization was not necessary.

2.9.8 Determination of Radioactive Incorporation

To determine the extent of radioactive incorporation in labelling reactions, two 1 ul aliquots were pipetted onto Whatman DEAE 81 discs (2.5cm diameter) before the addition of the enzyme, and

1 ul at the termination of the reaction. This third disc and one of the original discs were washed five times, each for five minutes, with 0.5 M disodium hydrogen orthophosphate, twice for one minute with distilled water, and finally rinsed with acetone. After drying the radioactivity on each was counted with a Series 900 mini-monitor (Mini-Instruments Ltd). The percentage incorporation relative to radioactive input could then be calculated. Only probes with an incorporation of greater than 50 % were used.

2.9.9 Bacterial Strains and Plasmid Vectors

Strain JM83 (details requiring different type face) of Escherichia coli was used as the recipient for the Bluescript KS+ plasmid (NBL). It was grown on minimal medium plates and stored sealed with Parafilm at 4°C and the plates were restreaked every 1-2 months. Transformants were maintained on 2xTY plates containing ampicillin and stocks of host strains and transformants were also stored frozen at -70°C in 2xTY made 20 % (v/v) with glycerol.

Bluescript KS+ vector allows recombinant clones to be identified by colour selection. The plasmid contains a non-functional fragment of the E. coli lac gene (including operator, promoter and N-terminal alpha-peptide of beta-galactosidase), which can be induced by isopropyl-beta-D-thiogalactopyranoside (IPTG) and can complement the defective beta-galactosidase gene of JM83 (Gronenborn and Messing, 1978). This activity can be detected using 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (X-gal), a colourless lactose analogue, which is hydrolysed and results in the colonies produced being blue in colour. When a fragment of foreign DNA is inserted into the polylinker cloning site of the vector this disrupts the alpha-peptide and destroys its ability to complement the defective beta-galactosidase activity. Thus the colonies resulting from the transformation of recombinant plasmids are colourless.

2.9.10 Ligation of plasmid DNA

Following digestion with the appropriate enzyme the plasmid vectors were treated with phosphatase to reduce self ligation. After 30-60 min, calf-intestinal phosphatase (BCL) was added to the restriction digest (0.1 U/ul digestion volume) and the mixture incubated for a further 30 min. Reactions were terminated by phenol extraction and precipitation, the pellet being resuspended in TE. The ligation reaction was carried out by addition of the following components: 10 ng phosphatased vector, 10 ng insert DNA, 1 ul 50mM ATP, 1 ul 10x ligase buffer and 1 U ligase, made up to 10 ul with ster. dist. water. The mixture was left to overnight at 4°C prior to transformation.

2.9.11 Standard Transformation Protocol

The method of Hanahan (1985) was used to prepare competent cells for cloning experiments.

A single bacterial colony was picked from a minimal agar plate and used to inoculate 5 ml of 2xTY broth in a 10 ml Sterilin tube. This was then shaken overnight in a 37°C incubator. A 150 ul aliquot of this culture was used to inoculate 30 ml of SOB medium in a 250 ml conical flask and this was shaken at 37°C for 2-3 h, until the optical density at 550 nm had reached 0.45-0.55. The cells were poured into three 10 ml Sterilin tubes, chilled on ice for 10-15 min and then harvested by centrifugation at 1000 g for 10 min at 4°C. The pelleted cells in each tube were drained thoroughly, resuspended in 3 ml of ice-cold TFB and incubated on ice for 10 min before being recentrifuged and resuspended in 0.8 ml ice cold TFB. DnD (1 M DTT (Sigma), 90 % DMSO (BDH), 10 mM KAc pH 7.5 stored at -20°C) was added to 3.5 % (28 ul) and the cells mixed and incubated on ice for a further 10-20 min before aliquoting 200 ml samples into chilled Eppendorf tubes for transformation as desribed below.

2.9.12 Transformation of Cells with Plasmid DNA

Up to 20 ng of DNA in a 10 ul volume was added to 200 ul of competent cells in a 1.5 ml Eppendorf tube. The mixture was incubated on ice for 20-40 min, heat shocked at 42°C for 90 sec and chilled briefly. Then 800ul of 2xTY medium was added and the cells incubated at 37 C with shaking, for 30 minutes. 200 ul samples were aliquoted into 1.5 ml Eppendorf tubes and 50 ul of X-gal was added prior to spreading. The plates were allowed to dry before being inverted and incubated at 37°C overnight. Recombinants were identified by the formation of white colonies as opposed to blue colonies formed by bacteria containing religated Bluescript vector.

2.10 Fungal Isolates

The following isolates were provided by C. M. Brasier (CMB) the isolate numbers being those of the Dutch elm disease culture collection of the Forest Research Station, Farnham, Surrey, UK. The isolates were originally isolated by a number of different workers designated by the following initials:

CMB = Dr. C. M. Brasier. DH = Dr. D. Houston. JG = Dr. J. Gibbs.
MM = Dr. M. Meulemans. ES = Dr. E. Smalley. HH = Dr. H. Heybroek.
JP = Dr. J. Pinon. AM = Dr. A. Mangan. GBO = Dr. G. B. Oullette.
GM = Dr. G. Milbrath. MN = Dr. McNabb. LRS = Dr. L. R. Schreiber.
LB = Dr. L. Bernier. SS = Dr. S. Sumer. HAJ = Mr. H. A. Jorgenson.
HK = Dr. H. Kryukova. LM = Dr. L. Mittempergher. BR = Dr. B. Rossnev.
MK = M. Keyserlingk. DE = Dr. H. Evans. PS = Dr. P. Scard.
JFW = Dr. J. F. Webber. DW = Dr. D. Wainhouse. JJ = Dr. J. Jamnicky.

Countries Be=Belgium Fr=France GB=Great Britain Dk=Denmark Ei=Ireland
Po=Poland Ro=Romania Sp=Spain SU=Soviet Union Yu=Yugoslavia
US=USA Ca=Canada Ho=Holland Tu=Turkey It=Italy Hu=Hungary
Pg=Portugal Bu=Bulgaria Ge=Germany Cz=Czechoslovakia
In=Iran Id=India

2.10.1 Non-aggressive isolates of *Ophiostoma ulmi*

Isolate	Mating Type	Location	Date of Isolation	Provided By
Initial broad geographical sample:				
TR65	B	E. of Giresun, Tu.	1980	CMB
W9	B	GB.	1971	JG
H830	A	Wallingford, Vermont, US.	1980	DH
H812	A	Millinocket, Maine, US.	1983	DH
H173	A	Durham, New Hampshire, US.	1977	JG
PG381	?	Tomar, Pg.	1986	CMB

Yu99	A	S. of Belgrade, Yu.	1980	CMB
R21	A	Bozovici, Ro.	1986	CMB
I170	B	Bronte, Sicily, It.	1979	CMB
Tchat1d	B	Tchatcal, SU.	1986	CMB
H358	?	Jodoigne, Be.	1980	MM

2.10.2 NAN isolates of Ophiostoma ulmi

Isolate	Mating Type	Location	Date of Isolation	Provided By
Initial broad geographical sample:				
H670	A	Lincoln, Maine, US.	1977	ES
H669	B	Berkeley, California, US.	1981	ES
H937	B	Millinocket, Maine, US.	1986	DS
H161	B	Philip, Nova Scotia, Ca.	1977	JG
H6	B	Sloten, Ho.	1972	CMB/HH
C112	B	Chichester, GB.	1982	CMB
O101	B	Orsett, GB.	1983	CMB
H172	A	Keene, New Hampshire, US.	1977	JG
H106	B	Paris, Fr.	1977	JP
H312	B	Gironde, Fr.	1979	JP
ES376	B	Ventosa, Sp.	1984	CMB
H351	A	Tournai, Be.	1980	MM
PG402	B	Tomar, Pg.	1986	CMB
T223	B	Tewkesbury, GB.	1983	CMB
H297	B	Edenbury, County Offaly, Ei.	1978	AM
Selected sample of bark isolates from Tomar, Portugal:				
TOMIj10	B	Tomar, Pg.	1986	CMB
TOMIm4	B	Tomar, Pg.	1986	CMB
TOMIIe5	B	Tomar, Pg.	1986	CMB
TOMIIc4	B	Tomar, Pg.	1986	CMB
TOMIId10	B	Tomar, Pg.	1986	CMB

Selected sample of twig isolates from Mafra, Portugal:

PG461	B	Mafra, Pg.	1986	CMB
PG470	B	Mafra, Pg.	1986	CMB
PG473	A	Mafra, Pg.	1986	CMB
PG493	A	Mafra, Pg.	1986	CMB
PG506	B	Mafra, Pg.	1986	CMB

Selected sample of bark isolates from Mafra, Portugal:

MAFa3	A	Mafra, Pg.	1986	CMB
MAFa12	A	Mafra, Pg.	1986	CMB
MAFc3	A	Mafra, Pg.	1986	CMB
MAFf20	B	Mafra, Pg.	1986	CMB
MAFg4	B	Mafra, Pg.	1986	CMB

Selected sample from Tewkesbury, Great Britain:

T210	B	Tewkesbury, GB.	1983	CMB
T219	B	Tewkesbury, GB.	1983	CMB
T231	B	Tewkesbury, GB.	1983	CMB
T235	B	Tewkesbury, GB.	1983	CMB
T237	B	Tewkesbury, GB.	1983	CMB
T247	B	Tewkesbury, GB.	1983	CMB
T244	B	Tewkesbury, GB.	1983	CMB
T252	B	Tewkesbury, GB.	1983	CMB
T264	B	Tewkesbury, GB.	1983	CMB
T267	B	Tewkesbury, GB.	1983	CMB
T220	B	Tewkesbury, GB.	1983	CMB
T222	B	Tewkesbury, GB.	1983	CMB
T223	B	Tewkesbury, GB.	1983	CMB
T228	B	Tewkesbury, GB.	1983	CMB
T236	B	Tewkesbury, GB.	1983	CMB
T247	B	Tewkesbury, GB.	1983	CMB
T253	B	Tewkesbury, GB.	1983	CMB
T254	B	Tewkesbury, GB.	1983	CMB
T255	B	Tewkesbury, GB.	1983	CMB
T265	B	Tewkesbury, GB.	1983	CMB
T202	B	Tewkesbury, GB.	1983	CMB

T213	B	Tewkesbury, GB.	1983	CMB
T239	A	Tewkesbury, GB.	1983	CMB
T248	A	Tewkesbury, GB.	1983	CMB
T257	A	Tewkesbury, GB.	1983	CMB
T261	B	Tewkesbury, GB.	1983	CMB
T201	B	Tewkesbury, GB.	1983	CMB
T204	B	Tewkesbury, GB.	1983	CMB
T205	B	Tewkesbury, GB.	1983	CMB
T206	B	Tewkesbury, GB.	1983	CMB
T208	B	Tewkesbury, GB.	1983	CMB
T209	B	Tewkesbury, GB.	1983	CMB
T214	B	Tewkesbury, GB.	1983	CMB
T217	B	Tewkesbury, GB.	1983	CMB
T218	B	Tewkesbury, GB.	1983	CMB
T230	B	Tewkesbury, GB.	1983	CMB
T232	B	Tewkesbury, GB.	1983	CMB
T238	B	Tewkesbury, GB.	1983	CMB
T262	B	Tewkesbury, GB.	1983	CMB
T216	B	Tewkesbury, GB.	1983	CMB
T224	B	Tewkesbury, GB.	1983	CMB
T225	B	Tewkesbury, GB.	1983	CMB
T233	B	Tewkesbury, GB.	1983	CMB
T234	B	Tewkesbury, GB.	1983	CMB
T240	B	Tewkesbury, GB.	1983	CMB
T243	B	Tewkesbury, GB.	1983	CMB
T246	B	Tewkesbury, GB.	1983	CMB
T250	B	Tewkesbury, GB.	1983	CMB
T7	A	Tewkesbury, GB.	1973	CMB
T40	A	Tewkesbury, GB.	1973	CMB

Selected sample from Orsett, Great Britain:

096	B	Orsett, GB.	1983	CMB
0103	B	Orsett, GB.	1983	CMB
0127	B	Orsett, GB.	1983	CMB
0138	B	Orsett, GB.	1983	CMB
0169	B	Orsett, GB.	1983	CMB

0182	B	Orsett, GB.	1983	CMB
0121	B	Orsett, GB.	1983	CMB
0109	A	Orsett, GB.	1983	CMB
0128	A	Orsett, GB.	1983	CMB
0145	B	Orsett, GB.	1983	CMB
0174	B	Orsett, GB.	1983	CMB
Selected sample from Chichester, Great Britain.				
C80	A	Chichester, GB.	1982	CMB
C81	B	Chichester, GB.	1982	CMB
Selected sample from Eire:				
H220	B	Dublin, Ei.	1978	AM
H295	B	County Louth, Ei.	1978	AM
H296	B	Eddenburg, Ei.	1978	AM
H298	B	Eddenburg, Ei.	1978	AM
H301	B	Castleknock, Dublin, Ei.	1978	AM
Selected sample from the Guadalahara area, Spain:				
ES122	B	Guadalahara, Sp.	1984	CMB
ES135	B	Guadalahara, Sp.	1984	CMB
ES174	B	Guadalahara, Sp.	1984	CMB
ES598	B	Guadalahara, Sp.	1984	CMB
ES239	B	Guadalahara, Sp.	1984	CMB
ES669	A	Guadalahara, Sp.	1984	CMB
ES78	B	Casa de Campo, Sp.	1984	CMB
ES193	B	Aranjuez, Sp.	1984	CMB
ES204	B	N. of La Guardia, Sp.	1984	CMB
Selected sample from the Avila area, Spain:				
ES113	A	Avila, Sp.	1984	CMB
ES698	A	Avila, Sp.	1984	CMB
ES706	B	Avila, Sp.	1984	CMB
ES736	A	Avila, Sp.	1984	CMB
ES105	A	Santa Celicia, Sp.	1984	CMB

ES382	A	Ventosa, Sp.	1984	CMB
ES697	B	10km N. of San Raphael, Sp.	1984	CMB
Selected sample from Portugal:				
PG34	B	E. of Beringel, Pg.	1985	CMB
PG115	B	N. of Caldas, Pg.	1985	CMB
PG137	B	E. of Porto (Dairo Valley), Pg.	1985	CMB
PG235	B	Porto da Lage, nr. Tomar, Pg.	1985	CMB
PG67	B	Lousa, Pg.	1985	CMB
PG74	B	Malviera, Pg.	1985	CMB
PG94	B	Mafra, Pg.	1985	CMB
PG276	B	Monsanto Park, Lisbon, Pg.	1985	CMB
Selected sample from North America:				
H166	B	Ellsworth, Maine, US.	1977	JG
H170	B	Sandstone, Minnesota, US.	1977	JG
H175	B	Lyons Falls, New York, US.	1977	JG
H667	B	Manhatten, Kansas, US.	1970	ES
H672	A	Minneapolis, Minnesota, US.	1977	ES
H678	B	Brown County, Minnesota, US.	1970	ES
H684	A	Milwaukee, Wisconsin, US.	1982	ES
H842	A	West Bridgewater, Vermont, US.	1983	DH
H2001	B	Salem, Oregon, US.	1988	GM
RR2	B	Toronto, Ca.	1973	CMB
H2076	B	Madison, Wisconsin, US.	1989	ES
H2077	?	Madison, Wisconsin, US.	1989	ES
H2078	B	Madison, Wisconsin, US.	1989	ES
H2079	B	Madison, Wisconsin, US.	1989	ES
H2080	B	Madison, Wisconsin, US.	1989	ES
H2081	B	Madison, Wisconsin, US.	1989	ES
H2082	B	Madison, Wisconsin, US.	1989	ES
H2083	B	Madison, Wisconsin, US.	1989	ES
H2085	B	Iowa, US.	1989	MN
H2086	B	Minnesota, US.	1989	MN
H2087	B	Minnesota, US.	1989	MN
H2090	B	Delaware, Ohio, US.	1989	LRS

H2091	A	Delaware, Ohio, US.	1989	LRS
H2092	B	Delaware, Ohio, US.	1989	LRS
H2094	A	Delaware, Ohio, US.	1989	LRS
H2095	B	Delaware, Ohio, US.	1989	LRS
H2096	A	Powell, Ohio, US.	1989	LRS
H2097	B	Toronto, Ca.	1989	LB
H2098	B	Virginia, US.	1989	LB
H2090	B	Delaware, Ohio, US.	1989	LRS
H2091	A	Delaware, Ohio, US.	1989	LRS

2.10.3 EAN isolates of Ophiostoma ulmi

Isolate	Mating Type	Location	Date of Isolation	Provided By
Initial broad geographical sample:				
P135	B	E. of Stettin, Po.	1980	CMB
H346	B	Bolu, Tu.	1979	SS
H224	B	Odense, De.	1978	HAJ
CA4	B	Tashkent, SU.	1986	CMB
Yu141	B	W. of Skopje, Yu.	1980	CMB
H285	B	Co. Cork, Ei.	1978	AM
H648	B	Tashkent, SU.	1981	HK
H226	B	Regensburg, Ge.	1978	HH
H327	A	Brezno-Nizke Tatry, Cz. (From Ulmus)	1979	JJ
AST27	B	In.	1977	CMB
R136	B	Near Black Sea Coast, Ro.	1980	CMB
M3	A	Exhibition Centre, Moscow, SU.	1979	CMB
Selected sample from Romania:				
R51	B	Riminicu Sarat, Ro.	1980	CMB
R58	B	Racaciuni, Ro.	1980	CMB
R67	B	Filipesti, Ro.	1980	CMB
R74	B	Miroslavesti, Ro.	1980	CMB
R94	B	Horlaceni, Ro.	1980	CMB
R112	B	S. of Botosani, Ro.	1980	CMB

R130	B	SE. of Iasi, Ro.	1980	CMB
R135	B	Nr. Ghidigeni, Ro.	1980	CMB
R139	B	S. of Babadag Ro.	1980	CMB
R148	B	Slava Cercheza, Ro.	1980	CMB
R59	A	Racaciuni, Ro.	1980	CMB
R118	A	S. of Botosani, Ro.	1980	CMB
R140	A	Slava Cercheza, Ro.	1980	CMB

Further broad geographical sample:

H50	A	Nr. Florence, It.	1976	LM
H225	B	Regensburg, Ge.	1978	HH
H255	?	Moscow, SU. (bark isolate)	1978	HH
H276	B	Sofia, Bu.	1978	BR
H281	B	Cork, Ei.	1978	AM
H328	B	Gottschalk, nr. Charkov, SU.	1978	HH
H329	B	Demirkoy, Tu.	1979	SS
H345	A	Moscow, SU.	1979	HH
H371	B	Rhenen, Ho.	1980	HH
H590	B	Gatow, West Berlin, Ge.	1980	HH
H986	B	Tbilisi, Georgia, SU.	1986	HE
H987	A	Borjomi, Georgia, SU.	1986	HE
H2024	B	Lillafured, Hu.	1979	CMB
V1	A	Volgograd, SU.	1979	CMB
V3	B	Volgograd, SU.	1979	CMB
P11	B	Warsaw, Po.	1980	CMB
TR67	B	E. of Giresun, Tu.	1980	CMB
AST20	A	In.	1977	CMB
CKT11	B	In.	1977	CMB
H246	B	Volgograd, SU.	1978	HH
P129	H	N. of Stettin, Po.	1980	CMB

Selected sample from Ireland:

H215	B	Co. Clare, Ei.	1978	AM
H287	B	Co. Clare, Ei.	1978	AM
H294	B	Roscrea, Co. Tipperary, Ei.	1978	AM
H299	B	Co. Rosscommen, Ei.	1978	AM

2.10.4 Other Fungal Isolates

Isolate	Mating Type	Location	Date of Isolation	Provided By
Himmalayan isolate:				
H666	B	Baba Reshi, Kashmir, Id.	1960	HH
Ophiostoma piceae isolates:				
H920		ex oak, GB.	1985	JG
H1042		ex oak, GB.	1987	PS
H988		ex elm bark, Tadjikistan, SU.	1986	CMB
H1039		ex oak, GB.	1987	PS
H926		ex elm bark, Sp.	1984	JFW
H917		ex conifer bark, GB.	1985	DW
H916		IMI 200580		
H1045		ex oak, GB.	1987	PS
H1044		ex oak, GB.	1987	PS
H1043		ex oak, GB.	1987	PS
H1041		ex oak, GB.	1987	PS
H1040		ex oak, GB.	1987	PS
Ophiostoma minor isolates:				
O/910				
O/912				
Ceratocystis brunneo-ciliata isolates:				
I11				
II13A				
Ceratocystis coerulescens isolates:				
O/901				
O/906				

3 RESULTS I - MITOCHONDRIAL DNA POLYMORPHISMS.

CsCl-purified mt DNA (Fig. 3.1) from an initial broad geographical range of isolates from the three O. ulmi subgroups (see Section 2.10) was digested with a number of available restriction enzymes. Those employed were AccI, ApaI, BalI, BamHI, BclI, BglII, ClaI, EcoRI, EcoRV, Eco0109, HindIII, HincII, HpaI, KpnI, MluI, PstI, PvuI, PvuII, SacI, SalI, SmaI, TaqI, TthIII-I, XbaI and XhoI. Three enzymes, PvuII, EcoRI and BclI, were identified as producing informatively polymorphic fragments within the size range of approximately 1 to 20 kilobase pairs (kb), a range allowing maximum resolution of bands on a large agarose gel. The other enzymes produced either too few fragments to be particularly informative or so many that the fragments were too small and faint to be distinguished.

As can be seen in Figs. 3.2, 3.3 and 3.4, there was considerable polymorphism between isolates using all three of the selected enzymes. These figures represent a small selection of a large number of restriction digests in which agarose gels were run for short, intermediate, and long periods of time to achieve optimum separation of all the restriction fragments. Some isolates were run on more than one gel in order to provide controls. In addition possible plasmid bands of around 2.2 kb were present in isolates H358 (Belgium) and H312 (France) when digested with PvuII (Fig. 3.2) and around 2.7 kb in isolate H830 (Vermont, US) when digested with BclI (Fig. 3.3). The true nature of these putative plasmids, their possible connection with d-infection and relationship to mt DNA is currently under investigation in our laboratory (N. Charter, pers. comm.). There is a further band of around 3.5 kb in size in the PvuII digest of isolate H358 (see Fig. 3.2). This band is somewhat fainter than the putative plasmid of around 2.2 kb in the same digest and its nature is unclear, though it may be a separate plasmid or the linear counterpart of the smaller plasmid.

Fainter contaminant ribosomal DNA (r DNA) bands, arising from traces of satellite n DNA maybe seen in the mt DNA digests (Garber and Yoder, 1983). The r DNA bands slightly below the upper mt DNA and

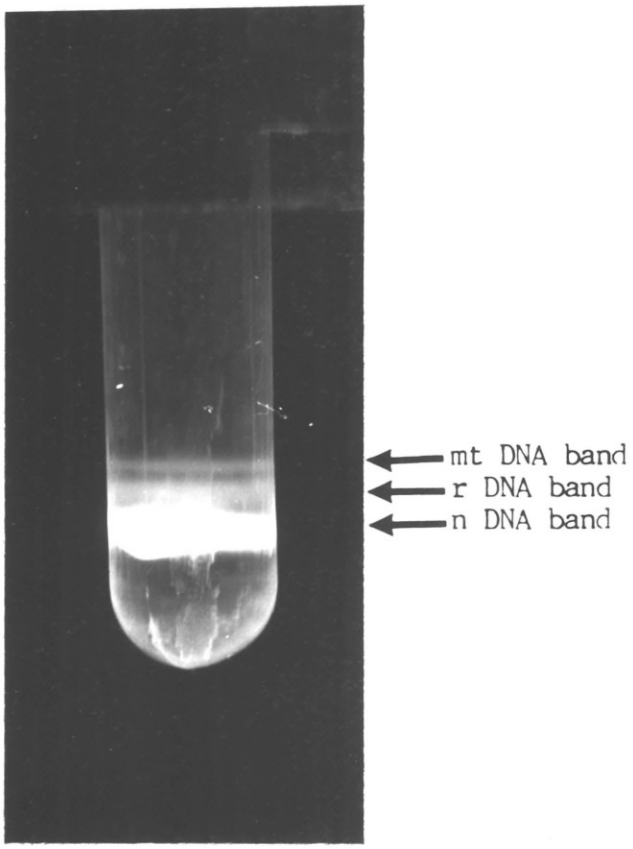

Fig. 3.1. CsCl gradient separation of O. ulmi DNA

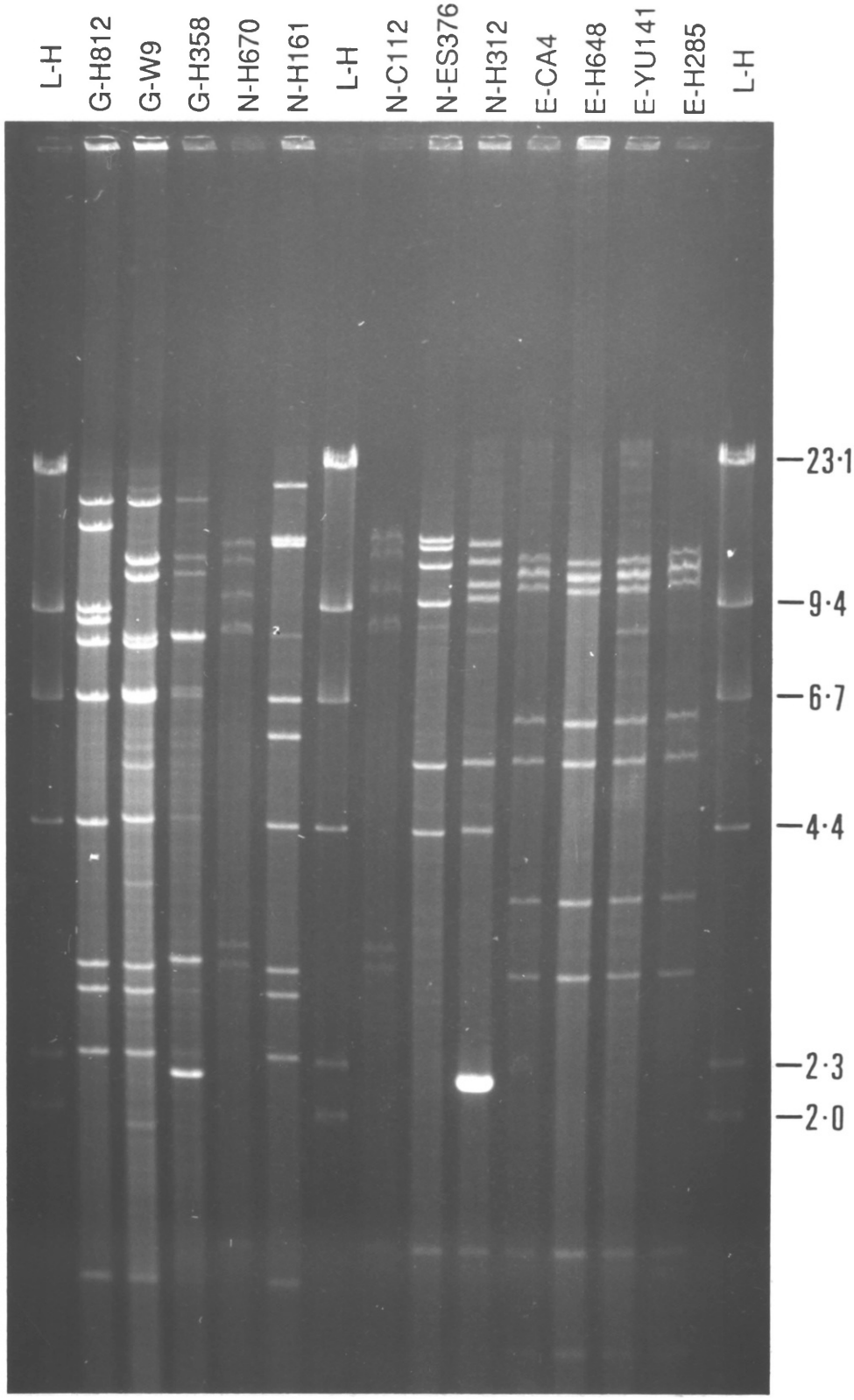

Fig. 3.2. Mitochondrial DNA from a broad geographical range of 12 non-aggressive, NAN and EAN isolates digested with PvuII. L-H denotes HindIII digested lamda DNA markers and the numbers to the right the the sizes of the fragments in kilobases.

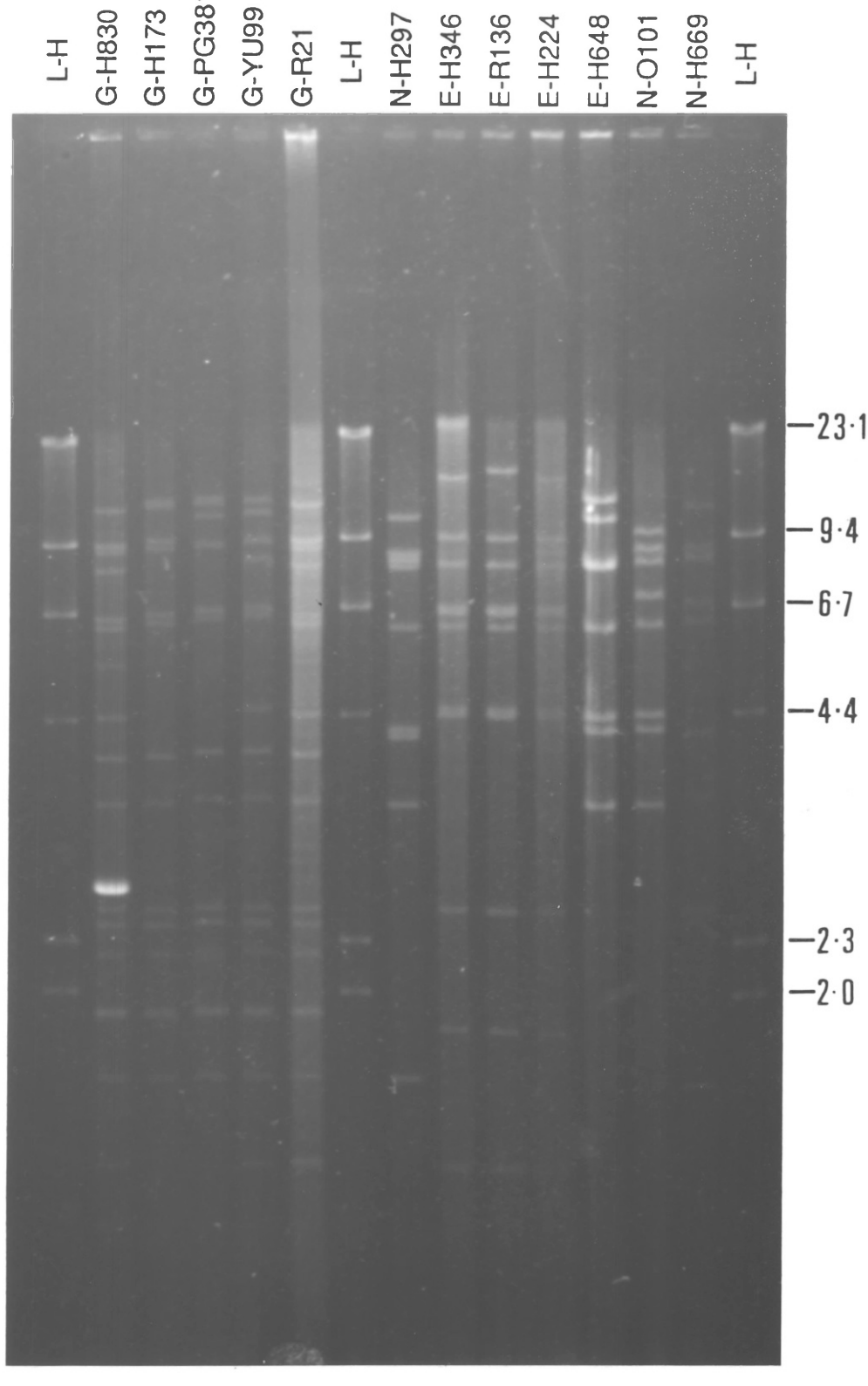

Fig. 3.3. Mitochondrial DNA from a broad geographical range of 12 non-aggressive, NAN and EAN isolates digested with BclI. L-H denotes HindIII digested lamda DNA markers and the numbers to the right the the sizes of the fragments in kilobases.

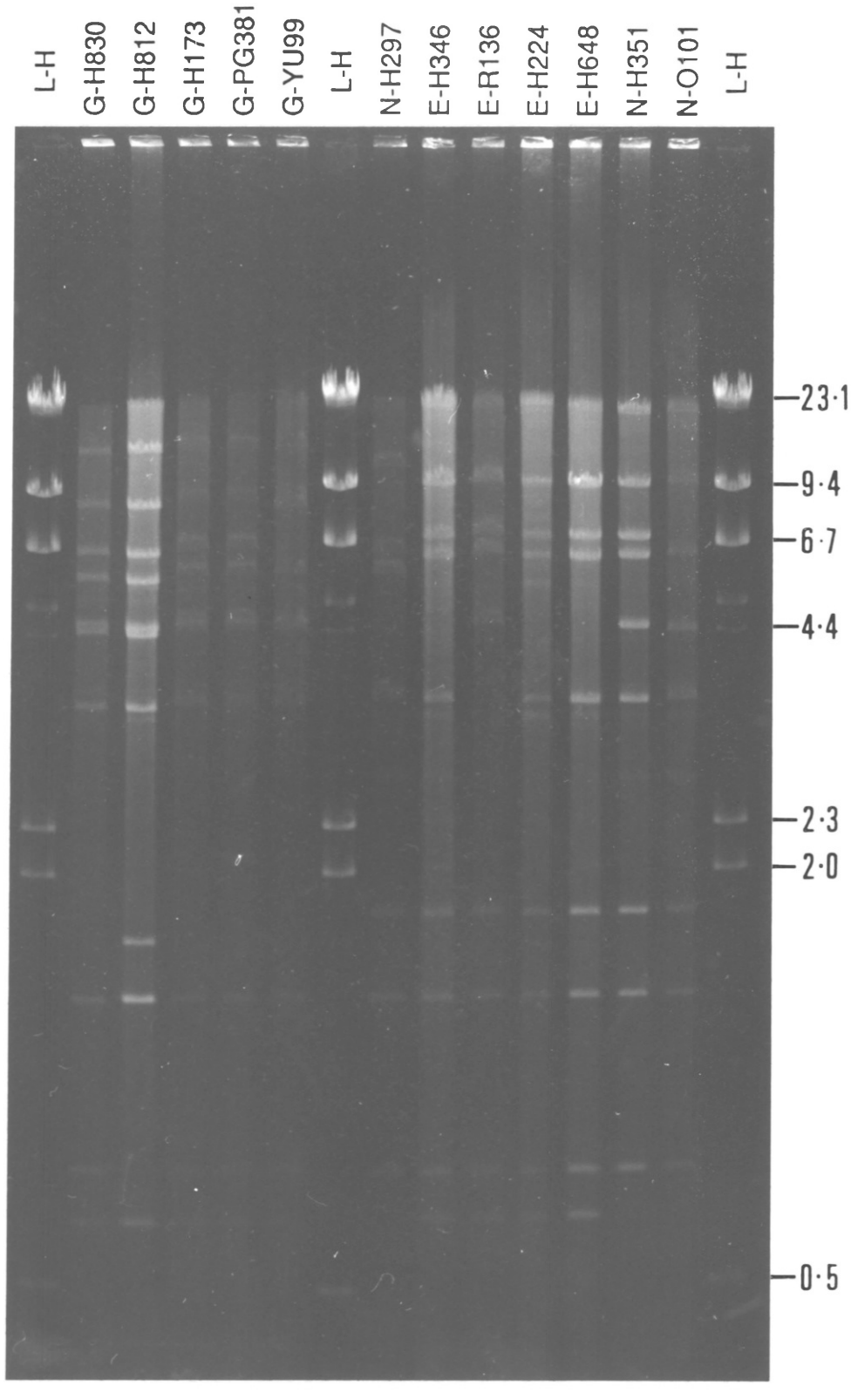

Fig. 3.4. Mitochondrial DNA from a broad geographical range of 12 non-aggressive, NAN and EAN isolates digested with EcoRI. L-H denotes HindIII digested lamda DNA markers and the numbers to the right the the sizes of the fragments in kilobases.

above the lower n DNA band in CsCl gradients (Fig. 3.1) and is consequently difficult to separate from the mt DNA. Contaminant r DNA bands on agarose gels of digested mt DNA were identified by probing Southern blots of mt DNA digests with a clone (pDM238) of the Drosophila r DNA tandem repeat - donation of Prof. D.M. Glover. Fig. 3.5 shows PvuII digested mt DNA from a range of isolates which were then probed with clone pDM238 (Fig. 3.6). Other very faint bands in the digests of some isolates are probably also due to n DNA contamination or to incomplete digestion of the samples.

RFLPs of the mt DNA detected by agarose gel electrophoresis wer e recorded according to the presence or absence of bands for each isolate (Tables 3.1, 3.2 and 3.3). Interpretations of the bands exhibited by the example isolates shown in Figs. 3.2, 3.3 and 3.4 are given in Figs. 3.7, 3.8, and 3.9 respectively. Hierarchical cluster analysis of each of the three data sets was performed using the SPSS-X release 2.0 program from Northwestern University to cluster the isolates according to their polymorphisms using average linkage between groups (Figs. 3.10, 3.11 and 3.12). The hierarchical cluster analysis program yields dendrograms with a scale of 0 - 25, which may be viewed as being a rough gauge of 'genetic similarity' between any particular pair of isolates. However, since the mt DNA has not been restriction mapped it is impossible to determine whether polymorphisms are caused by a single point mutation or one or more larger scale insertions, deletions or rearrangements. Consequently, the scale of the dendrograms cannot be said to accurately represent a real genetic distance between any two isolates and can only be used as a guide to the general inter-relatedness of the different subgroups and isolates. (Though it would be of interest to determine the detailed genetic structure of O. ulmi mt DNA, this was not the aim of the present study. Rather it was intended to study the genetic variability of field populations of the pathogen in order to gain an insight into its population structure and epidemiology.)

The dendrograms generated for the three data sets show excellent grouping of the EAN, NAN and non-aggressive isolates with the exception of NAN isolates H161 (Nova Scotia, Canada) and H937 (Maine, USA) which appear to be generally more similar to, though

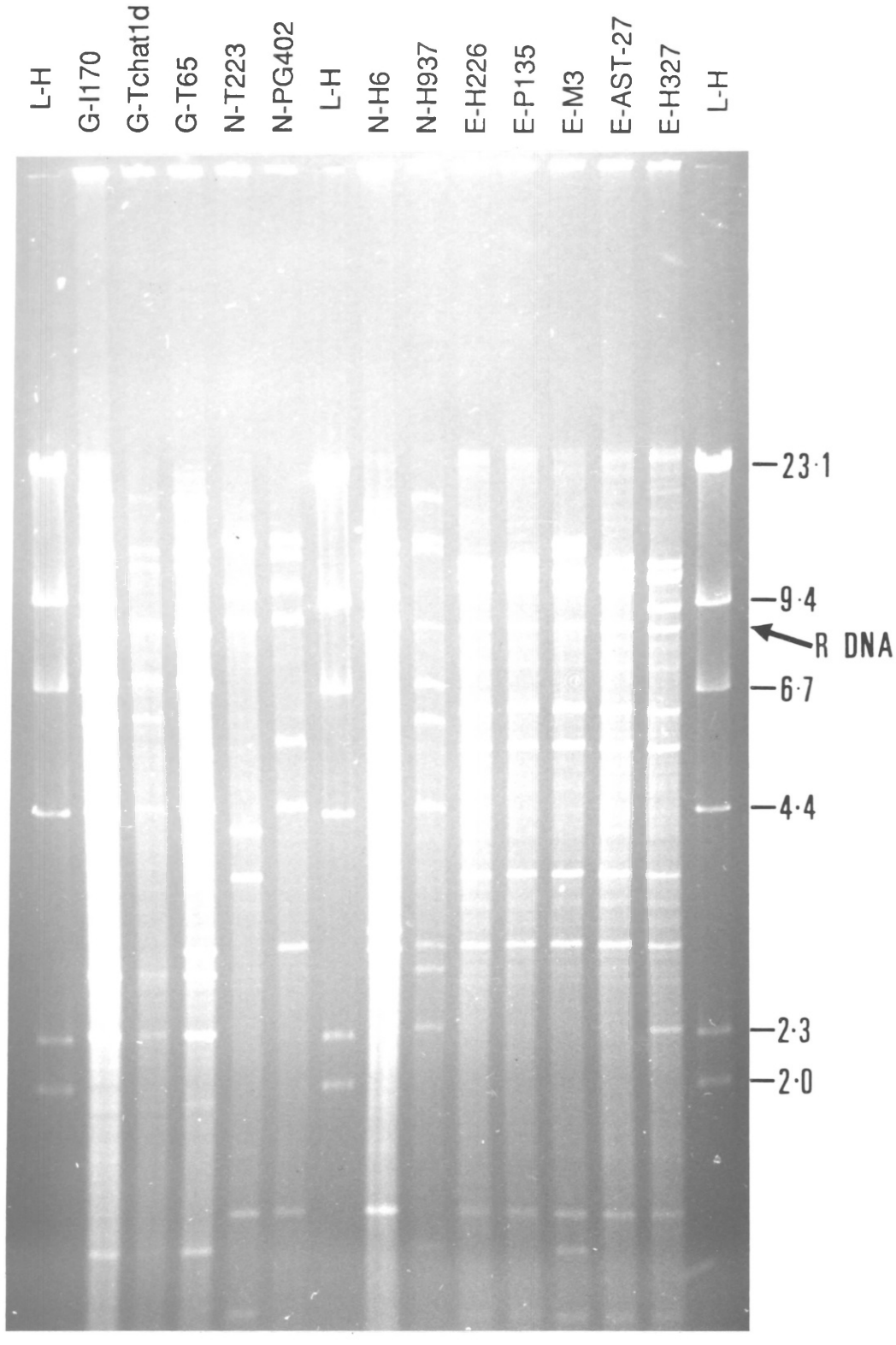

Fig. 3.5. Mitochondrial DNA from a broad geographical range of non-aggressive, NAN and EAN isolates digested with PvuII and showing putative r DNA bands of around 8.0 kb. L-H denotes HindIII digested lamda DNA markers and the numbers to the right the sizes of the fragments in kilobases

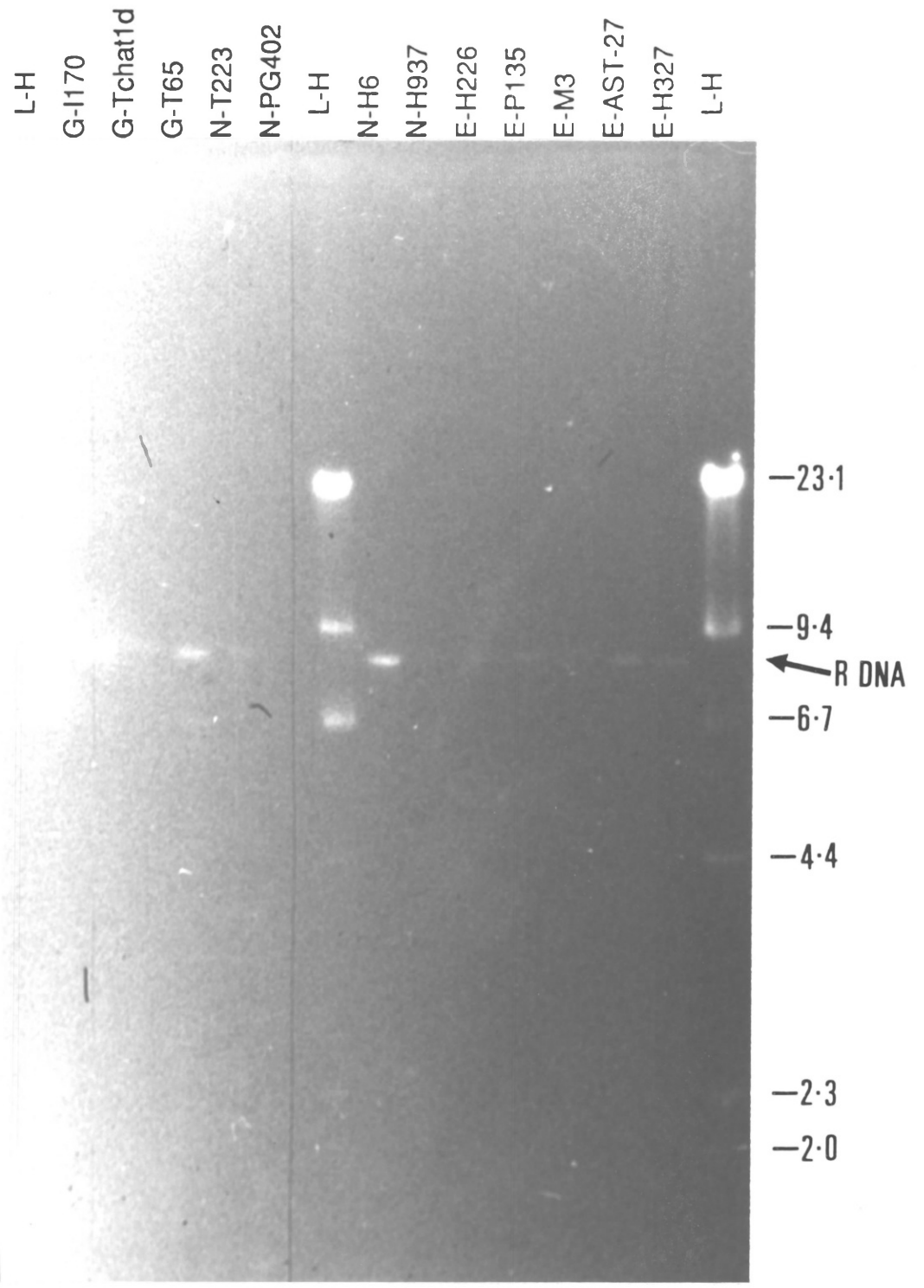

Fig. 3.6. Autoradiograph of a Southern blot of the agarose gel shown in Fig. 3.5 probed with clone pDM238 containing a tandem repeat of Drosophila r DNA. L-H denotes HindIII digested lamda DNA markers and the numbers to the right the sizes of the fragments in kilobases.

		1.2	1.4	1.5	2.3	2.8	3.0	3.2	3.6	4.2	4.7	5.4	6.0	6.3	6.8	6.9	7.5	8.1	8.8	8.9	9.2	9.4	9.8	10.5	10.7	10.7	11.2	11.2	11.2	11.9	12.3	12.4	13.2	13.2	13.8	15.1	15.1	17.6	19.5	TOTAL SIZE
NAG	H173	0	1	0	1	1	1	0	0	0	1	0	0	0	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	88.6
NAG	PG381	0	1	0	1	1	1	0	0	0	1	0	0	0	1	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1	0	79.4
NAG	YU99	0	1	0	1	1	1	0	0	0	1	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0	79.9
NAG	R21	0	1	0	1	1	1	0	0	0	1	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	77.2
NAG	H812	0	1	0	1	1	1	0	0	0	1	0	0	0	1	0	0	1	0	1	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	1	1	0	0	80.1
NAG	W9	0	1	0	1	1	1	0	0	0	1	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	1	0	0	77.2
NAG	H358	0	1	0	1	1	1	0	0	0	1	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	0	77.2
NAG	I170	0	1	0	1	1	0	0	0	0	1	0	1	0	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	78.4
NAG	TCHAT1D	0	1	0	1	1	0	0	0	0	1	0	1	0	1	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	0	80.2
NAG	T65	0	1	0	1	1	1	0	0	0	1	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	1	0	77.2
EAN	CA4	1	0	1	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	66.2
EAN	H346	1	0	1	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	66.2
EAN	R136	1	0	1	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	67.4
EAN	H224	1	0	1	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	66.2
EAN	H648	1	0	1	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	66.2
EAN	YU141	1	0	1	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	66.2
EAN	H285	1	0	1	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	66.2
EAN	H226	1	0	1	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	66.2
EAN	P135	1	0	1	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	66.2
EAN	M3	1	1	1	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	71.1
EAN	AST27	1	0	1	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	67.2
EAN	H327	1	0	1	1	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	66.3
NAN	0101	0	0	1	0	0	1	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	51.4
NAN	H351	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	57.2
NAN	H297	1	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	56.1
NAN	H670	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	51.4
NAN	H161	0	1	0	1	1	1	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	73.5
NAN	C112	0	0	1	0	0	1	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	1	51.4
NAN	ES376	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	60.3
NAN	H312	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0	0	1	1	0	0	0	0	0	57.2
NAN	T223	1	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	56.1
NAN	PG402	0	0	1	0	0	1	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	58.3
NAN	H6	0	0	1	0	0	1	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	51.4
NAN	H937	0	1	0	1	1	1	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	73.5

Table 3.1 Summary of the mt DNA PvuII digest data for a broad geographical range of isolates. The size of each band is given in kilobases as is the total size estimate for each isolate.

		1.3	1.4	1.7	1.7	1.9	1.9	1.9	2.2	2.2	2.4	2.4	3.3	3.3	3.9	3.9	4.1	4.2	4.4	4.4	4.5	5.1	5.1	6.0	6.1	6.3	6.4	6.9	7.9	7.9	8.0	8.1	8.9	9.4	9.6	9.6	9.7	9.8	10.6	10.6	11.6	11.6	11.6	11.7	14.5	15.4	15.5	TOTAL SIZE
NAG	H830II	0	1	1	0	0	1	1	0	1	1	1	0	1	0	1	0	0	0	1	0	0	0	0	1	0	1	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	76.1
NAG	H173	0	0	1	0	0	1	1	1	1	1	1	0	1	0	1	0	0	0	1	0	0	0	0	1	0	1	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	74.0
NAG	PG381	0	0	1	0	0	1	1	1	1	1	1	0	1	0	1	0	0	0	1	0	0	0	0	1	0	1	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	77.2
NAG	YU99	0	1	1	0	0	1	1	1	1	1	1	0	1	0	1	0	0	0	1	0	0	0	0	1	0	1	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	77.1
NAG	R21	0	1	1	0	0	1	1	1	1	1	1	0	1	0	1	0	0	0	1	0	0	0	0	1	0	1	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	76.1
NAG	H812II	0	0	1	0	0	1	1	1	1	1	1	0	1	0	1	0	0	0	1	0	0	0	0	1	0	1	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	79.9
NAG	W9	0	1	1	1	0	0	1	1	0	1	1	0	1	0	1	0	0	0	1	0	0	0	0	1	0	1	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	75.9
NAG	TCHAT1D	0	1	0	0	0	1	1	1	1	1	1	0	1	0	1	0	0	0	1	0	0	0	0	1	0	1	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	74.0
NAG	TR65	0	1	1	0	0	1	1	1	1	1	1	0	1	0	1	0	0	0	1	0	0	0	0	1	0	1	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	76.1
EAN	CA4	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	65.1
EAN	H346	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	65.1
EAN	R136	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	65.1
EAN	H224	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	66.0
EAN	H648II	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	65.1
EAN	YU141	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	65.1
EAN	H285	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	65.1
EAN	H226	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	65.1
EAN	P135	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	65.1
EAN	M3	0	1	1	0	1	1	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	65.1
EAN	AST27	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	69.2
EAN	H327	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	65.1
NAN	0101	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	65.1
NAN	H351II	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	50.2
NAN	H297	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	55.9
NAN	H670	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	53.9
NAN	H161II	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	50.2
NAN	C112	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	70.4
NAN	ES376	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	50.2
NAN	H312	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	54.4
NAN	T223	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	48.0
NAN	PG402	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	53.9
NAN	H6	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	52.3
NAN	H937	0	0	1	0	0	1	0	0	0	1	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	69.2

Table 3.2. Summary of the mt DNA BclI digest data for a broad geographical range of isolates. The size of each band is given in kilobases as is the total size estimate for each isolate.

		0.5	0.7	0.7	0.8	1.3	1.6	1.8	3.1	3.3	3.5	4.3	4.5	4.6	5.1	5.6	6.1	6.1	6.3	6.5	6.9	8.0	8.3	8.8	8.8	10.0	10.0	11.4	11.4	11.6	13.3	16.5	18.8	?	?	?	?
NAG	H830II	0	0	1	1	1	0	0	0	1	0	0	1	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0
NAG	H173	0	0	1	1	1	0	0	0	1	0	0	1	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0
NAG	PG381	0	0	1	1	1	0	0	0	1	0	0	1	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0
NAG	YU99	0	0	1	1	1	0	0	0	1	0	0	1	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0
NAG	R21	0	0	1	1	1	0	0	0	1	0	0	1	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0
NAG	H812II	0	0	1	0	1	1	0	0	1	0	0	1	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0
NAG	W9	0	0	1	1	1	0	0	0	1	0	0	1	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0
NAG	H358	0	0	1	1	1	0	0	0	1	0	0	1	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0
NAG	I170	1	1	1	1	1	0	0	0	1	0	0	1	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0
NAG	TCHAT1D	1	1	1	1	1	0	0	0	1	0	0	1	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0
NAG	TR65	0	0	1	1	1	0	0	0	1	0	0	1	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0
EAN	CA4	0	0	1	1	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0
EAN	H346	0	0	1	1	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0
EAN	R136	0	0	1	1	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0
EAN	H224	0	0	1	1	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0
EAN	H648II	0	0	1	1	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0
EAN	YU141	0	0	1	1	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0
EAN	H285	0	0	1	1	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0
EAN	H226	0	0	1	1	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0
EAN	P135	0	0	1	1	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0
EAN	M3	0	0	1	1	1	0	1	1	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0
EAN	AST27	0	0	1	1	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0
EAN	H327	0	0	1	1	1	0	1	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0
NAN	0101	0	0	0	1	1	0	1	0	0	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0
NAN	H351II	0	0	0	1	1	0	1	0	0	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	1	0	0
NAN	H297	0	0	0	1	1	0	1	0	0	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	1	0	0
NAN	H670	0	0	0	1	1	0	1	0	0	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	1	0	0
NAN	H161II	0	0	1	1	1	0	1	0	0	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	1	0	0
NAN	C112	0	0	0	1	1	0	1	0	0	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	1	0	0
NAN	ES376	0	0	0	1	1	0	1	0	0	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	1	0	0
NAN	H312	0	0	0	1	1	0	1	0	0	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	1	0	0
NAN	T223	0	0	0	1	1	0	1	0	0	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	1	0	0
NAN	PG402	0	0	0	1	1	0	1	0	0	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	1	0	0
NAN	H6	0	0	0	1	1	0	1	0	0	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	1	0	0
NAN	H937	0	0	1	1	1	0	0	0	1	0	0	1	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1

Table 3.3. Summary of the mt DNA EcoRI digest data for a broad geographical range of isolates. The size of each band is given in kilobases.

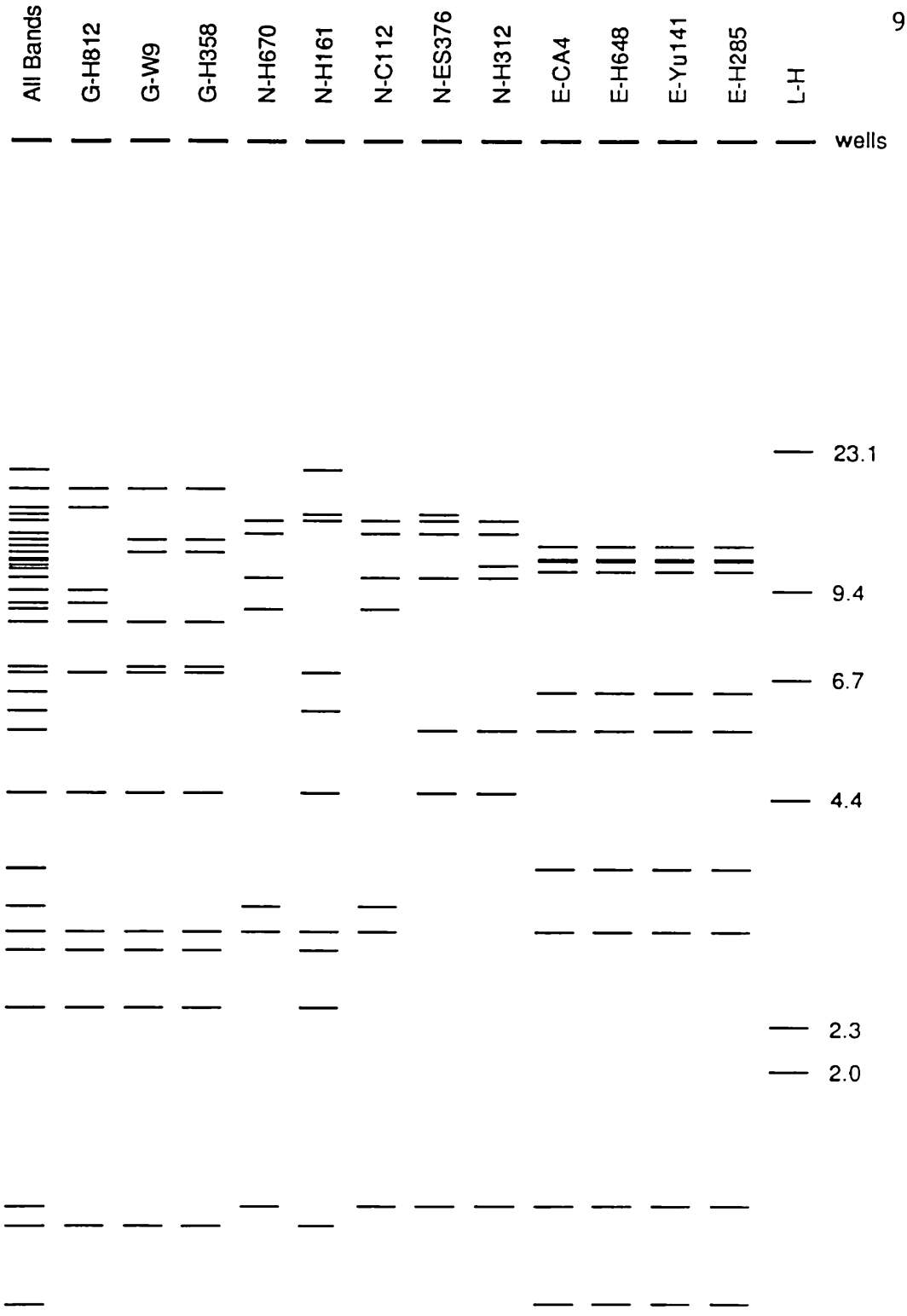

Fig. 3.7. Interpretation of banding patterns of the PvuII digested mt DNA shown in Fig. 3.2. L-H denotes HindIII digested lamda DNA markers and the numbers to the right the sizes of the fragments in kilobases.

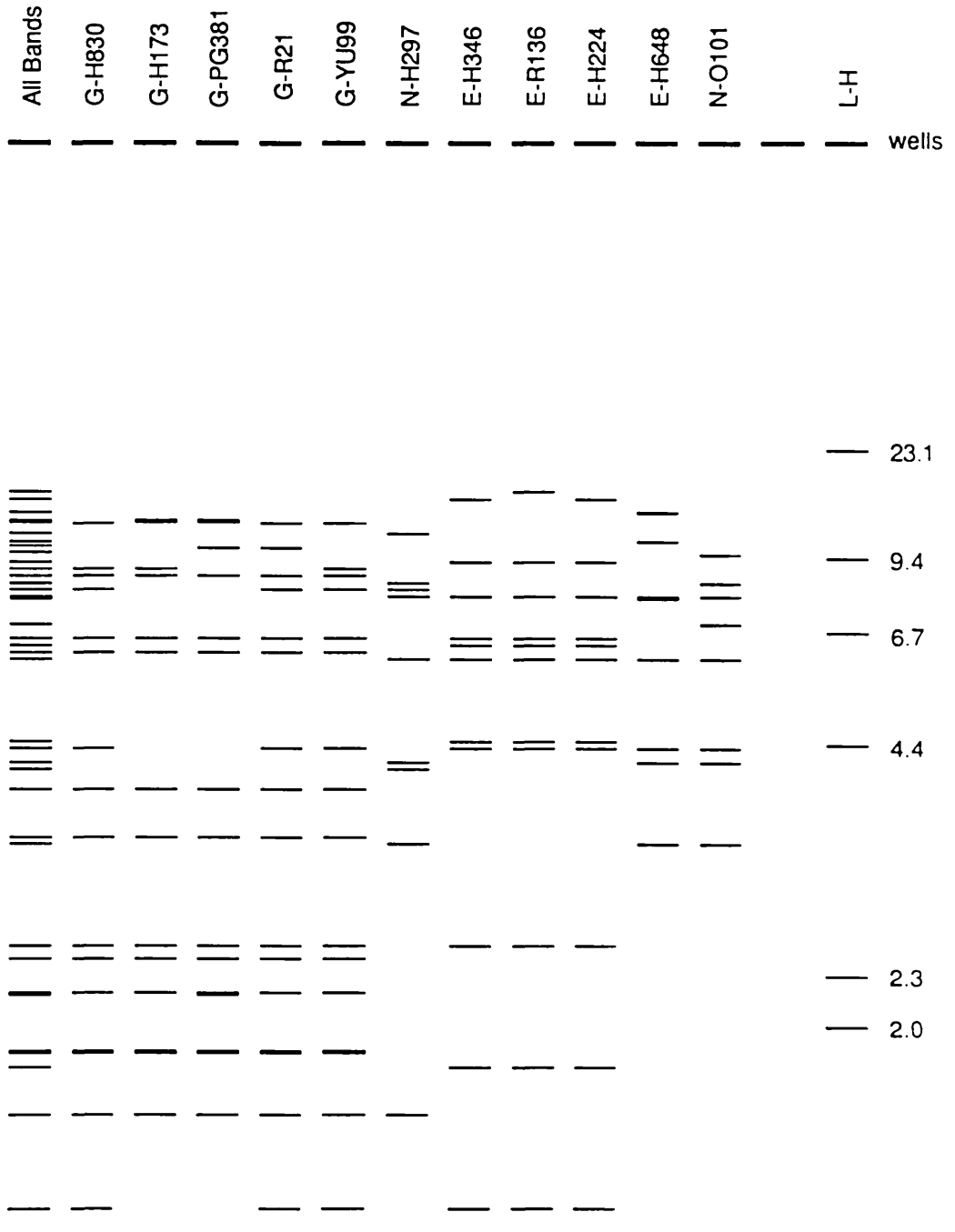

Fig. 3.8. Interpretation of banding patterns of the BclI digested mt DNA shown in Fig. 3.3. L-H denotes HindIII digested lamda DNA markers and the numbers to the right the sizes of the fragments in kilobases.

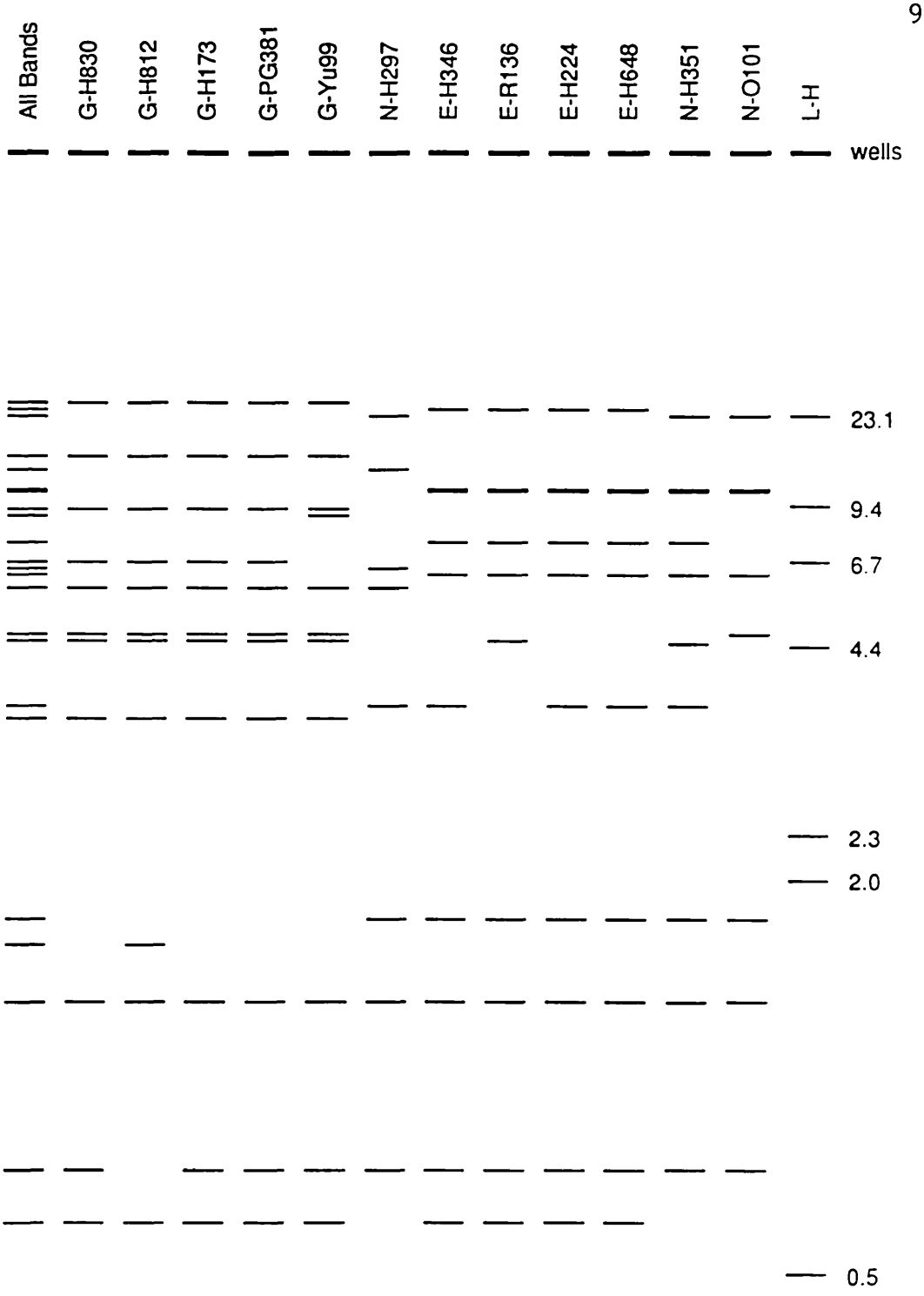

Fig. 3.9. Interpretation of banding patterns of the EcoRI digested mt DNA shown in Fig. 3.4. L-H denotes HindIII digested lamda DNA markers and the numbers to the right the sizes of the fragments in kilobases.

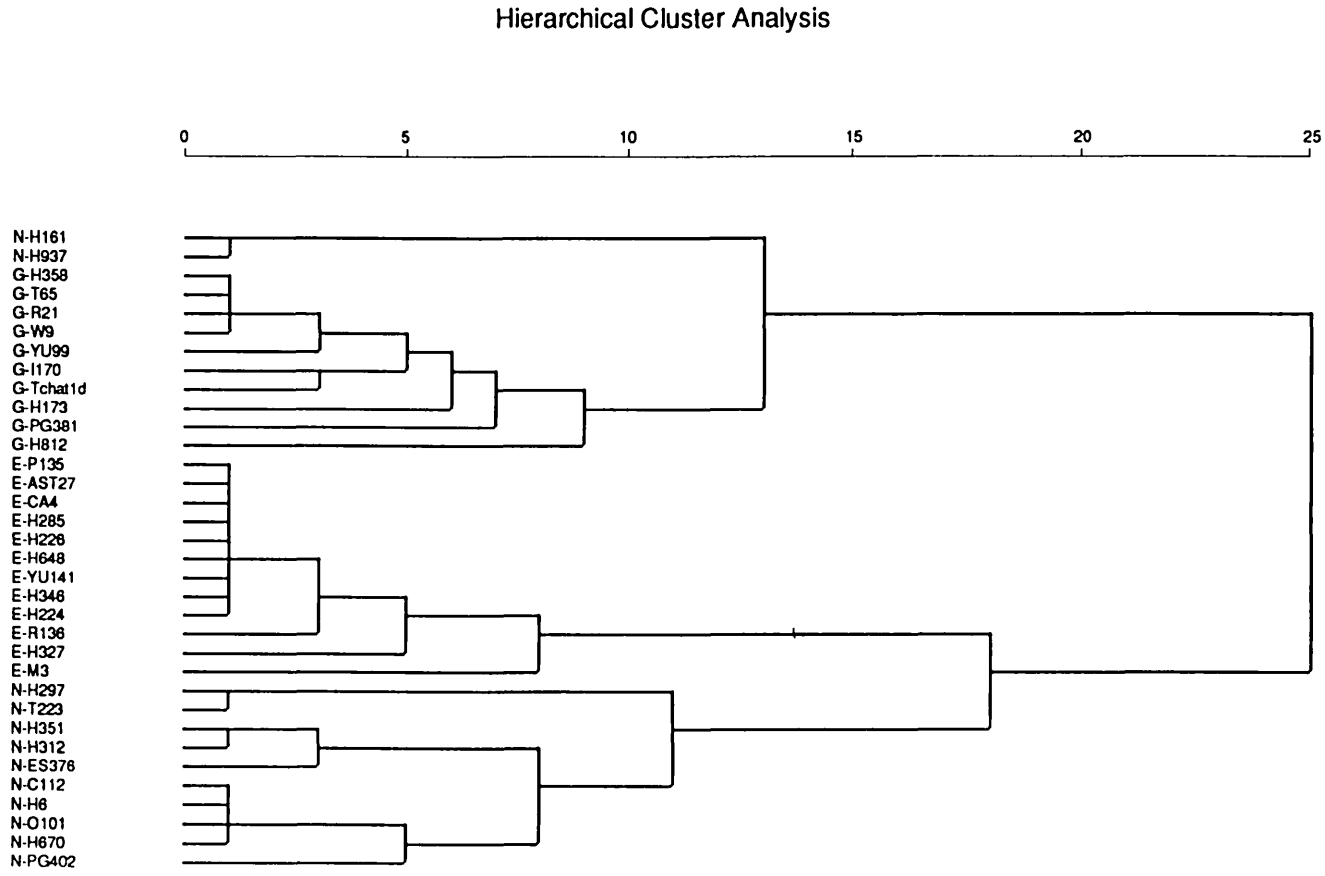

Fig. 3.10. Hierarchical cluster analysis of the mt DNA PvuII digest data summarised in Table 3.1.

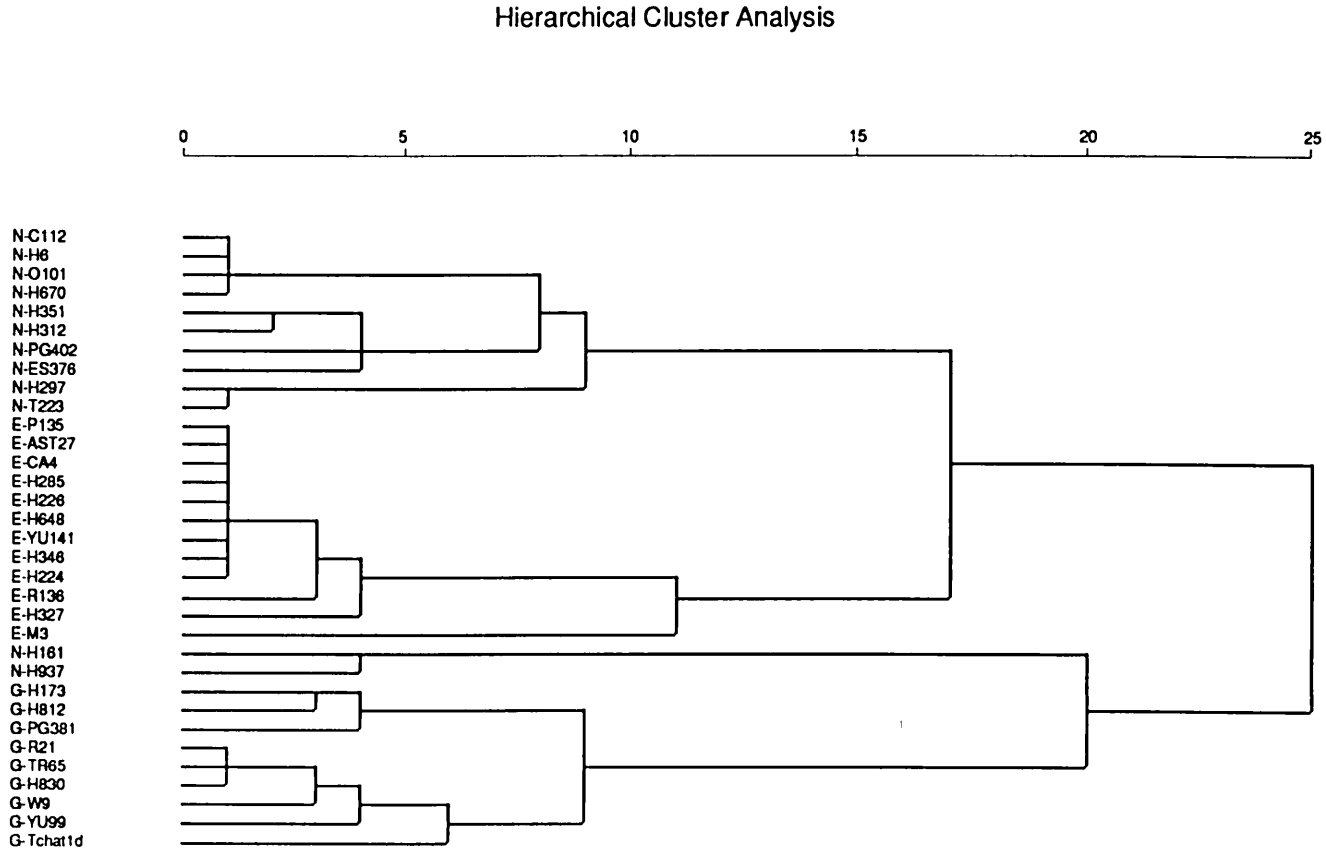

Fig. 3.11. Hierarchical cluster analysis of the mt DNA BclI digest data summarised in Table 3.2.

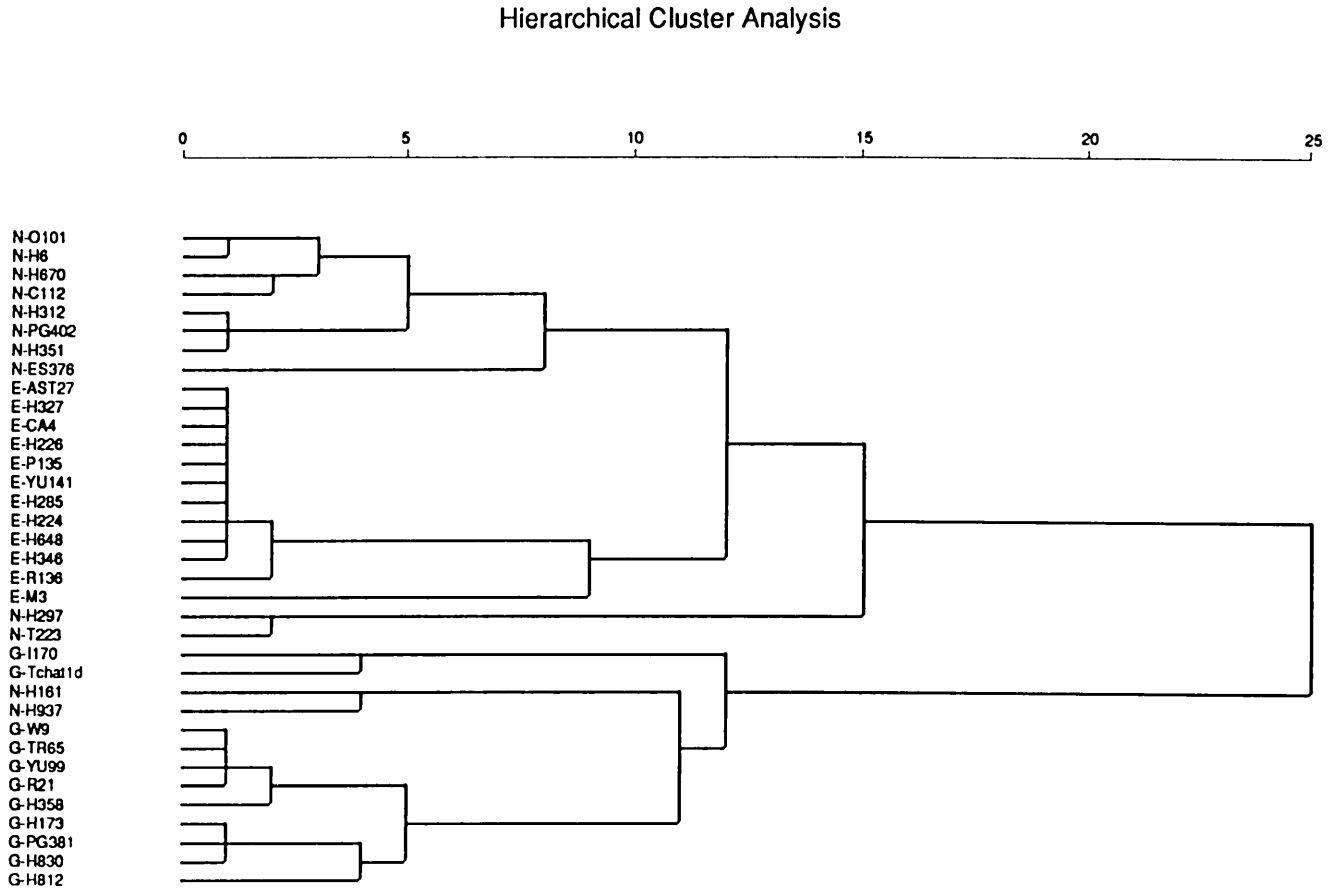

Fig. 3.12. Hierarchical cluster analysis of the mt DNA EcoRI digest data summarised in Table 3.3.

still significantly distinct from, the non-aggressive isolates except in the case of EcoRI where isolates I170 and Tchat1d appear less closely related to the other non-aggressives than H161 and H937. Isolates T223 and H297, both of which belong to the Tewkesbury / Guadalajara vc group are the next furthest outlying NAN isolates with all three enzymes. In contrast to the other two digests however when these two isolates when digested with EcoRI they appear as distantly related to the main group of NANs as to the EAN isolates.

For each of the three enzymes it may be seen that the EAN and NAN aggressive isolates are clearly more closely related to each other than to the non-aggressive isolates. Furthermore, the size of the mt DNAs of these isolates was calculated for the enzymes PvuII and BclI by comparing the fragments with a log graph of lamda-HindIII marker fragment molecular weights. The sizes obtained for the isolates of the three subgroups, with the exception of the unusual isolates H161 and H973, are entirely distinct with the range of values for the non-aggressive, EAN and NAN isolates being 74 - 88 kb, 65 - 71 kb and 48 - 60 kb respectively (Tables 3.1, 3.2 and 3.3). It was felt that the size of the mt DNA could not be calculated from EcoRI fragments as all the isolates in this study exhibited a large fragment of around 20 kb or greater with this enzyme. An accurate determination of the size of the mt DNA of O. ulmi by means of double enzyme digests was beyond the scope of this study, however the results suggest that the size of the mt DNAs may be diagnostic for the three subgroups. It is of interest that the sizes of the mt DNA of the unusual isolates H161 and H937, at around 69 - 73 are approximately intermediate between the range of sizes for the EAN and non-aggressive isolates and completely distinct from that of other 'typical' NAN isolates.

Within the other 'typical' NAN isolates it is of particular interest that isolates of the same or related vegetative compatibility groups are grouped together. T223 (Tewkesbury, UK) and H297 (Eire) which are both members of the Tewkesbury / Guadalajara vc group (see Section 5.1 and Table 3.4) share identical mt DNA restriction patterns with each of the three enzymes and are consequently closely grouped on each of the three dendrogams. The

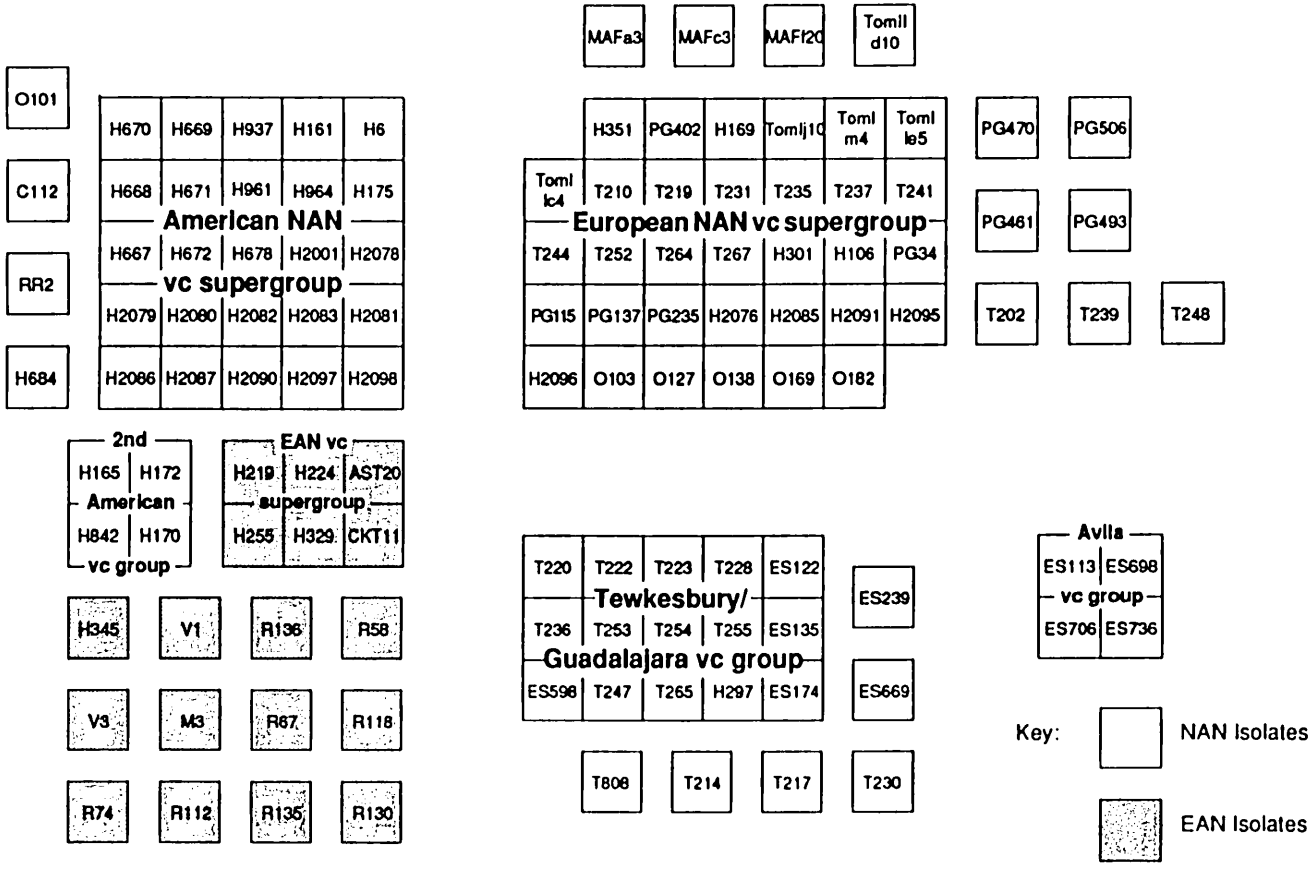

Table 3.4 Summary of the known vc relationships of most isolates utilised in this thesis. Adjacent squares indicate isolates of the same vc group. Squares separated by half a square indicate isolates which give an n reaction to each other. Squares separated by two squares indicate isolates which give a w reaction to each other. The arrangement of the subgroups in the Table is purely subjective and is not meant to signify any particular relationship other than vegetative compatibility.

same applies to isolates H6 (Holland) and 0101 (Orsett, UK), and they are in turn closely grouped with isolates H670 (Maine, USA) and C112 (Chichester, UK). All four of these isolates share the same w allele and isolates H6 and H670, despite their highly disparate geographical origins are both members of the American NAN vc supergroup.

The mt DNA patterns of the EAN isolates are considerably less diverse than those of the NAN or non-aggressive isolates. In fact with each of the three enzymes all of the isolates with the exception of isolates M3 (Moscow, USSR), R136 (Romania), and for two enzymes H327 (Czechoslovakia), exhibit identical patterns. This is of particular interest since M3 and R136 are highly unusual EAN isolates in that they exhibit several typical non-aggressive n DNA polymorphisms when probed with a number of different n DNA clones (see Section 6.2). Furthermore, when digested with PvuII isolate M3 appears to share band 2 with all of the non-aggressive isolates and the unusual NAN isolates H161 and H937.

4 RESULTS II - NUCLEAR DNA POLYMORPHISM.

CsCl-purified nuclear DNA (Fig. 3.1) of non-aggressive isolate H173 was digested with BamHI and 2 to 3.5 kb fragments were isolated with 'Geneclean' from ultra-pure low melting point agarose. These fragments were ligated into the plasmid vector Bluescript-KS+ which had been digested with BamHI, and transformed into E.coli strain JM83. Small scale plasmid isolations were made from 87 positive colonies and the plasmids were digested with BamHI. Agarose gel electrophoresis of restricted plasmids confirmed that they did indeed contain inserts (Figs. 4.1 and 4.2). A dot blot of the restricted plasmids, and total mt DNA, total n DNA, and vector DNA controls was probed first with total mt DNA to show whether any of the clones actually contained contaminant mt DNA BamHI fragments rather than n DNA BamHI fragments (Fig. 4.3). It can be seen that only clone 25 gave a positive signal and this was therefore discarded. EcoRI-digested n DNA from a range of isolates was then probed with random n DNA clones to detect polymorphisms. Figs. 4.4 and 4.5 show autoradiographs of n DNA from an initial broad geographical range of non-aggressive, NAN and EAN isolates digested with HindIII and BamHI and probed with clones 125 and 137 respectively. Both clones were found to accurately discriminate between the aggressive and non-aggressive isolates in this sample but did not distinguish between the NAN and EAN races.

In order to detect nuclear polymorphisms between EAN and NAN isolates samples of total n DNA of an EAN isolate (M3) and an NAN isolate (C112) were digested with HindIII and BamHI, and electrophoresed adjacently on a 1 % agarose gel, Southern blotted and probed separately with all 86 random n DNA clones (eg. Fig. 4.6). Thirteen clones revealed polymorphisms between M3 and C112 and these were then used as probes against the same initial broad geographical range of non-aggressive, NAN and EAN isolates (see Section 2.11) to determine whether they were characteristic probes for the three different sub-groups, merely distinguished between the EAN and the NAN, or revealed intra subgroup variability.

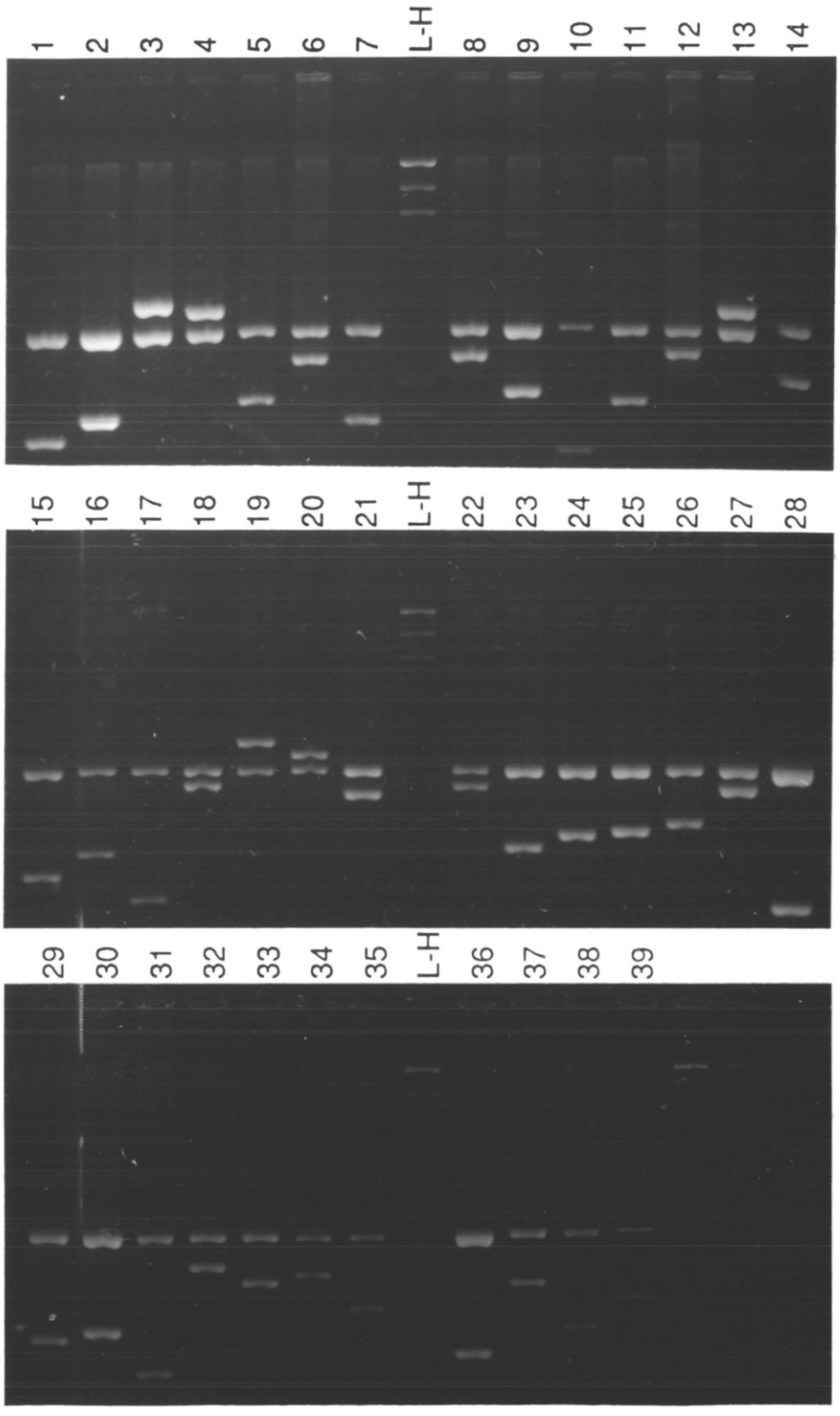

Fig. 4.1. BamHI digests of random n DNA clones 1-39 showing the presence and size of inserts. L-H denotes HindIII digested lamda DNA markers.

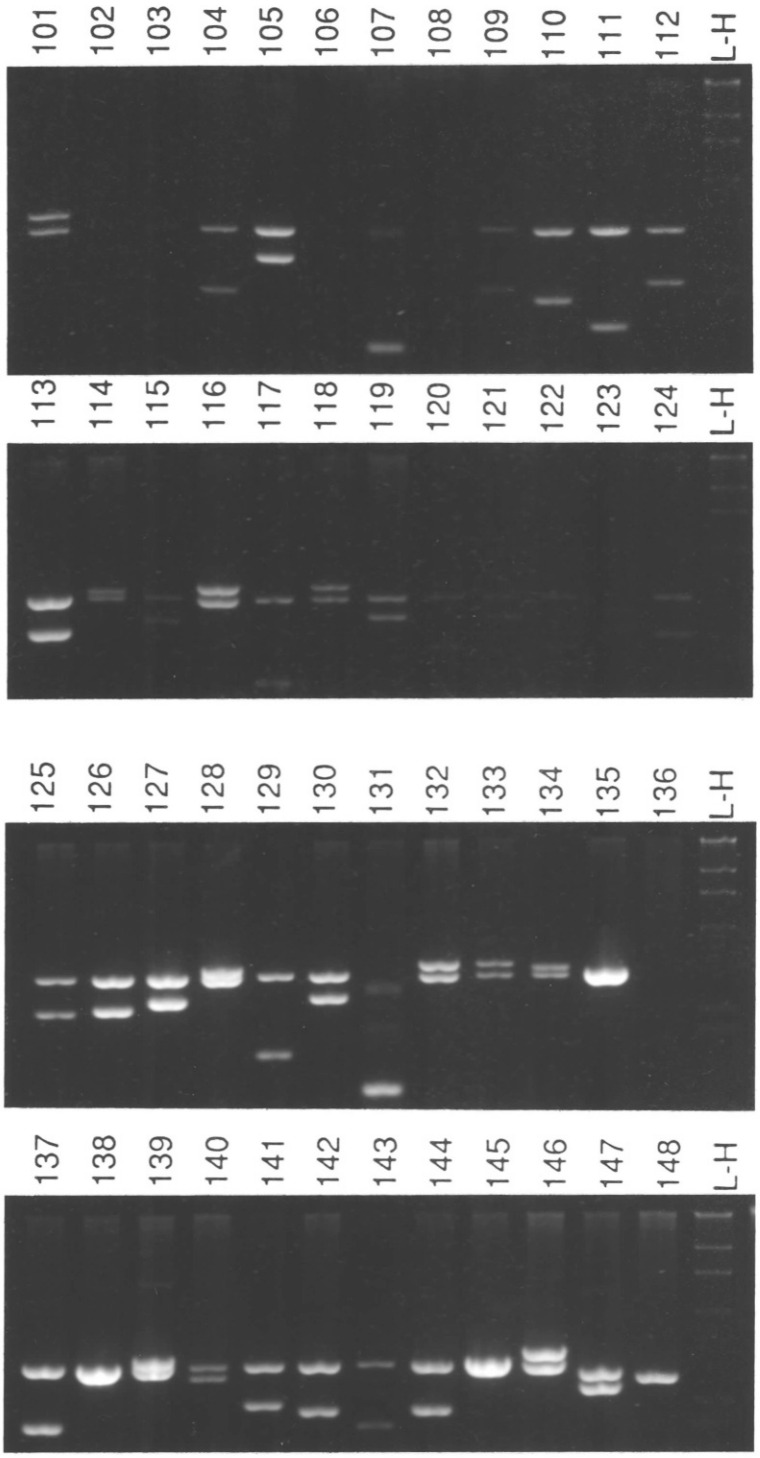

Fig. 4.2. BamHI digests of random n DNA clones 101-148 showing the presence and size of inserts. L-H denotes HindIII digested lamda DNA markers.

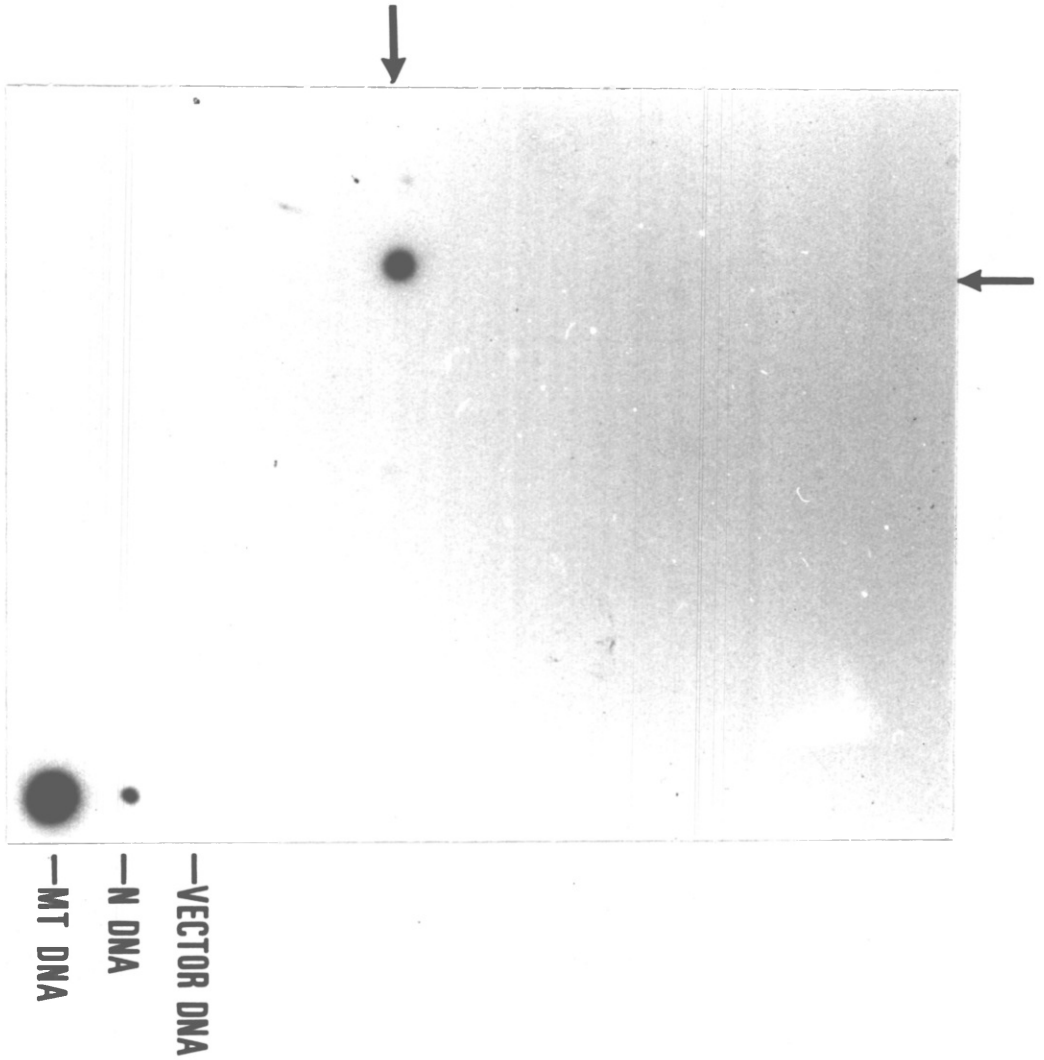

Fig. 4.3. Autoradiograph of a dot blot of putative n DNA clones 1-39 and 101-148 probed with labelled mt DNA. Arrows denote the position of clone 25.

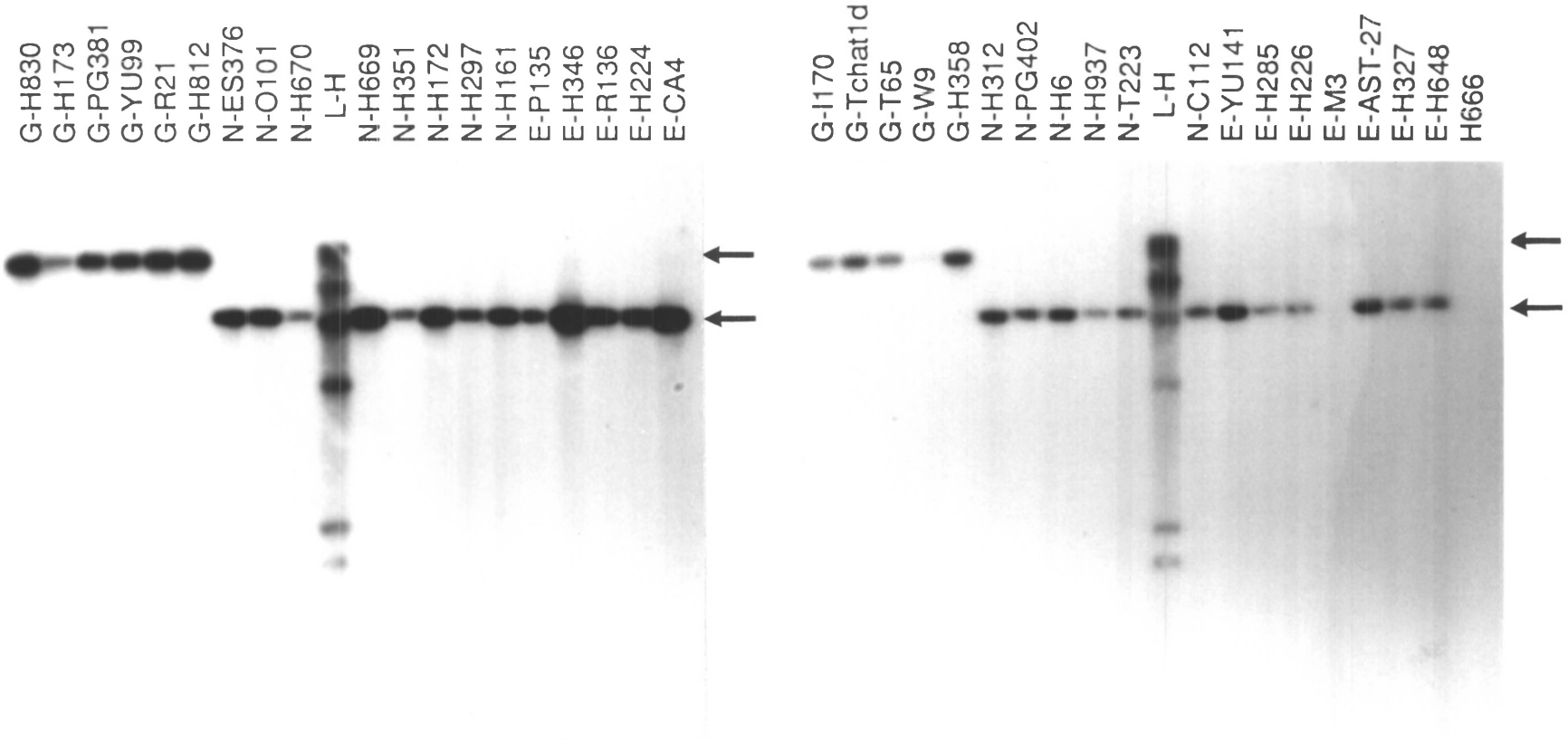

Fig. 4.4. HindIII digested n DNA of an initial broad geographical range of EAN, NAN and non-aggressive isolates and isolate H666 probed with random n DNA clone 125. The letter before each isolate denotes the subgroup of which it is a member; G = non-aggressive; N = NAN; E = EAN. L-H denotes HindIII digested lamda DNA size markers. Arrows mark the position of polymorphisms.

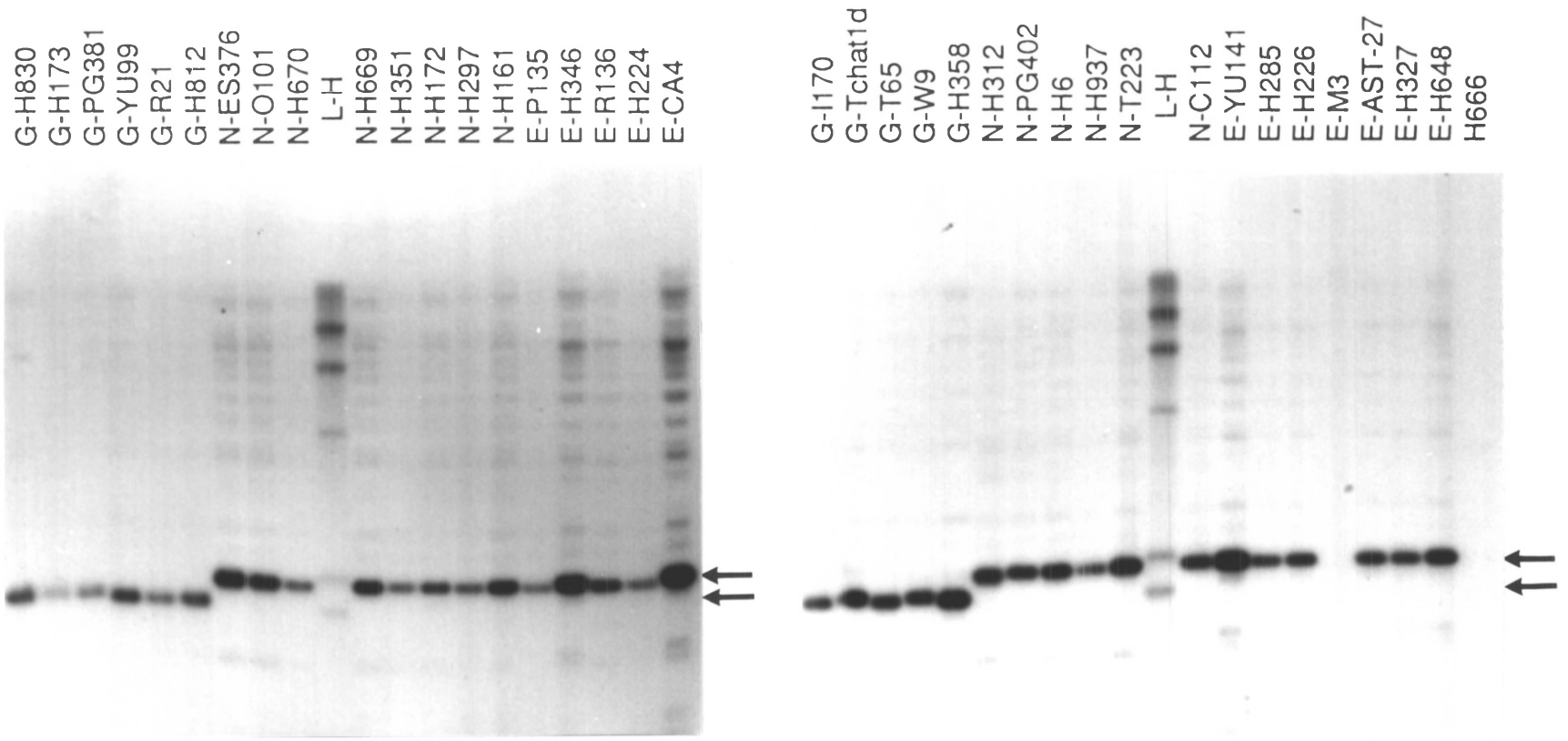

Fig. 4.5. BamHI digested n DNA of an initial broad geographical range of EAN, NAN and non-aggressive isolates and isolate H666 probed with random n DNA clone 137.

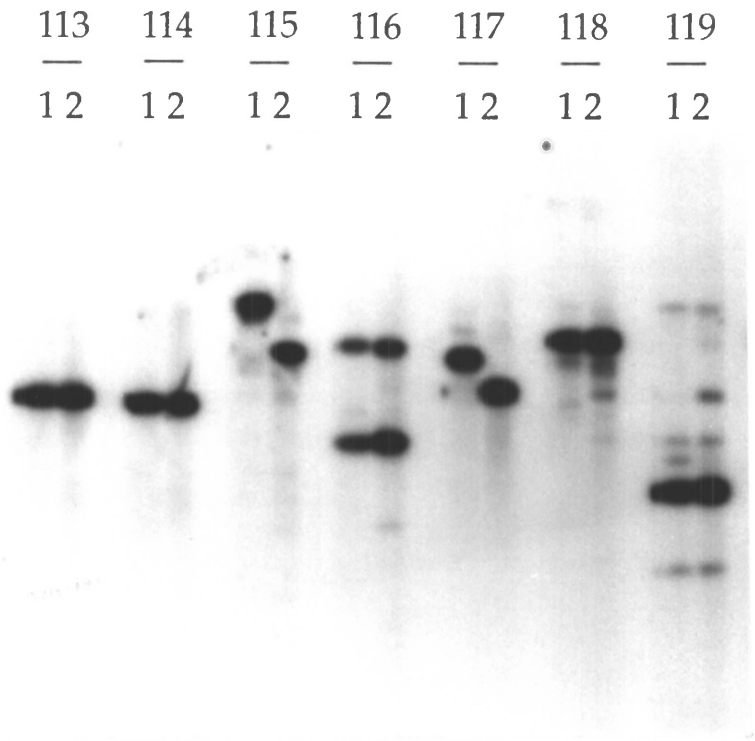

Fig 4.6. HindIII, digested n DNA from paired NAN isolate C112 (1) and EAN isolate M3 (2) probed with random n DNA clones 113 - 119.

These results are summarised in Fig. 4.7 where the polymorphisms exhibited by the different isolate/enzyme/probe combinations are represented by colours. The typically non-aggressive polymorphisms of non-aggressive isolates are represented by blue as are the identical polymorphisms when exhibited by EAN and NAN isolates. Polymorphisms typically exhibited by EAN isolates, and which differ from the typical non-aggressive polymorphisms are represented by green as are the identical polymorphisms exhibited by 50 % or less of NAN isolates. Polymorphisms typically exhibited by more than 50 % of NAN isolates, and which differ from those exhibited by EAN and non-aggressive isolates are represented by red. Where over 50 % of NAN and EAN isolates exhibit the same polymorphisms and there are no NAN specific polymorphisms they are represented by yellow. Apparently random novel polymorphisms are represented by white.

When viewed as a whole, this initial data set demonstrates a good general distinction between the three subgroups. The non-aggressive isolates in particular, may be readily distinguished from the aggressive isolates. Particularly striking is that probes selected for their prospective ability to distinguish between EAN and NAN isolates were, without exception, able to discriminate between the non aggressive isolates and the overwhelming majority of aggressive isolates.

While a number of probes efficiently distinguish between all three sub-groups, eg. HindIII digested n DNA probed with clone 115 and BamHI digested n DNA probed with clones 6, 29 and 103 (eg. Figs. 4.7, 4.8 and 4.9), a number of probes revealed that certain NAN isolates exhibited what were typically EAN polymorphisms as opposed to typically NAN polymorphisms, eg. isolates T223 and H297 when HindIII digested n DNA is probed with clones 20 and 117 (Figs. 4.7, 4.10 and 4.11), and possibly isolates H172, H312, ES376, H351, PG402, T223 and H297 when BamHI digested n DNA is probed with clone 128 (eg. Figs. 4.7 and 4.12). Clones 36, 110 and 122 probed against HindIII digested DNA and clones 23 and 124 probed against BamHI digested n DNA revealed a number of aggressive isolates which apparently exhibit typical non-aggressive polymorphisms (eg. Figs. 4.7 and 4.13). In addition, a number of other clones such as 110 and 117 probed against

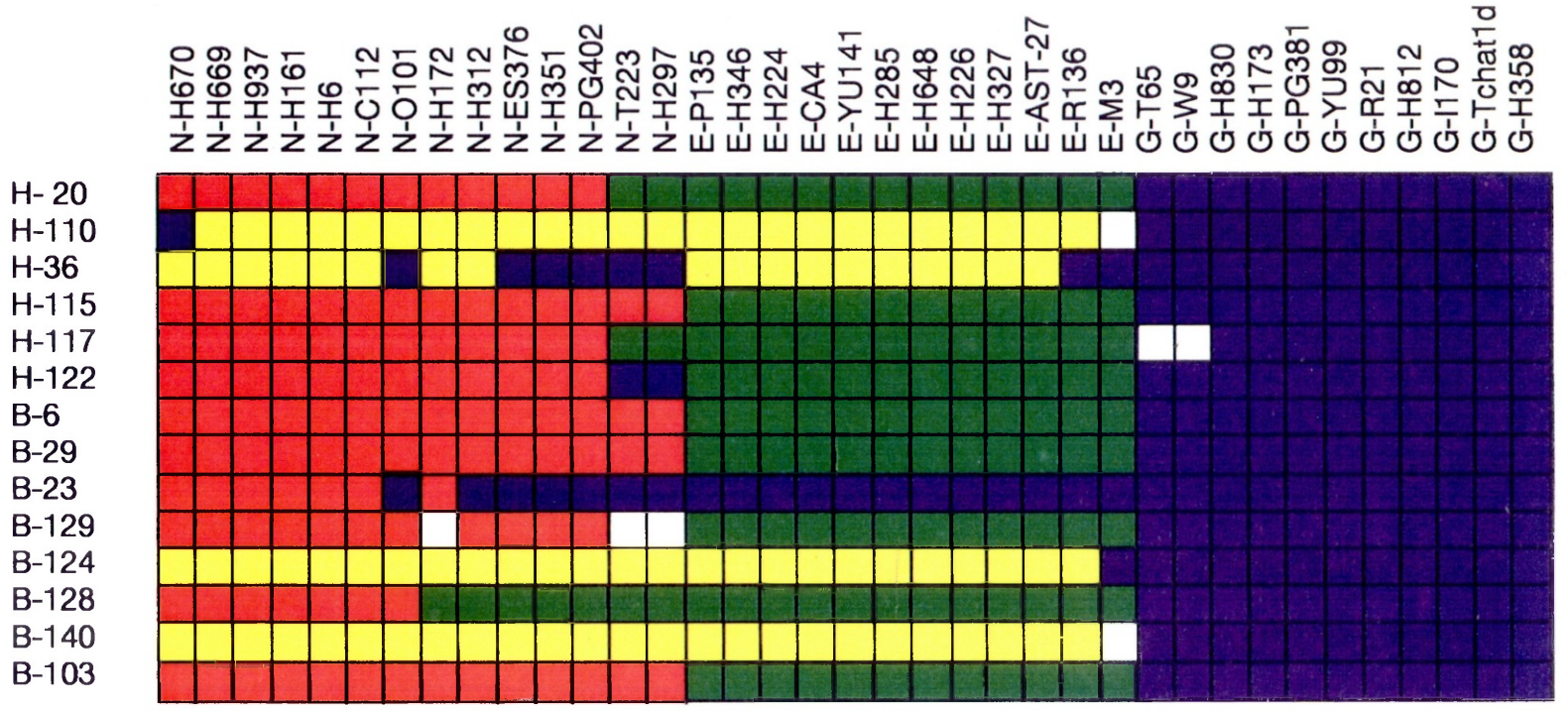

Fig. 4.7. Summary of the n DNA polymorphisms exhibited by a broad geographical range of non-aggressive, EAN and NAN isolates when their n DNA was digested with HindIII or BamHI and probed with a number of n DNA clones which had been shown to distinguish between isolates M3 and C112. The letter before each isolate denotes the subgroup of which it is a member; G = non-aggressive; N = NAN; E = EAN. H and B prior to the clone numbers denote the enzymes HindIII and BamHI respectivley with which the n DNA was digested. For the key to the colours see text.

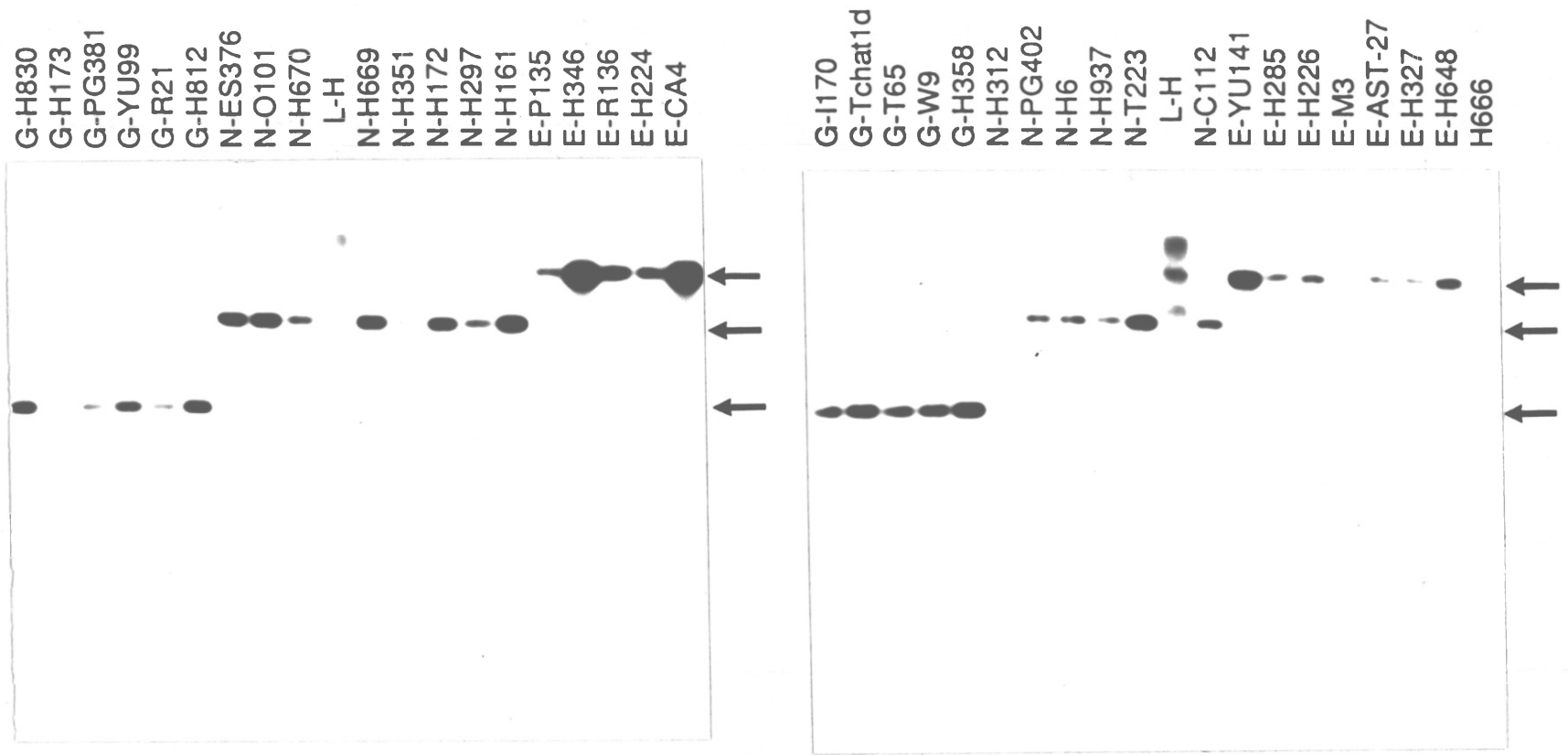

Fig. 4.8. BamHI digested n DNA of an initial broad geographical range of EANN, NAN and non-aggressive isolates and isolate H666 probed with random n DNA clone 6.

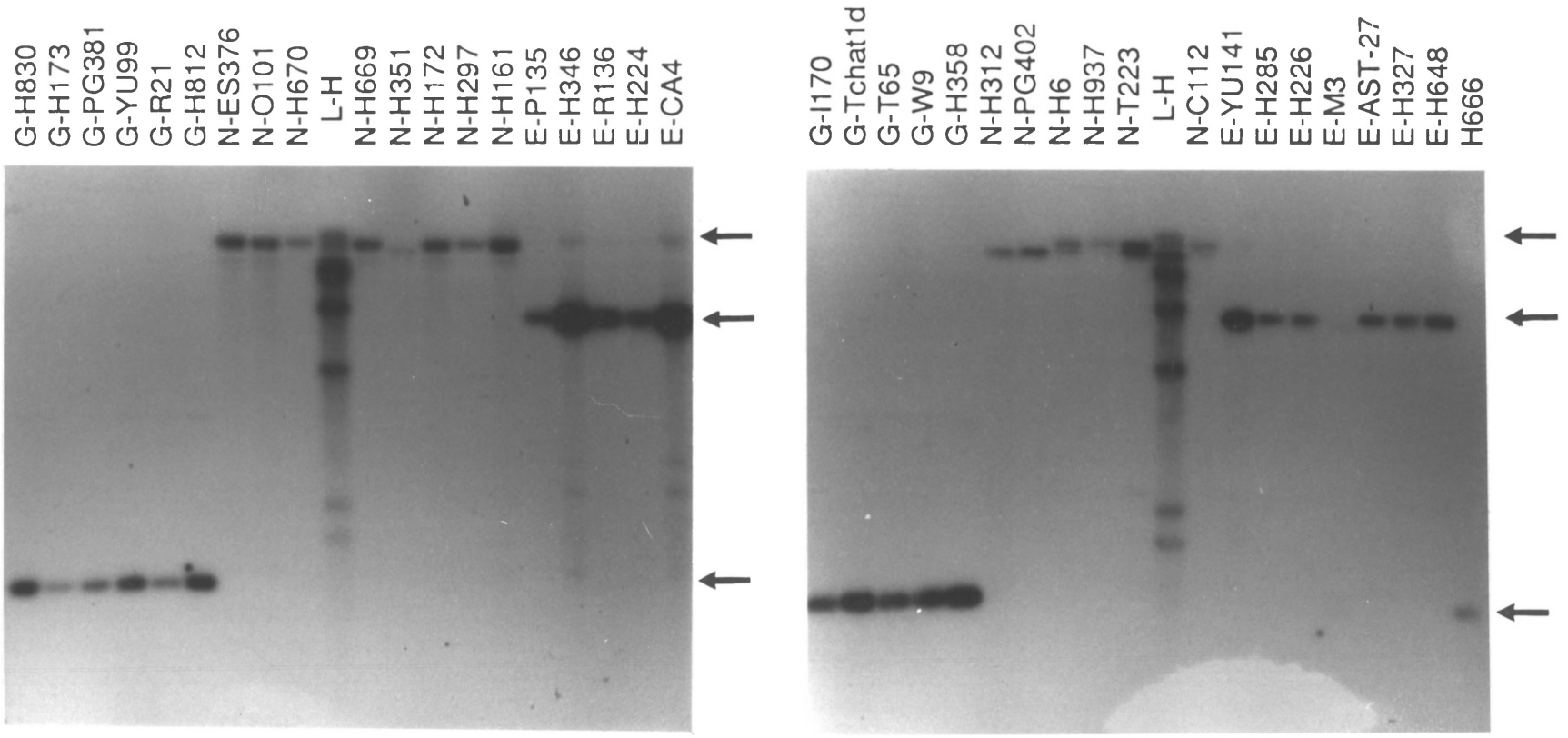

Fig. 4.9. BamHI digested n DNA of an initial broad geographical range of EAN, NAN and non-aggressive isolates and isolate H666 probed with random n DNA clone 103.

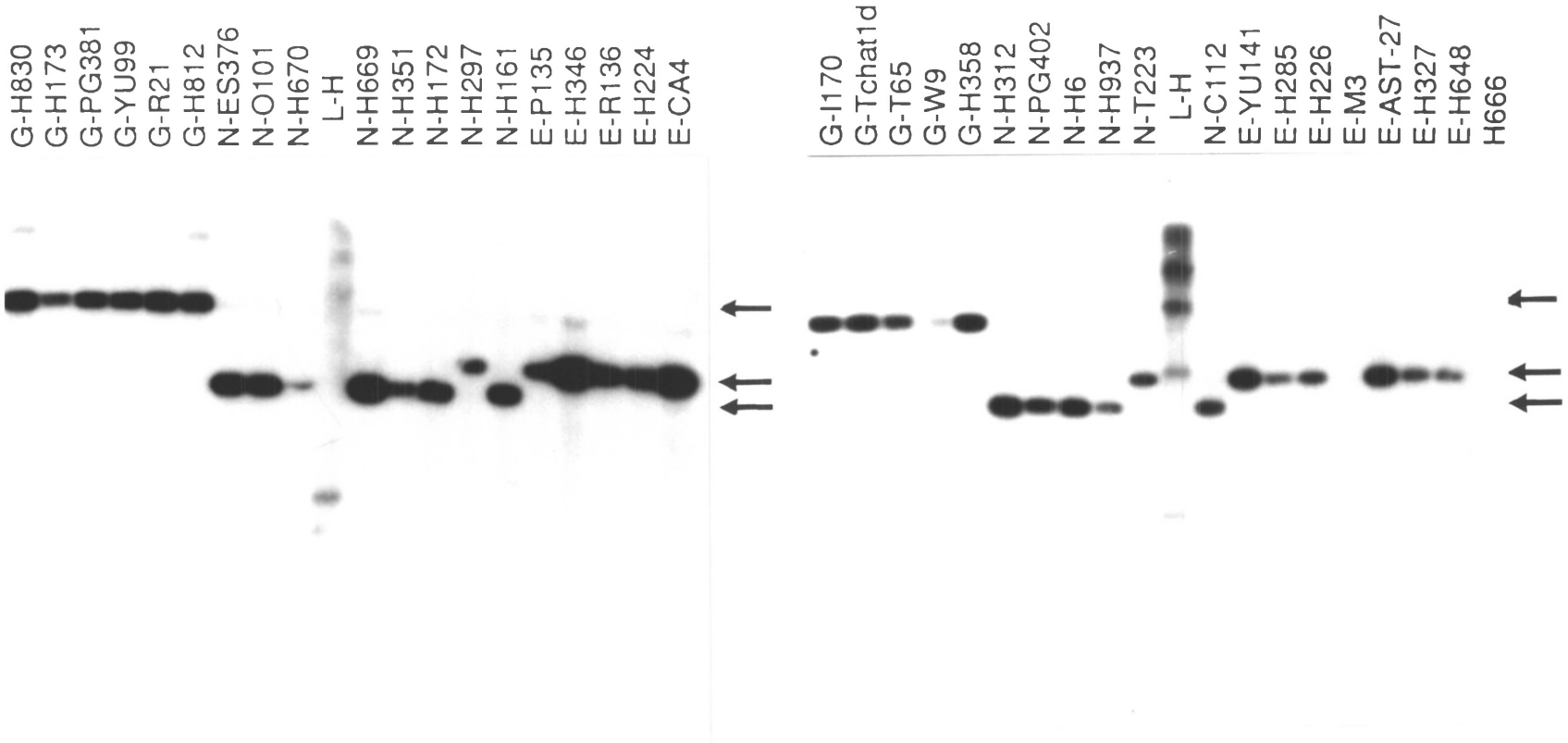

Fig. 4.10. HindIII digested n DNA of an initial broad geographical range of EAN, NAN and non-aggressive isolates and isolate H666 probed with random n DNA clone 20.

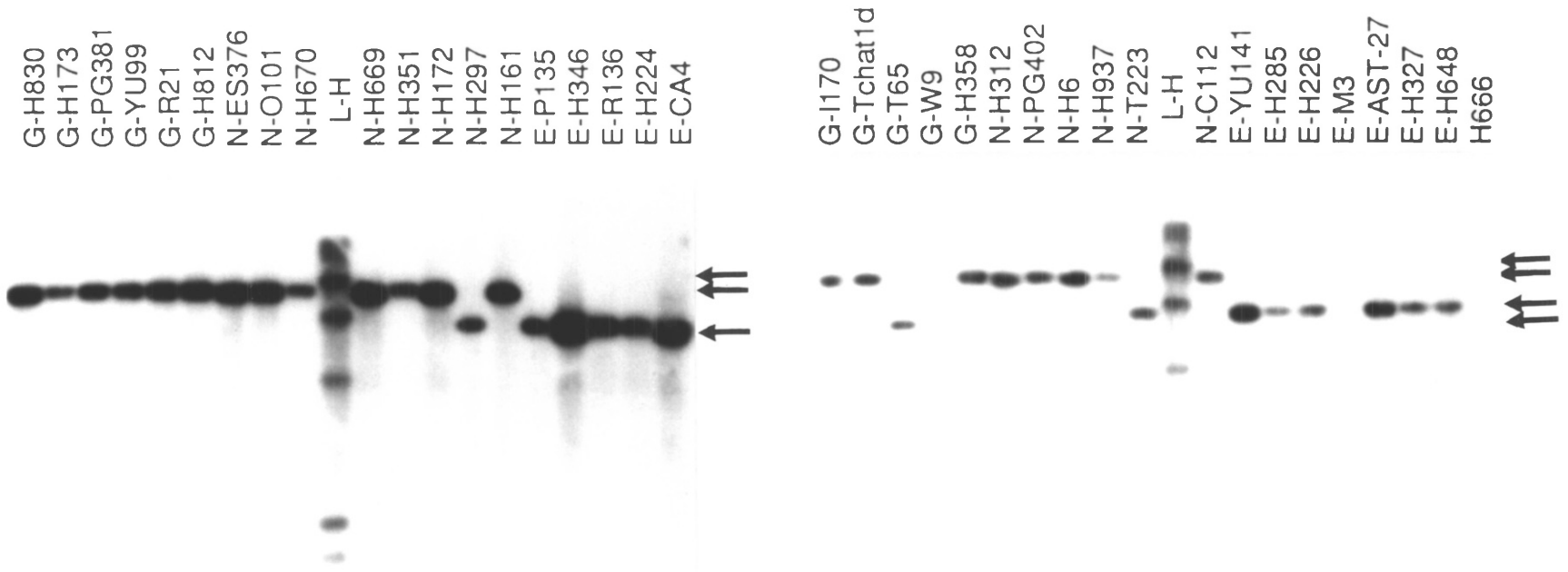

Fig. 4.11. HindIII digested n DNA of an initial broad geographical range of EAN, NAN and non-aggressivee isolates and isolate H666 probed with random n DNA clone 117.

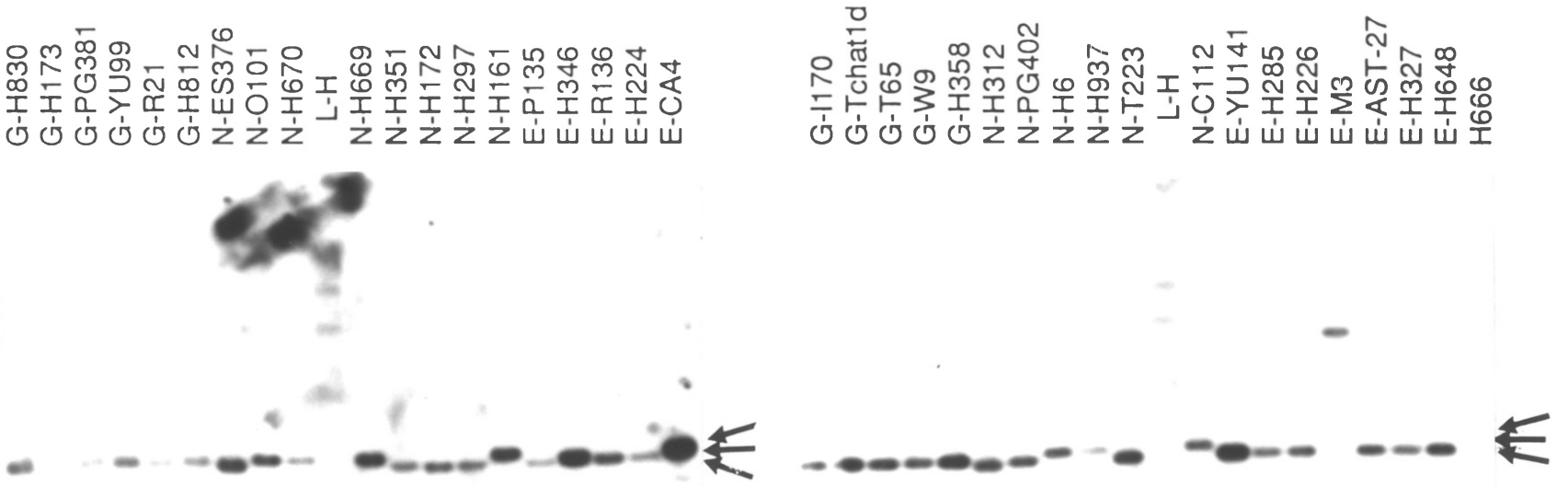

Fig. 4.12. BamHI digested n DNA of an initial broad geographical range of EAN, NAN and non-aggressive isolates and isolate H666 probed with random n DNA clone 128.

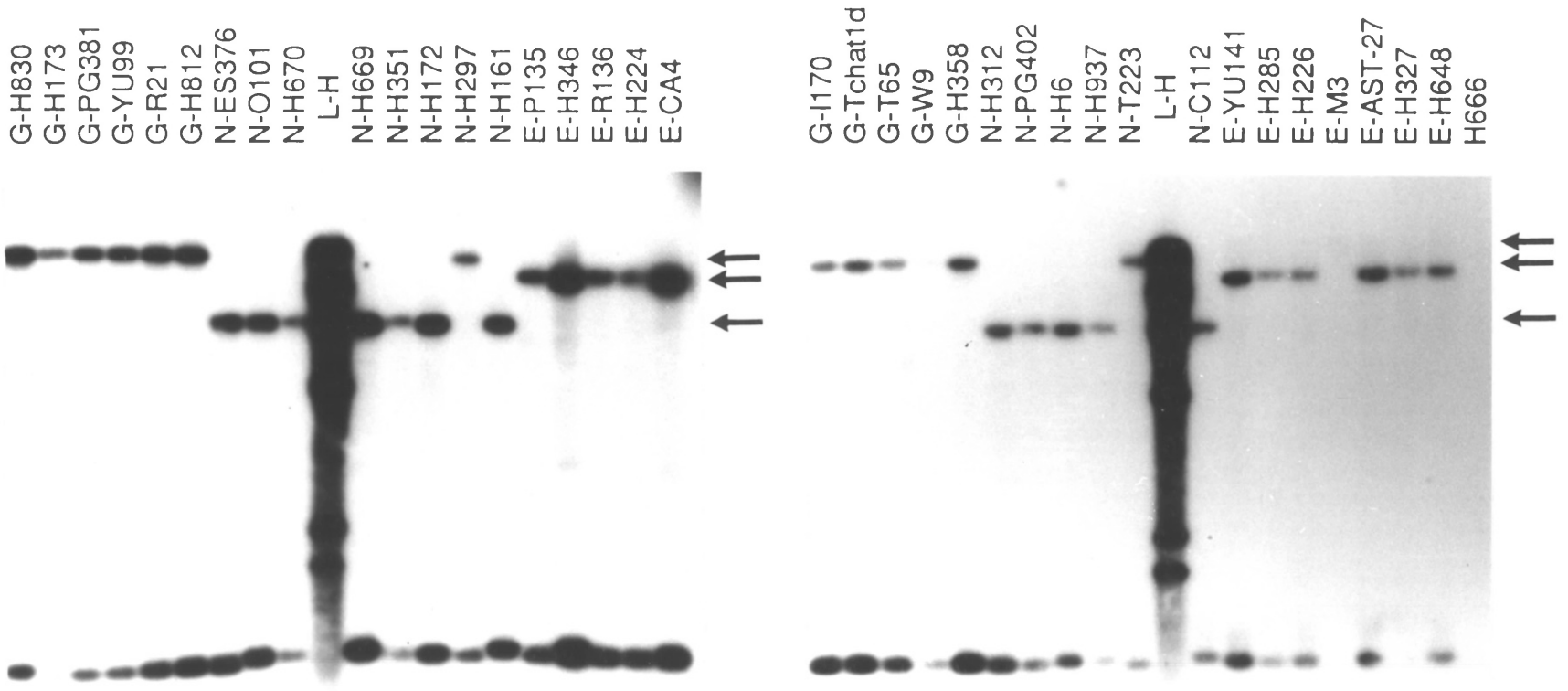

Fig. 4.13. HindIII digested n DNA of an initial broad geographical range of EAN, NAN and non-aggressive isolates and isolate H666 probed with random n DNA clone 122.

HindIII digested n DNA and clones 129 and 140 probed against BamHI digested n DNA, revealed several novel polymorphisms (eg. Figs. 4.7 and 4.14).

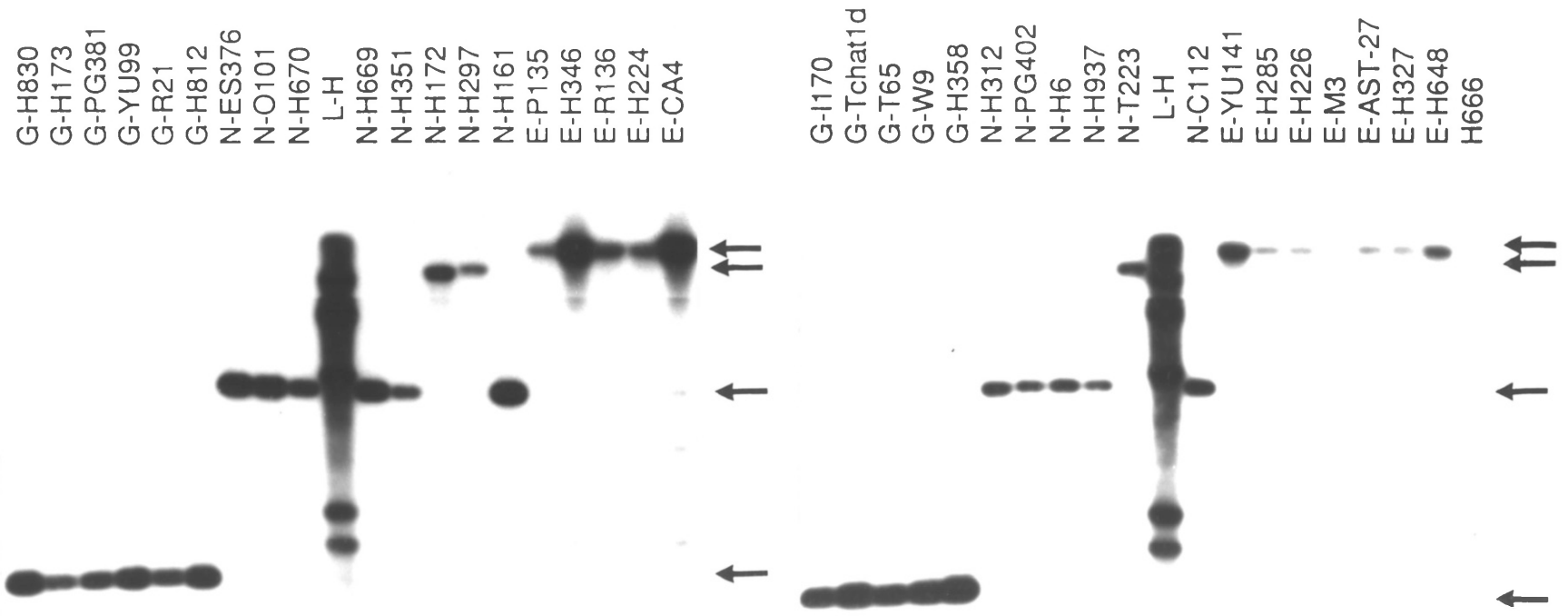

Fig. 4.14. BamHI digested n DNA of an initial broad geographical range of EAN, NAN and non-aggressive isolates and isolate H666 probed with random n DNA clone 129.

5 RESULTS III - VEGETATIVE COMPATIBILITY GROUPS, NUCLEAR DNA POLYMORPHISM AND POPULATION STRUCTURE.

5.1 Population Structure at Tewkesbury, Great Britain.

Tewkesbury is one of the three originally recognised outbreak areas of the NAN epidemic in the UK along with Chichester and Orsett. These three sites were first sampled by Gibbs and Brasier in 1971 (Gibbs and Brasier, 1973) and the Tewkesbury sample was found to be much the slowest growing of the three with a mean growth rate of 3.17 mm/day when grown on MEA at 20°C, compared with 3.61 and 3.84 for Orsett and Chichester respectively. In addition the Tewkesbury sample was thought to be distinctive in that the isolates exhibited some unusual EAN-like morphological and growth rate characteristics. Their growth was somewhat uneven and they were less regularly striate than other NANs when grown on MEA at 20°C (Brasier, pers. comm.). This distinctive Tewkesbury type was still recognizable in 1975 when Brasier collected 2,500 isolates from across England and Wales south of Birmingham, and it was then found to extend north to Wolverhampton, west to Monmouth, south to Chippenham and west to Stratford and Nottingham (Brasier, pers. comm.). At around this time there was also an explosion of variation in vc groups among the NAN isolates in this area similar to that which has been well documented at Tomar and Mafra (see Sections 1.8 and 1.9) and is now thought to be part of the normal transition in population structure from epidemic front to post epidemic areas.

The area around Tewkesbury was resampled by Brasier in 1983 and vc testing revealed that although the dominant European NAN vc supergroup was common at around 16 % of the sample, there existed a second major vc group also comprising around 16 % of the sample, which produced 'w' reactions in pairings with the European NAN vc supergroup and was named the 'Tewkesbury 2nd vc group'. The remaining 67 % of the sample consisted of a heterogeneous vc group component (Table 1.2). Isolates of the 2nd vc group exhibited the unusual 'Tewkesbury' phenotype which had been observed over ten years

previously at the same site - see above. Vegetative compatibility analysis of samples taken from the other initial outbreak sites of Chichester and Orsett in the same year did not reveal the presence of this second major vc group (Table 1.2).

One of the most striking features of Fig. 4.7 is that two independent probes, namely clones 20 and 117, when probed against HindIII digested n DNA produced essentially identical infraracial polymorphisms. As described in Section 4, the Tewkesbury isolate T223 and the Irish isolate H297 exhibited clear cut characteristically EAN banding patterns with these two clones although both isolates appeared to be otherwise typical NAN isolates with respect to most other mt DNA polymorphisms and n DNA polymorphisms (Figs. 3.10, 3.11, 3.12 and 4.7).

NAN isolate T223 was from the 1983 Tewkesbury sample and had been shown to be a member of the second major vc group (Fig. 3.4). This raised the question of whether these characteristically EAN polymorphisms correlated with vegetative compatibility. To determine whether this was the case it was decided to screen blind a further 19 isolates from this Tewkesbury sample along with two Chichester isolates, two Orsett isolates and the potentially hybrid isolate H106 with the same two probes. Jeng et al (1988) suggested that isolate H106 is a hybrid between the EAN and NAN races on the basis of a range of cultural and electrophoretic characteristics. The readings for mycelial fresh weight, dry weight, acetone powder and water content for this isolate, which had been assigned to the NAN on the basis of discriminant functions, fell either within the normal EAN range or were intermediate between the EAN and the NAN. Isoenzyme and soluble protein patterns on polyacrylamide gradient gels for a number of isolates were assessed and in the electrophoretic patterns of tetrazolium oxidase for example, H106 exhibited one band specific to the EAN subgroup and two bands characteristic of NAN isolates.Ten NAN vc supergroup isolates and a further nine from the 2nd major vc group (see Section 2.10.2) were thus examined and as can be seen from Table 5.1 and Figs. 5.1 and 5.2 the correlation was found to be complete; isolates of the 2nd major vc group exhibiting solely EAN type polymorphisms and isolates of the NAN vc supergroup exhibiting solely

Table 5.1. Summary of initial Tewkesbury and other control isolates and the polymorphisms exhibited when probed with clones 20 and 117.

European NAN vc supergroup isolates:

	T210	T219	T231	T235	T237	T241	T244	T252	T264	T265
20H	X	X	X	X	X	X	X	X	X	X
117H	X	X	X	X	X	X	X	X	X	X

Tewkesbury 2nd vc group isolates:

	T220	T222	T223	T228	T236	T247	T253	T254	T255	T265
20H	O	O	O	O	O	O	O	O	O	O
117H	O	O	O	O	O	O	O	O	O	O

Control Chichester (C) and Orsett (O) isolates, which give 'w' reactions to the European NAN vc supergroup, and the possible EAN/NAN hybrid isolate H106:

	C80	C81	O96	O109	H106
20H	X	X	X	X	X
117H	X	X	X	X	X

X = typical NAN polymorphism, O = typical EAN polymorphism
20H = clone 20 probed against HindIII digested DNA.
117H = clone 117 probed against HindIII digested DNA.

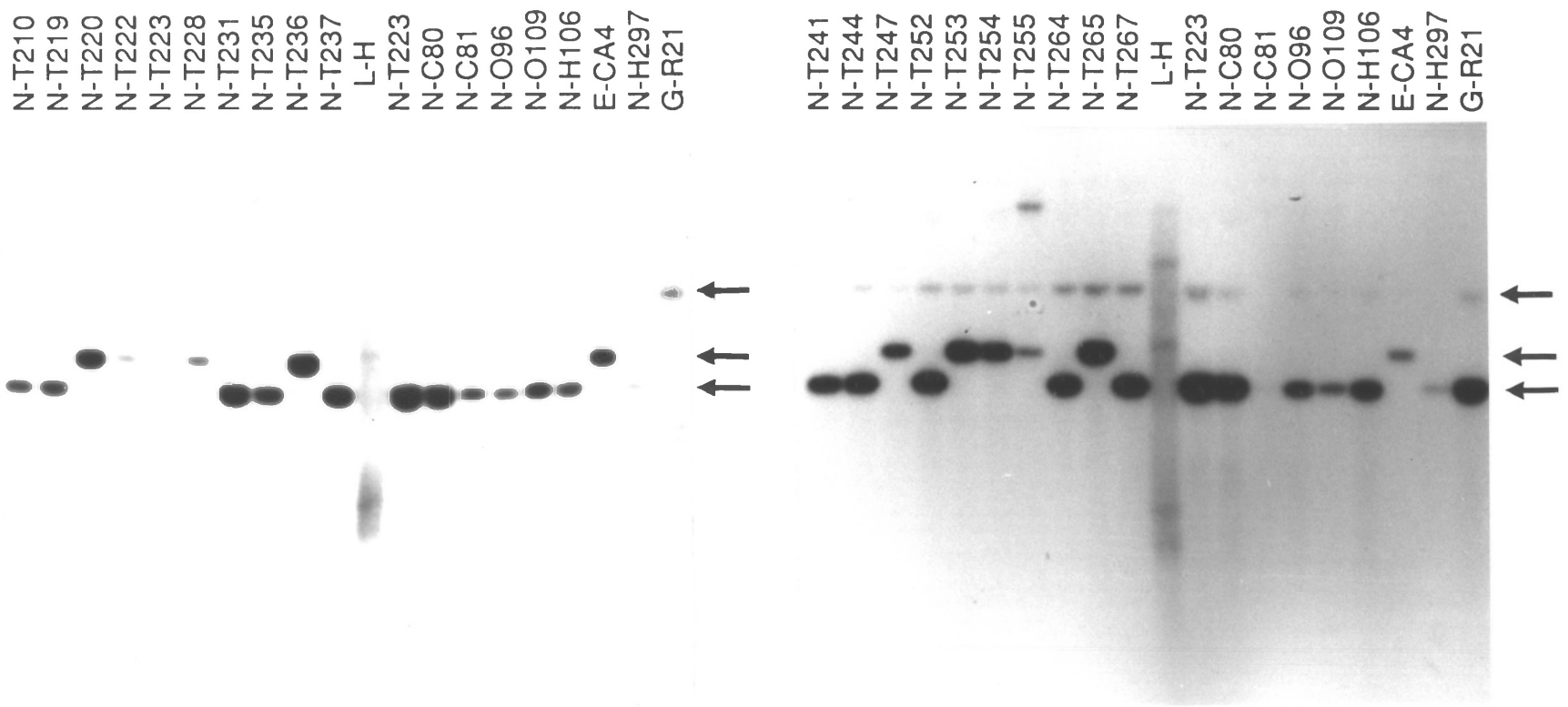

Fig. 5.1. HindIII digested total DNA of a range of European NAN vc supergroup and Tewkesbury 2nd vc groupp isolates probed with n DNA clone 20.

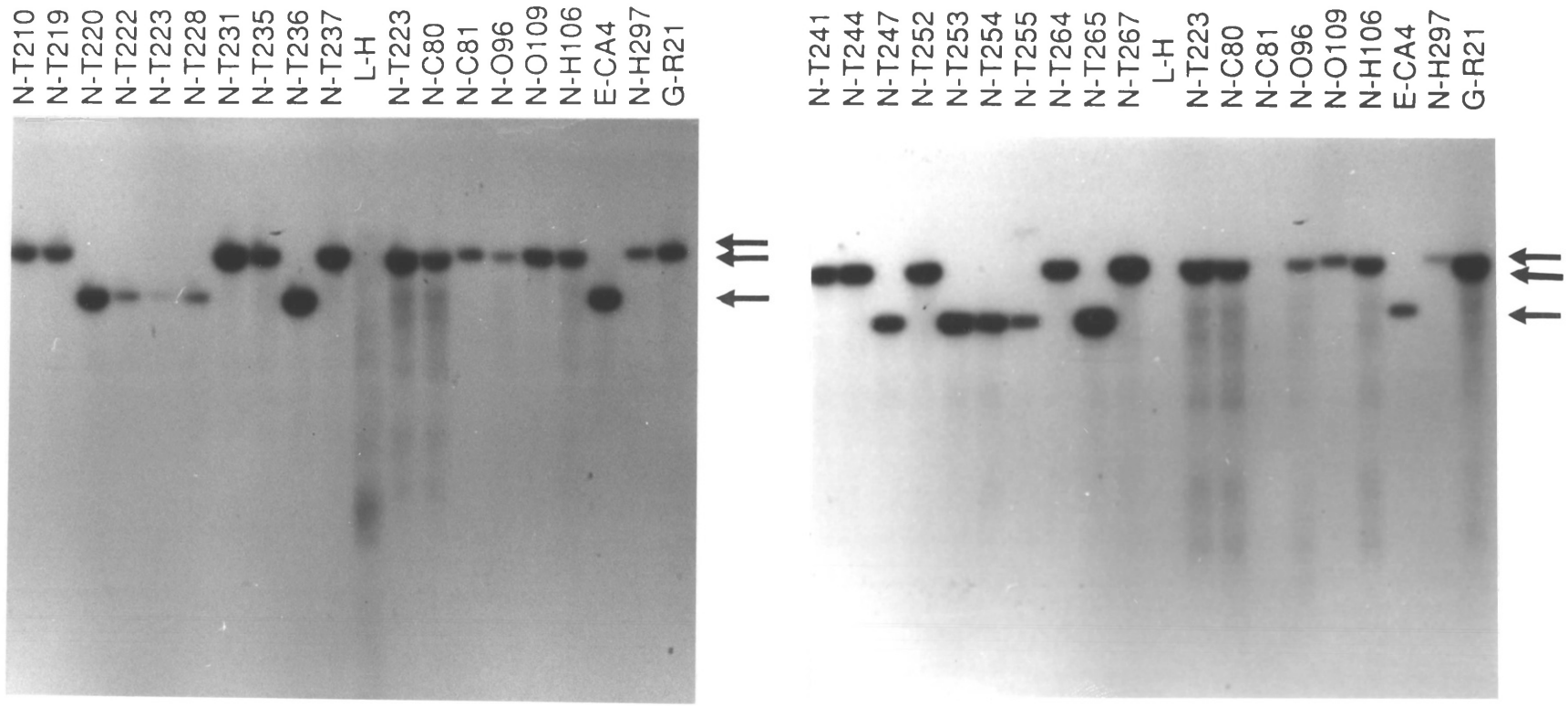

Fig. 5.2. HindIII digested total DNA of a range of European NAN vc supergroup and Tewkesbury 2nd vc group isolates probed with n DNA clone 117.

NAN type polymorphisms. All four Chichester and Orsett isolates and isolate H106 exhibited solely NAN type polymorphisms, though little can be deduced from such small samples. (In this and all further n DNA polymorphism experiments small scale total DNA preparations were used rather than large scale CsCl n DNA preparations.)

At the time of sampling in 1983 the Tewkesbury area had been post epidemic for over ten years and apart from the unusual presence of a 2nd major vc group, was in many ways typical of such an area (Brasier, 1988): There was a considerably reduced percentage of major vc group isolates and a large heterogeneous component comprising vc groups giving both 'n' and 'w' reactions to the two major vc groups (Brasier, 1988). These additional vc groups are thought to have been generated from the originally, almost clonal epidemic front population by the processes of outcrossing, introgression and pseudoselfing described in Sections 1.6 and 1.7.

In order to further investigate the relationship between n DNA polymorphisms and vc groups it was then decided to screen a further 29 Tewkesbury isolates from the heterogeneous component of the 1983 sample (see Section 2.10.2 and Table 5.2). Six of the isolates gave 'n' reactions to the European NAN vc supergroup, thirteen isolates gave 'n' reactions to the Tewkesbury 2nd vc group, and ten isolates gave 'w' reactions to both these major vc groups. Utilizing the same two probes all possible combinations of polymorphisms were obtained (Table 5.2 and Figs. 5.3 - 5.8) indicating probable free recombination of these n DNA fragments between the two major vc groups and other genotypes. In addition two isolates T7 and T40, which were sampled at Tewkesbury in 1973, soon after the initial outbreak of the disease at this site, exhibited consistently EAN type polymorphisms with the same two probes (Table 5.2 and Figs. 5.3 - 5.8).

Isolates T7 and T40 are unique isolates in that they have a rather unusual ragged appearance in culture and were in fact the first A mating type isolates found in Britain, earlier sampling in 1971 having failed to reveal any A mating type isolates (Brasier and Gibbs, 1975). When normal A type isolates are selfed they produce progeny which fall into an approximately 1:1 ratio with respect to A

Table 5.2. Summary of a further sample of Tewkesbury isolates and the polymorphisms exhibited when probed with clones 20 and 117.

Isolates giving 'n' reactions to the European NAN vc supergroup (isolates T202, T239 and T248 give 'n' reactions to each other, rest unknown):

	T202	T213	T239	T248	T257	T261
20H	O	O	X	O	X	X
117H	X	O	X	X	X	O

Isolates giving 'n' reactions to the Tewkesbury 2nd vc group (isolates T208, T214, T217 and T230 give 'n' reactions times each other, rest unknown):

	T201	T204	T205	T206	T208	T209	T214	T217	T218	T232	T238	T262	T263
20H	X	O	X	X	X	O	O	X	X	O	O	O	X
117H	X	O	X	X	X	O	O	X	O	O	X	O	X

Isolates giving 'w' reactions to both major vc groups:

	T216	T224	T225	T233	T234	T240	T243	T246	T250	T259
20H	X	X	X	O	O	O	X	X	X	O
117H	X	X	X	O	O	O	X	X	X	X

Isolates sampled in 1973 soon after initial outbreak at Tewkesbury:

	T7	T40
20H	O	O
117H	O	O

X = typical NAN polymorphism, O = typical EAN polymorphism
20H = clone 20 probed against HindIII digested DNA.
117H = clone 117 probed against HindIII digested DNA.

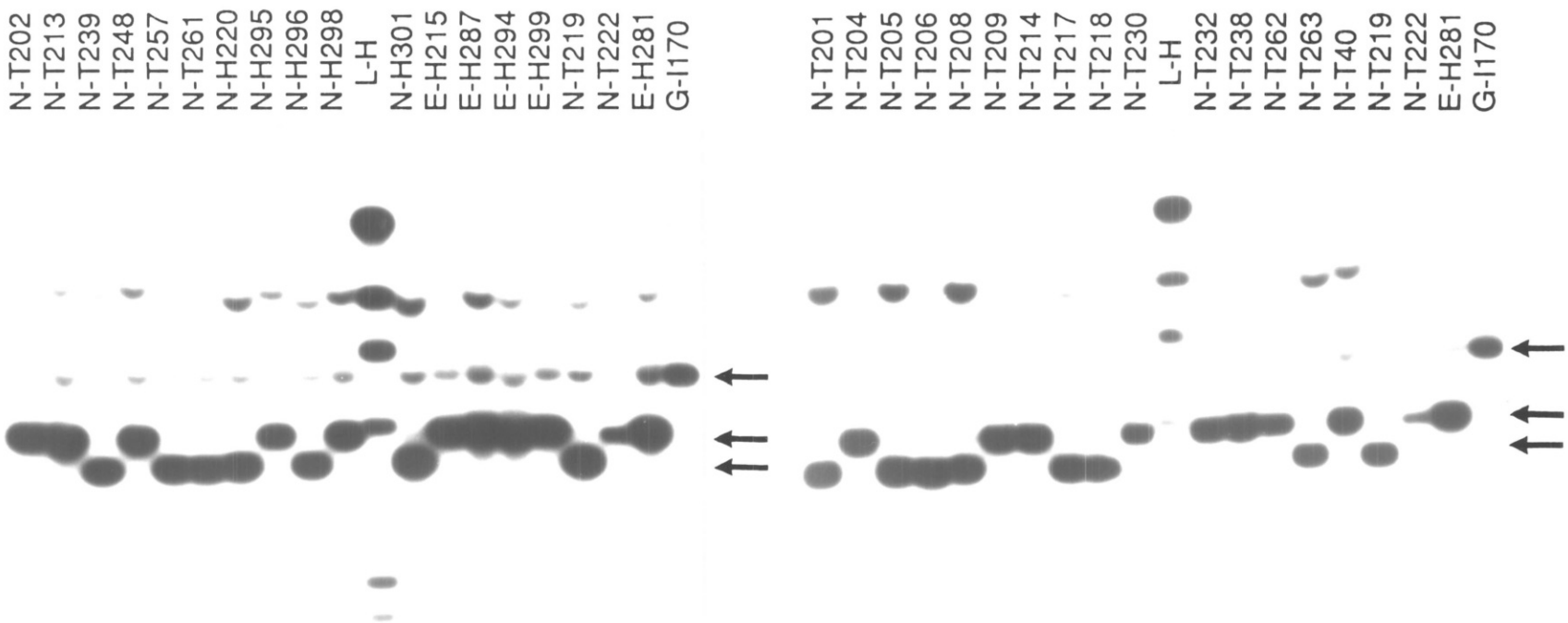

Fig. 5.3. HindIII digested total DNA of isolates from Britain, Eire and the Iberian peninsula probed with n DNA clone 20.

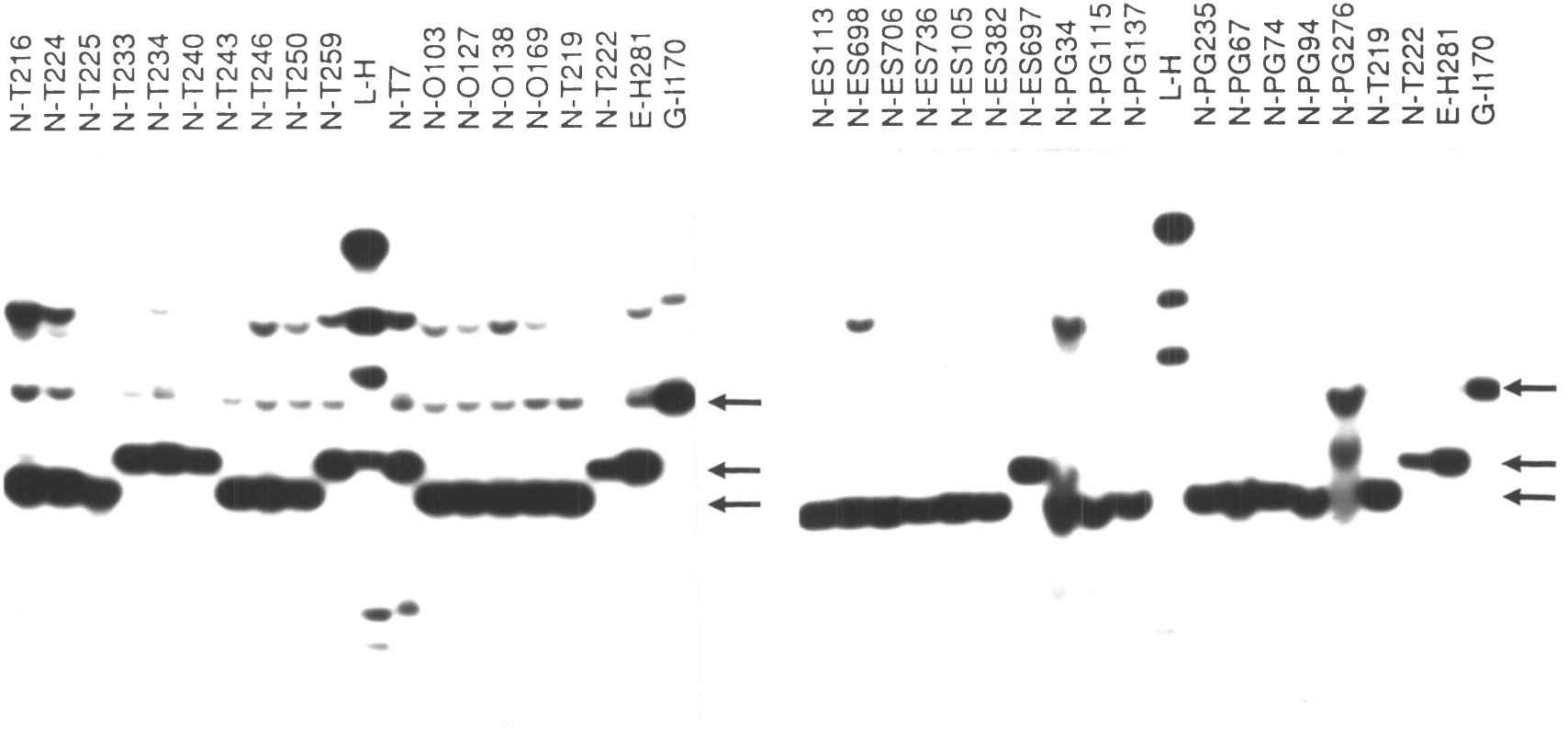

Fig. 5.4. HindIII digested total DNA of isolates from Britain, Eire and the Iberian peninsula probed with n DNA clone 20.

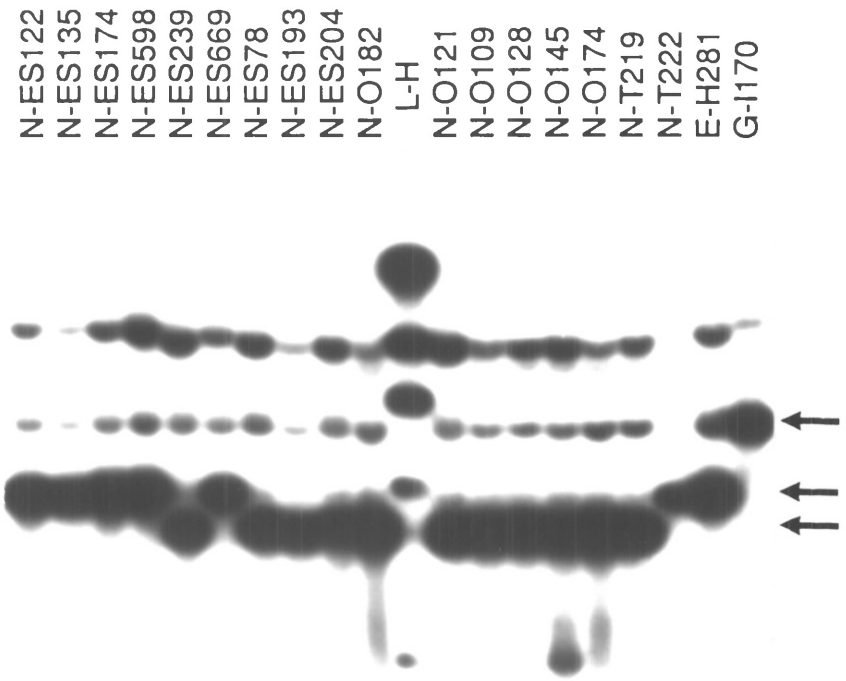

Fig. 5.5. HindIII digested total DNA of isolates from Britain, Eire and the Iberian peninsula probed with n DNA clone 20.

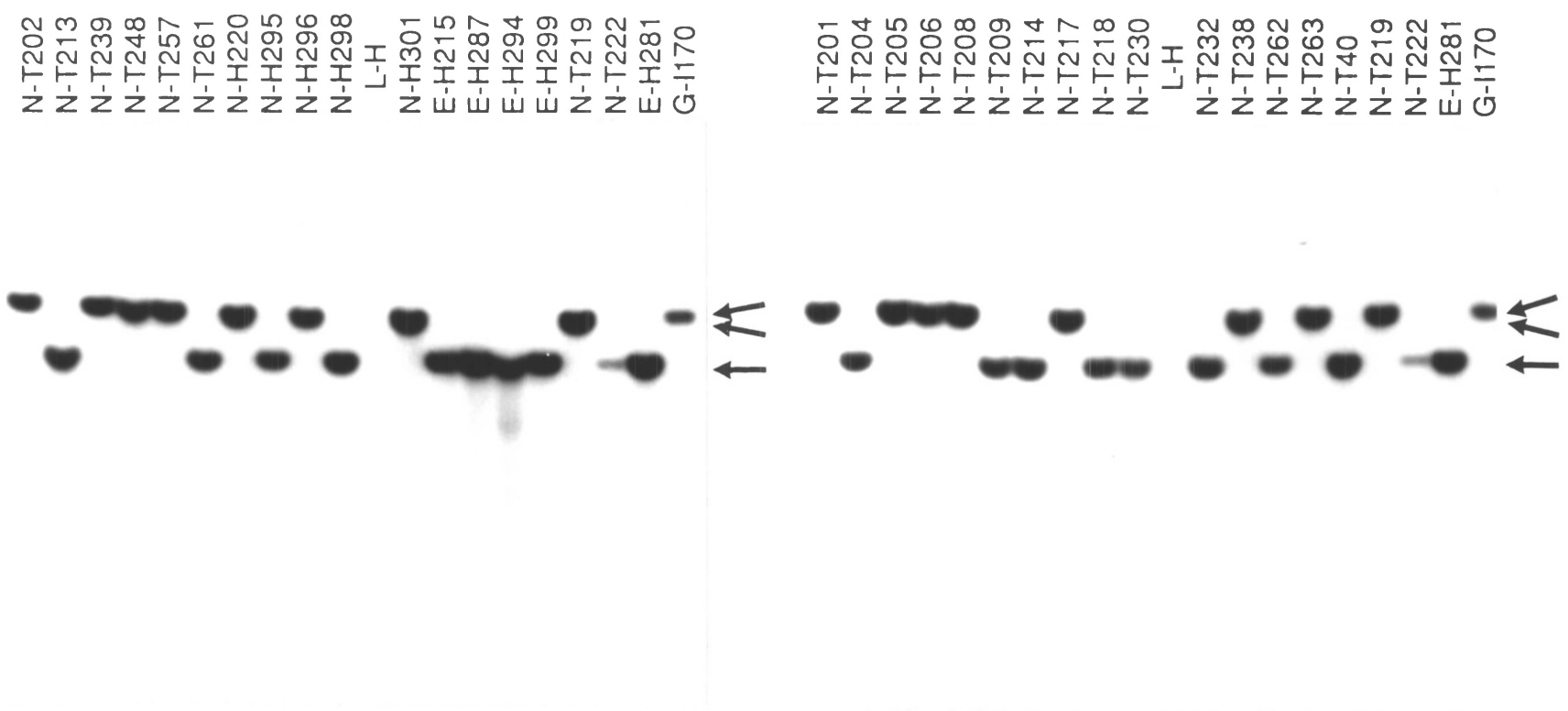

Fig. 5.6. HindIII digested total DNA of isolates from Britain, Eire and the Iberian peninsula probed with n DNA clone I17.

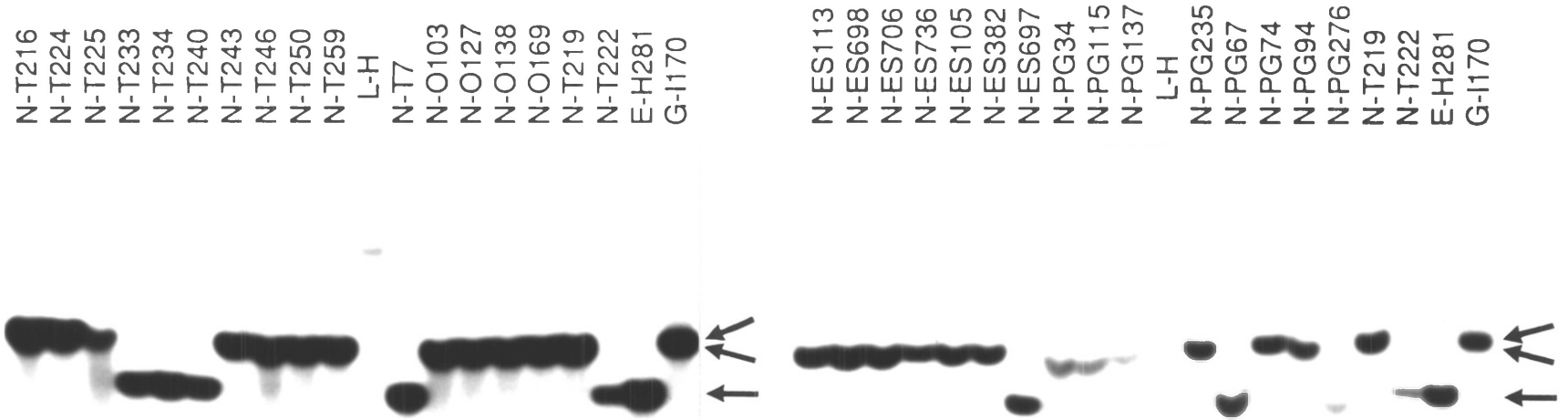

Fig. 5.7. HindIII digested total DNA of isolates from Britain, Eire and the Iberian peninsula probed with n DNA clone 117.

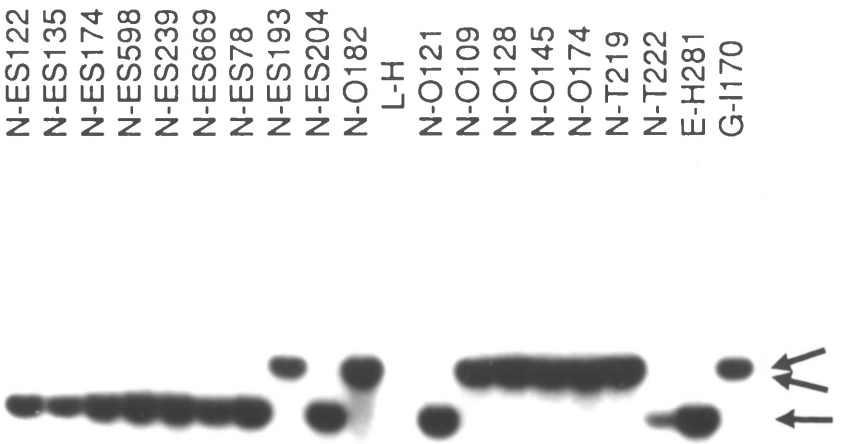

Fig. 5.8. HindIII digested total DNA of isolates from Britain, Eire and the Iberian peninsula probed with n DNA clone I17.

and B types, and which exhibit significantly variable growth rates, though they still fall within the usual range of aggressive isolates. Isolates T7 and T40 on the other hand, are completely separated with the A types growing very much more slowly than the B types and well below normal for NANs (Brasier and Gibbs, 1975). It would be of great interest to probe isolates with a range of other clones to assess whether they exhibit other unusual polymorphisms.

The presence of the EAN type polymorphisms in T7 and T40 along with the anomalous characteristics of the initial clonal population at Tewkesbury suggests an early presence of an at least partially heterogeneous population. The origin of these apparently typically EAN type n DNA fragments remains a matter of supposition, though given that the outbreak in Britain is thought to be the first appearance of the NAN aggressive subgroup in Western Europe (Brasier, 1979) and the fact that the importation of the NAN aggressive subgroup from North America is well documented (Brasier and Gibbs, 1973), it seems likely that the origin of these EAN type polymorphisms is North America. Thus, the currently different population structures of the three initial UK outbreak sites may well reflect the genetic profile of the isolates making up the initial founder populations.

The correlation of these characteristically EAN type polymorphisms with vegetative compatibility groups suggested screening other samples of NAN isolates with the same probes in order to gain further information about the distribution of genetic variability within the aggressive population structure. It was thus decided to examine samples of NAN isolates from areas where field surveys had been undertaken and especially where major vc groups other than the European NAN vc supergroup were present.

5.2 Population Sructure in Ireland.

In Ireland two aggressive outbreaks appear to have occurred around 1977, with an EAN outbreak centred initially around Cork and an NAN outbreak centred initially around Dublin. These events have been well documented by Walsh and Mangan (1977) and Brasier (1979).

As mentioned above Irish NAN isolate H297 exhibited identical n DNA banding patterns to T223 (Fig. 4.7), and was also a member of the same vc group. A widespread sample of Irish isolates had been collected by A. Mangan in 1978 and it was therefore decided to investigate further the extent of these EAN type polymorphisms in Ireland. The same two probes were used to screen four Irish EAN and five Irish NAN isolates of unknown vc groups (see Section 2.10). The four Irish EAN isolates exhibited consistently EAN type polymorphisms while of the five Irish NAN isolates, two gave typically NAN type polymorphisms, two gave typically EAN type polymorphisms and one isolate gave a different polymorphism with each of the two probes (Table 5.3 and Figs. 5.3 - 5.8).

The presence of typically EAN and NAN polymorphisms in these Irish NAN isolates indicates that the population structure among NAN isolates may be similar to that found in the Tewkesbury area, although the vc groups of these isolates have yet to be determined. Since these outbreaks are thought to have begun around 1977 these results provide supporting circumstantial evidence for the proposition that the at least partially heterogeneous NAN population may have originated from the British western outbreak area centred around Tewkesbury. There remains however the possibility that the NAN outbreak in Ireland may have originated directly from North America or even elsewhere. Neither can the possibility of recent EAN/NAN hybridisation in Ireland be entirely excluded.

5.3 Population Structure at Orsett, Great Britain.

At Orsett in Essex, another of the original three UK outbreak areas, initial sampling in 1972 revealed the typical clonal population structure of an epidemic front and the uneven morphological and slower growth rate characteristics of isolates at Tewkesbury were absent. Furthermore a vc analysis of isolates collected from the Orsett area by Brasier in 1983 revealed the dominance of the NAN vc supergroup and the apparent absence of the Tewkesbury 2nd vc group (Brasier, 1988). At this time the European NAN vc supergroup component comprised 13 % of the sample while the

Table 5.3. Summary of Irish isolates and the polymorphisms exhibited when probed with clones 20 and 117.

EAN isolates:

	H215	H287	H294	H299
20H	0	0	0	0
117H	0	0	0	0

NAN isolates:

	H220	H295	H296	H298	H301
20H	0	0	X	0	X
117H	X	0	X	0	X

X = typical NAN polymorphism, 0 = typical EAN polymorphism
20H = clone 20 probed against HindIII digested DNA.
117H = clone 117 probed against HindIII digested DNA.

remaining 87 % was made up of a heterogeneous vc group component (Table 1.2).

If one is to explain the difference betwen the population structure at Tewkesbury and other sites even partly in terms of founder effects it would be of interest to know whether the characteristically EAN n DNA polymorphisms found at Tewkesbury were also present within the Orsett population. Consequently it was decided to screen a number of isolates from Orsett with the same two probes. Ten isolates sampled by Brasier in 1983 (see Section 2.10.2) were screened with probes 20 and 117. Five isolates were members of the European NAN vc supergroup while a further five isolates gave w reactions to this group. The former five isolates exhibited consistently NAN type polymorphisms while of the latter five isolates four again gave NAN type polymorphisms while the fifth gave an EAN type polymorphism when probed with clone 117 (Table 5.4 and Figs. 5.3 - 5.8).

Though only a very limited sample from this area, it is sufficient to demonstrate that EAN type polymorphism is present in this population at least by 1983. The normal NAN type morphological and growth rate characteristics of initial samples collected at Orsett in 1972 may indicate that isolates similar to those of the Tewkesbury 2nd vc group were not initially widespread. Thus, the presence of EAN polymorphism within the 1983 sample suggests either later interaction with the Tewkesbury centred population or a lower initial presence of EAN type polymorphisms in the original founder population and which has been kept at a low level by selection pressures.

5.4 Population Structure at Guadalajara, Spain.

In 1984 an active front was located by Brasier in the Guadalajara area of Central Spain. Here another major vc group is dominant and it was shown by Brasier (1988) to be the same vc group as the second vc group at Tewkesbury. At the epidemic front in Guadalajara itself, this 'Tewkesbury / Guadalajara' vc group comprised 95 % of a sample of 39 NAN isolates with only two other

Table 5.4. Summary of Orsett isolates and the polymorphisms exhibited when probed with clones 20 and 117.

European NAN vc supergroup isolates:

	0103	0127	0138	0169	0182
20H	X	X	X	X	X
117H	X	X	X	X	X

Isolates giving 'w' reactions to the European NAN vc supergroup and to each other:

	0121	0109	0128	0145	0174
20H	X	X	X	X	X
117H	O	X	X	X	X

X = typical NAN polymorphism, O = typical EAN polymorphism
20H = clone 20 probed against HindIII digested DNA.
117H = clone 117 probed against HindIII digested DNA.

isolates of different vc groups being found (Table 1.2). The situation in the Guadalajara area in Spain, despite the apparent absence of the European NAN vc supergroup, is already undergoing an explosion of new vc groups similar to that which has occurred at Tewkesbury and elsewhere.

Given the almost total dominance of the Tewkesbury / Guadalajara vc group in this area it was decided to determine whether the characteristically EAN type polymorphisms of the isolates at Tewkesbury correlated with the same vc group in this area, and whether the normal NAN type polymorphisms were present at all. A sample of nine isolates collected from the area in 1985 (see Section 2.10.2) were therefore screened with probes 20 and 117. Four isolates which were members of the Tewkesbury / Guadalajara vc group were from the epidemic front at Guadaljara itself, as were two isolates which gave n reactions to the European NAN vc group. In addition there were three isolates some distance from the actual front at Guadalajara, which gave w reactions both to the Tewkesbury / Guadalajara vc group and to the European NAN vc supergroup (Table 5.5). The four Tewkesbury / Guadalajara vc group isolates again showed EAN type polymorphisms with both probes. Of the two isolates giving 'n' reactions to the 2nd vc group, one exhibited consistently EAN type polymorphisms while the other exhibited one EAN and one NAN type polymorphism. Of three isolates giving w reactions to the Tewkesbury / Guadalajara vc group one isolate gave consistently NAN type polymorphisms and the other two exhibited one EAN and one NAN type polymorphism (Table 5.5 and Figs. 5.3 - 5.8).

As at Tewkesbury therefore, the EAN type polymorphisms correlate completely with the Tewkesbury / Guadalajara vc group. However despite the almost completely clonal nature of the population at the Guadalajara site, and the apparent absence of the European NAN vc supergroup, there is already a mixture of EAN and NAN type polymorphisms within the small newly emerging heterogeneous component.

Since there is considerable similarity with the population structure at Tewkesbury, it is also tempting to suggest the Guadalajara outbreak may have originated following the introduction

Table 5.5 Summary of Guadalahara isolates and the polymorphisms exhibited when probed with clones 20 and 117.

Guadalahara (Tewkesbury) vc group isolates:

	ES122	ES135	ES174	ES598
20H	0	0	0	0
117H	0	0	0	0

Isolates giving 'n' reactions to the Guadalahara (Tewkesbury) vc group and to each other:

	ES239	ES669
20H	X	0
117H	0	0

Isolates giving 'w' reactions to the Guadalahara vc group. ES78 x ES193 gives an 'n' reaction. ES204 gives a 'w' reaction against the other two:

	ES78	ES193	ES204
20H	X	X	X
117H	0	X	0

X = typical NAN polymorphism, 0 = typical EAN polymorphism
20H = clone 20 probed against HindIII digested DNA.
117H = clone 117 probed against HindIII digested DNA.

of the aggressive subgroup from Ireland or the UK mainland. Once again, however, the source may lie elsewhere and the evidence is far from unequivocal.

5.5 Population Structure Around Avila, Spain.

In 1984 Brasier (Brasier, 1988) also located a second active front site in central Spain which covered the Segovia - Avila - Penaranda region. He found that once again there was a dominant major vc group which in this case gave a w reaction to both the European NAN vc supergroup and the Tewkesbury / Guadalajara vc group. This 'Avila' vc group makes up 88 % of the sample whereas the heterogeneous component accounts for only 12 %, and as at Guadalajara the European NAN vc supergroup is apparently absent (Table 1.2). The Avila vc group is not one which has been observed elsewhere though the overall population structure with respect to vc groups is remarkably similar to that found at Guadalajara (Table 1.2). Of particular note however is that Avila vc group has an A mating type frequency of 40 %, whereas the supergroup component of both epidemic and post epidemic populations normally comprises purely B type isolates.

Seven isolates, four which were members of the Avila vc group, and three giving w reactions to this vc group and the European NAN vc supergroup (see Section 2.10.2), were again screened with probes 20 and 117 following HindIII digestion. The four isolates belonging to the Avila vc group gave NAN type polymorphisms without exception, while of the three isolates which gave 'w' reactions to this major vc group and to each other, two showed consistently NAN type polymorphisms and the third a consistently EAN pattern (Table 5.6 and Figs. 5.3 - 5.8). Thus, at this site, though the major vc group is different again from either the European NAN vc supergroup or the Tewkesbury / Guadalajara vc group the same overall pattern has emerged.

Here though the dominant vc group contains consistently NAN type polymorphisms suggesting a different origin and/or genetic profile for the initial founder population to that of the nearby

Table 5.6. Summary of Avila isolates and the polymorphisms exhibited when probed with clones 20 and 117.

Avila vc group isolates which give a 'w' reaction to the European NAN vc supergroup:

	ES113	ES698	ES706	ES736
20H	X	X	X	X
117H	X	X	X	X

Isolates giving 'w' reactions to the Avila vc group. They all give 'w' reactions to the European NAN vc supergroup and to each other.

	ES105	ES382	ES697
20H	X	X	O
117H	X	X	O

X = typical NAN polymorphism, O = typical EAN polymorphism
20H = clone 20 probed against HindIII digested DNA.
117H = clone 117 probed against HindIII digested DNA.

Guadalajara region. Alternatively selection pressures and/or genetic drift may partially account for the different population structure at these two sites.

3.3.6 Population Structure in Portugal.

A number of Portugese sites were sampled by Brasier in 1985 and eight isolates (see Section 2.10.2) were again screened with the two clones 20 and 117. Four of the isolates were members of the European NAN vc supergroup while four gave w reactions to the supergroup and to each other (Table 5.7). The four NAN vc supergroup isolates all exhibited exclusively NAN polymorphisms while among the four heterogeneous component isolates, though two exhibited only NAN type polymorphisms, the other two exhibited an EAN type polymorphism when probed with clone 117 (Table 5.7 and Figs. 5.3 - 5.8). The situation at most Portugese sites is similar to that found at Orsett, UK, with the European NAN vc supergroup the sole supergroup present, though in Portugal the outbreaks have occurred much more recently. Once again a typical explosion of variation has occurred (Brasier, 1988) and the presence of the EAN type polymorphism in the heterogeneous component is demonstrated, though its origin must remain a matter of supposition as at Orsett.

Table 5.7. Summary of Portugese isolates and the polymorphisms exhibited when probed with clones 20 and 117.

European NAN vc supergroup isolates:

	PG34	PG115	PG137	PG235
20H	X	X	X	X
117H	X	X	X	X

Isolates giving 'w' reactions to the European NAN vc supergroup and to each other:

	PG67	PG74	PG94	PG276
20H	X	X	X	X
117H	O	X	X	O

X = typical NAN polymorphism, O = typical EAN polymorphism
20H = clone 20 probed against HindIII digested DNA.
117H = clone 117 probed against HindIII digested DNA.

6 RESULTS IV - INTROGRESSION, EPIDEMIOLOGY AND EVOLUTION.

6.1 Non-aggressive Nuclear DNA Polymorphism in the NAN Aggressive at Epidemic Fronts.

As early as 1976, Brasier and Gibbs had shown that sexually compatible isolates of the aggressive and non-aggressive subgroup could be obtained from a single piece of diseased elm bark, and they suggested that epidemic front populations of the aggressive subgroup might be involved in rare crossing events with the non-aggressive (Brasier and Gibbs, 1976). Brasier (1987a) went on to put forward a variety of evidence to suggest that following such a hybridization event in nature, any resulting progeny would be unlikely to survive. He had shown that the progeny of forced crosses have extremely unusual cultural characteristics and have generally low pathogenicity (Brasier and Gibbs, 1976). In addition laboratory experiments had shown that reproductive barriers largely prevented the aggressive subgroup from acting as a recipient in forced crosses (Brasier, 1977), while other experiments using mites to reproduce field conditions suggested the barrier would be virtually total in the wild (Brasier, 1978).

In a more detailed study of the fitness characters of the progeny of an aggressive x non-aggressive cross, Kile and Brasier (1990) studied their inheritance patterns and interrelationships. They demonstrated continuous inheritance for elm colonising ability, in vitro ceratoulmin wilt toxin production, linear growth rate at 20°C and vascular wilt ability. In contrast the inheritance of mycelial density on elm sapwood discs was skewed towards the NAN parental type whereas the opposite was true for synnematal and protoperithecial production. Growth temperature response and fertility of the progeny as sexual donors on an NAN recipient both followed a bimodal inheritance pattern coinciding with the parental types, the NAN-like type being predominant in each case. As recipients the majority of the progeny were sexually sterile indicating strong genetic disruption of the parent systems.

Both the pathogenic and saprophytic phases of the disease cycle are highly competetitive, and the low competitive fitness of hybrids, as well as the effect of the non-aggressive to aggressive fertility barrier, had been postulated as the explanation for the fact that no isolates with the characteristics of hybrids had yet been found in nature (Brasier, 1983a; 1986b; 1987a). Kile and Brasier (1990) also concluded that although progeny showing good performance in some characters were detected, the characters were mainly inherited at random, and thus that the chance of a hybrid genotype being sufficiently fitted overall to compete with the aggressive subgroup must be low. Though they felt that fitness disadvantages of the hybrids were probably too great to allow most more than a transient existence, any which sporulated should have had a > 50 % chance of being able to freely fertilise an aggressive recipient. It was suggested that as a very rare event, such repeated fertilisations could lead to sporadic introgression of non-aggressive genes into the increasingly dominant aggressive gene pool.

That mt DNA transfer between the two subgroups may occur at the epidemic front has already been indicated (see Section 3), though this may occur via hyphal anastomosis allowing only the transfer of cytoplasm and not nuclei. It is at the epidemic front that the two strains have the greatest opportunity for interaction since the non-aggressive is rapidly displaced by the aggressive (Brasier and Gibbs, 1976; Brasier, 1986a). At the epidemic front the frequency of A mating types among the aggressive population is at its lowest (Brasier, 1988a) and this must enhance the opportunity for crosses between the subgroups to occur. Finally there is evidence that although the aggressive population is almost clonal in structure at the epidemic front, an increasing amount of variability is soon generated.

At the frontal sites of Tomar and Mafra in Portugal, extensive surveys had been undertaken by Brasier in 1985 and 1986 and the general population structure at these sites has already been discussed in Sections 1.8 and 1.9. Of particular interest at these sites however was that a small number of morphologically highly unusual isolates were found. Thirteen out of around 400 isolates

collected were very fast growing, waxy looking isolates when grown in culture with widely ranging pathogenicities. In addition a larger number of isolates which exhibited more of a phenotypic continuum had a flat, frosty looking or suede-like NAN appearance in culture (Brasier, pers. comm.).

It was consequently decided to screen a number of isolates sampled from the current epidemic front site at Tomar and the recent epidemic front site at Mafra for the possible presence of non-aggressive n DNA polymorphisms. The probes were selected for their frequent elucidation of polymorphism and/or their repetitive distribution within the genome. In all, fifteen NAN isolates from the two areas were selected (see Section 2.10.2) on the basis of their unusual morphology. Of the five bark isolates selected from the epidemic front site at Tomar four were members of the dominant European NAN vc supergroup of which the clonal component at this site is comprised. The fifth isolate gave an n reaction against the four European NAN vc supergroup isolates. The ten isolates from Mafra, of which five are from the pathogenic twig phase and five are from the saprophytic bark phase, are members of the newly generated heterogeneous component which now forms the majority of the sample populations in both phases, and thus none are members of the European NAN vc supergroup, though some share the same w allele (Table 3.4).

Table 6.1 and Figs. 6.1 - 6.6 show that one of the fifteen selected isolates, TomIId10 exhibits typically non-aggressive polymorphisms with a whole range of different probes, while another isolate MAFg4 also exhibits some main band non-aggressive polymorphisms when HindIII digested DNA is probed with clone 115 and when BamHI digested DNA is probed with clone 137. In several other cases there is variability between isolates particularly in their secondary bands, however it is difficult to attribute these as non-aggressive polymorphisms and they may alternatively be random variation. TomIId10, along with TomIIc4, is one of the so-called 'fast waxy' isolates, is the only one of the five selected isolates from Tomar not to be a member of the European NAN vc supergroup, and having been isolated from the saprophytic bark phase is likely to be one of the earliest heterogeneous vc group isolates generated at the

Table 6.1. Summary of the novel or typical non-aggressive polymorphisms exhibited by the 15 morphologically unusual NAN isolates selected from Tomar and Mafra.

Clones	H23	H36	H109	H115	H119	B6	B124	B126	B137	B140
Isolates										
Portugese bark isolates from the epidemic front site at Tomar.										
TomIj10	0	0	S	0	0	0	0	0	0	0
TomIm4	0	0	S	0	0	0	0	0	0	0
TomIIe5	0	0	0	0	0	0	0	0	0	0
TomIIc4	0	0	0	0	0	0	0	0	0	0
TomIId10	S	S	S	X	S	X	X	X	X	S
Portugese twig isolates from the recent epidemic front site at Mafra.										
PG461	0	0	0	0	0	0	0	0	0	0
PG470	0	0	0	0	0	0	0	0	0	0
PG473	0	0	0	0	0	0	0	0	0	0
PG493	0	0	S	0	0	0	0	0	0	0
PG506	0	0	S	0	0	0	0	0	0	0
Portugese bark isolates from the recent epidemic front site at Mafra.										
MAFa4	0	0	0	0	0	0	0	0	0	0
MAFa12	0	0	S	0	0	0	0	0	0	0
MAFc3	0	S	0	0	0	0	0	0	0	S
MAFf20	0	S	0	0	0	0	0	0	0	0
MAFg4	0	0	0	X	0	0	0	0	X	0

X = typical non-aggressive main band polymorphism(s)

S = typical non-aggressive secondary band polymorphism(s)

0 = typical NAN polymorphism(s)

H = clone probed against HindIII digested DNA.

B = clone probed against BamHI digested DNA.

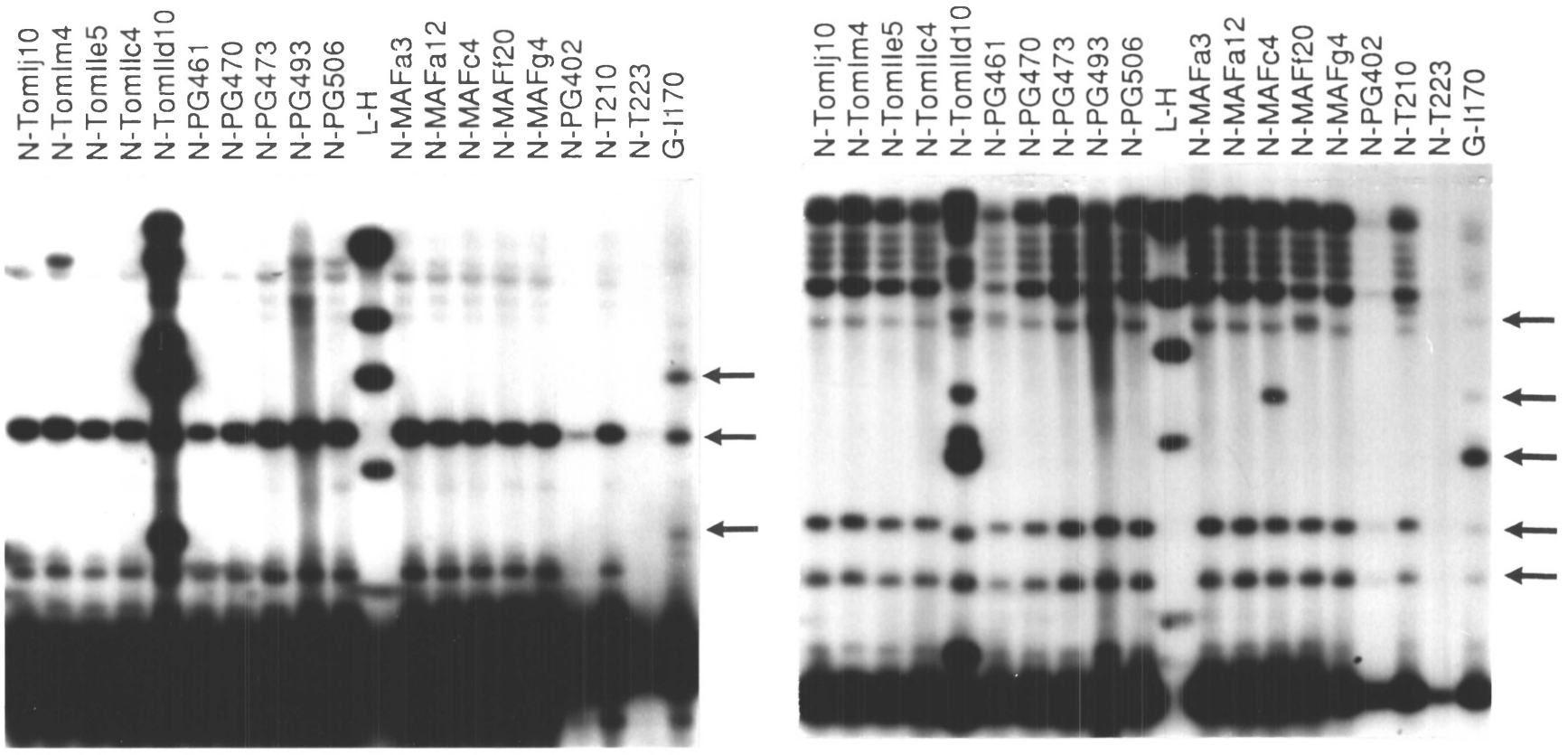

Fig. 6.1. HindIII digested total DNA of morphologically unusual epidemic front isolates from Tomar and Mafra, Portugal, probed with n DNA clones 23 (left) and 36 (right).

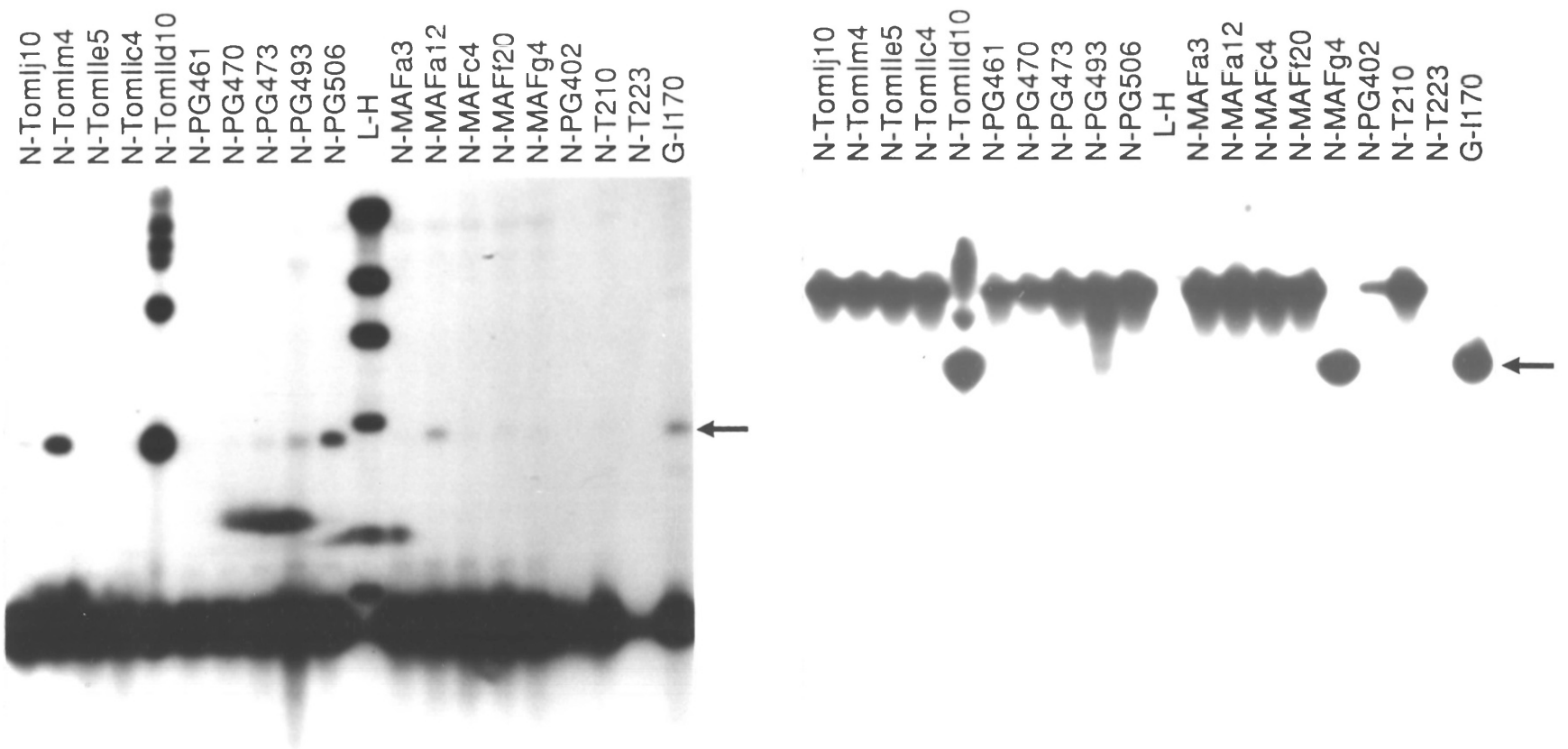

Fig. 6.2. HindIII digested total DNA of morphologically unusual epidemic front isolates from Tomar and Mafra, Portugal, probed with n DNA clones 109 (left) and 115 (right).

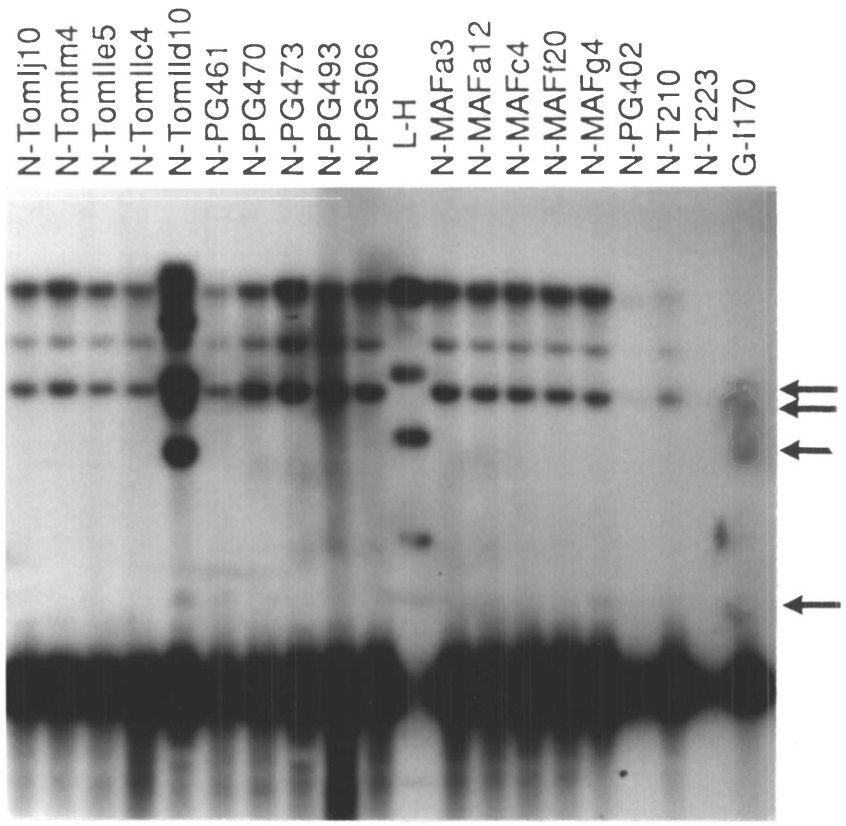

Fig. 6.3. HindIII digested total DNA of morphologically unusual epidemic front isolates from Tomar and Mafra, Portugal, probed with n DNA clone 119.

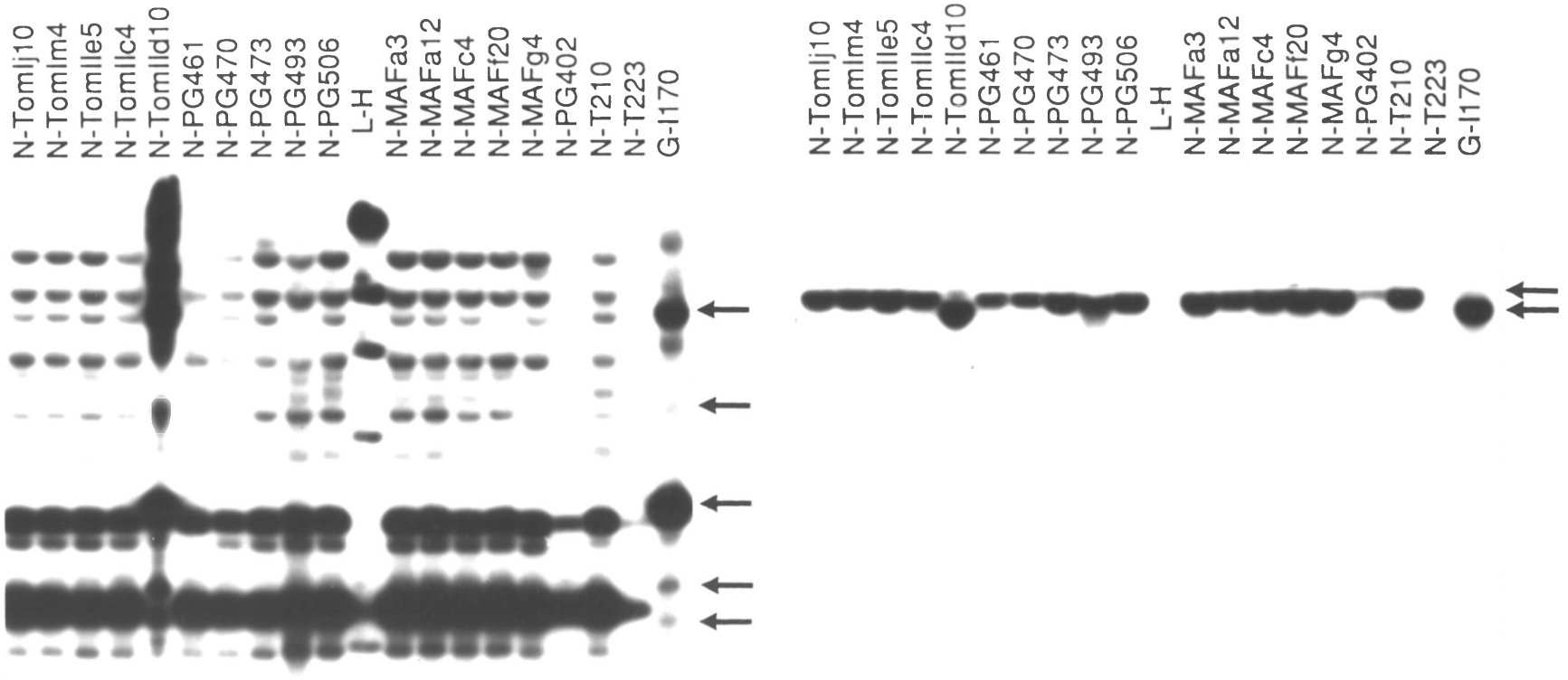

Fig. 6.4. BamHI digested total DNA of morphologically unusual epidemic front isolates from Tomar and Mafra, Portugal, probed with n DNA clones 6 (left) and 124 (right).

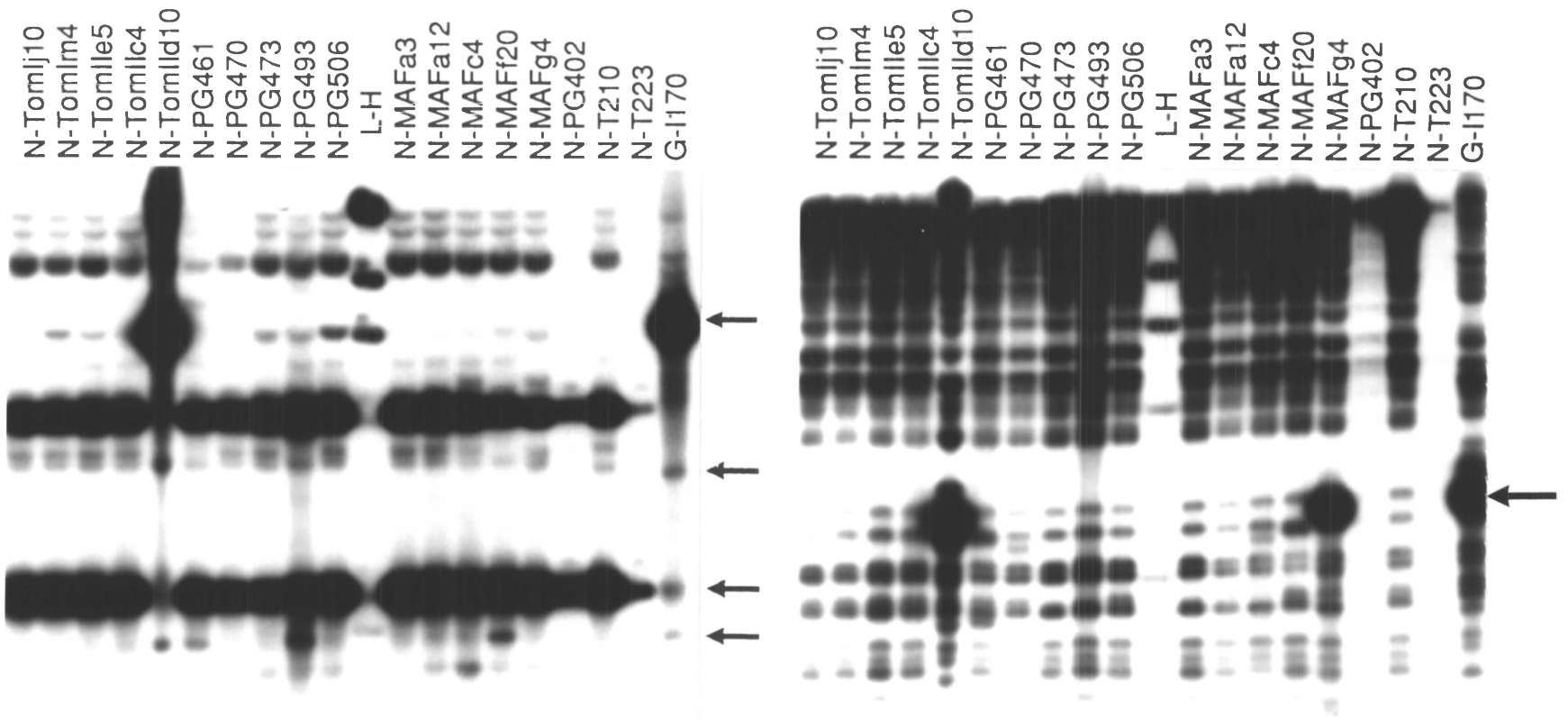

Fig. 6.5. BamHI digested total DNA of morphologically unusual epidemic front isolates from Tomar and Mafra,, Portugal, probed with n DNA clones 126 (left) and 137 (right).

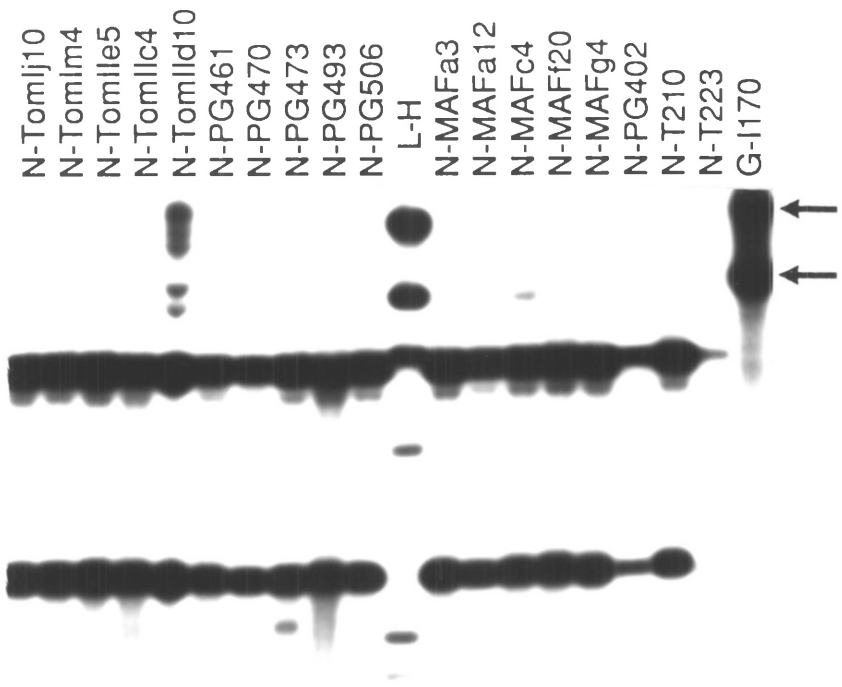

Fig. 6.6. BamHI digested total DNA of morphologically unusual epidemic front isolates from Tomar and Mafra, Portugal, probed with n DNA clone 140.

epidemic front. Since the isolate exhibiting by far the greatest amount of typically non-aggressive polymorphism is from the epidemic front site at Tomar, while the isolates from the recent epidemic front site at Mafra, incuding another 'fast waxy' isolate PG470, exhibit almost entirely typical NAN polymorphisms suggests that TomIId10 may be one of the F1 progeny of an initial NAN x non-aggressive cross.

6.2 The Presence of Non-aggressive Nuclear DNA Polymorphisms in the EAN.

A single twig isolate P129, from an epidemic front site in Poland, is the only isolate collected from within the geographical range of the EAN which cannot be assigned to either the aggressive or non-aggressive subgroups. It does however have a distinctly 'fast waxy' appearance very similar to isolates such as TomIId10 at Tomar and Mafra at the epidemic front in Portugal. Given the distinctive typically non-aggressive polymorphisms exhibited by the TomIId10 it was decided to screen P129 with the same range of probes as had been employed to screen the Tomar and Mafra samples (see Section 3.4.1). Isolate P129 also exhibited some typical non-aggressive secondary bands when digested with HindIII and BamHI and probed with clones 115 and 6 respectively (Figs. 6.39 - 6.42). Because of the considerable similarity with the situation at Tomar and Mafra this would seem to provide further evidence of non-aggressive n DNA introgression as a result of rare crossing events, this time into the EAN subgroup.

The initial broad geographical range of isolates employed in the experiments described in Section 4 had been screened with clones chosen for their prospective ability to distinguish between EAN and NAN isolates M3 and C112. However, these experiments had shown that clone 36 when probed against HindIII digested DNA EAN isolates M3 and R136, from Moscow and Romania respectively, exhibited typical non-aggressive n DNA polymorphisms (see Figs. 4.7 and 6.7 or Fig. 6.19). A similar result was found for M3 when clone 124 was probed against BamHI digested DNA (Figs. 4.7 and 6.8 or Fig. 6.27). This raised the

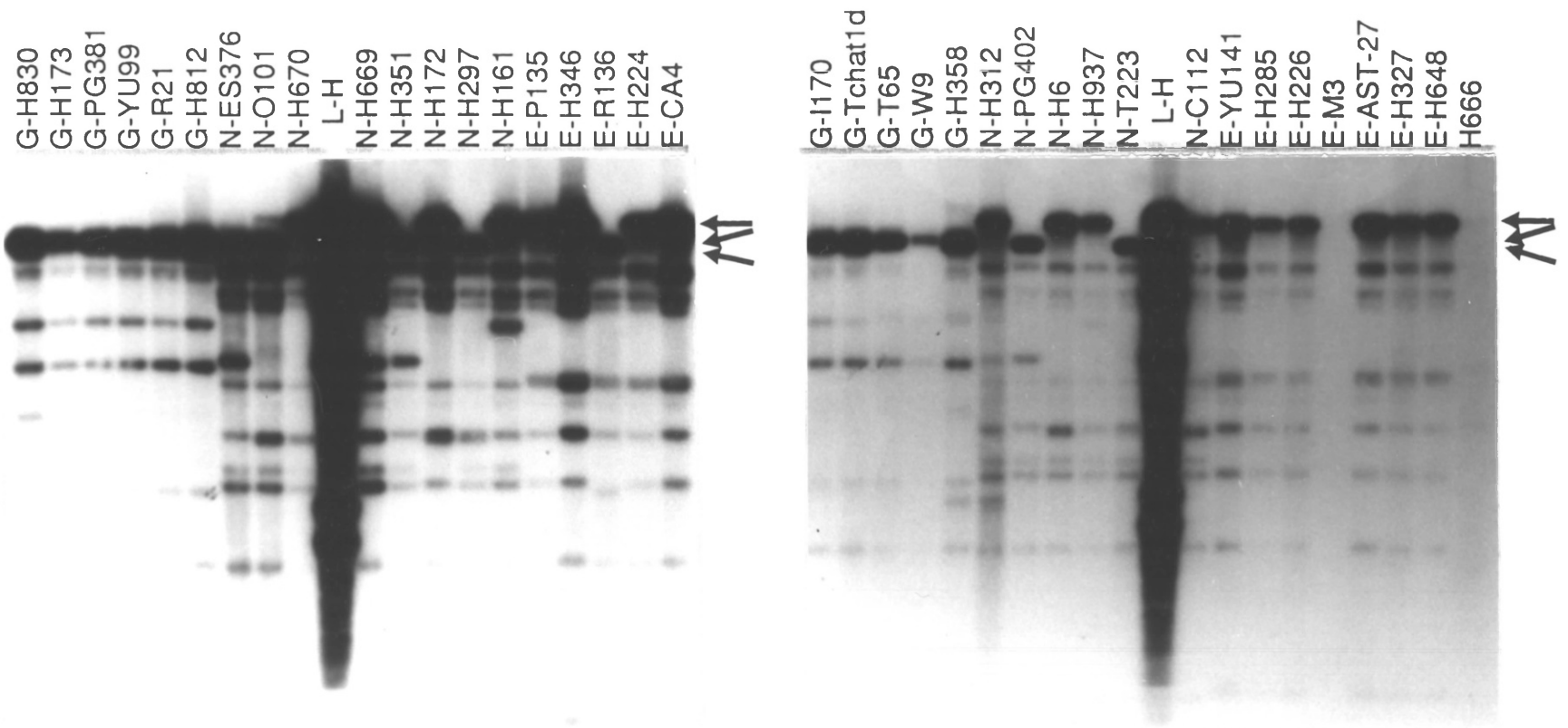

Fig. 6.7. HindIII digested total DNA of an initial broad geographical range of EAN, NAN and non-aggressive isolates and isolate H666 probed with random n DNA clone 36.

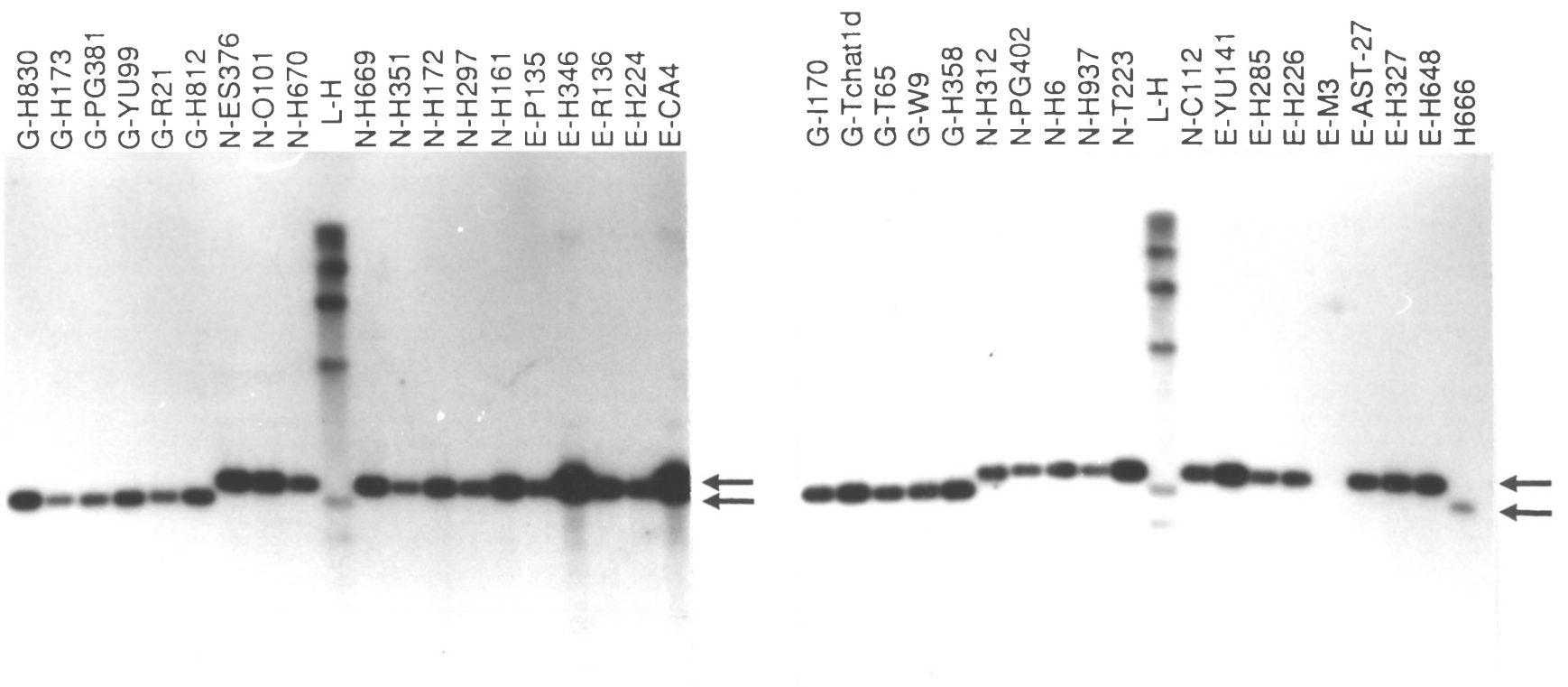

Fig. 6.8. BamHI digested total DNA of an initial broad geographical range of EAN, NAN and non-aggressive isolates and isolate H666 probed with random n DNA clone 124.

question of the extent and origin of non-aggressive nuclear DNA polymorphism in the EAN.

The range of clones apparently able to distinguish between EAN and NAN isolates had been exhausted, though detecting polymorphisms between the two races of the aggressive strain they would seem to be the most likely clones to additionally distinguish between the non-aggressive and EAN sub-groups, and thus discover further evidence of introgression. It would have been possible, though extremely laborious and expensive, to screen pairs of a non-aggressive isolate and for example isolate M3 or another EAN isolate, with a further range of clones in order to discover potentially useful probes. However, there was nothing to suggest that M3 in particular should exhibit non-aggressive introgression. In addition, evidence from mt DNA polymorphisms (Figs. 3.10, 3.11 and 3.12), n DNA main band polymorphisms (Fig. 4.7) and the n DNA secondary band polymorphisms (eg. Fig. 6.1), concerning the relatedness of the aggressive races to each other relative to the relatedness of the aggressive and non-aggressive sub-groups, suggested that many of the other clones were likely to reveal polymorphism between the non-aggressive and EAN isolates. Consequently it was considered that it would be more efficient to continue the screening of the initial broad geographical range of isolates with a series of randomly selected clones.

Such an approach was shown to be justified as over half of the random clones employed efficiently distinguished between the aggressive and non-aggressive subgroups (Fig. 6.9). In addition however, clones 23, 105, 119, 141 and 146 when probed against HindIII digested DNA, and clone 105 when probed against BamHI digested DNA revealed a non-aggressive type polymorphism for either R136 from Romania or AST-27 from Iran (see Figs. 6.9 and 6.10 - 6.14). These results suggested a significant measure of non-aggressive n DNA polymorphism within the EAN. Such polymorphisms however, could have at least two possible causes. The first, already alluded to above, is that they may be the product of rare crosses at epidemic front sites, between isolates of the EAN and non-aggressive subgroups followed by a gradual dilution of the non-aggressive component through genetic drift and/or selection pressures. Alternatively, these polymorphisms

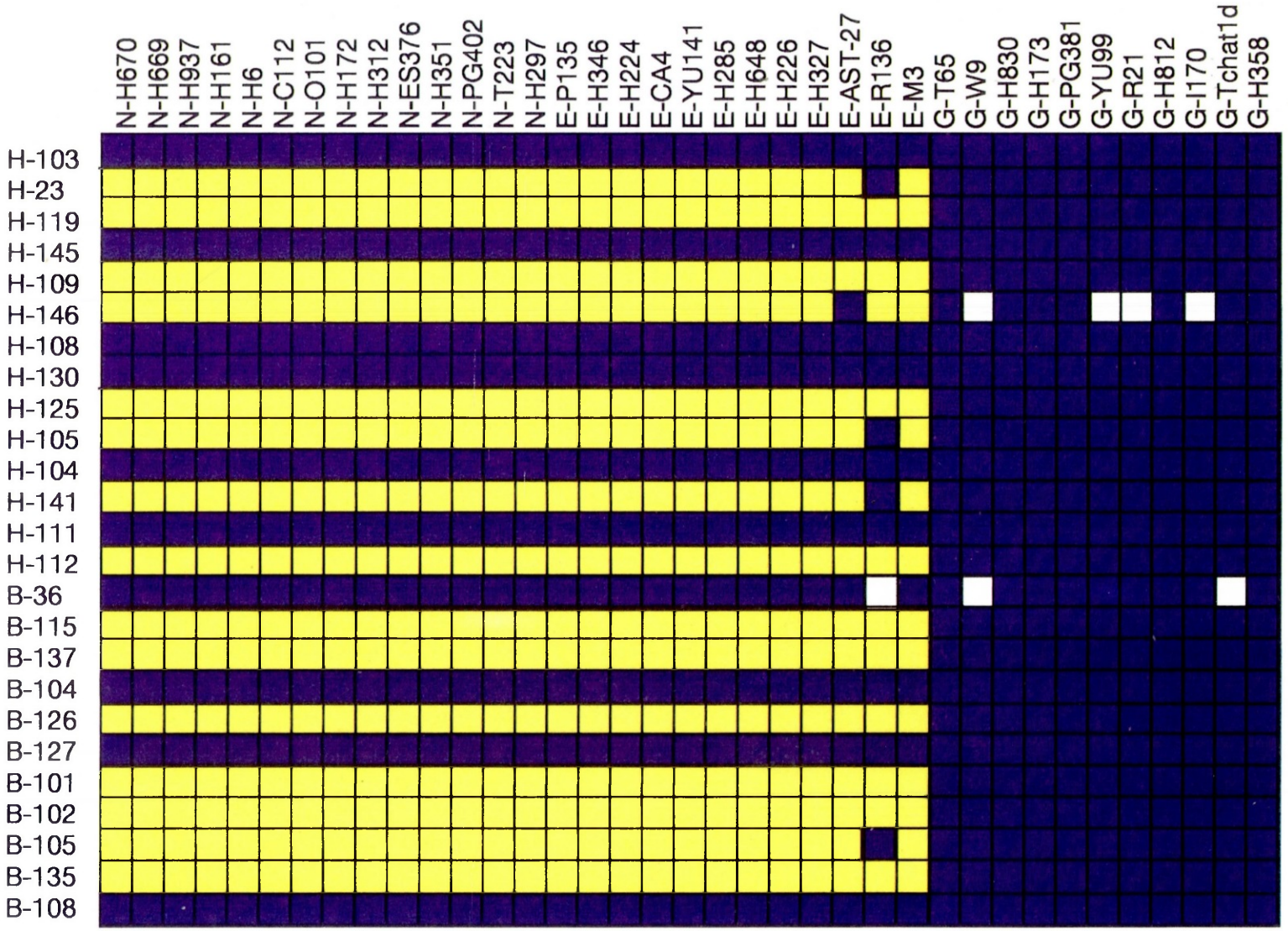

Fig. 6.9. Summary of the n DNA polymorphisms exhibited by a broad geographical range of non-aggressive, EAN and NAN isolates when their n DNA was digested with HindIII or BamHI and probed with a number of random n DNA clones. The letter before each isolate denotes the subgroup of which it is a member; G = non-aggressive; N = NAN; E = EAN. H and B prior to the clone numbers denote the enzymes HindIII and BamHI respectivley with which the n DNA was digested. The key to the colours is as for Fig. 4.7.

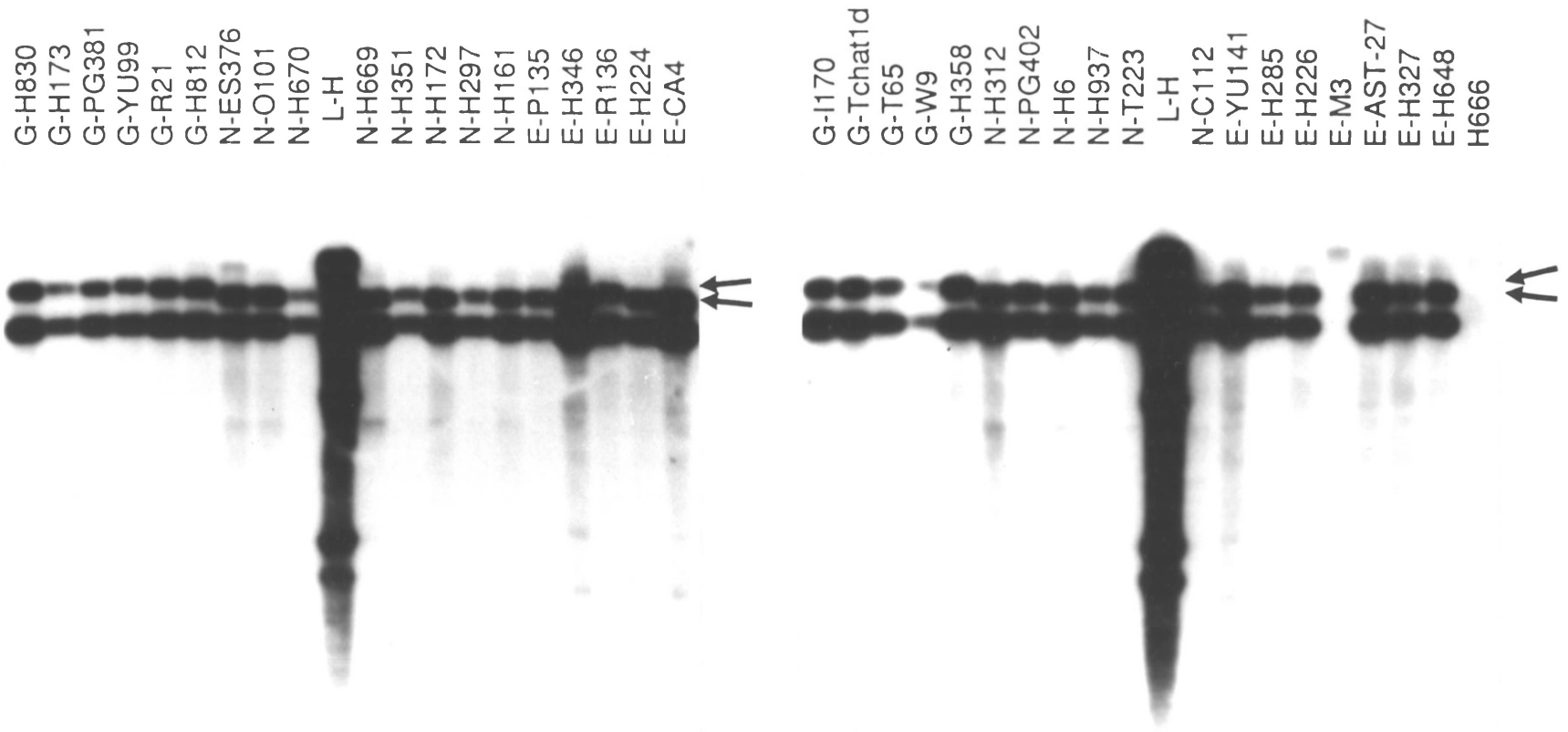

Fig. 6.10. HindIII digested total DNA of an initial broad geographical range of FAN, NAN and non-aggressive isolates and isolate H666 probed with random n DNA clone 23.

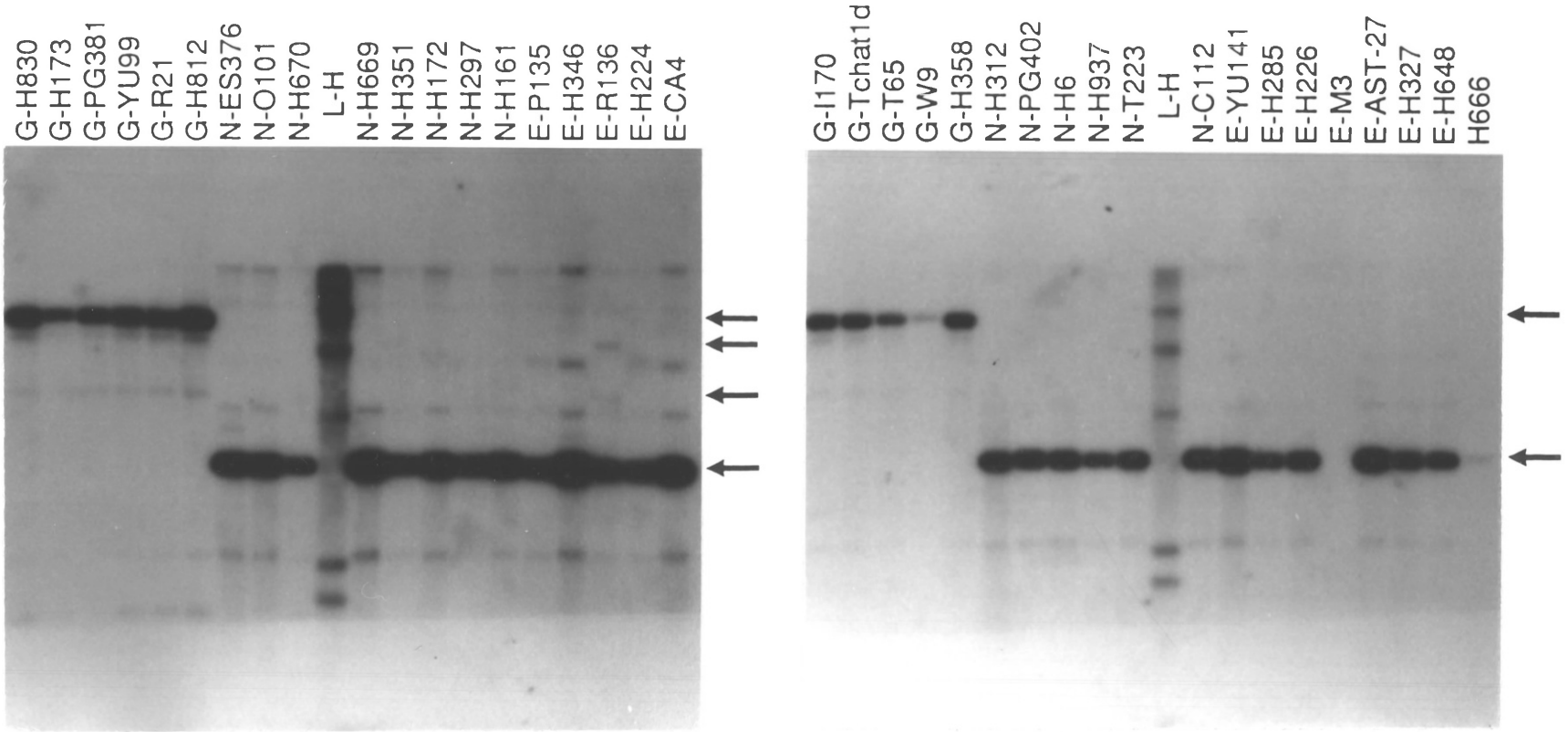

Fig. 6.11. HindIII digested total DNA of an initial broad geographical range of EAN, NAN and non-aggressive isolates and isolate H666 probed with random n DNA clone 119.

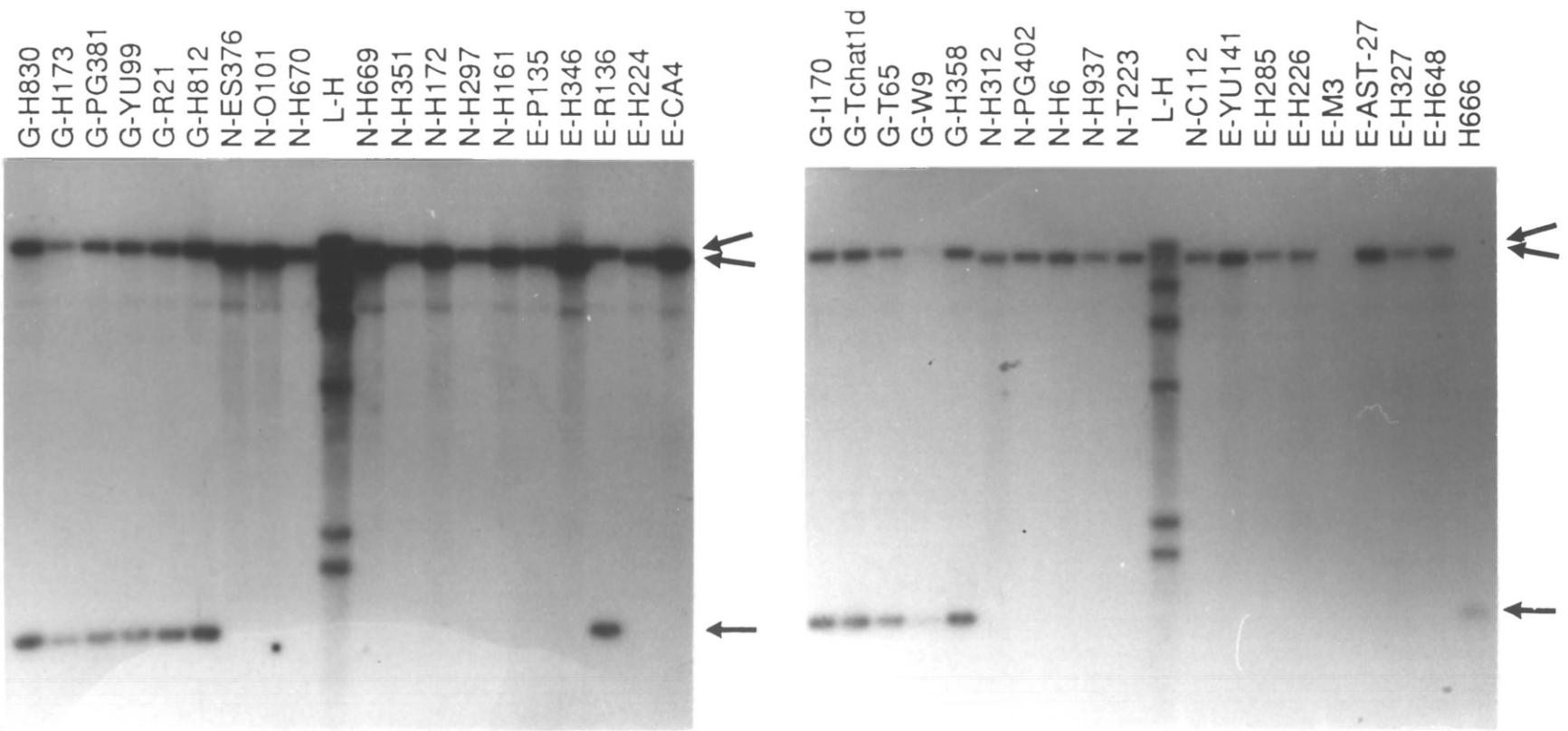

Fig. 6.12. HindIII digested total DNA of an initial broad geographical range of EAN, NAN and non-aggressiveee isolates and isolate H666 probed with random n DNA clone 141.

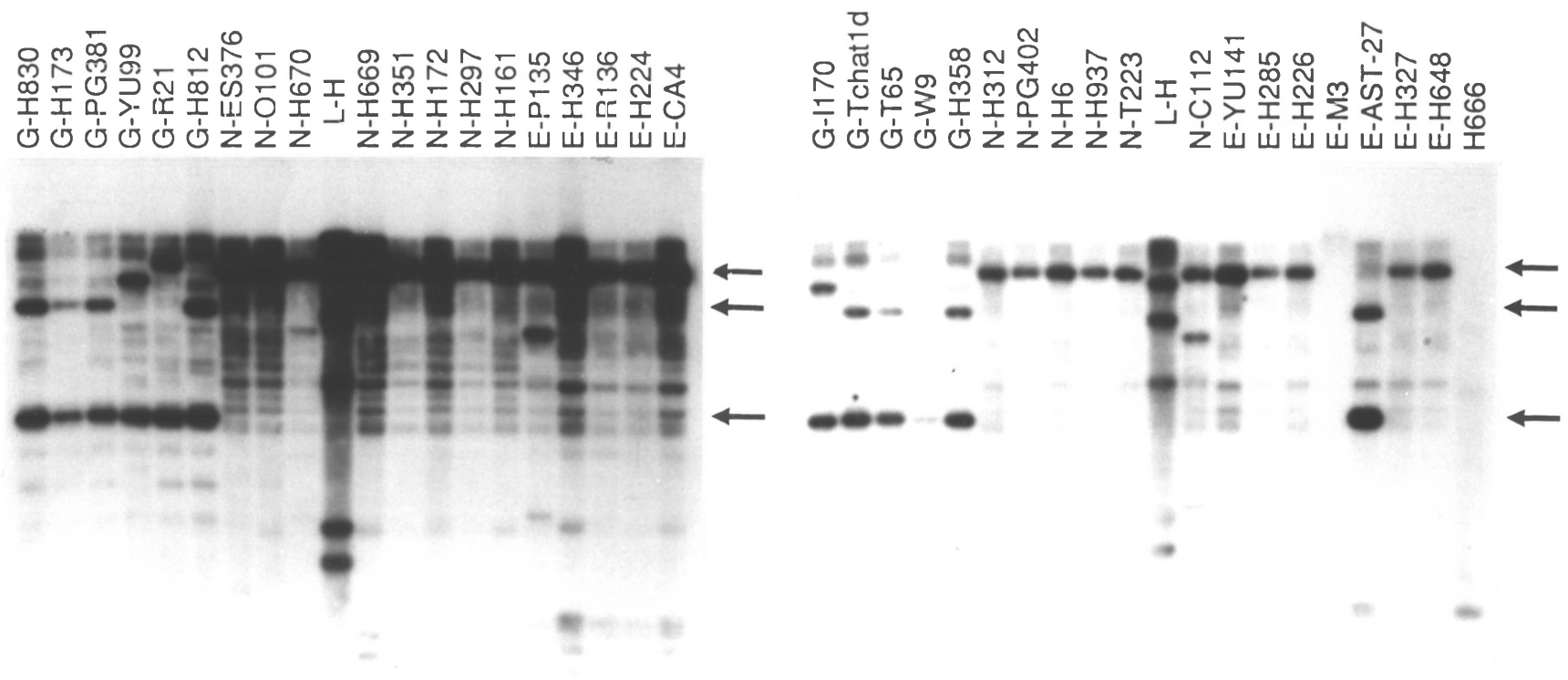

Fig. 6.13. HindIII digested total DNA of an initial broad geographical range of EAN, NAN and non-aggressive isolates and isolate H666 probed with random n DNA clone 146.

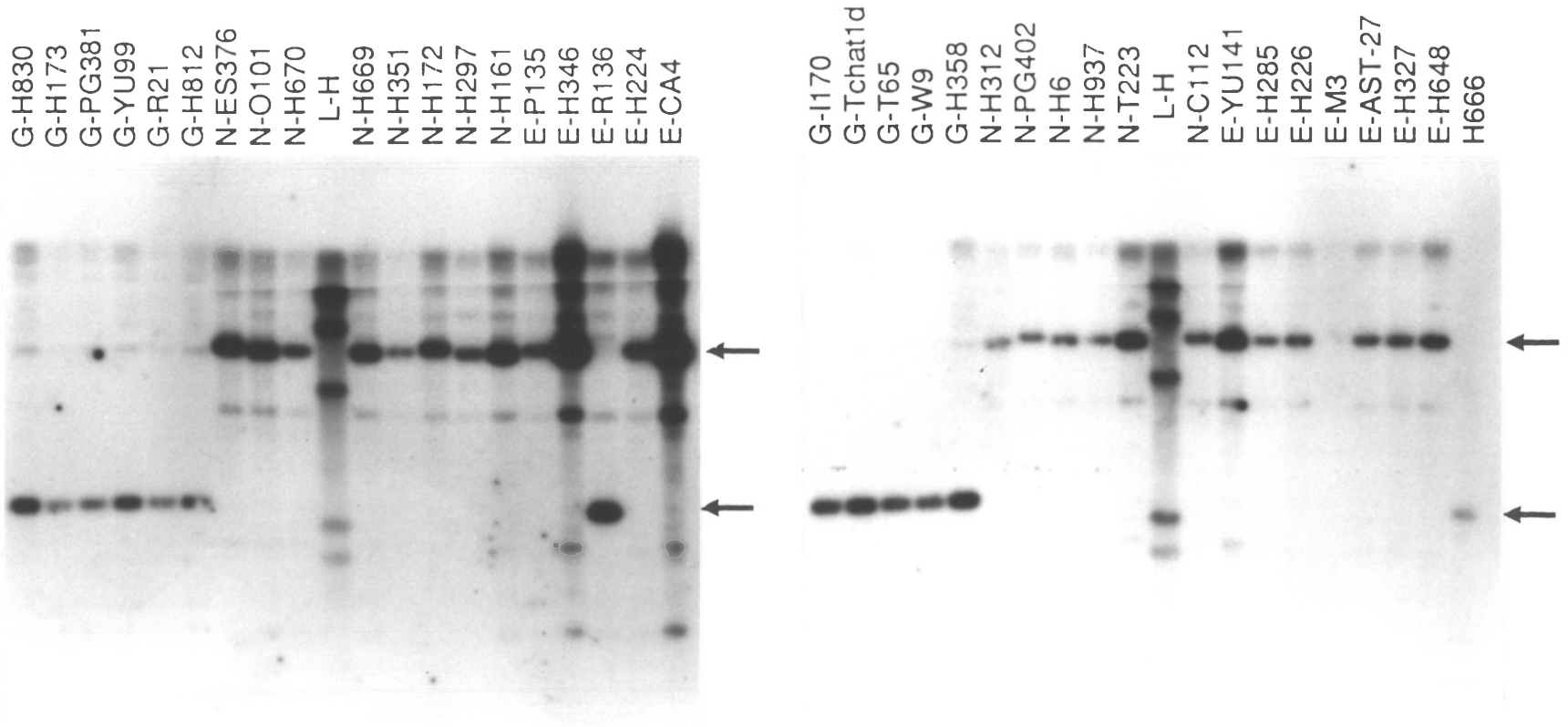

Fig. 6.14. BamHI digested total DNA of an initial broad geographical range of EAN, NAN and non-aggressive isolates and isolate H666 probed with random n DNA clone 105.

may represent remnant non-aggressive polymorphisms from the evolution of the aggressive sub-group from the non-aggressive.

As mentioned in Sections 1.5, northeast Romania is thought to be the possible area of initial outbreak of the EAN aggressive subgroup. The earliest published indication of the EAN outbreak appears to be in northeastern Romania in the early 1950s (Petrescu, 1963; Brasier, 1983a; 1990a) and by extrapolating felling records Brasier has suggested that the appearance of the EAN in this area may date back to the mid 1940's. If these non-aggressive type polymorphisms were to represent the descendants of the isolates involved in evolution of the aggressive subgroups from the non-aggressive then one might hope to find further Romanian EAN isolates, in addition to R136, which exhibit non-aggressive polymorphisms. It was therefore decided to screen a concentrated sample of EAN isolates collected in northeast Romania in 1980 for non-aggressive polymorphisms, and compare it with a broad geographical sample of EAN isolates especially from the adjacent areas of the Soviet Union and central and western Europe (see Section 2.10.3).

BamHI and HindIII digested DNA of these isolates was probed with a number of different n DNA clones which had revealed typical non-aggressive or novel polymorphisms against EAN isolates in the initial broad geographical sample. As can be seen from Table 6.2, and Figs. 6.15-6.29, a number of these additional isolates also revealed typical non-aggressive or novel polymorphisms. Of the additional thirteen Romanian EAN isolates screened only isolate R112, which along with R136 gives an n reaction to the EAN vc supergroup, exhibited several typical non-aggressive polymorphisms (Table 6.2). However, isolates R130, R93, R148, R118, R140, H50 (Italy), and H986 (Georgia, USSR) also exhibited typical non-aggressive polymorphisms when digested with BamHI and probed with n DNA clone 140. It may also be seen that isolates H328 (Nr. Charkov, USSR), H987 (Georgia, USSR) and V1 (Volgograd, USSR), in addition to isolates M3, H345 and AST-27, reveal occasional typically non-aggressive and/or novel polymorphisms. None of the other isolates exhibited non-aggressive or novel polymorphisms and as may be seen from Table 6.2, R112 and R136

among this sample.

Finally, it may also be seen from Table 6.2 that isolate AST-27 (Iran) is the only EAN isolate to exhibit a non-aggressive polymorphism when digested with HindIII and probed with n DNA clone 146. This is particularly interesting in that although AST-27 is a typical EAN isolate in every other respect it has a considerably lower pathogenicity than most aggressive isolates and is known to carry a single gene for reduced aggressiveness (Brasier, 1986c). It may be that this polymorphism is linked to a lower pathogenic ability.

Table 6.2. Summary of those EAN isolates exhibiting novel or typical non-aggressive type polymorphisms with a range of different clones.

		NAG I170	EAN H224	EAN R112	EAN R136	EAN H328	EAN H987	EAN V1	EAN M3	EAN H345	EAN AST-27
HindIII											
Clones	Bands										
23	Main	X	0	0	X	0	0	0	0	0	0
36	Main	X	0	X	X	+	X	0	X	X	0
105	Main	X	0	X	X	0	0	0	0	0	0
105	2ndry	X	0	0	0	0	0	0	*	*	0
110	Main	X	0	0	0	0	0	0	*	*	0
115	Main	X	0	0	0	*	*	0	0	0	0
119	Main	X	0	0	0	0	*	0	0	0	0
119	2ndry	X	0	X	X	0	0	0	0	0	0
141	Main	X	0	X	X	0	0	0	0	0	0
146	Main	X	0	0	0	*	0	0	0	0	X
BamHI											
Clones	Bands										
6	Main	X	0	0	+	0	0	*	0	0	0
36	Main	X	0	0	*	0	0	0	0	0	0
103	Main	X	0	0	0	*	*	*	*	*	0
105	Main	X	0	X	X	0	0	0	0	0	0
124	Main	X	0	0	0	X	X	0	X	X	0
137	2ndry	X	0	0	0	0	*	0	*	*	0
140	Main	X	0	X	0	0	0	0	*	*	0

X = typical non-aggressive polymorphism

0 = typical EAN polymorphism

* and + = novel polymorphisms

NB. DNA digested with BamHI and probed with clone 140 also revealed a typical non-aggressive polymorphism with isolates R130, R93, R148, R118, R140, H50 and H986.

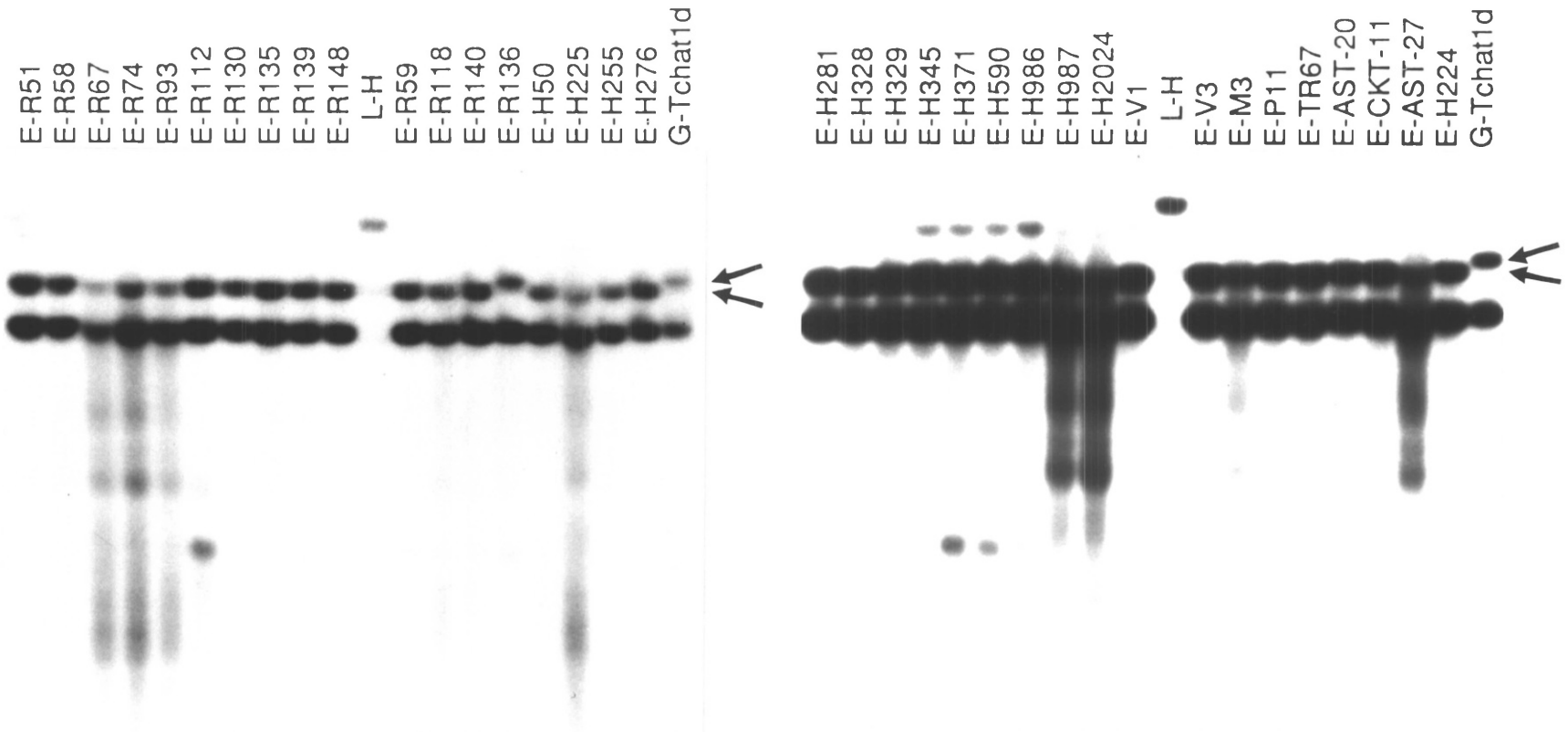

Fig. 6.15. HindIII digested total DNA of a range of FAN isolates probed with n DNA clone 23.

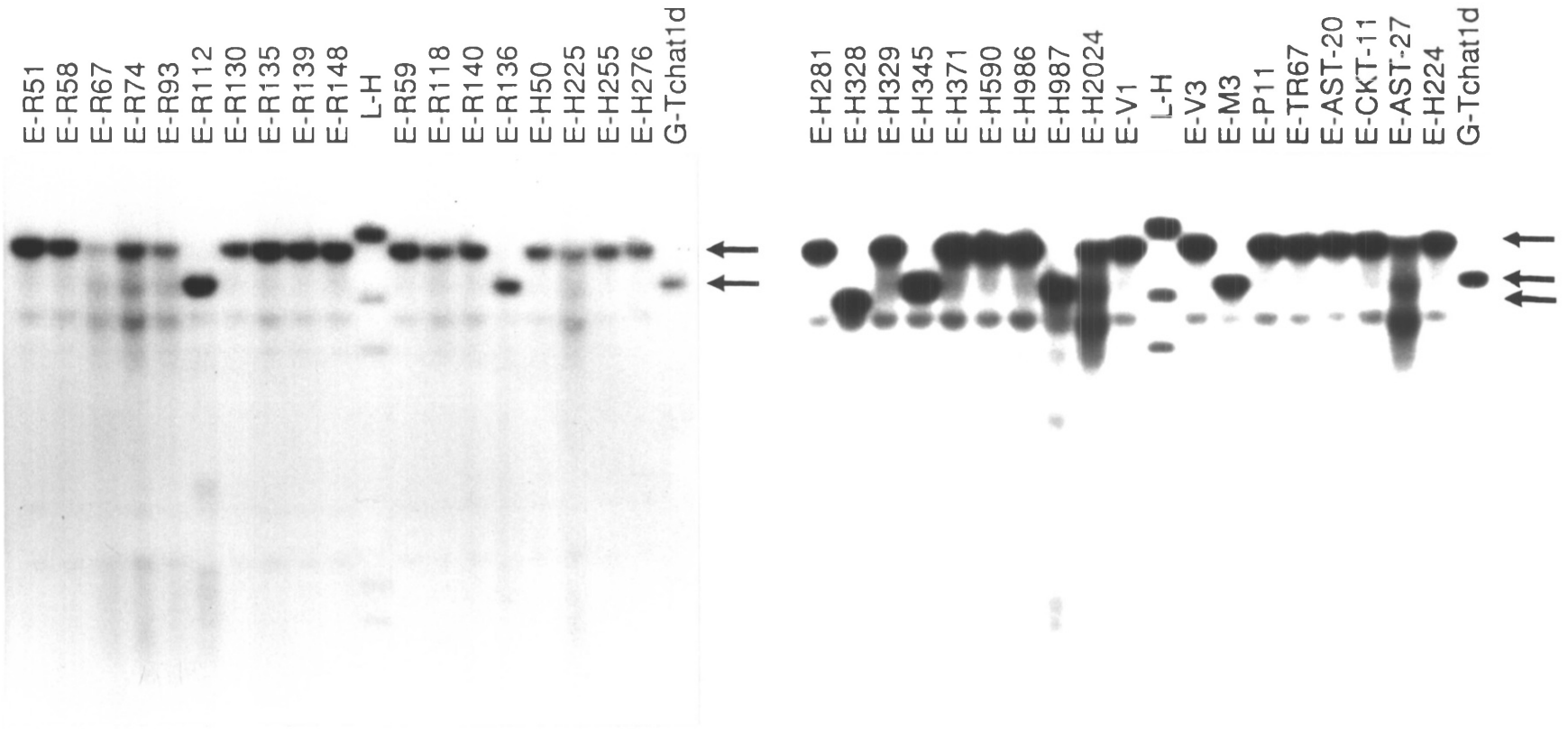

Fig. 6.16. HindIII digested total DNA of a range of EAN isolates probed with n DNA clone 36.

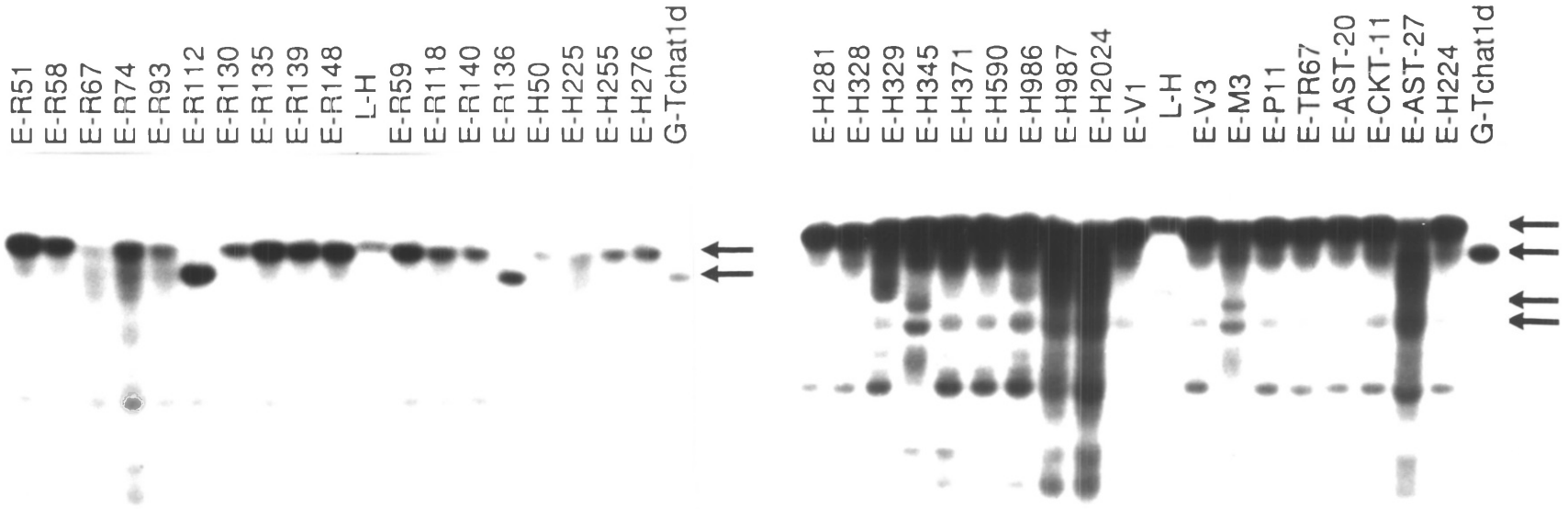

Fig. 6.17. HindIII digested total DNA of a range of EAN isolates probed with n DNA clone 105.

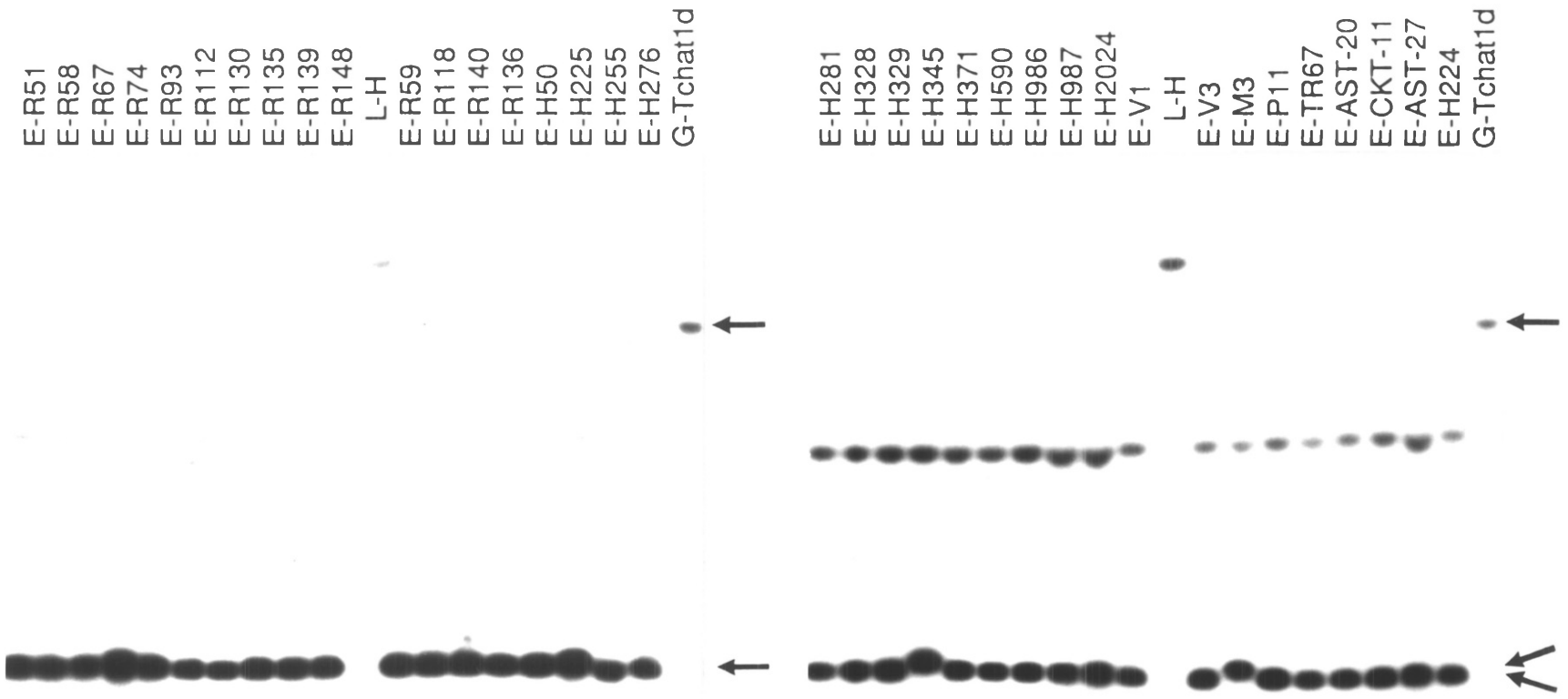

Fig. 6.18. HindIII digested total DNA of a range of EAN isolates probed with n DNA clone 110.

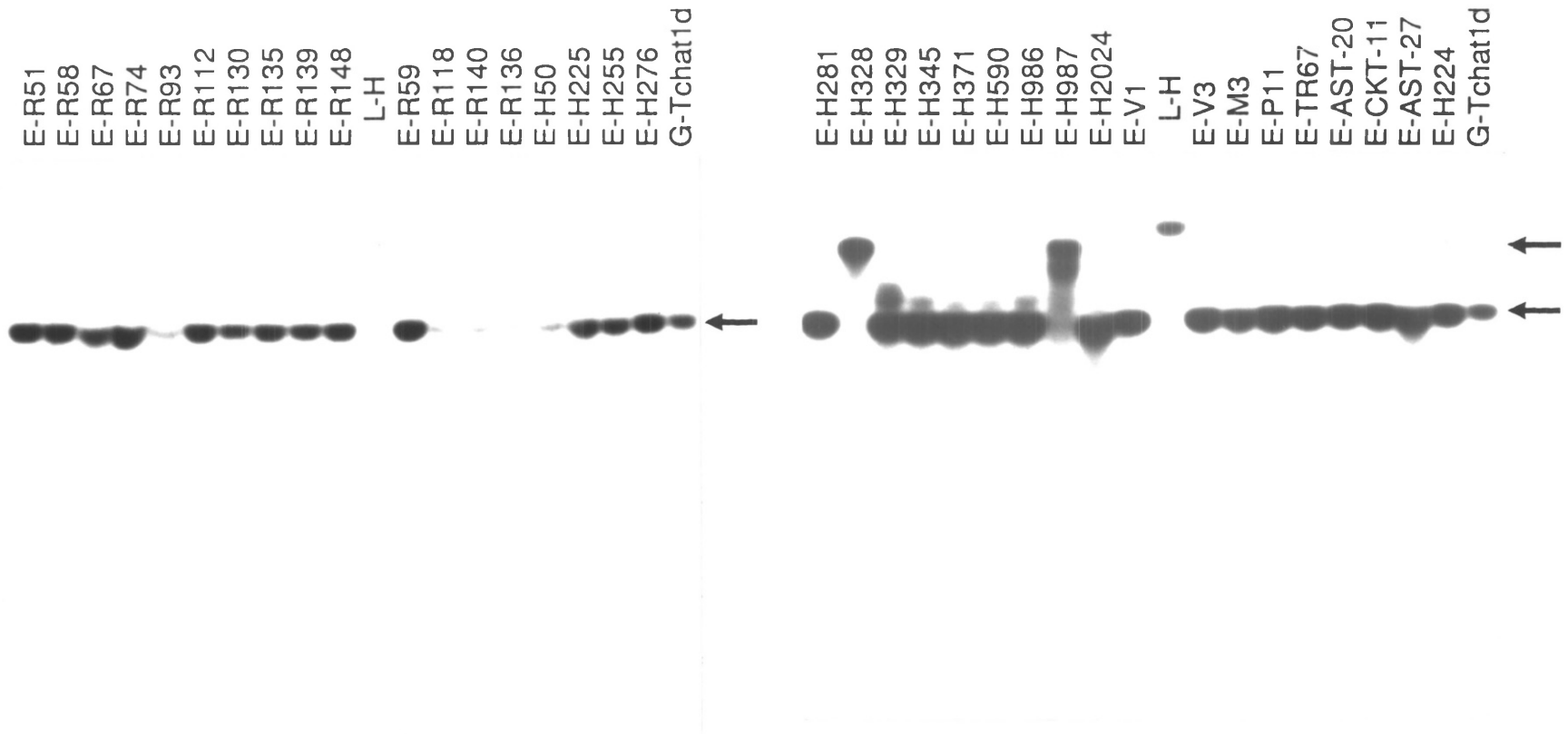

Fig. 6.19. HindIII digested total DNA of a range of FAN isolates probed with n DNA clone 115.

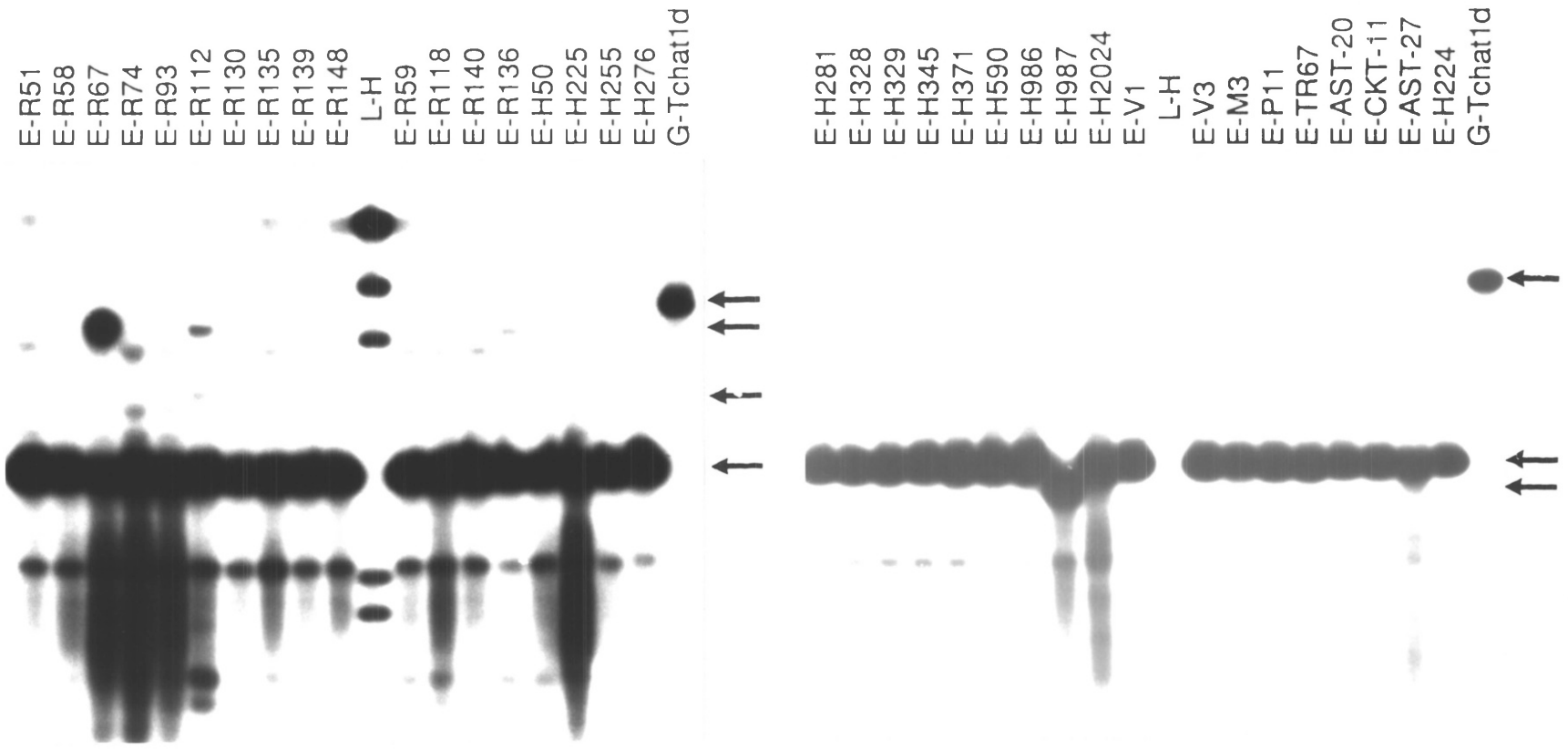

Fig. 6.20. HindIII digested total DNA of a range of EAN isolates probed with n DNA clone 119.

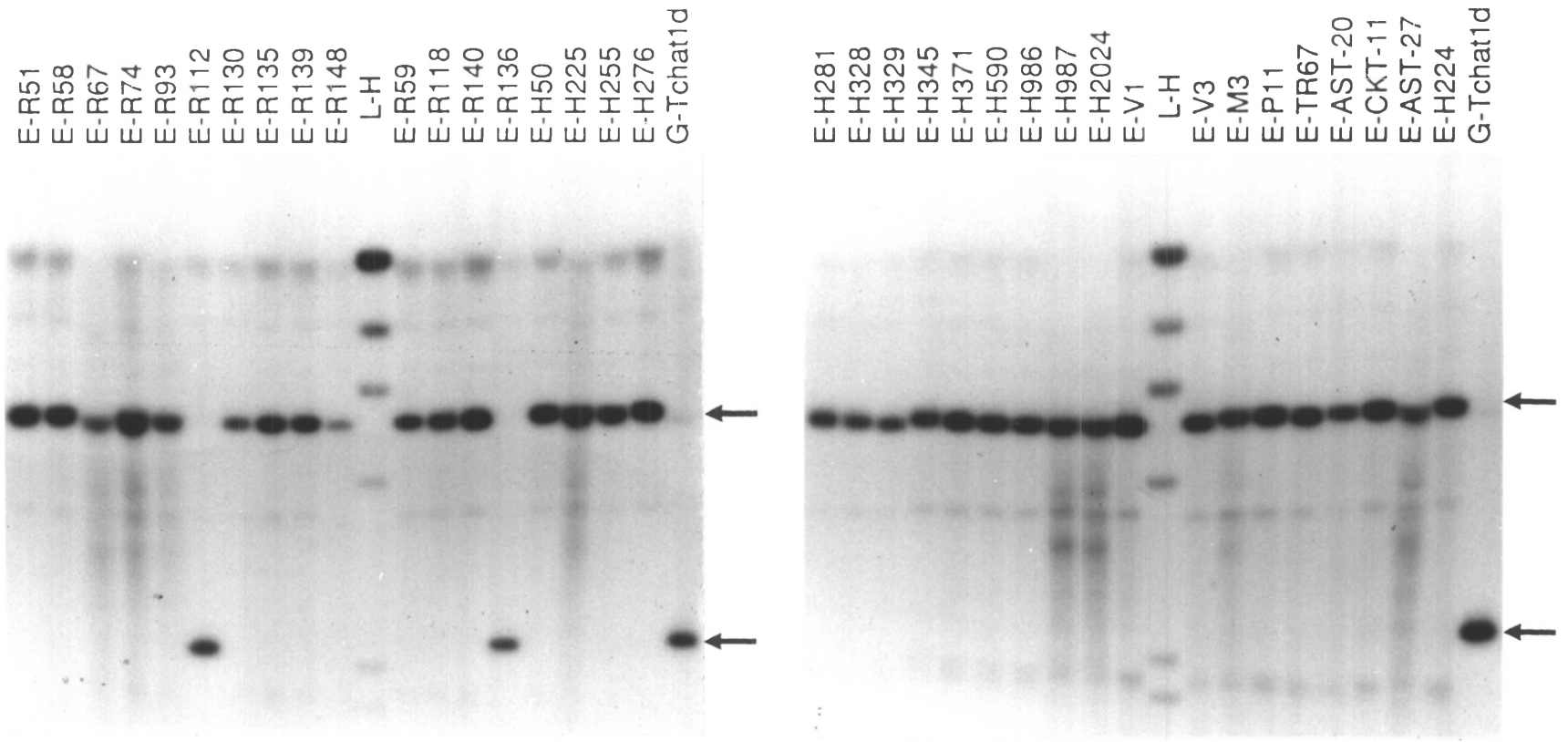

Fig. 6.21. HindIII digested total DNA of a range of FAN isolates probed with n DNA clone 141.

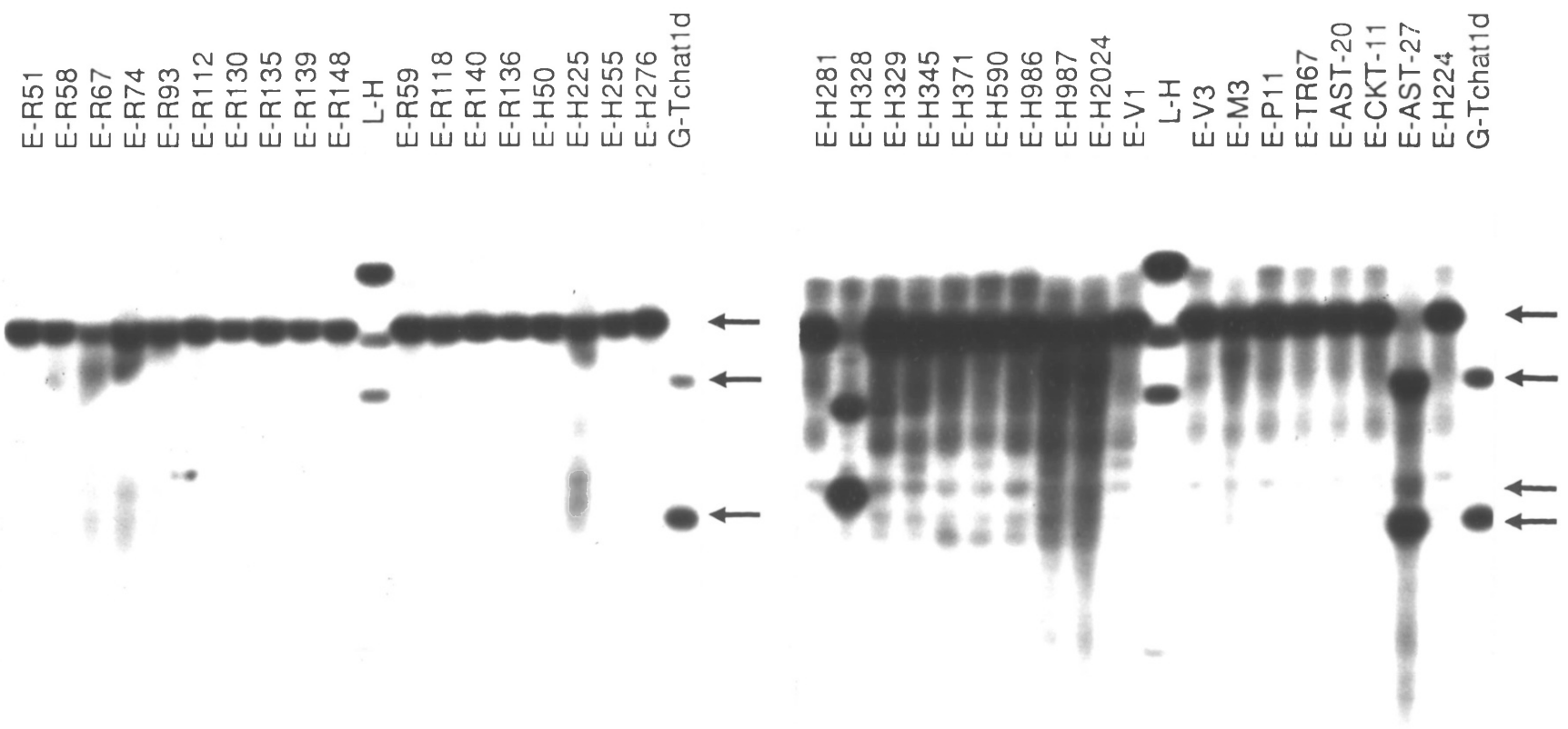

Fig. 6.22. HindIII digested total DNA of a range of EAN isolates probed with n DNA clone 146.

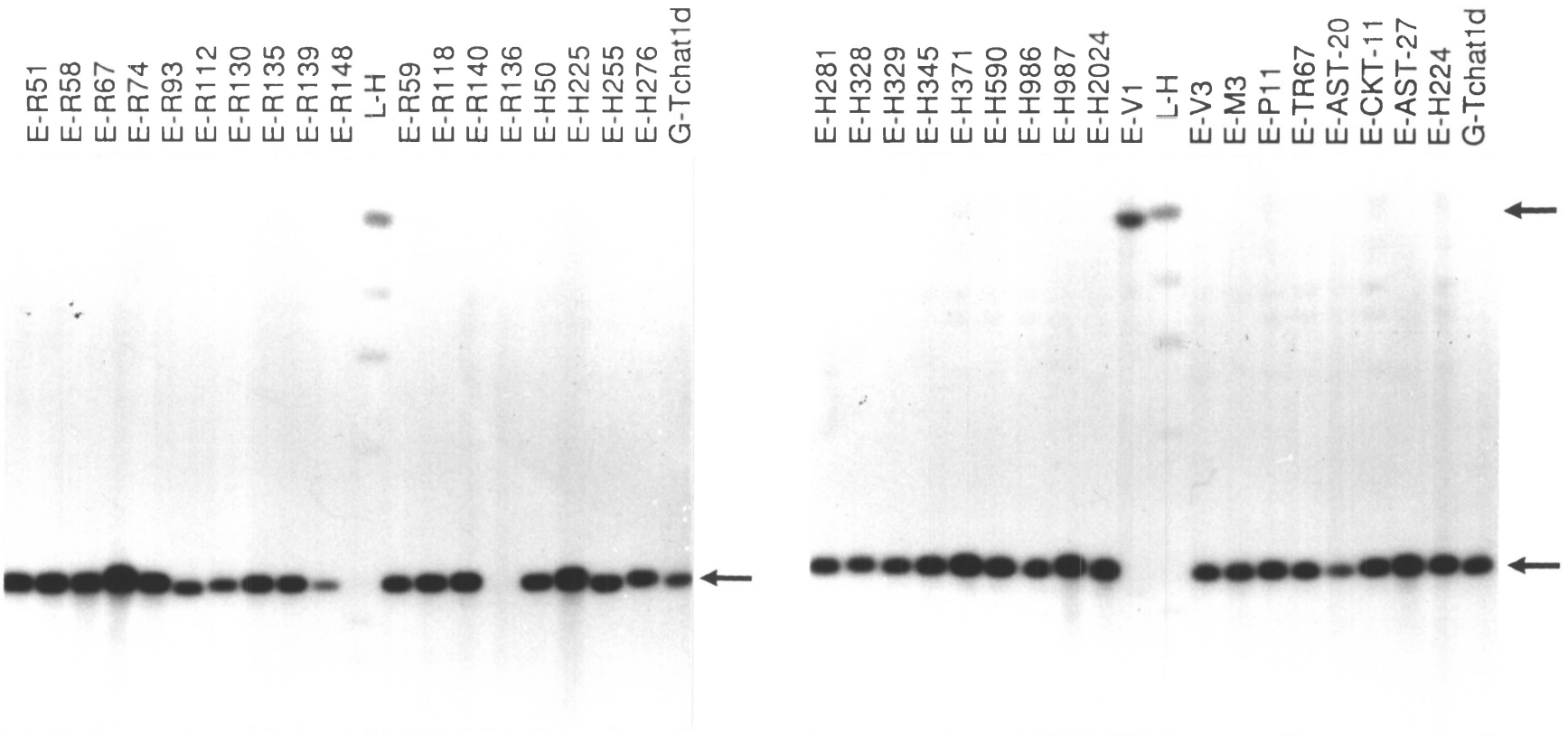

Fig. 6.23. BamHI digested total DNA of a range of EAN isolates probed with n DNA clone 6.

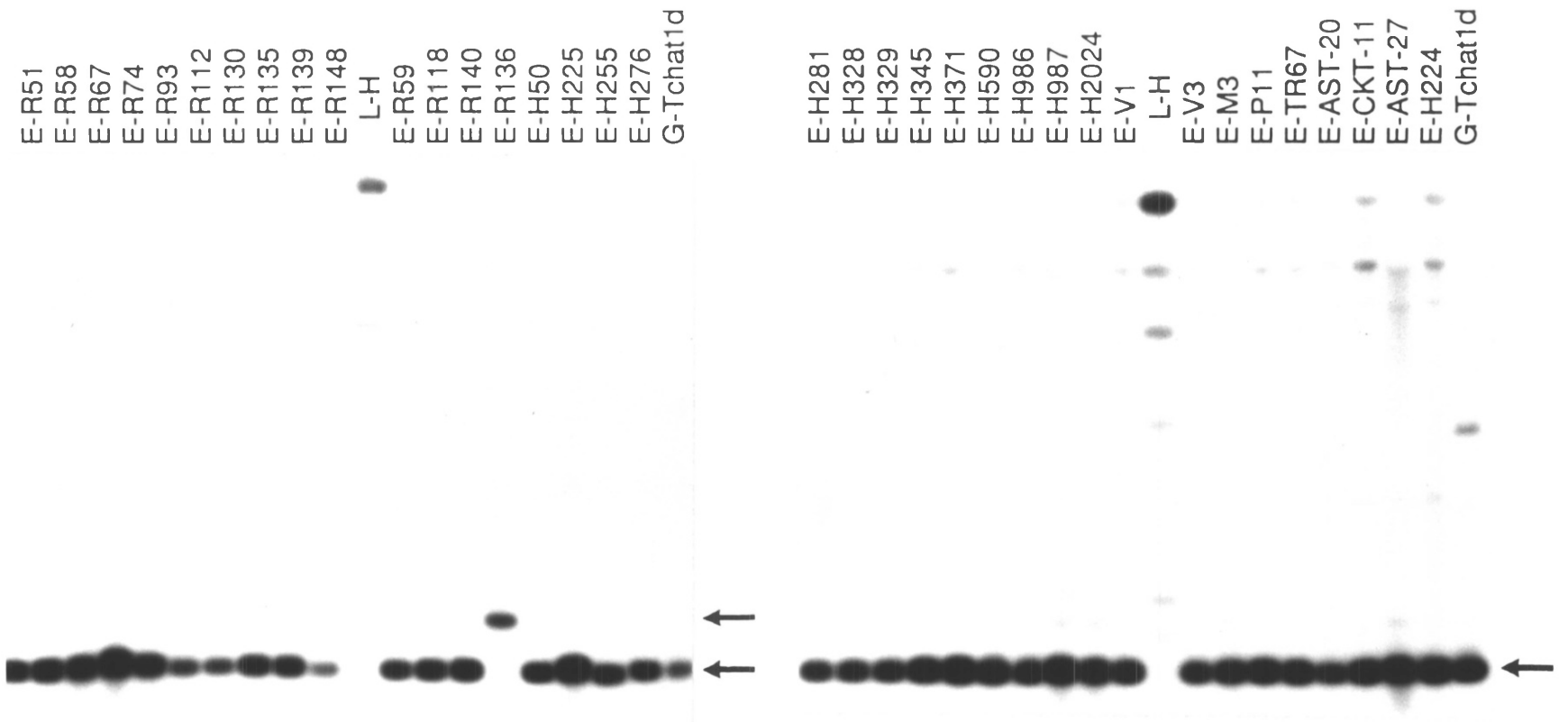

Fig. 6.24. BamHI digested total DNA of a range of EAN isolates probed with n DNA clone 36.

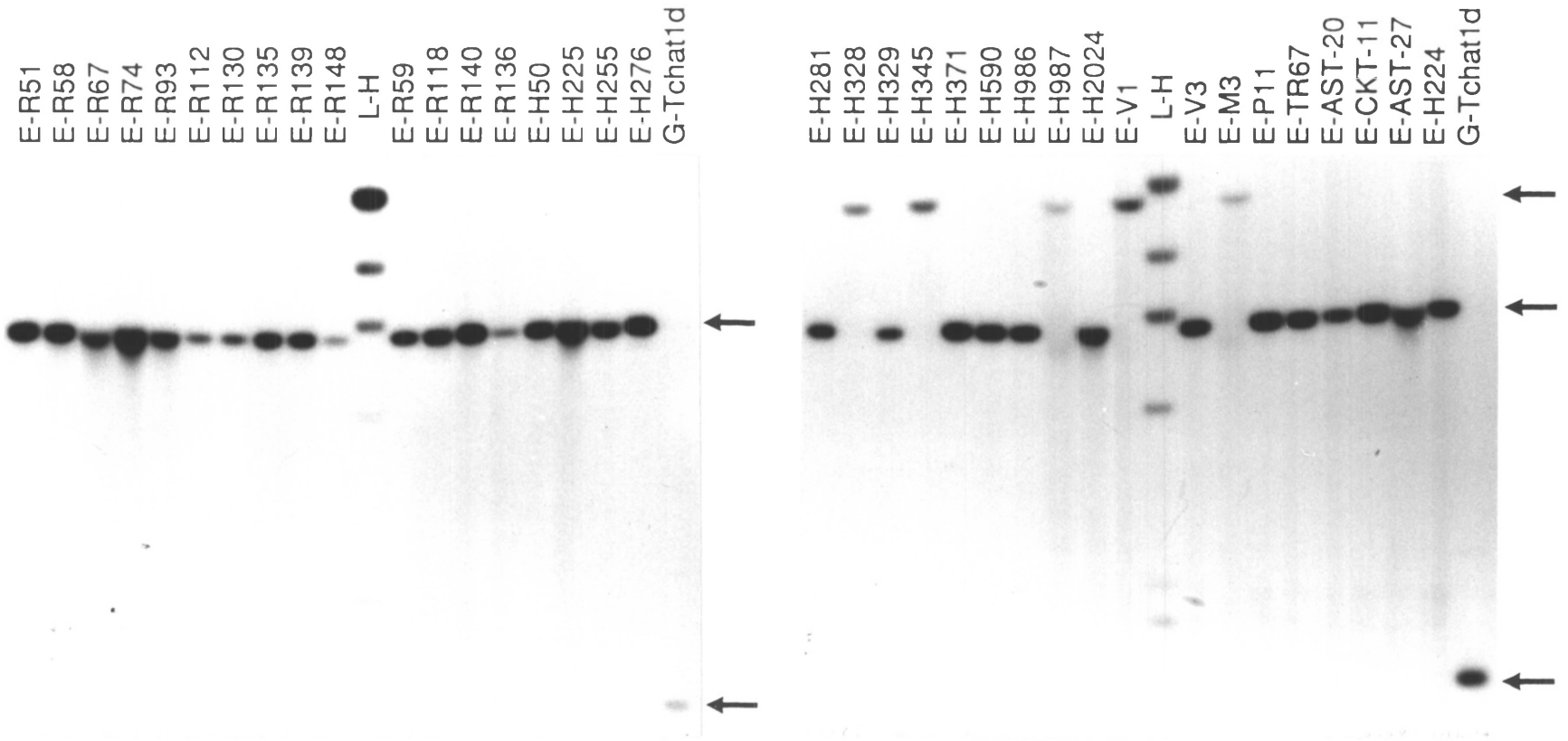

Fig. 6.25. BamHI digested total DNA of a range of EAN isolates probed with n DNA clone 103.

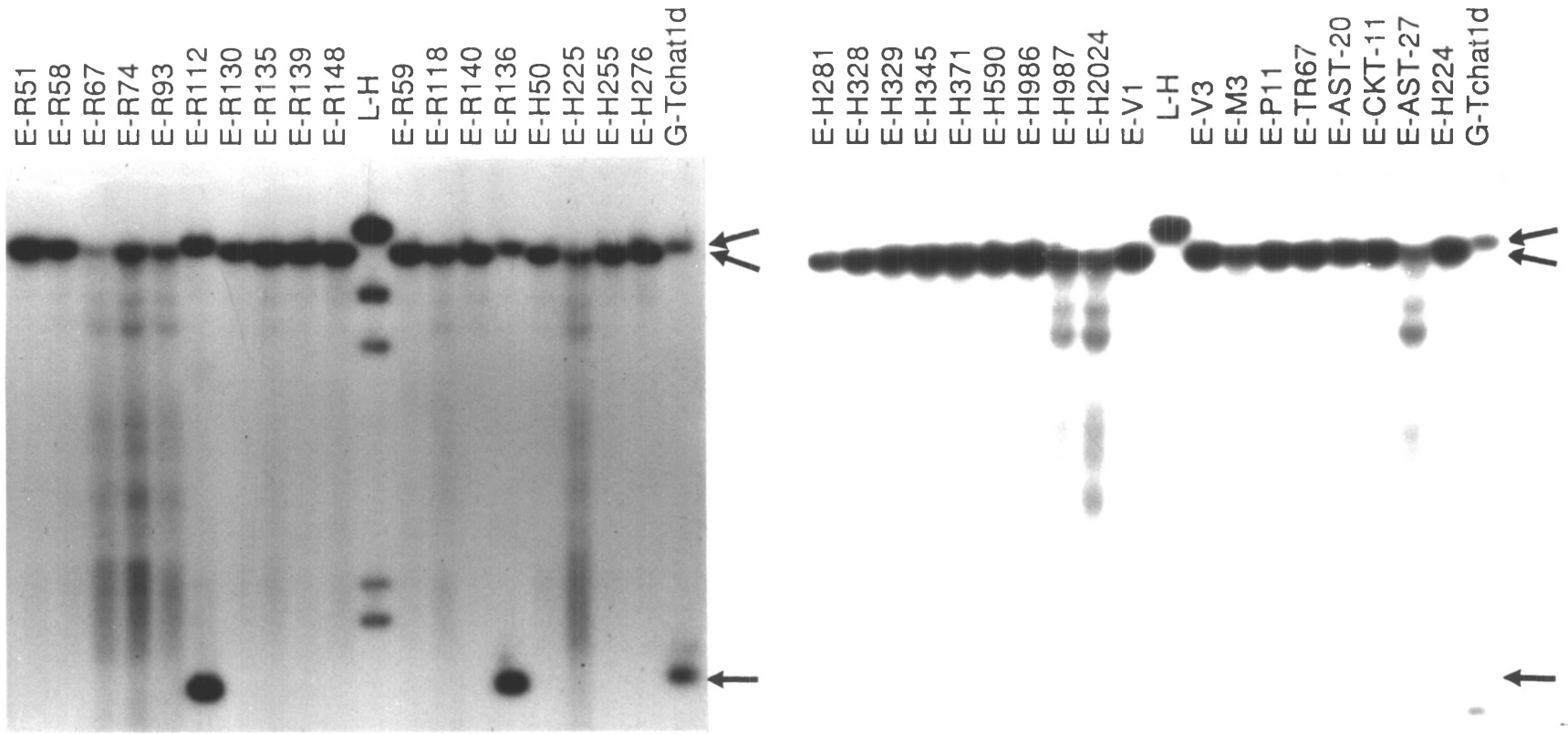

Fig. 6.26. BamHI digested total DNA of a range of EAN isolates probed with n DNA clone 105.

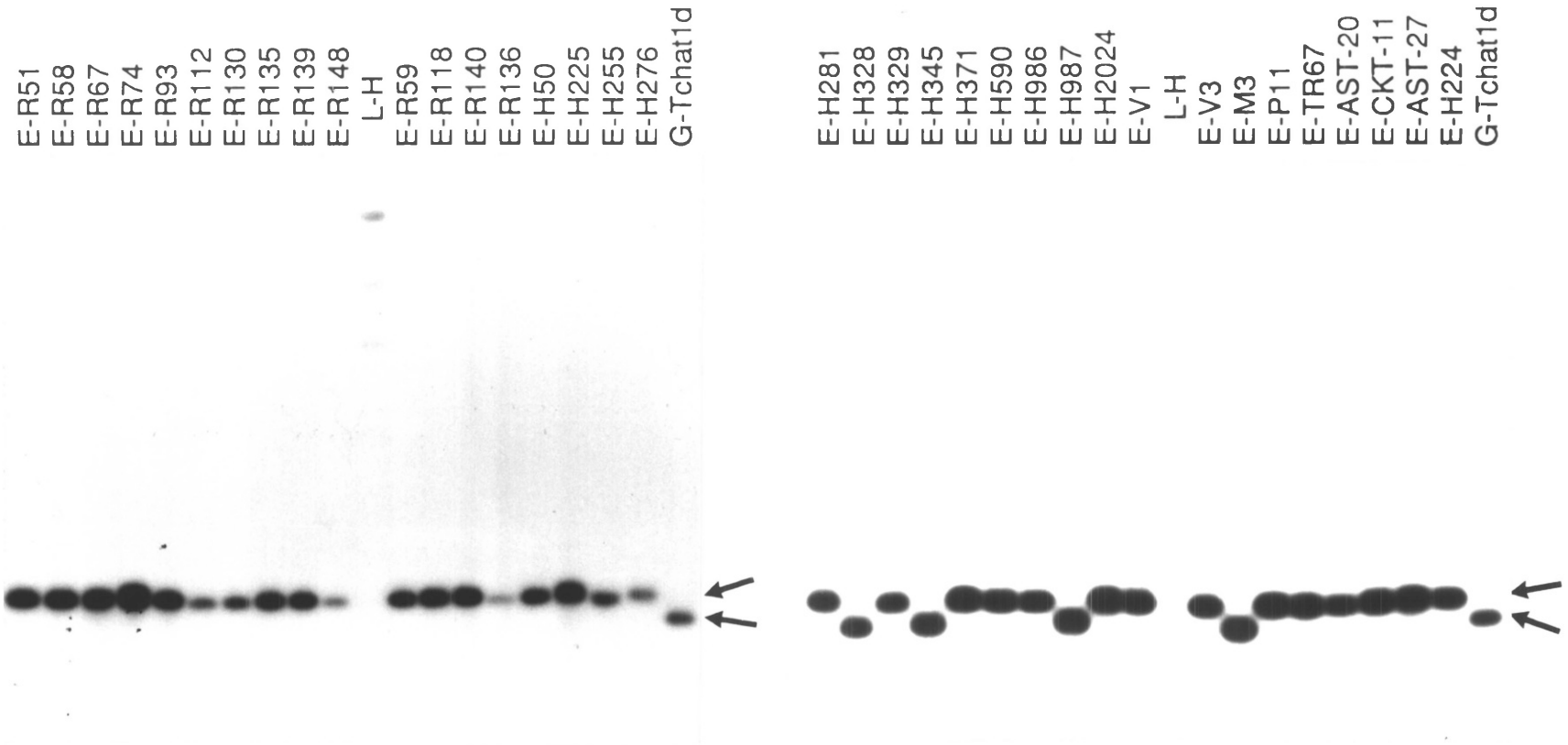

Fig. 6.27. BamHI digested total DNA of a range of EAN isolates probed with n DNA clone 124.

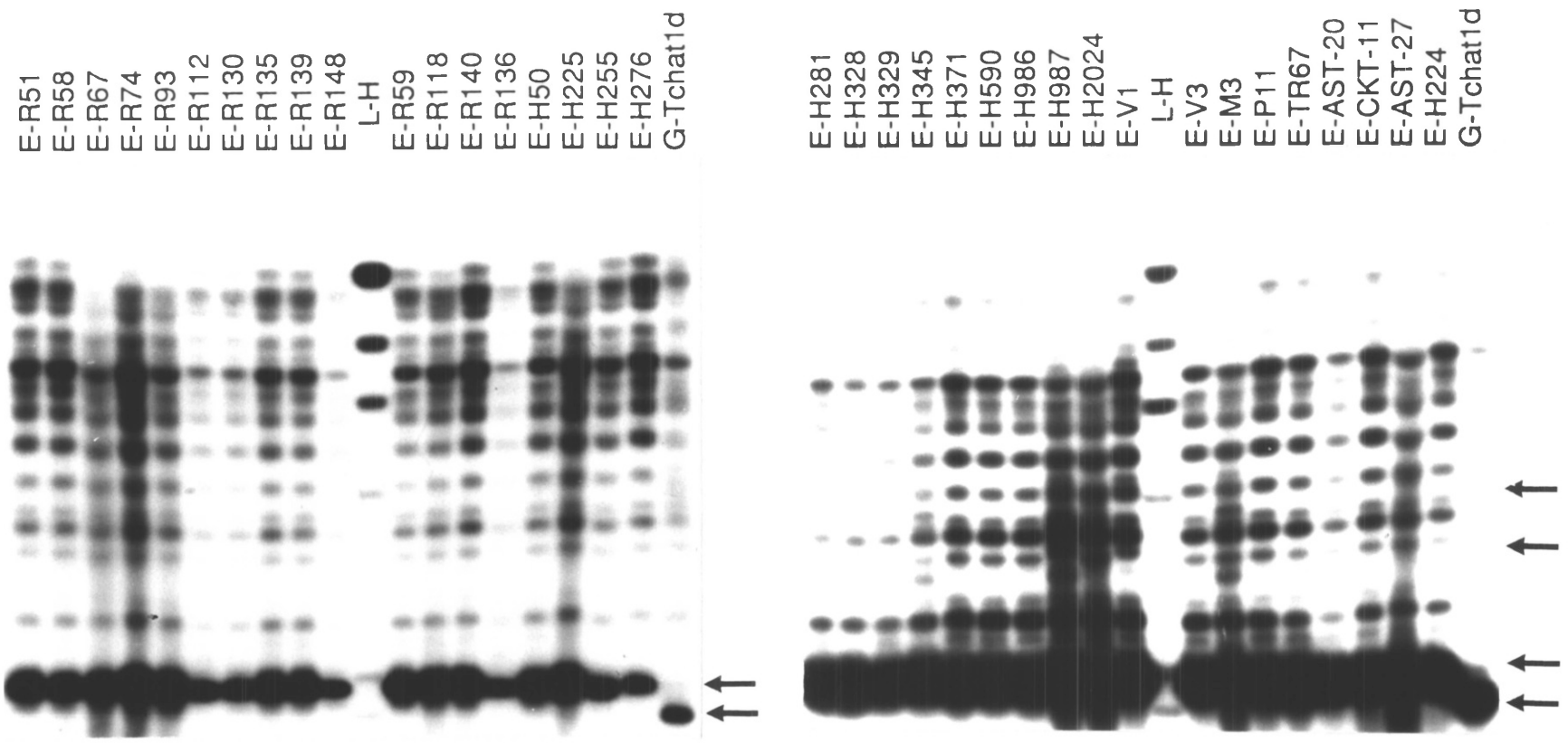

Fig. 6.28. BamHI digested total DNA of a range of EAN isolates probed with n DNA clone 137.

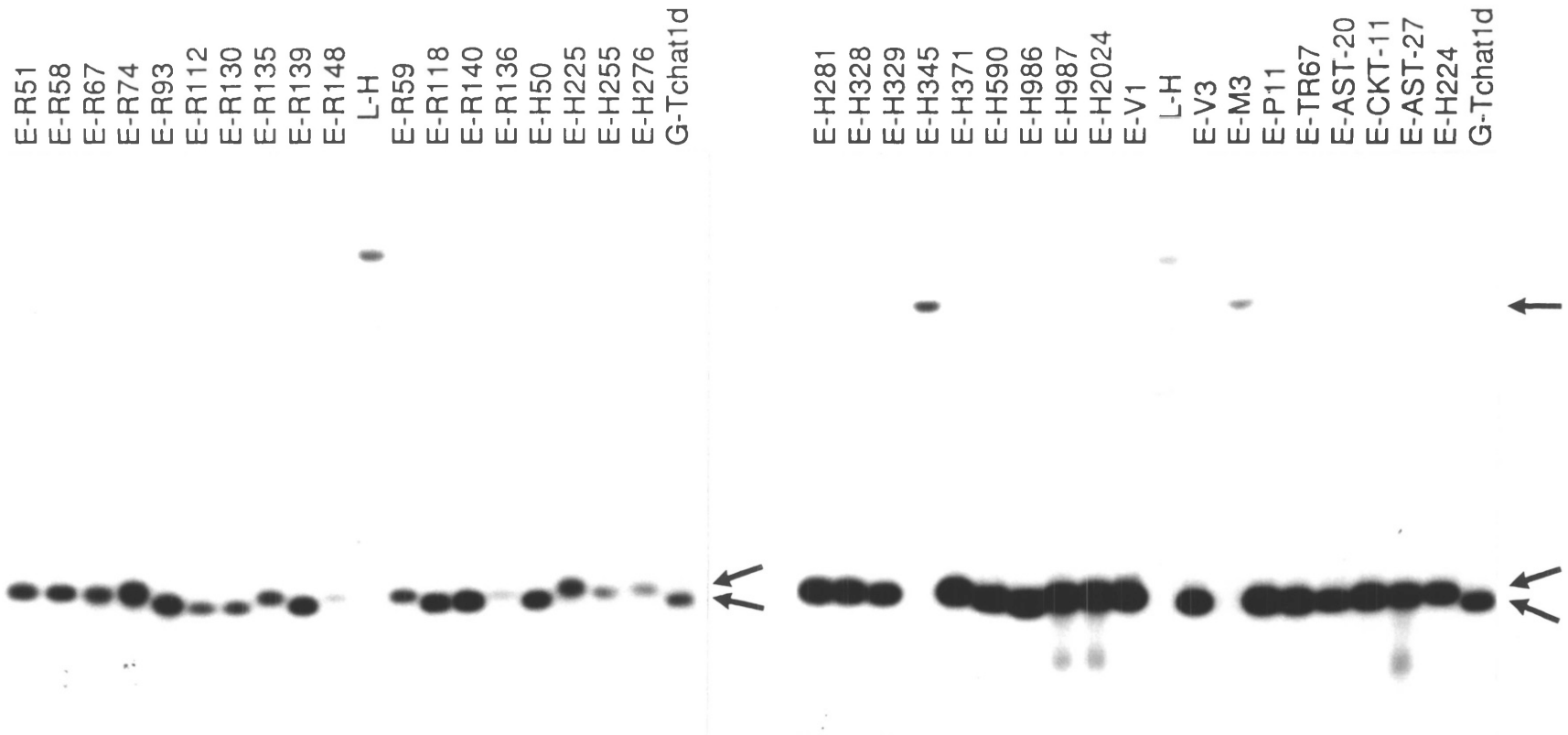

Fig. 6.29. BamHI digested total DNA of a range of EAN isolates probed with n DNA clone 140.

6.3 A Possible Evolutionary Relationship Between the EAN and NAN subgroups.

There is circumstantial evidence to suggest that an evolutionary relationship may exist between the EAN and NAN. Brasier (1989) is of the view that a founder population of EAN-infected elm material may have been introduced into the United States during the 1940's, possibly into the northern mid-West via the Great Lakes (McNabb, 1974; Gibbs et al, 1979), where it then evolved into the NAN as a result of adaptation to the large and highly susceptible American elm population (see Section 1.4). This may have resulted in the loss of the 'up mut' factor, responsible for the dimorphism and generally greater instability of the EAN subgroup, and the consistently faster mean growth rate and higher mean pathogenicity of NAN isolates compared with EAN isolates (Brasier, 1986c).

In order to investigate this evolutionary hypothesis it was decided to screen a number of recently sampled isolates from around the southern region of the Great Lakes along with a broader geographical range of North American NAN isolates (see Section 2.10.2). It is worth noting that this was not an ideal sample in that the vc analysis of these isolates carried out by Brasier and Kirk was contemporary with the molecular studies. The combination of probes and enzymes used were those which had revealed novel polymorphisms and typical non-aggressive polymorphisms within Soviet and eastern European EAN isolates, so that the results might be compared with both these isolates and the initial broad geographical range of EAN isolates examined in Sections 3 and 4.

The structure of the North American NAN aggressive population in terms of vegetative compatibility interactions of the aggressive isolates mentioned in this thesis is summarised in Table 3.4 (Brasier and Kirk, in press and pers. comm.). A striking feature of this table is that the clonal component of the North American aggressive population actually consists of not one, but three major vc groups; the NAN vc supergroup which is predominant throughout Europe and has been designated the European NAN vc supergroup comprises only around 9 % of a total North American sample of 110 isolates; a separate vc

group which appears to be the predominant vc group in North America, and has been designated the American NAN vc supergroup comprises around 58 % of this sample; and finally a vc group which gives an n reaction when paired with American NAN vc supergroup isolates and has been designated the 2nd American vc group. The percentage of the latter vc group is around 20 %. There is also a highly heterogeneous vc group component similar to those found at European post epidemic sites, though in North America it makes up only around 13 % of the sample compared with around 70 to 90 % of samples from European post epidemic sites of both the EAN and NAN. The distribution of vc groups in North America among a sample of around 100 NAN isolates is summarised in Table 6.3.

The American NAN vc supergroup is not restricted to North America, as isolate H6 from Holland is also a member of this vc group and isolates 0101 from Orsett, UK, and C112 from Chichester, UK, give n reactions to the American NAN vc supergroup and may therefore be derived from it. Thus it would appear that both the American NAN vc supergoup and the European NAN vc supergroup, and perhaps also the progenitors of the Tewkesbury / Guadalajara vc group and the Avila vc group as well, have been imported into Europe from North America such that the make up of the present day NAN aggressive population structure in Europe is likely to be a result of a combination of factors including the genetic profile of the founder populations, genetic drift and selection pressures (Brasier, 1984). One might suspect selection pressures to be of considerable importance since the extent of the American NAN vc supergroup in Europe appears to be extremely limited, whereas the European NAN vc supergroup is predominant as the major vc group. This is in direct contrast to North America where the American NAN vc supergroup is predominant and may well reflect differences in a whole range of selection factors such as climatic differences and probably most importantly the greater resistance of the European elm population.

A second feature of considerable significance revealed by Table 3.4 is that EAN vc supergroup isolates, and the unusual EAN isolates V1, M3 and H345 which give n reactions to the EAN vc supergroup, produce n reactions when paired against American NAN vc

	Population components			
	Dominant vc clone (North American supergroup)	Secondary vc clone ('n x ' North American supergroup)*	Tertiary vc clone (European NAN vc supergroup)	Heterogeneous component : 10 other vc groups
Component as percentage of sample (101 isolates total)	58.4% (59)	19.8% (20)	8.9% (9)	12.9% (13)
% A mating-types in each component	20.3%	45.0%	22.2%	53.8%

+ The North American vc supergroup was present at almost all sample sites including Kansas, Minnesota, Iowa, Wisconsin, Illinois, Ohio, Maryland + Virginia, Vermont, Maine; Toronto, Montreal and Quebec; California and Oregon.

* 'n x' : gives an 'n-reaction' against the North American vc supergroup, and is probably derived from the latter.

Table 6.3. Structure of NAN aggressive population in North America. Courtesy of Dr. C.M. Brasier (Brasier and Kirk, 1990 -in press).

supergroup isolates. The fact that these groups share the same w allele is strong evidence for the evolutionary relationship between the EAN and NAN subgroups discussed above. If one assumes that the European NAN vc supergroup was at least a component of the original founder population which was imported into Britain in the 1960's, then the continued presence of a sizeable population of the European NAN vc supergroup in North America in the face of competition from the predominant American NAN vc supergroup suggests that both groups may have different, particular selectable advantages just as the heterogeneous non-supergroup component of the aggressive subgroups appears to have at post epidemic sites (see Section 1.8). Actual proof of selection however would be difficult to find, and the possible major involvement of founder effects and genetic drift in the presence of limited selection pressures cannot yet be eliminated. Indeed it may be that the European NAN vc supergroup will eventually be eradicated from North America altogether.

The range of main and secondary band polymorphisms revealed by the screening of the selected sample of North American NAN isolates are shown in Figs. 6.31 - 6.42 and summarised in Fig. 6.30. All 21 American NAN vc supergroup isolates tested share an identical profile of n DNA polymorphisms when probed with the clones employed in this study and the same is true of all 12 European NAN vc supergroup isolates tested. This would appear to indicate strong selectable advantages for these two groups of isolates. European NAN vc supergroup isolates however, when digested with HindIII and probed with clone 36 or digested with BamHI and probed with clones 128 and 23 exhibit main band polymorphisms which are shared by some or all EAN isolates. This would appear to suggest a possible chronological series of evolutionary steps from the initial EAN founder population to the European NAN vc supergroup and then to the American NAN vc supergroup. However, it is important to note that with clone 36 all EAN isolates with the exception of the five EAN isolates R136, H328, H345, M3 and H987 exhibit the same main band polymorphism as American NAN vc supergroup isolates as well as some isolates which give n reactions to the American NAN vc supergroup. This suggests that the initial founder population contained at least a certain amount of

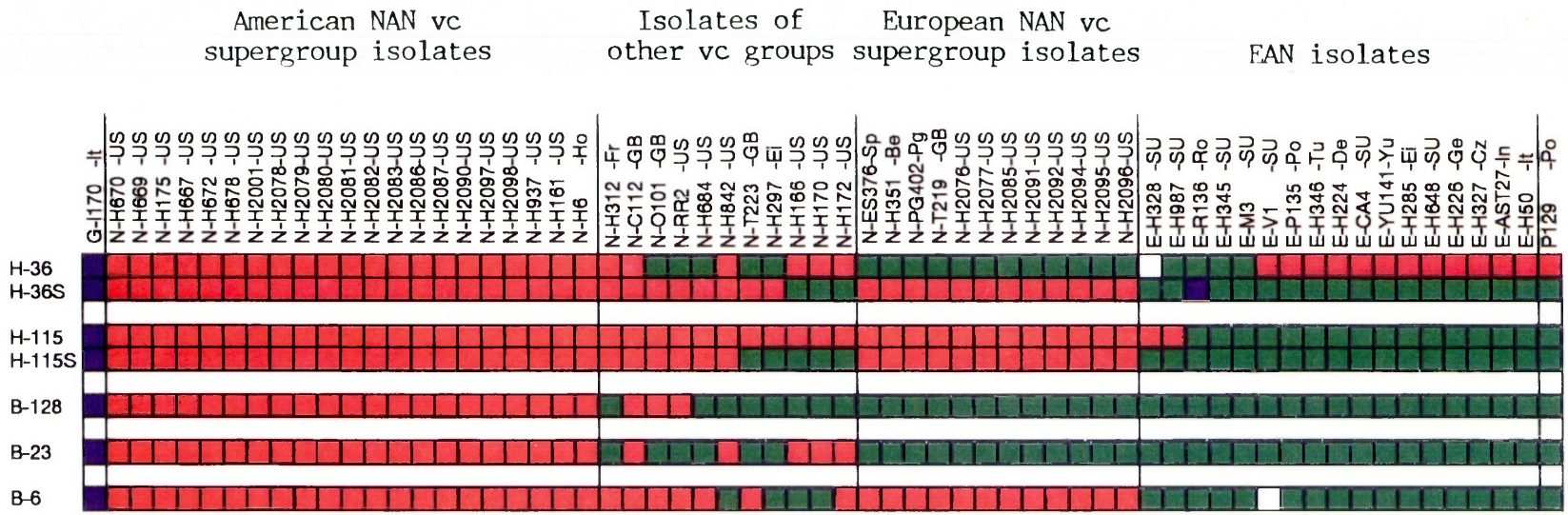

Fig. 6.30. Summary of the n DNA main and secondary (S) band polymorphisms exhibited by a range of American NAN and other assorted isolates when their n DNA was digested with HindIII or BamHI and probed with a number of n DNA clones. The letter before each isolate denotes the subgroup of which it is a member; G = non-aggressive; N = NAN; E = EAN. H and B prior to the clone numbers denote the enzymes HindIII and BamHI respectively with which the n DNA was digested. The key to the colours is as for Fig. 4.7.

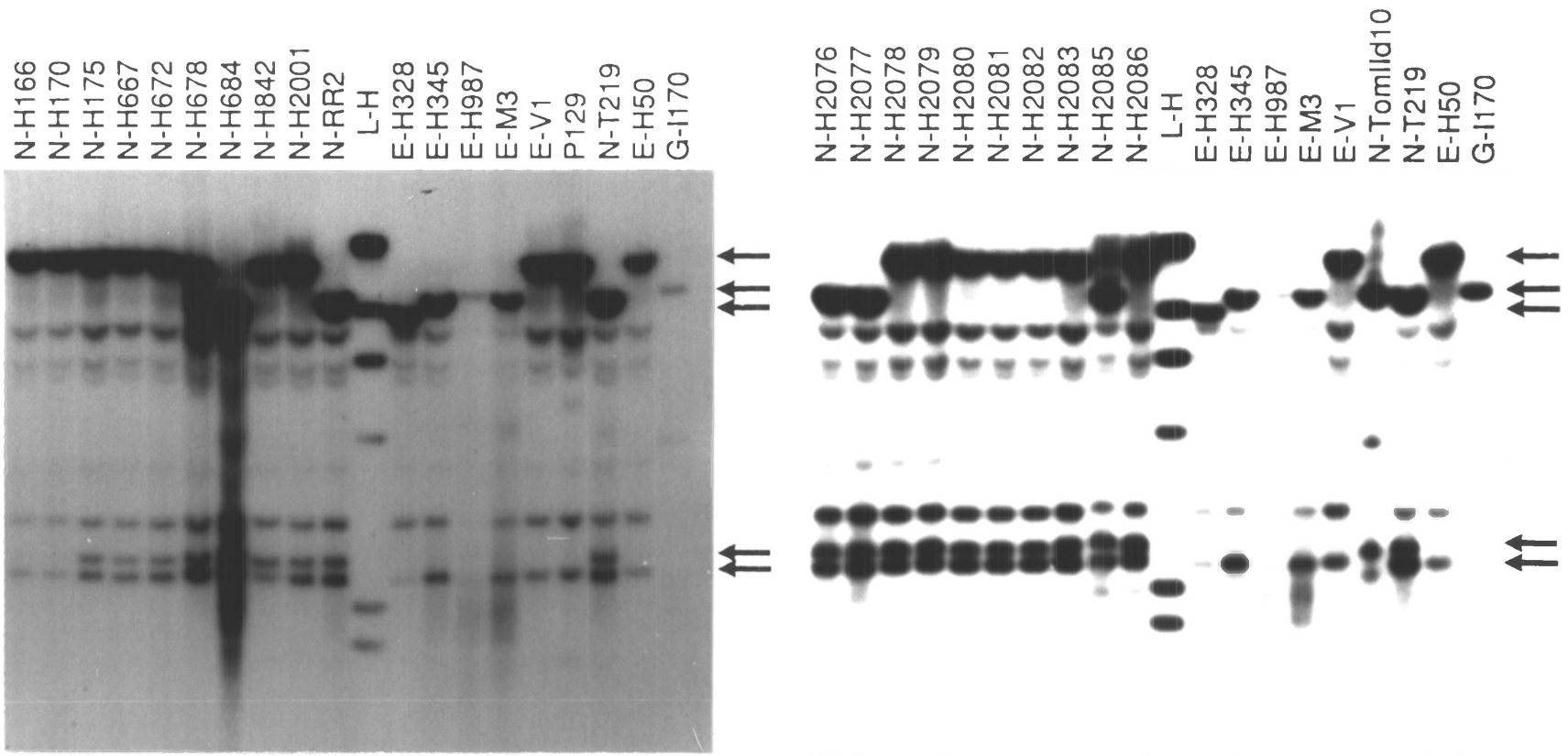

Fig. 6.31. HindIII digested total DNA from a concentrated sample of North American NAN isolates probed with n DNA clone 36.

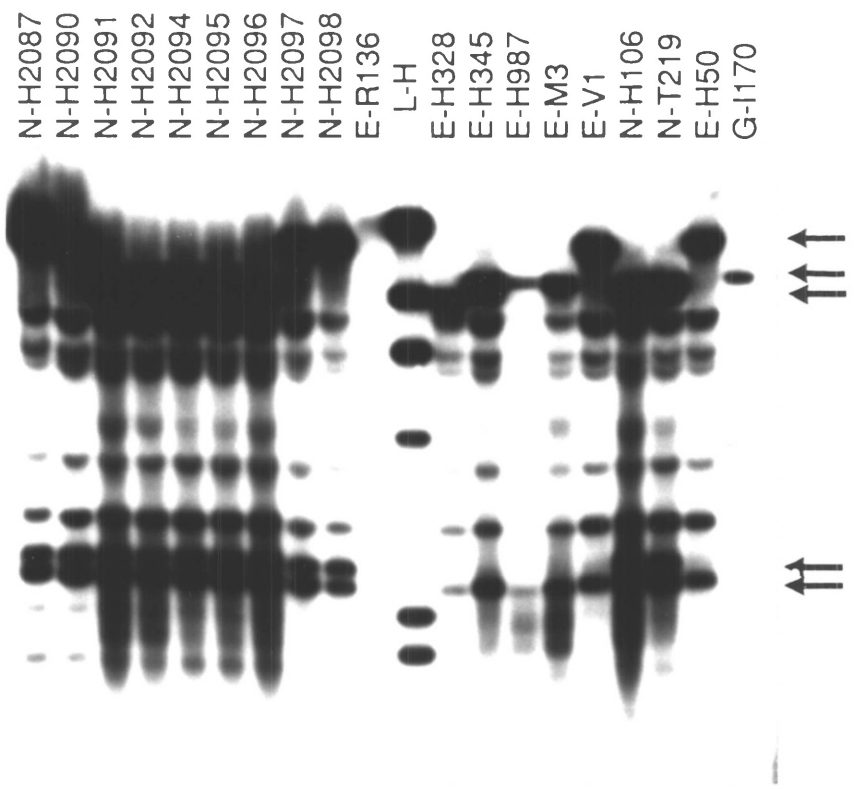

Fig. 6.32. HindIII digested total DNA from a concentrated sample of North American NAN isolates probed with $\overline{n}$ DNA clone 36.

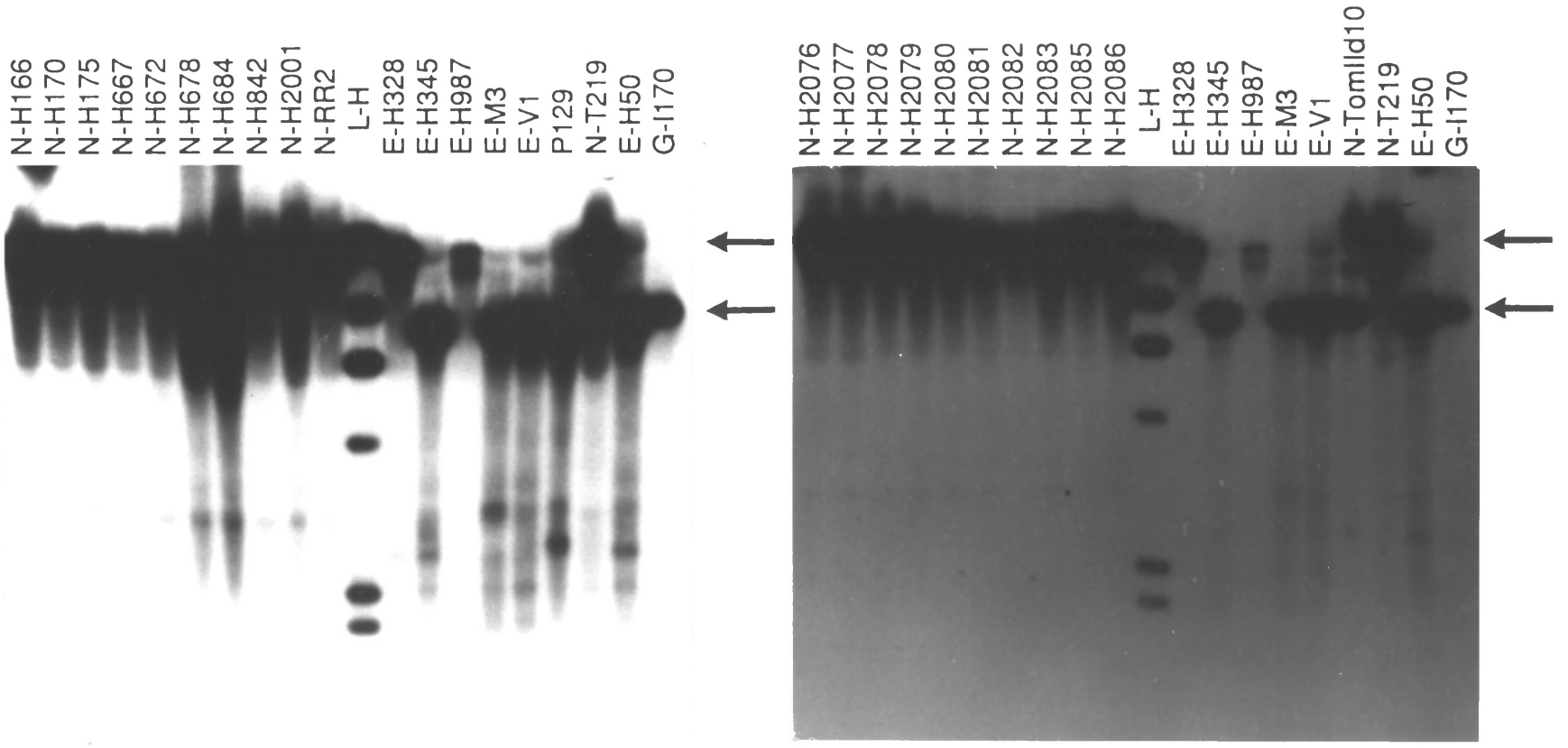

Fig. 6.33. HindIII digested total DNA from a concentrated sample of North American NAN isolates probed with n DNA clone 115.

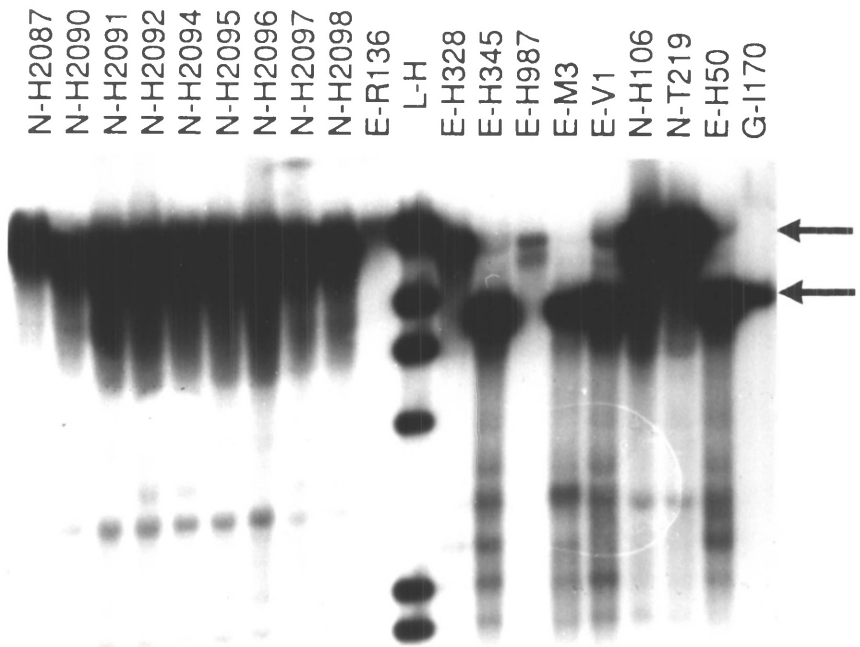

Fig. 6.34. HindIII digested total DNA from a concentrated sample of North American NAN isolates probed with n DNA clone 115.

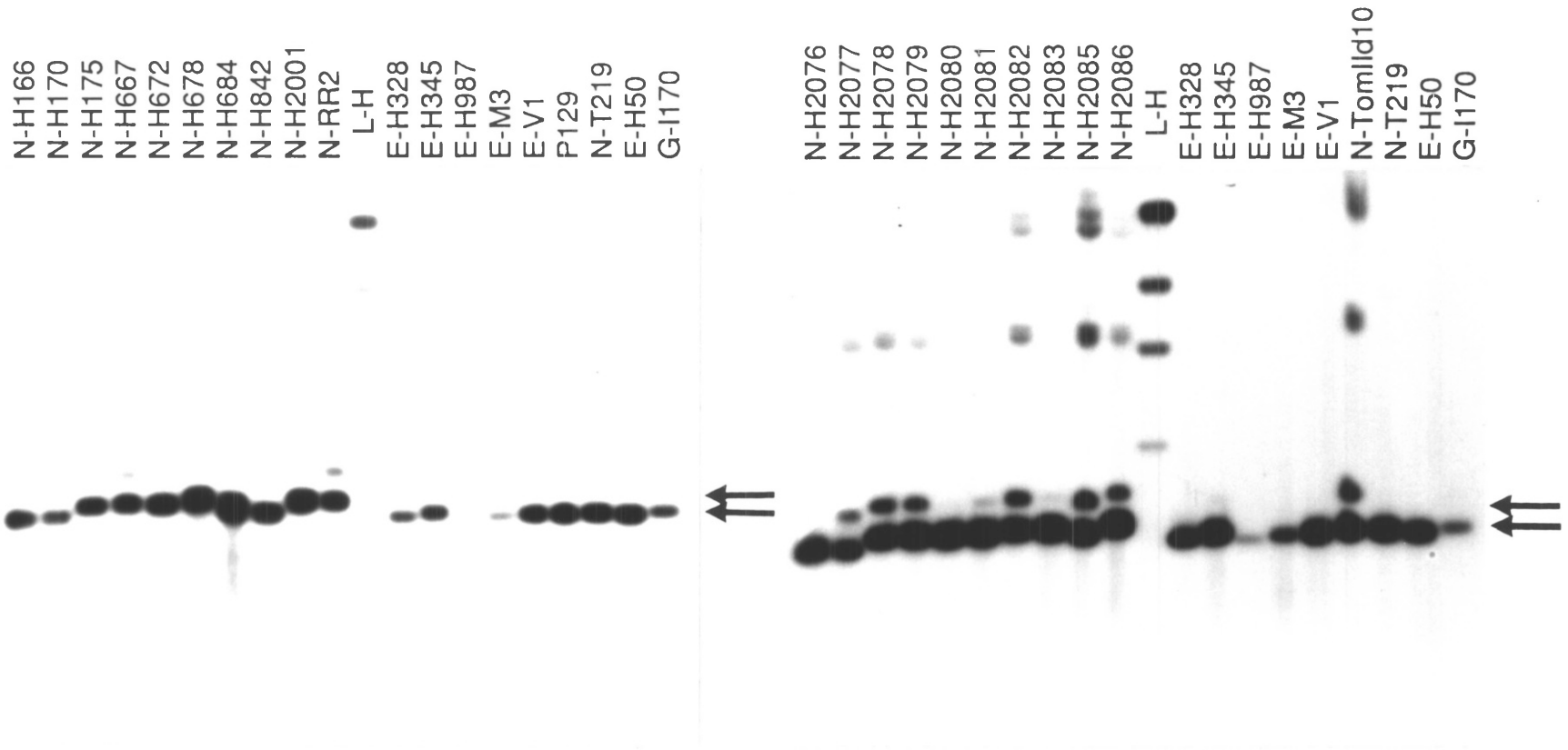

Fig. 6.35. BamHI digested total DNA from a concentrated sample of North American NAN isolates probed with n DNA clone 128.

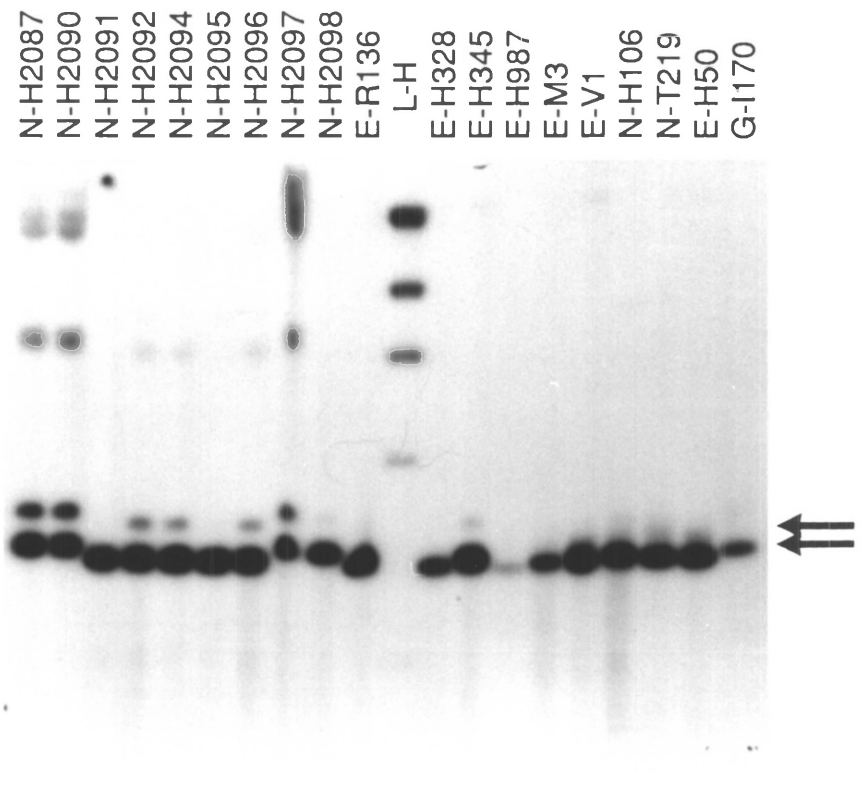

Fig. 6.36. BamHI digested total DNA from a concentrated sample of North American NAN isolates probed with n DNA clone 128.

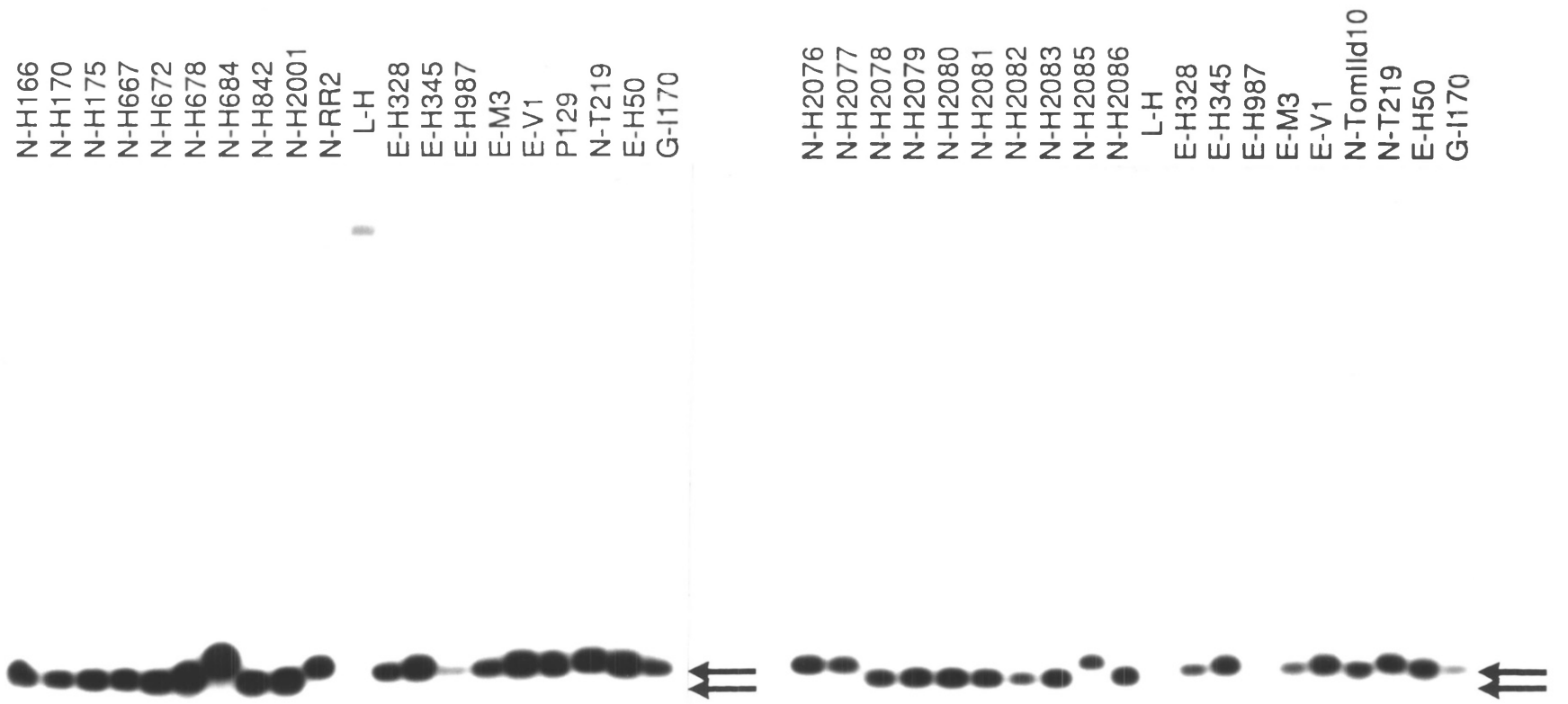

Fig. 6.37. BamHI digested total DNA from a concentrated sample of North American NAN isolates probed with n DNA clone 23.

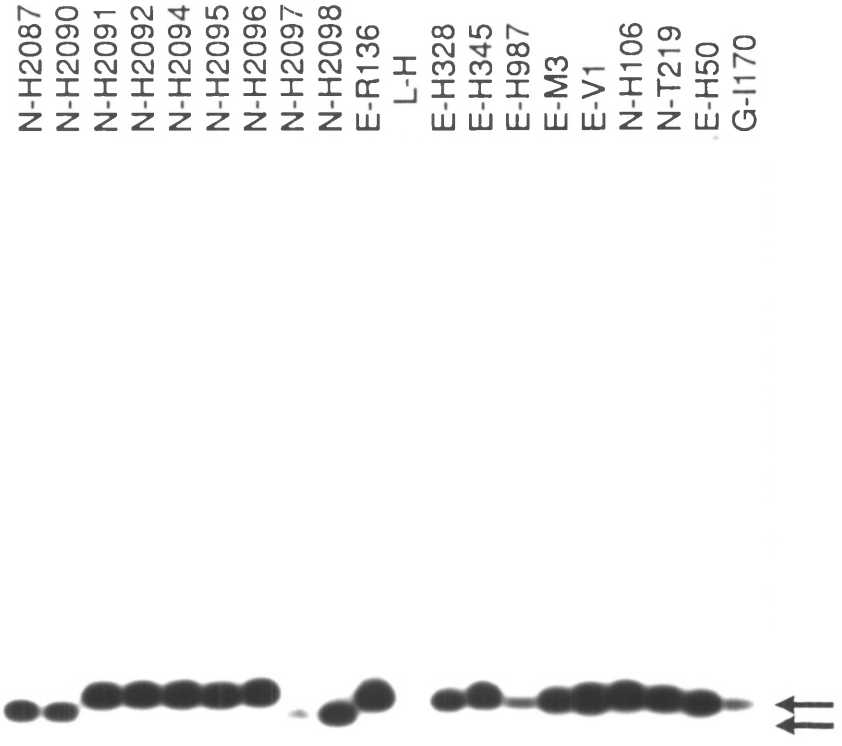

Fig. 6.38. BamHI digested total DNA from a concentrated sample of North American NAN isolates probed with n DNA clone 23.

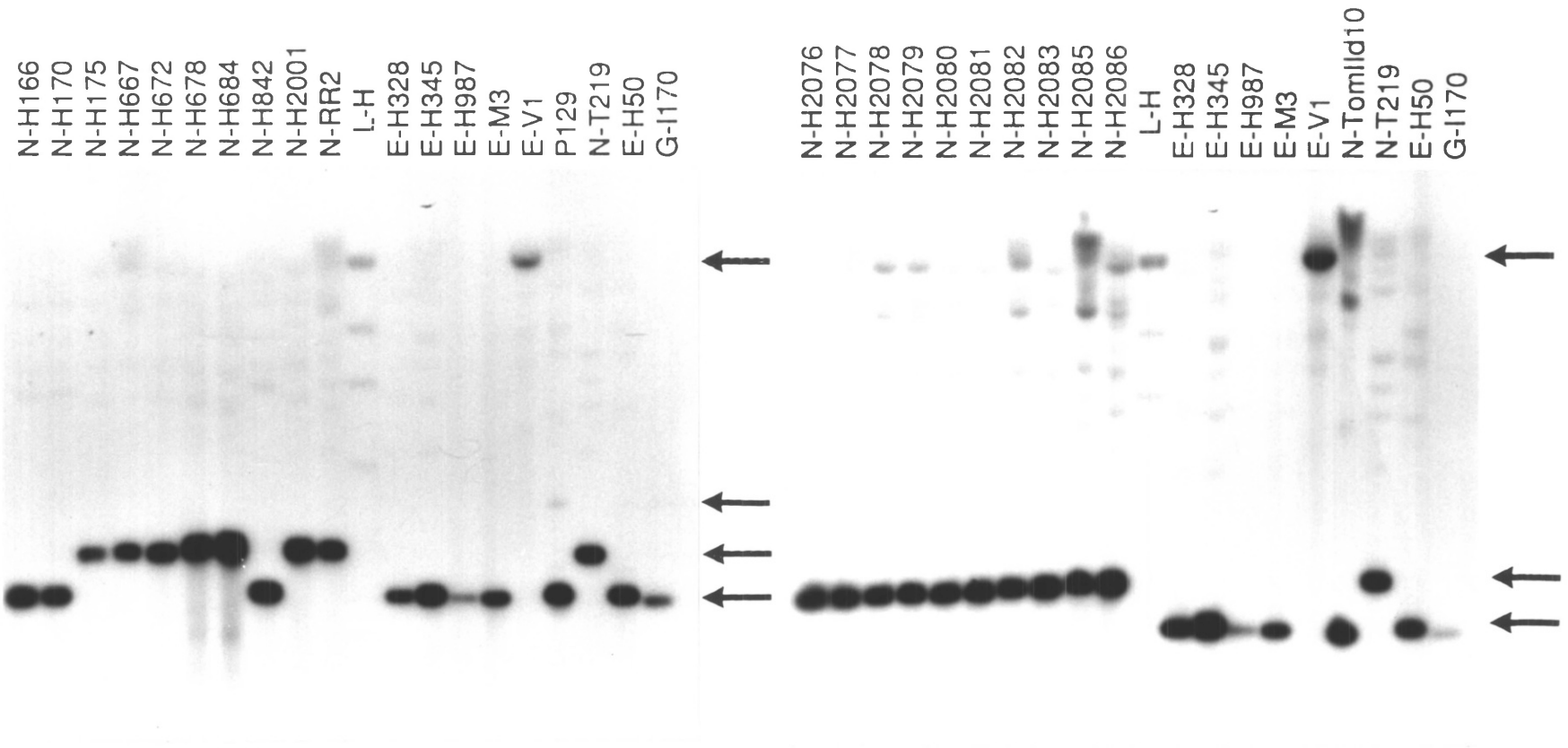

Fig. 6.39. BamHI digested total DNA from a concentrated sample of North American NAN isolates probed with n DNA clone 6.

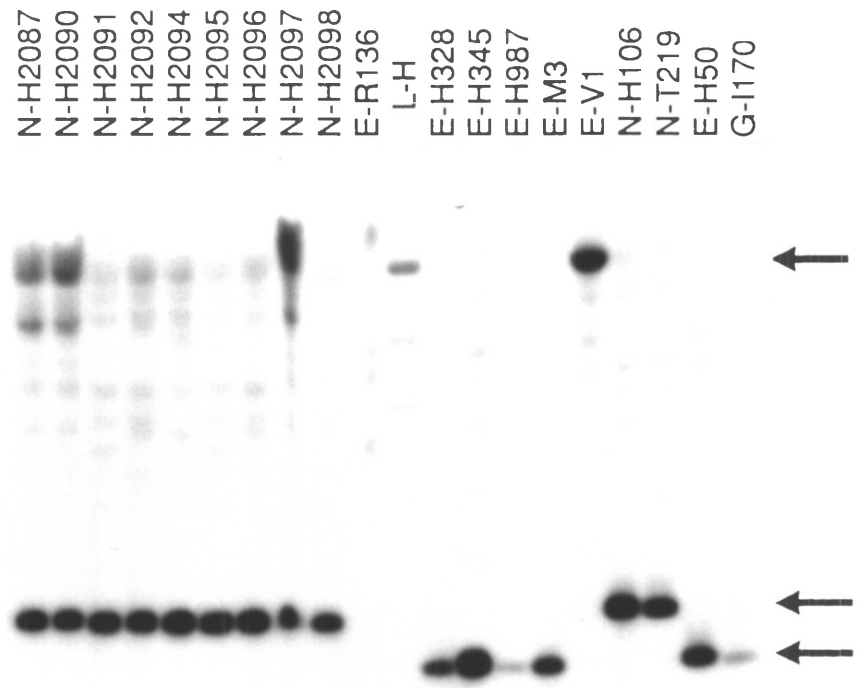

Fig. 6.40. BamHI digested total DNA from a concentrated sample of North American NAN isolates probed with n DNA clone 6.

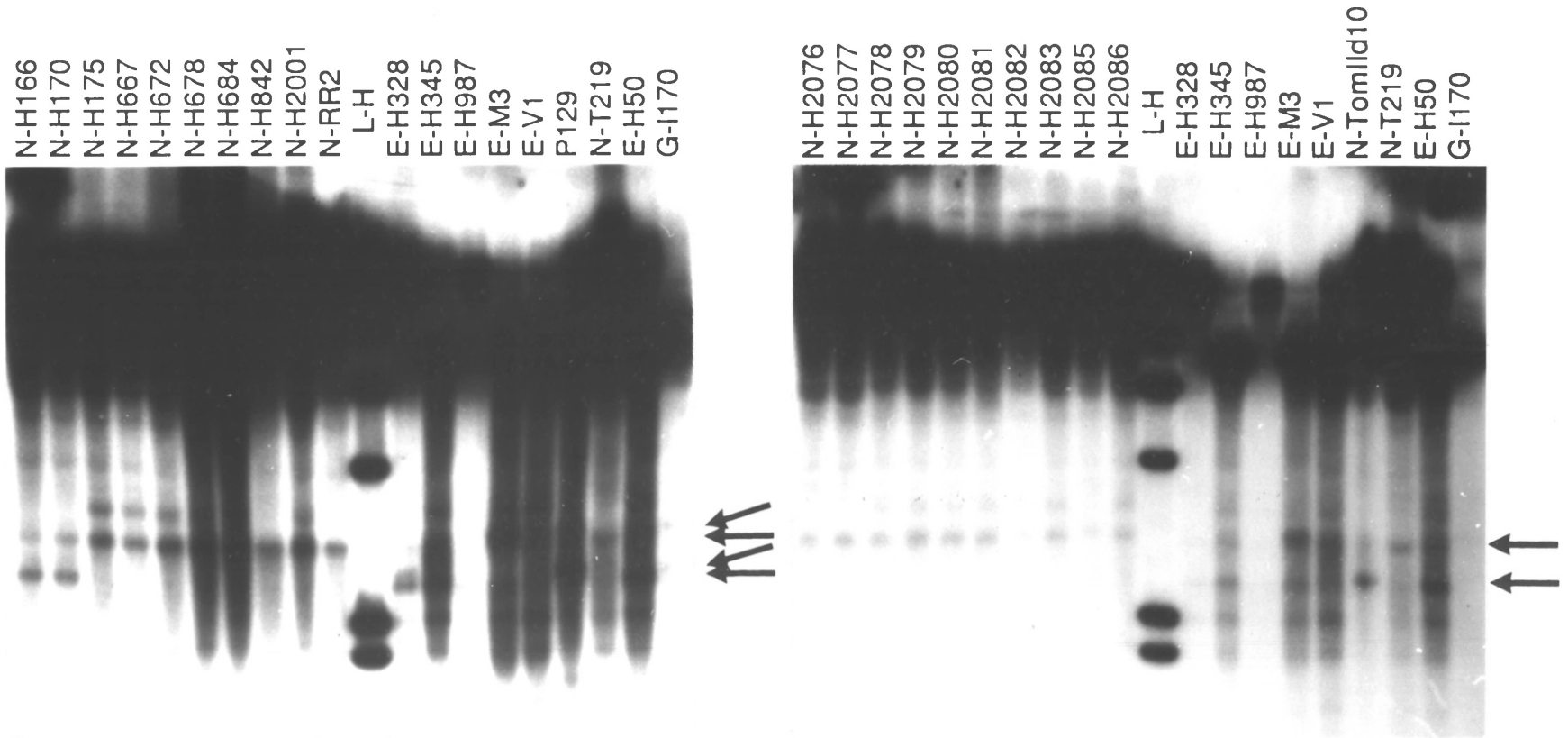

Fig. 6.41. HindIII digested total DNA from a concentrated sample of North American NAN isolates probed with n DNA clone 115.

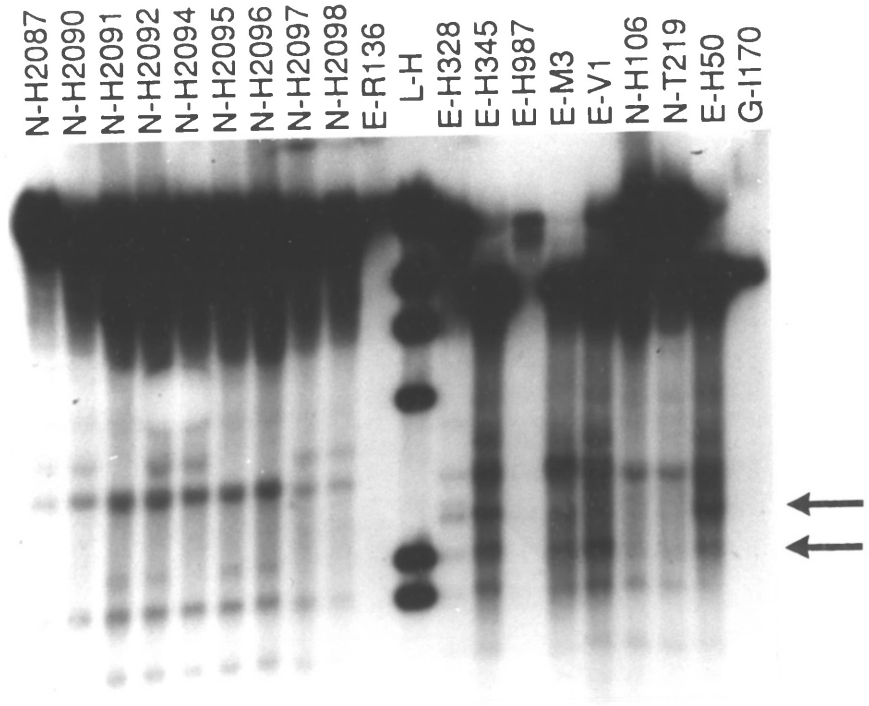

Fig. 6.42. HindIII digested total DNA from a concentrated sample of North American NAN isolates probed with n DNA clone 115.

genetic variability and was not completely clonal.

In contrast to the European NAN vc supergroup and American NAN vc supergroup isolates all the isolates which give 'n' and 'w' reactions to the American NAN vc supergroup, with the exception of C112, are in some way intermediate between these two major vc groups. They exhibit some main band polymorphisms typical of the European NAN vc supergroup and some main band polymorphisms typical of the American NAN vc supergroup. This may be the result of sexual crosses between European NAN vc supergroup and American NAN vc supergroup isolates with the segregation of the different polymorphisms into the progeny, and could thus be explained without invoking an evolutionary explanation. However, the situation is more complex because two, possibly three, 2nd American vc group isolates H172, H170 and H166 and two Tewkesbury vc group isolates T223 and H297 exhibit typical EAN secondary bands which are not found in any of the European NAN vc supergroup or American NAN vc supergroup isolates tested. Furthermore, the unusual EAN isolates H328 and H987 from the USSR exhibit a main band polymorphism which is found in every NAN isolate screened and is absent in every other EAN isolate tested. The existence of such combinations of polymorphisms cannot be explained in anything other than evolutionary terms. One can only suggest that such isolates are likely to be similar to those isolates involved in the initial outbreak of the aggressive subgroup in North America and may bear similar combinations of polymorphisms. Indeed similar isolates may have made up the original aggressive founder population from which the European NAN vc supergroup and American NAN vc supergroup evolved.

6.4 Other evolutionary hypotheses.

In addition to the broad initial geographical range of EAN, NAN and non-aggressive isolates examined in Section 3.2, a further isolate, H666, was also screened with the same selection of probes. H666 is the only sample of the disease found so far in the Himalayas. It was collected in 1960 at Srinagar, Kashmir (Heybroek, 1966). In a cultural and pathogenicity comparison with the three known subgroups, isolate H666 was shown to have a number of unusual characteristics (Brasier, pers. comm.) It has a unique morphological appearence in culture, although like the aggressive isolates its optimum growth rate is around 20°C. This particular isolate is a B type and has a pathogenicity intermediate between that of the aggressive and non-aggressive subgroups. It will not freely hybridise with any of the three subgroups when they are acting as recipients and the progeny of forced crosses between H666 and aggressive isolates reveal an even stronger negative interaction than crosses between aggressive and non-aggressive isolates. It is reasonably stable in culture even after 30 years and does not exhibit the dimorphism typical of EAN isolates.

No detailed survey of the Himalayas for O. ulmi has ever been undertaken, however this geographically isolated region with a unique elm flora has recently been proposed as a possible area of origin for Dutch elm disease by Brasier (1990). Though cautious of overinterpretation from a single isolate which has been in culture for such a long period of time, he suggested that H666 might represent a fourth possibly ancestral form of O. ulmi (Brasier, 1983; 1988; 1990).

In culture H666 often senesces rapidly, and as a result difficulty was experienced in obtaining undegraded DNA from the isolate for RFLP analysis. When probed with this range of n DNA clones the signals produced were generally faint and occasionally undetectable so the polymorphisms have been tabulated separately. (Attempts to isolate mt DNA from this isolate proved unsuccessful.) It may be seen from Fig. 6.43 that with a number of probes H666 exhibits novel polymorphisms which are not found among any of the

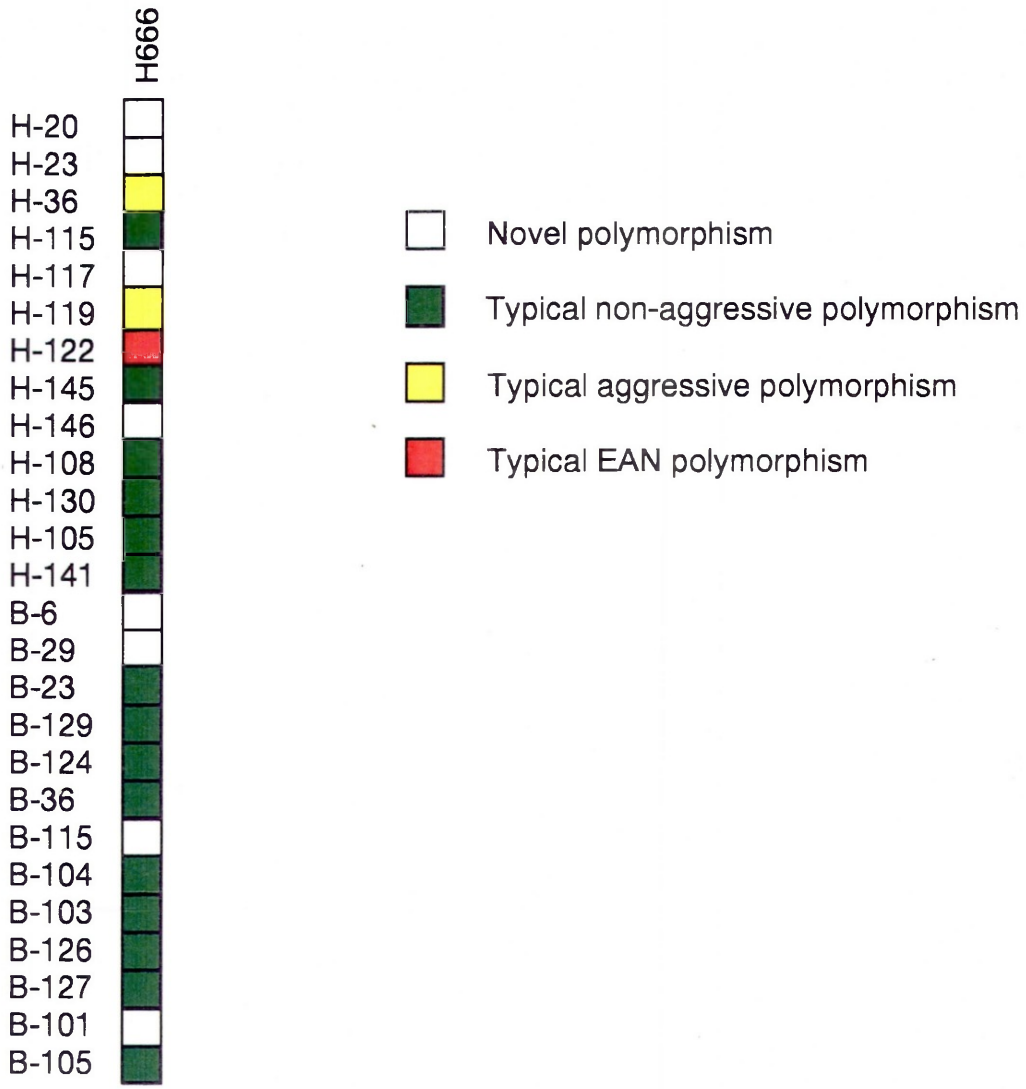

Fig. 6.43. Summary of the n DNA polymorphisms exhibited by isolate H666 when digested with HindIII or BamHI and probed with a number of n DNA clones. H and B prior prior to the clone number denote the enzymes HindIII and BamHI respectively with which the n DNA was digested. The key to the colours is as for Fig. 4.7.

isolates from the three main subgroups, while others detect some typical aggressive polymorphisms or more frequently typical non-aggressive polymorphisms.

It is of interest that H666 exhibits a majority of typically non-aggressive bands possibly suggesting a less recent origin than the aggressive subgroups, and the presence in addition of some typically aggressive polymorphisms may indicate that such isolates have some role in the evolution of the disease. These are only preliminary results however, and the question of the true status of this isolate remains open. A full scale survey of the Himalayan region and further biological and especially molecular studies of any isolates collected would be highly desirable.

An alternative hypothesis for the origin of Dutch elm disease put forward by Brasier (1990a) is its rapid recent evolution within Europe from another fungus such as Ophiostoma piceae. Brasier postulated that during the last century a number of exceptional environmental factors may have combined to cause sufficient stress to elm and other tree populations that an explosion in their associated bark beetle population occurred. He suggested that as a result a saprotrophic or weakly parasitic Ophiostoma associate of the beetles, such as an O. piceae-like fungus may have evolved into a more pathogenic fungus like the non-aggressive.

Brasier (1990a) also noted that from the 1930s onwards an epidemic of an oak vascular mycosis complex occurred in central and eastern Europe which involved O. roboris, believed by Brasier to be synonymous with O. piceae. O. ulmi and O. piceae share broadly similar conidial, synnematal and ascospore states and are therefore likely to be closely related species. Brasier (1990a) goes on to suggest that through 'host jumping' beetle vectors an oak fungus such as O. piceae may have transferred to elm and evolved into the EAN aggressive or hybridised with the resident non-aggressive to give rise to the EAN.

To see whether any evidence could be found to support these hypotheses it was decided to screen several O. piceae isolates (see Section 2.10.4) with a range of O. ulmi n DNA clones which had revealed inter-subgroup variability. Unfortunately, as with H666,

difficulty was experienced in obtaining undegraded DNA and the signals obtained were unreliable and are not presented here. Those bands which could be discerned bore no similarity at all with those exhibited by any O. ulmi isolate. DNA extractions from a number of other related species, namely O. minor, Ceratocystis brunneo-ciliata and O. coerulescens were also attempted but produced only degraded or undigestable samples. Given the poor quality of the DNA extracted from these isolates it would be preferable to begin these experiments afresh with a broader range of isolates and probes.

GENERAL DISCUSSION

The results presented in Sections 3 and 4 demonstrate that infra-specific divisions within O. ulmi, previously defined on the basis of mating behaviour and other cultural and physiological characteristics, are also supported by evidence from mt and n DNA polymorphisms. The three dendrograms produced by cluster analysis of mt DNA polymorphisms (see Figs. 3.10, 3.11, and 3.12) each demonstrate the closer relationship of the EAN and NAN subgroups to each other than to the non-aggressive. Out of 86 random n DNA clones eight, or around 9 %, were able to distinguish between a majority of the EAN and NAN isolates of the initial broad geographical sample (Fig. 4.7). All the fourteen clones, around 16 % of the total, which were selected for their prospective ability to distinguish between EAN and NAN isolates were additionally able to distinguish between the overwhelming majority of aggressive and non-aggressive isolates (Fig. 6.9). In addition 14 out of 22, or around 64 % of the n DNA clones randomly selected from the remaining 72 clones revealed polymorphism between a majority of aggressive and non-aggressive isolates within the same sample (see Fig. 6.9 and Section 6.2). Thus preliminary rough estimates for the frequency of aggressive / non-aggressive and EAN / NAN polymorphism are around 80 % and 9 % respectively. Both the mt and n DNA polymorphisms therefore provide further evidence in support of a common origin for the aggressive subgroups (Brasier, 1988). The n DNA polymorphisms between the aggressive and non-aggressive provide further evidence of their genetic divergence so apparent in the genetic disharmony seen in forced crosses (Brasier and Gibbs, 1976; Brasier, 1977; Kile and Brasier, 1990).

Considerable mt DNA size variation and RFLPs between biological species of fungi is well documented (eg. Bruns and Palmer, 1986; Collins and Lambowitz, 1981; Weber, 1986; Croft, 1987; Smith and Anderson, 1989; Foerster et al., 1987). However very little variation has been observed below the species level in Aspergillus nidulans and A. nidulans var. echinulatus (Croft, 1987), Cochliobolus

heterostrophus (Garber and Yoder, 1984), Phytophthora infestans (Carter et al., 1990) or Bremia lactucae (Hulbert and Michelmore, 1988), and only within Schizophyllum commune (Specht et al., 1983), Saccharomyces cerevisiae (Sanders et al., 1977) and Neurospora species (Taylor, 1986) has extensive variation been found.

Overall O. ulmi mt DNA is shown here to exhibit considerable size variation and frequent RFLPs both within and between the three main subgroups, and particularly between the aggressive and non-aggressive. Indeed these differences are of a similar scale to those found between biological and morphological species in other fungal genera (Foerster et al., 1988; Brums et al., 1988; Smith and Anderson, 1989; Taylor and Natvig, 1989). It is particularly striking that isolates H161 and H937 exhibit many 'typical' non-aggressive mt DNA bands when digested with all three of the enzymes employed in these studies suggests that mt DNA introgression may have occurred. Both these isolates originate from northeastern North America (see Section 2.10.2) and appear to be entirely typical NAN aggressive isolates. They are members of the dominant American NAN vc supergroup component of the North American population and the n DNA banding patterns produced by probing their total DNA with over 30 randomly selected n DNA clones revealed no evidence of non-aggressive n DNA introgression or even typical EAN polymorphisms (see Figs. 4.7, 6.9 and 6.30).

Both these isolates were sampled from a recent epidemic front region where there is considerable opportunity for non-aggressive and aggressive mycelia to come into close physical contact, and, as described in Section 6.1, non-aggressive n DNA introgression into the aggressive appears to occur under such circumstances. As there is no evidence for n DNA introgression in this case, mt DNA introgression alone may have occurred possibly via hyphal anastomosis followed by mitochondrial but not nuclear transfer. The fact that the size of the mt DNA of these isolates is somewhat lower than that of 'typical' non-aggressive isolates may indicate that such molecules have arisen by recombination between NAN and non-aggressive mt DNAs following transfer.

Mitochondrial DNA transfer would appear to be the most likely

explanation, though the polymorphism data contains no conclusive evidence that recombination has occurred. The possibility that the presence of non-aggressive mt DNAs in otherwise typical NAN aggressive isolates represents not introgression but the evolutionary origin of the NAN from the non-aggressive via the EAN would seem to be remote, however given the mode of inheritance of mt DNA and the reasonably limited size of the samples employed in these studies this possibility cannot be entirely excluded.

Without undertaking more detailed restriction mapping it is impossible to determine whether the size variation found here in O. ulmi mt DNA results from the presence or absence of optional introns as found within the 'nidulans' group of Aspergillus species (Croft, 1987), Neurospora crassa (Burger at al., 1982) Coprinus cinereus (Economou, et al., 1987) and Saccharomyces cerevisiae (Hengsgens et al., 1983), and whether or not such fragments recombine in or out of the mt DNA as a result of senescence, cytoplasmic infection or other stress factors as has been described in Podospora anserina (Kuck, 1989). When considered in conjunction with the range of morphological and cultural differences the characteristic size variation presented here provides a further indication that the designation of the aggressive subgroup as O. ulmi should perhaps be re-evaluated. Brasier (1990a) suggested they might be considered as sibling or biological species. Additional molecular analysis of the mitochondrial genome of O. ulmi would provide information about the cause of size variation and RFLPs, and might cast further light upon the evolutionary relationships of the three main subgroups and related species.

The vc supergroup dominating at different NAN early and late post epidemic sites has been shown to vary (Brasier, 1988a). However, a characteristic feature of every site examined would appear to be the maintenance, apparently through some sort of selection, of a single, or in the case of Tewkesbury, two asexually reproducing clonal vc groups, even in the face of the generation and maintenance of an outcrossing highly heterogeneous component. Eventually a balance between the clonal and heterogeneous components is reached (Brasier, 1988a).

The morphological and physiological uniformity of O. ulmi isolates within a single vc group at epidemic fronts, as noted by Brasier (1984; 1988a), shows a certain similarity with the situation found in Aspergillus species, even though the two systems have distinctly different dynamics. Whereas in O. ulmi the aggressive constitutes an invading epidemic population Aspergillus is probably relatively evolutionarily stable. Grindle (1963) first noted that in Aspergillus nidulans members of a single heterokaryon compatibility (h-c) group tended to exhibit a similar general morphology and radial growth rate, a point clearly confirmed by Jinks et al. (1966). Butcher et al. (1972) produced similar evidence of h-c group uniformity in response to different environments. Croft and Jinks (1977) examined of a number of characters in a broad geographical sample of wild UK isolates. They revealed greater variation between h-c groups than within h-c groups for every character, and the authors concluded that the members of any one h-c group were clonally related to each other. Such partial genetic isolation mechanisms could promote sympatric speciation and may have played a significant role in the evolution of many fungal genera and species including Aspergillus and O. ulmi (Croft and Jinks, 1977; Brasier, 1987). However, it is also thought that such incompatibility systems may provide provide a check on the spread of cytoplasmic infection (Caten, 1972; Brasier, 1984). Given the potentially highly disruptive effects of cytoplasmic infection it seems likely that such a role would be a strong selective force behind the evolution of such systems, while their effects as genetic isolation mechanisms may be merely a consequence of their existence, but which then promoted further evolutionary divergence through sympatric speciation.

The results presented in Sections 5 and 6 provide strong molecular evidence of the clonal nature of wild isolates within a single vc group. In none of the samples of a single vc group examined were n DNA polymorphisms between isolates observed even when the samples were collected from widely dispersed locations. This agrees well with the morphological and cultural data. Similar results were obtained by Croft (1987) who in a sample of nine A. nidulans isolates, found no variation within an h-c group using two random

genomic n DNA clones. By contrast isolates within the same vc group have been demonstrated to exhibit mt DNA polymorphism - see isolates H351 and PG402 of the European NAN vc supergroup and isolates H670, H161, H937 and H6 of the American NAN vc supergroup. This may well reflect the more rapid evolution of mt DNA compared to n DNA and/or mitochondrial transfer and possible recombination as mentioned above.

The conflict between collectivism and individualism has been a dominant feature of discussions concerning the nature of wild mycelial populations and the role of somatic incompatibility systems (eg. Gregory, 1984; Brasier, 1984; Rayner et al., 1984). As discussed in Section 1.8.1, the discovery of somatic incompatibility systems in many fungi has meant that it is no longer tenable to consider widespread and unrestricted heterokaryosis within mycelial populations as a general phenomenon in wild fungal populations. As a result the collectivism championed by group selectionists has given way to the individualistic theories of those who view mycelial mosaics in terms kin-selection, selfish genes and the action of selection on the individual.

Brasier (1984), translating the mycelial mosaic population structure of O. ulmi into behavioural terms, interpreted the 'essentially antagonistic aspect of vegetative incompatibility interactions' in terms of defence of an individual mycelium's territory, along the lines proposed by Todd and Rayner (1980) and Rayner et al. (1984) as a result of studies of the Basidiomycotina. Brasier (1984) went on to state that such an explanation appeared to be in line with the 'selfish gene' concept expounded by Dawkins (1976).

In O. ulmi variation in morphology and cultural characteristics with at least a partial genetic basis occurs in post-epidemic populations of a single vc group (Brasier, pers. comm.) and indeed mt DNA variation is reported here. However, the fact that no n DNA polymorphism within a single vc group was observed in these studies provides further evidence that vegetative incompatibility mechanisms are able to delimit somatic individuals of O. ulmi though they in no way preclude sexual conjugation. The results presented here thus support the statement of Gregory that 'membership of the co-op is

restricted to members of the clone' (Gregory, 1984).

Somatic collectivism in O. ulmi would therefore seem to operate only between individuals of the same vc group, however the discovery of an EAN isolate which gives a compatible vc reaction with the European NAN vc supergroup (Brasier, pers. comm.) demonstrates that it is not restricted to genetically closely related individuals.

The typically non-aggressive polymorphisms exhibited by isolate P129 and the Tomar and Mafra isolates (see Sections 6.1 and 6.2) lend considerable support to the suggestion that non-aggressive n DNA introgression into the aggressive through rare recombination events may occur at epidemic fronts. The majority of progeny generated from such a cross are likely to be at a severe competitive disadvantage from what is known of the progeny of experimental crosses of non-aggressive and aggressive parents (Gibbs and Brasier, 1976; Brasier, 1987b; Kile and Brasier, 1990). The results of Kile and Brasier in particular demonstrate that the progeny of these crosses exhibit poor performance in potential fitness characters such as vascular wilt ability and bark colonising ability, or are sexually sterile. However, some progeny may indeed survive, generating variation in vc groups and other characteristics, and they may be stabilised by back-crossing with aggressive isolates. Such introgression may at least partially account for the rapid emergence of a heterogeneous vc group component at epidemic fronts. In this respect it would be particularly interesting to study the developing population structure where the aggressive subgroup invades an area in which the non-aggressive is absent, and it would appear that such an opportunity may have recently arisen in New Zealand (Brasier, pers. comm.). The fact that rare crossing events may occur in nature between the aggressive and non-aggressive does not of itself preclude the consideration of these two subgroups as discrete species since these two 'subgroups' otherwise remain entirely distinct entities with the non-aggressive being essentially displaced by the aggressive. While transient hybrids may occur, perhaps acting as a vehicle for gene flow, no truly hybrid forms survive in post epidemic regions and no evidence of extensive introgression has been found at the n DNA level.

A proposed pattern of spread of the non aggressive and EAN and NAN aggressive during the first and second epidemics of Dutch elm disease is summarised in Figs. 1.3 and 1.4. It has been proposed by Brasier (1990a) that the EAN may have arisen from the non-aggressive in northeast Romania/Ukraine/Moldavia region where the first outbreak of the aggressive was recorded, and further that a form of O. ulmi close to the EAN aggressive may have arrived in North America in the 1940's and subsequently evolved into the NAN. The detection of some typically non-aggressive n DNA polymorphisms among EAN isolates and particularly isolates R136 and R112 provides some, if tenuous support for the hypothesis that the EAN may have originated from the non-aggressive in the northeast Romania region. Furthermore the relatively large number of novel polymorphisms exhibited by these isolates and some other isolates from the USSR (see Table 6.2 and Fig. 7.1) may be the remnants of recombination events which occurred during the possible initial emergence of the EAN from the non-aggressive subgroup. An evolutionary relationship between the non-aggressive and the EAN is also indicated by the fact that they both exhibit colony instability caused by the 'up-mut' factor which is absent in the NAN. In addition it should be noted that the range of values for EAN mt DNA size is intermediate between those of the NANs and non-aggressives, and might also reflect the evolution of the NAN from the non-aggressive via the EAN.

Despite the occurrence of non-aggressive n DNA polymorphisms in NAN isolates from epidemic front isolates there is no evidence for their presence among a great many NAN isolates isolated from post epidemic regions. This may indicate their eradication over time through genetic drift and/or selection pressures. By contrast non-aggressive n DNA polymorphisms have been found among EAN isolates from an epidemic front and also from the relatively small sample from the post epidemic Romania/Moldavia/Ukraine region, an area from which the non-aggressive has apparently been absent for many years. It would be difficult to demonstrate conclusively that the non-aggressive polymorphisms among the isolates of this region represent evolutionary remnants. However, given the apparent absence of non-aggressive polymorphisms from all other post epidemic sites examined

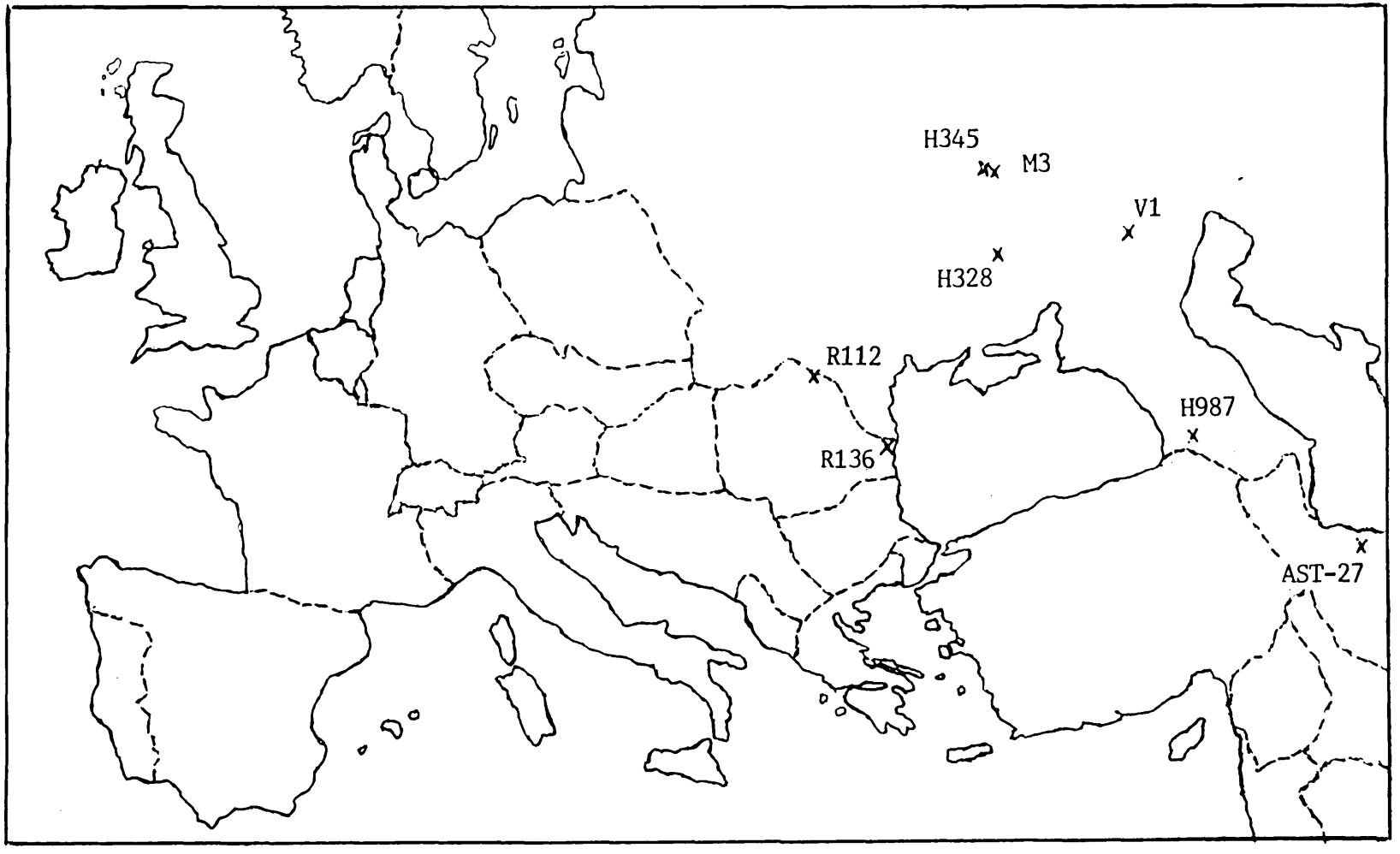

Fig. 7.1. Map showing the geographical distribution of those EAN isolates which exhibit novel or typical non-aggressive polymorphisms when probed with a number of random nuclear DNA probes (see Table 6.2).

the screening of larger samples of isolates from other post epidemic and epidemic front sites with a greater number of probes might provide reasonable evidence to distinguish between introgression and evolutionary explanations.

The origin of the founder population thought to have given rise to the NAN in North America remains in question but the results presented in Sections 6.2 and 6.3 provide some evidence that the source may lie within the Romania/Moldavia/Ukraine region from which isolates exhibiting relatively highly frequent novel polymorphisms for such a small sample have been collected. Screening larger samples from both this region and North America may cast further light on this hypothesis. Certainly there is evidence to suggest that wherever the founder population originated it contained at least some variability upon which selection then acted to promote the emergence of the major North American vc supergroups.

The results of the work with isolate H666 and Ophiostoma piceae were preliminary only, and yielded little by way of firm evidence. Even so, the frequent occurrence of novel n DNA polymorphisms among a small sample of EAN isolates collected from a region thought to be the possible place of origin of the EAN suggests that an extensive survey of this region may reveal isolates exhibiting polymorphisms linking them to isolate H666, O. piceae or other possible species involved in the generation of the aggressive subgroup. The range of n DNA polymorphisms exhibited by H666 (Fig. 6.43) make it a particularly interesting isolate for further research.

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