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Characterisation of the European pathogen population of *Magnaporthe grisea* by DNA fingerprinting and pathotype analysis

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Summary

The genetic variability among 41 isolates of the blast pathogen (*Magnaporthe grisea*) from five European rice growing countries was studied. The genealogy of the isolates was investigated by DNA fingerprinting and the results compared to the degree of similarity for (a)virulence factors. Fingerprinting grouped the isolates into five discrete lineages, that typically showed less than 65% band similarity. Within each lineage, two or more haplotypes were detected with a band similarity of 80% or higher. Each lineage showed a characteristic virulence pattern. All isolates of lineage 'E5' belonged to the same pathotype. The other lineages were composed of clusters of closely related pathotypes that showed variation for virulence to cultivars with certain known resistance genes, while remaining invariably (a)virulent to others. In most cases, lineage classification of an isolate could be easily inferred by its pathotype. Certain resistance genes and certain lineage-excluding resistance gene combinations appear to provide protection against all of the virulence factors sampled.

Introduction

Blast disease of rice, caused by the fungal pathogen *Magnaporthe grisea* (*Pyricularia oryzae*) is endemic to all European rice growing areas. The majority of these areas have a Mediterranean climate, characterised by hot and dry weather conditions for most part of the growing season, and unfavourable for blast disease development (CIHEAM, 1996). Leaf blast epidemics are rare, although nearly all commercially grown European rice cultivars are susceptible to the disease. Towards the end of the season, conditions become more favourable, with a higher risk of prolonged periods of humid weather. Also, dew periods grow longer as the days grow shorter. Panicle infection is therefore much more common, sometimes causing economic loss. In Italy, by far the most important European rice growing country, preventive spraying with fungicides is widely practised.

The use of fungicides could be reduced by breeding blast resistant rice cultivars that are adapted to European growing conditions. A number of major genes conferring a high level of resistance to rice blast are known (McCouch et al., 1994), but most sources of resistance genes lack important characteristics needed for successful growth under European field conditions, notably for cold tolerance and growth duration. Moreover, successful introduction of a major gene does not guarantee a lasting resistance. In other temperate regions, such as Japan and Egypt, many cases of a rapid breakdown of the resistance in newly released cultivars have been documented (Ezuka, 1979; Horino et al., 1990). Considering the effort that is required to incorporate the resistance into a cultivar with acceptable agronomic performance, rationalising the choice of genes in resistance breeding efforts is needed.

Recent studies done elsewhere in the world show that blast pathogen populations typically contain a number of closely related, 'clonal' isolate groups called lineages, that each have a restricted virulence pattern (Levy et al., 1993; Correa-Victoria et al., 1994). More importantly, the data indicate that an entire

lineage may be invariably avirulent to cultivars with certain resistance genes. Zeigler et al. (1994) proposed to exploit this phenomenon in resistance breeding: durable resistance might be obtained by combining those resistance genes that together confer resistance to all the lineages in the pathogen population. A combined lineage and pathotype assessment of isolates from different European countries might help us identify such resistance genes for use in European rice breeding programs. This paper presents the first study of genetic variability of the blast pathogen in Europe.

Materials and methods

Blast isolates. Isolates were collected in different years and from various rice cultivars in five European countries. The date of collection, the host cultivar, and the geographic origin for each isolate are shown in Table 1. Isolates were cultured from a single conidiospore. Cultures were grown in Petri dishes on rice polish agar medium. Growth and sporulation of the pathogen was obtained by placing the Petri dishes at 27 °C under fluorescent light for eight days. For medium and long term storage, cultures were grown on the same medium covered with a filter paper (Valent et al., 1986). Filter papers with sporulating cultures were removed, dried, and vacuum sealed before storage at –20 °C.

Rice cultivars; experimental lay-out. To detect pathogenic variants, the isolates were inoculated to a set of 22 cultivars (Table 2), including several well characterised Japanese blast differentials (Yamada et al., 1976; Kiyosawa, 1978) at CIRAD, Montpellier, France. Preliminary tests with some of the Japanese differential isolates confirmed the differential reactions of the Japanese cultivars under our experimental conditions (Yamasaki and Kiyosawa, 1966). The cultivar 'Maratelli' was included as a susceptible check. The plants were grown in a greenhouse in plastic trays (45 × 30 × 7 cm) filled with a peat soil (Neuhaus no. 2). For each isolate, three trays were prepared. Per tray, 7 or 8 cultivars were sown in a single row of 15–20 plants each. Per trial, cultivars were randomly assigned to rows, keeping the same sort order of cultivars across trays for the different isolates. Ample nitrogen fertilizer was applied as described by Roumen (1992). Each isolate-host cultivar combination was assessed in two separate trials, carried out with at least a few weeks interval.

Table 1. Origin, year of collection, and host cultivar of European rice blast pathogen isolates (FR = France, HN = Hungary, IT = Italy, PR = Portugal, SP = Spain)

Isolate	Location	Year	Host-cultivar
FR1	Camargue	80	Delta
FR3	Camargue, Mas Combe	86	Smeraldo
FR5	Camargue, Mas Combe	86	Smeraldo
FR9	Camargue	86	unknown
FR10	Camargue, Domaine Gr. Badon	86	Belgioso
FR13	Camargue	88	Rocca
FR26	Camargue, Grand Romieu	90	Lido
FR27	Camargue, Mas Rousset	91	Thaibonnet (L202)
FR28	Camargue, Mas Rousset	91	Lido
FR32	Camargue, Mas Jasses	92	Koral
FR41	Camargue, Mas Adrien	94	Sariceltik
HN1	Szarvas	93	öki-2
HN2	Szarvas	93	öki-4
HN3	Szarvas	94	Ringola or Sandora
HN4	Szarvas	94	Ringola or Sandora
HN5	Szarvas	94	Ringola or Sandora
IT2	Vercelli	86	Vernia
IT3	unknown	86	Thaibonnet
IT6	Veneria	89	Ariete
IT10	Veneria	89	IAC 164
IT11	Castelmerlino	89	Lido
IT14	Sapise	89	Ariete
IT16	Veneria	90	unknown
IT20	Veneria	90	Thaibonnet (L202)
IT21	Rovasenda	90	Icario
IT22	Castello d'Agogna	91	Balilla
PR2	Mondego Quinta do Cal.	90	Pritz
PR3	Mondego Quinta do Cal.	90	Koral
PR13	Tejo Coruche Expl..	91	Thaibonnet (L202)
PR14	Tejo Coruche Expl..	91	Lido
PR16	Sado Alcacer Monte de Pedra	91	Onda
PR61	Mondego Ereira	91	Ringo
PR71	Tejo Coruche Romeiran	92	Koral
PR72	Tejo Coruche dos Coe.	92	Koral
PR76	Guadiana Herd. Alfaro	92	Thaibonnet (L202)
SP1	Valencia region	86	unknown
SP2	Delta de Ebro, Balada	94	Thaibonnet (L202)
SP3	Delta de Ebro, Partida Carlet	94	Bahia
SP4	Delta de Ebro, Salats	94	Bahia
SP5	Delta de Ebro, Salats	94	Lido
SP6	Aragon	94	Huesca

Table 2. Geographic origin, seed source, and reported resistance genes of differential rice cultivars used for pathotype analysis of European isolates of the rice blast pathogen

Cultivar	Origin	Seed Source	Resistance gene(s)
Aichi-asahi	Japan	NARC Tsukuba	*Pi-a*
Fujisaka-5	Japan	CIRAD	*Pi-i Pi-k^s*
Fukunishiki	Japan	CIRAD	*Pi-z Pi-sh*
Kusabue	Japan	CIRAD	*Pi-k Pi-sh*
Shin-2	Japan	CIRAD	*Pi-k^s Pi-sh*
BL-1	Japan	CIRAD	*Pi-b Pi-sh*
K-59	Japan	NARC Tsukuba	*Pi-t*
K-1	Japan	CIRAD	*Pi-ta*
Pi-no-4	Japan	CIRAD	*Pi-ta^2 Pi-sh*
Norin-22	Japan	NARC Tsukuba	*Pi-sh*
ST-1	Japan	NARC Tsukuba	*Pi-f*
Kanto-51	Japan	NARC Tsukuba	*Pi-k*
Nipponbare	Japan	NARC Tsukuba	*Pi-sh*
K-60	Japan	CIRAD	*Pi-k^p*
Reiho	Japan	NARC Tsukuba	*Pi-a Pi-ta^2*
Rico-1	USA	Beaumont Texas	unknown
Nato	USA	Beaumont Texas	unknown
Lido	Italy	CFR Arles	unknown
Thaibonnet (= L-202)	USA	CFR Arles	unknown
Gigante Vercelli	Italy	CFR Arles	unknown
Estrella	Portugal	CFR Arles	unknown
Maratelli	Italy	CIRAD	susceptible check

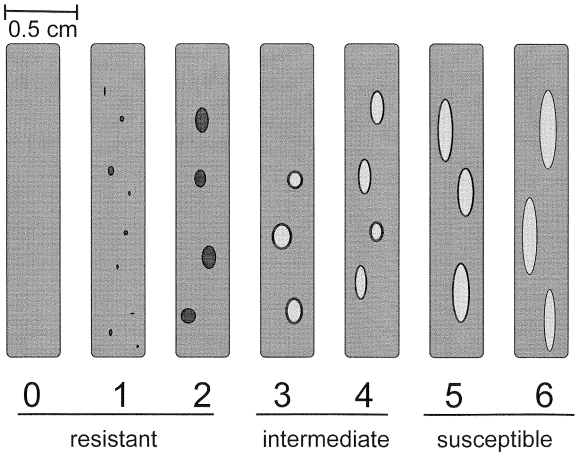


Figure 1. Schematic representation of the lesion type scale for the assessment of symptoms induced by the blast pathogen on rice leaves. 0–2: dark, resistant type lesions without sporulation. 3–5: sporulating type lesions with grey centre and a dark margin. 6: susceptible sporulating lesion type without dark margin.

Inoculation; scoring of symptoms. To obtain conidiospores for inoculations, pieces of colonized filter paper were taken out of storage and revived in Petri dishes containing rice polish agar. Spores were harvested as described by Bonman et al. (1986). Plants were inoculated when they had grown 5 leaves on the main culm. The trays were placed on a rotating table in a cage, and 30 ml of a suspension containing 50,000 spores ml^{-1} and 0.5% gelatine was sprayed to each tray of plants using an artist's paintbrush powered by compressed air. Spores that missed the plants were captured by air suction on a filter in the bottom of the cage, preventing cross-contamination. Following inoculation, the plants were kept in the dark inside a phytotron at 25 °C and 100% humidity for 16 h, and returned to the greenhouse the next day.

The infection type (IT) was scored six days after inoculation using a scale with 7 lesion type categories (Figure 1). The most common as well as the most susceptible lesion type that developed was noted for each isolate-cultivar combination using the cultivar rows as experimental unit. The IT was considered susceptible if the majority of the lesions that developed was type 5 or 6. Where no sporulating type lesions developed, the IT was judged as resistant. Any other combination of lesion types was categorized as an intermediate IT. Cultivars that did not show any differential reaction were dropped from the data matrix and data of cultivars that showed the same pattern to all isolates were combined in a single unit. The data were subsequently analysed by hierarchical cluster analysis using the average linkage method with the statistical software package SYSTAT version 3 (Wilkinson, 1986). Distances between isolates were calculated as the percentage disagreements using the three IT categories as input. Using this method, the calculated distance for pairs of isolates ranges from 0% in case of identical scores, to 100% if the IT falls into a different category for each of the differential cultivars.

Assessment by molecular markers. DNA fingerprinting of isolates was done at Purdue University, Indiana, USA, using the probe 'MGR586'. This probe corresponds to a repeated DNA sequence in the pathogen that is dispersed randomly through the genome, is not related to fitness, and typically produces a profile of 50 to 80 *Eco*RI bands (RFLPs) per isolate, making it highly suitable for fingerprinting purposes (Hamer et al., 1989; Levy et al., 1991). DNA was prepared from cultures grown in 2YEG (Valent et al., 1986), digested with *Eco*RI, fractioned on 0.8% agarose gels, and transferred to Hybond-N hybridization membranes (Amersham International) following the manufacturer's instructions. Blots were hybridized with the radioactively labelled probe 'MGR-586', then washed at 65 C in 0.1% SDS, 0.1% PPi, 0.2 × SSPE. The band pattern of isolates was visualized with auto radiography, and the degree of relatedness among the isolates was calculated according to the proportion of shared RFLPs for all pair-wise comparisons. UPGMA cluster analysis of isolate relatedness values sorted the isolates into groups that were portrayed in a UPGMA phenogram (Levy et al., 1993). The membership of each group was defined as containing all isolates whose inclusive average similarity was significantly greater than their average similarity to the next most similar isolate or group of isolates. The only exception to this definition was that all isolates with 90% or greater average similarity were assigned to the same group, to avoid defining every clonally related pair of haplotypes as a separate group. Each such UPGMA-defined group of isolates was considered as a separate genetic lineage (Levy et al., 1993).

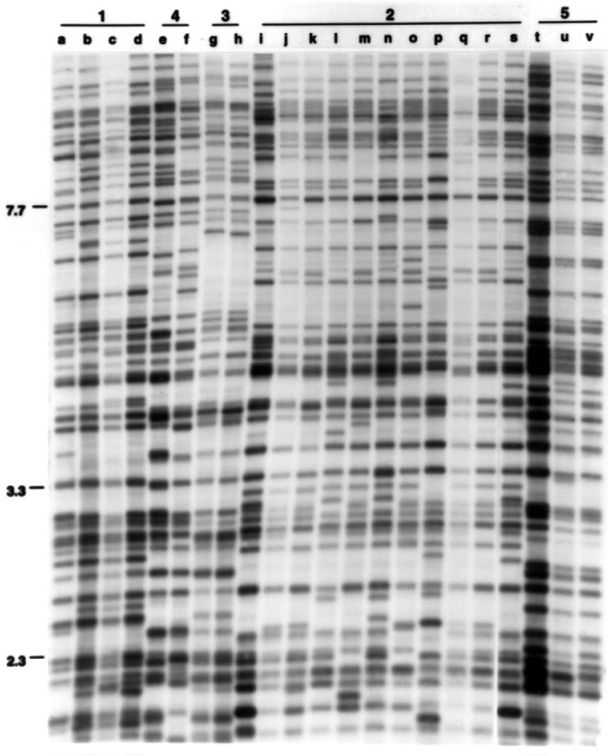


Figure 2. MGR586-DNA fingerprints of European rice blast fungus isolates. Marginal numbers indicate scale of fragment sizes in kilobases. Numeric column headings indicate lineage associations of the isolates that are loaded as follows: a) SP1, b) FR13, c) FR41, d) IT10, e) SP3, f) SP4, g) HN1, h) HN3, i) IT6, j) SP2, k) FR1, l) IT14, m) PR2, n) FR10, o) PR71, p) FR9, q) PR61, r) PR16, s) PR76, t) IT3, u) PR14 and v) IT20.

Results

MGR-DNA fingerprints revealed the presence of five lineages among the 41 isolates tested (Figure 2). The degree of band similarity among lineages was in the 55 to 65% range. Within lineages, similarity was 80% or higher (Figure 3). If we consider isolates that share more than 95% of the bands as a single haplotype, the number of haplotypes detected was 4, 13, 2, 2, and 3 for lineages 'E1' to 'E5', respectively. Lineage 'E2' seemed the most widespread and was detected in France, Italy, Portugal and Spain. Lineage 'E1' was found in France, Italy and Spain, while lineage 'E5' was detected in France, Italy and Portugal. Lineages 'E3' and 'E4' were found only in Hungary and the Spanish Delta de Ebro region, respectively. Comparison of the European fingerprints with those of other isolates fingerprinted at Purdue covering a total of 159 lineages showed that the European lineages are different from those so far detected in the Americas and Asia: all MGR-DNA lineages detected in Europe thus far express less than 50% similarity to any lineage observed on other continents (Levy, unpublished results).

The infection type (IT) scores of each cultivar-isolate combination were consistent between the two trials. The largest degree of fluctuation of symptoms across trials was observed for cultivar-isolate combinations with an intermediate IT: for example, a cultivar that showed a mixture of type 3 and 5 lesions in the first trial could just show type 3 lesions in the second, or vice versa. Isolate 'FR9' failed to produce a susceptible IT on any of the cultivars tested, including the susceptible check 'Maratelli'. 'FR9' also showed a markedly reduced growth and sporulation in Petri

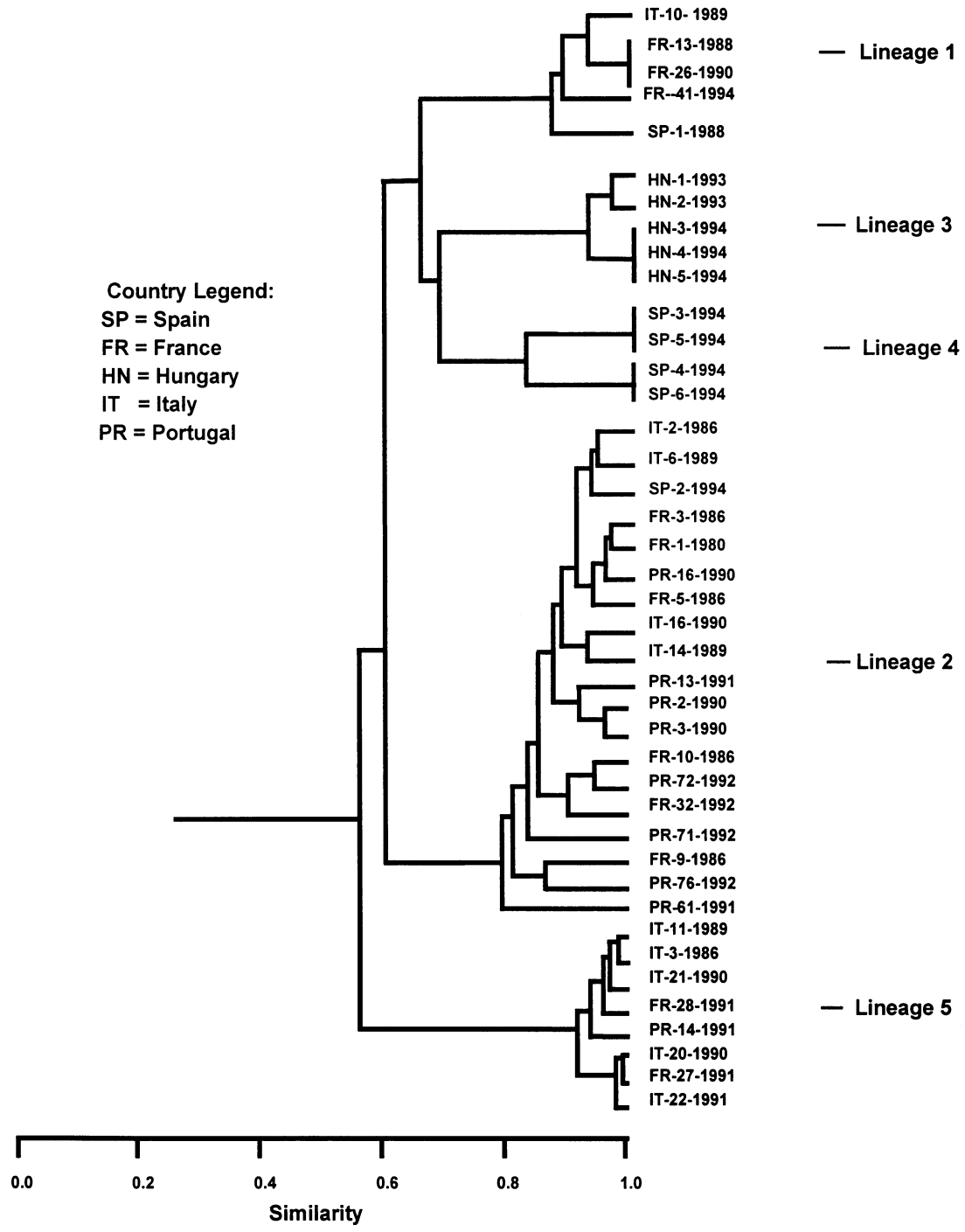


Figure 3. Rice blast pathogen lineage diversity in Europe. UPGMA phenogram of MGR586-fingerprint variation. The country and year of isolate collection is indicated.

dish culture, and may have lost some gene(s) required for normal development and infection. The susceptible check 'Maratelli' produced a susceptible IT (type 6 lesions) with all other isolates. Among the other differentials used, 'Reiho' (*Pi-a*, *Pi-ta*^{2}), 'Pi-no-4' (*Pi-sh*, *Pi-ta*^{2}), 'Bl-1' (*Pi-b*, *Pi-sh*), invariably showed complete resistance without symptom development. While 'Fukunishiki' (*Pi-sh*, *Pi-z*) also remained symptomless to most isolates, type 2 flecks developed with some isolates of lineage 'E1'. Completely resistant to all isolates were also the European cultivars 'Gigante Vercelli' and 'Estrella', but with the former typically developing many pin-point type 1 lesions and the latter type 2 flecks. All other cultivars showed clear differential reactions (Table 3). A susceptible IT was detected, at least once, for cultivars containing the resistance genes *Pi-a*, *Pi-f*, *Pi-i*, *Pi-k*, *Pi-k*^{p}, *Pi-k*^{s}, *Pi-sh*, *Pi-t*, and *Pi-ta*. The reaction patterns of the Japanese cultivars 'Nipponbare', 'Norin-22', 'Shin-2' and the US cultivar 'Rico-1' were highly similar for all isolates tested. The reaction pattern for cultivar 'Lido' was the same as that for 'Maratelli', whereas 'Kusabue' had the same pattern as 'Kanto-51'. In one of the tests, a single type 5 lesion developed on the cultivar 'Kusabue' after exposure to isolate 'FR13'. The isolate 'FR13-1' obtained from this lesion lesion had an MGR-DNA fingerprint identical to 'FR13' (indicating that it was not a simple contaminant) and behaved exactly like 'FR13', except for a markedly increased virulence to the cultivars 'Kusabue', and 'Kanto-51'. Both cultivars possess the *Pi-k* resistance gene.

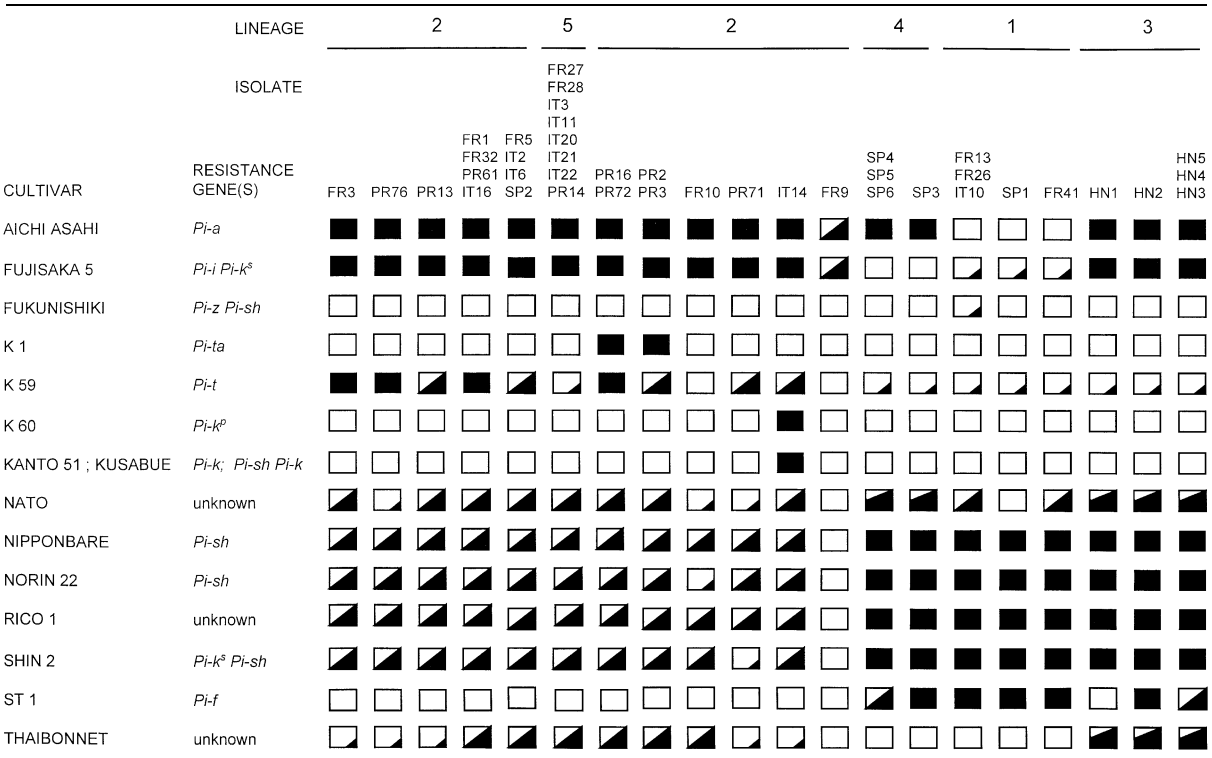
A single pathotype was detected in lineage 'E5'. The other lineages contained two or more pathotypes that were closely related, showing variation for virulence to cultivars with certain host-resistance genes while remaining invariably avirulent to others. Lineages that were present in more regions did not show marked differences of pathotype as a function of the country of origin, although it was noted that all lineage 'E2' isolates that are virulent to cultivar 'K-1' (*Pi-ta*) were from Portugal (Table 3). Cluster analysis of pathotypes revealed a tight relation between the lineage of an isolate and its virulence pattern (Figure 4). In most cases, the lineage of an isolate could be easily inferred by its pathotype. Isolates of the same lineage grouped together into distinct pathotype clusters for the lineages 'E1', 'E3' and 'E4'. However, the pathotype of lineage 'E5' isolates was included among isolates of lineage 'E2' (Figure 4, Table 3). These lineages could best be separated by their reaction on cultivar 'K-59'. On this cultivar, lineage 'E5' isolates induced a typical resistant, brown spindle shaped lesion type, whereas 'E2' isolates did not. The same typical lesion type, described as *Pi-t* related and called 'halo-type' by Kiyosawa (1972), was also observed for the lineages 'E1', 'E3' and 'E4'.

Discussion

In Europe, rice cultivation started less than 100 years ago in most areas and, compared to rice growing countries elsewhere, the total surface planted to rice is small. The most important production occurs in the Po valley of Italy with about 220,000 ha grown in 1995. The acreage planted in France, Portugal and Spain range between 20,000 and 50,000 ha each. Rice cultivation in Hungary plunged after adopting a market economy and was only about 5,000 to 6,000 ha in 1994 and 1995. Only one crop is grown per year and, with the exception of Hungary, prevailing weather is usually unfavourable for blast development until late in the growing season. The blast pathogen population is, thus, likely to go through a large bottleneck for prolonged periods, which favours loss of genetic variability. With five lineages present, the degree of lineage diversity in Europe is similar to that found in the USA, where rice cultivation also has a short history and where 8 lineages were detected by Levy et al. (1991). A much higher lineage diversity (more than 30 lineages) has been reported for tropical countries with a long history of rice cultivation on much larger acreage, such as the Philippines or India (Chen, 1993; Zeigler et al., 1995; Sivaraj, 1995).

Despite the relatively small sample size of isolates that were analyzed, we do not expect to find many additional lineages in the European countries studied, even if sampling is increased substantially. In general, the same lineages were detected in different years, from different cultivars, and 3 of the lineages were detected across different countries. On the other hand, the result that lineage 'E3' was only detected in Hungary while lineage 'E4' was found only in the Delta de Ebro region, may indicate some effective geographic barriers between regions. The best chance to find additional lineages may be in Spain. The isolates from the Delta de Ebro region were all sampled in the same year, while the Valencia rice growing area of this country was represented only by a single isolate (Table 1). No isolates were obtained from the Extramedura region near Sevilla, because practically no rice has been grown in this area in recent years due to prolonged drought.

Table 3. Differential reactions observed between rice cultivars and European isolates of the blast pathogen. Solid black boxes indicate a fully susceptible IT (lesion type 5–6). Boxes that are half or three quarters black indicate an intermediate IT (lesion type 3, 4 with a minor amount of type 5 possible). Boxes with a small black right corner indicate a resistant IT with type 2 lesions. Uncoloured boxes indicate a resistant IT without symptoms or with type 1 lesions. Results for cultivars that were invariably resistant (Bl-1, Pi-no-4, Reiho, Gigante-vercelli, Estrella) or susceptible (Maratelli, Lido) are not included



Adding further support to population studies done in rice growing countries elsewhere (Levy et al., 1991 and 1993; Correa-Victoria et al., 1994; Zeigler et al., 1995), the present study indicates lineages to have a restricted virulence spectrum. For example, the cultivar 'Aichi-asahi' was completely resistant to all isolates of lineage 'E1' regardless of origin (Spain, France, Italy). And none of the isolates belonging to lineages 'E2' or 'E5' could completely overcome the resistance present in the cultivars 'Nipponbare' or 'Shin-2', although they could produce sporulating lesions (Table 3). Interestingly, Latterell et al. (1965), who compared isolates from around the world in the early 1960's, reported the presence of three pathotypes in Italy with a virulence spectrum fitting that of lineages we detected at present. Two of these pathotypes were fully virulent to 'Aichi-asahi' (*Pi-a*) and 'Ishikari-shiroke' (*Pi-i*), while inducing an intermediate IT on cultivar 'Norin-22' (*Pi-sh*), which is typical of isolates belonging to the lineages 'E2' or 'E5'. The third pathotype was fully virulent to 'Norin-22', but completely avirulent to 'Aichi-asahi' and 'Ishikari-shiroke', which corresponds with a typical lineage 'E1' pathotype in out study. These data suggest that the same lineages may have been present in Italy for over more than 30 years with little change in virulence.

In general, the reaction patterns of the Japanese cultivars could be perfectly explained by their attributed major genes (Table 2). However, a difference was noted for cultivar 'ST-1'. Yunoki et al. (1970) reported that the Pi-f gene in this cultivar induced symptoms resembling partial resistance to many of the Japanese isolates tested. Although the reaction pattern of 'ST-1' to the European isolates confirms that this cultivar has a distinct resistance gene among these differentials (Table 3), its expression, a marked reduc-

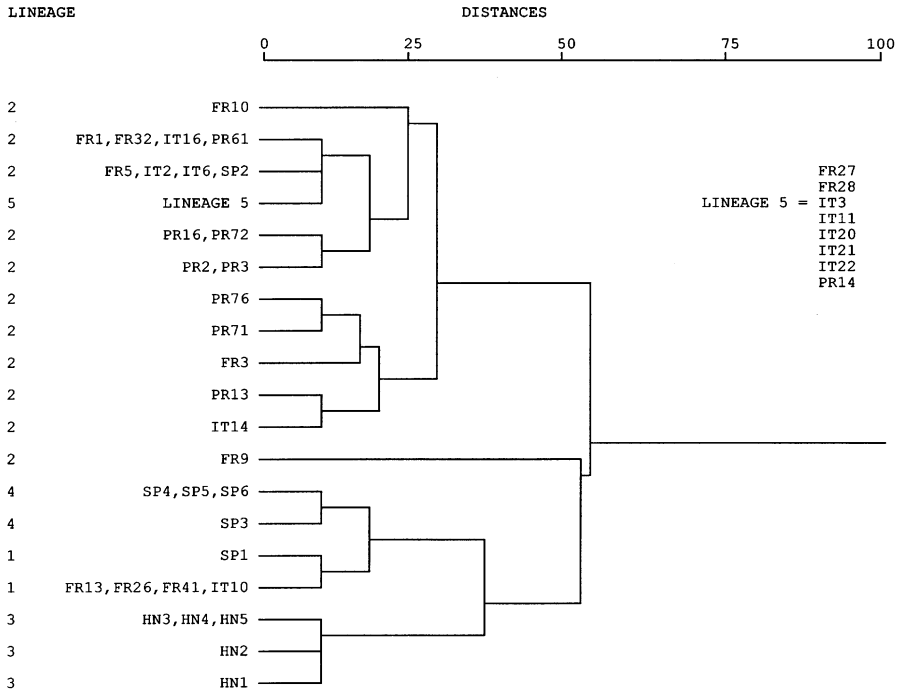


Figure 4. Rice blast pathogen pathotype diversity in Europe visualized by cluster analysis (Normalized percent disagreement; average linking method). The lineage is indicated in the left margin.

tion of IT, resembled race-specific resistance conferred by a typical major gene and not that of partial resistance.

The highly similar spectrum of resistance of the Japanese cultivars 'Shin-2', 'Norin-22', 'Nipponbare' and the US cultivar 'Rico-1' suggests that these cultivars have at least one resistance gene in common. A likely candidate is the Pi-sh gene, which was reported to be present in the 3 Japanese cultivars by Imbe and Matsumoto (1985). The characteristic symptoms of the European cultivars 'Gigante-Vercelli' and 'Estrella' suggests they contain major genes that are not yet identified. Such genes are probably also present in cultivars 'Nato' and 'Thaibonnet', which each showed a characteristic, lineage-related, pattern of intermediate and highly resistant reactions.

Although the present study is supportive of the hypothesis that lineages have a restricted virulence spectrum, the small sample size of most lineages does not allow for reliable prediction of the resistance genes that are more likely to confer durable resistance when used in breeding programs. Based on the definition of durable resistance by Johnson (1984), the best candidates are those resistance genes that continue to exclude lineages despite having been widely exposed to the population where their use is intended. Among the cultivars showing resistance genes of possible interest for use in Europe, this exposure has probably been most severe for the European cultivars 'Gigante Vercelli' and 'Estrella'. As donors of resistance, these cultivars have the additional advantage of being already agronomically adapted to the regional growing conditions. Other resistance genes of possible interest are *Pi-b*, *Pi-z* and *Pi-ta*^{2}, since the differentials carrying these genes showed complete resistance to all isolates tested regardless of lineage. Combinations of *Pi-f* with *Pi-ta* or *Pi-k* also appear to exclude all lineages. Prolonged exposure to high quantities of inoculum representing each of the lineages could perhaps be used to assess the risk of a rapid adaptation of the European pathogen population to specific resistance genes and resistance gene combinations.

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