PHENOTYPIC AND GENOTYPIC CHARACTERISATION OF ESTONIAN ISOLATES OF *PHYTOPHTHORA INFESTANS* IN 2004-2007

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SUMMARY

A collection of 432 single-lesion isolates of *Phytophthora infestans* collected from blighted potato foliage during 2004-2007 in Estonia, were analyzed for virulence (all isolates), mating type (424 isolates) and response to metalaxyl (412 isolates). The samples came from 25 fields comprising conventional production in central, northern, southern, south-eastern and south-western regions and from untreated experimental field trials at Jõgeva Plant Breeding Institute in eastern Estonia. Of the isolates 33% were A2 mating type. Both mating types were present in all fields; the frequency of A2 mating type varied from 3% to 71%. In the context of specific virulence, the Estonian population had a very low frequency of virulence against R5 (17%) and R9 (3%). The most common pathotype was 1.3.4.7.10.11. A subgroup of 57 isolates was assessed for mtDNA haplotype and RG57 fingerprints. Three mitochondrial DNA haplotypes, i.e. Ia (51%), IIa (42%) and IIb (7%), were found. Twenty-one RG57 fingerprints were detected. The four most common fingerprints represented more than half of the isolates (67%). On the basis of combined markers, thirty-three multilocus genotypes were identified, of which 81% were detected only once. Genotypic diversity measured by the normalized Shannon diversity index was 0.79. The data indicate that the Estonian population of *P. infestans* is diverse, having a large number of multilocus genotypes and both mating types within fields, with potential for sexual recombination and spread of fungicide resistance.

Key words: mating type, metalaxyl resistance, mitochondrial DNA haplotype, *Phytophtora infestans*, RG57 fingerprinting, virulence.

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INTRODUCTION

Phytophthora infestans (Mont.) de Bary is one of the most serious and economically important pathogens of potato worldwide. Under favourable conditions it can destroy the whole potato haulm and cause considerable yield loss. In Estonia, the average loss due to late blight can reach 20-25% and in untreated fields even more. Without control of potato late blight is not possible to achieve high high-quality yields. In Estonia, fungicides are used routinely in conventional potato production, but under favourable conditions for the disease, with heavy pathogen pressure, it is difficult to protect large areas.

Late blight is caused by the fungus-like oomycete *P. infestans*, which can reproduce both sexually and asexually. For sexual reproduction, *P. infestans* requires both A1 and A2 mating types to produce gametangia (Fry and Goodwin, 1997). The centre of diversity of this oomycete is in the highlands of Mexico (Fry and Goodwin, 1997), where both mating types have always been present. The population is especially virulent and remarkably diverse for neutral genetic markers (Fry *et al.*, 1993). Previous studies have shown that the population in Mexico is highly sub-structured (Fry *et al.*, 1992). At least two different migration events have occurred from Mexico. The first is postulated to have occurred before 1845, after which time *P. infestans* swept through Europe and Ireland resulting in the death of over one million people due to starvation, and emigration of 1.5 million people to other parts of Europe or North America (Drenth *et al.*, 1994). The second migration occurred in the 1970s to 1990s, bringing the A2 mating type out of Mexico and also containing genetically diverse and aggressive strains (Fry *et al.*, 1992; Fry *et al.*, 1993). Analyses of allozyme markers (Tooley *et al.*, 1985; Shattock *et al.*, 1986) and DNA fingerprints (Goodwin *et al.*, 1992a, 1992b; Drenth *et al.*, 1993) of isolates from a number of locations in the world supported the notion that the aforementioned changes resulted from displacement of an 'old' world-wide clonal lineage (US-1) by a new population (Spielman *et al.*, 1991; Fry *et al.*, 1993; Drenth *et al.*, 1994). The 'new genotypes' also proved to be generally more aggressive than the old clonal lineage (Carlisle

et al., 2002; Shattock, 2002). In Estonia, the A2 mating type was detected for the first time in 1987 (Vorobyeva *et al.*, 1991).

The control of potato late blight is massively dependent on the use of fungicides (Goodwin *et al.*, 1996). Resistance to metalaxyl was first recorded in *P. infestans* in Ireland and The Netherlands in 1980 (Davidse *et al.*, 1981; Dowley and O'Sullivan, 1981). Although the old population was largely sensitive to phenylamides, phenylamide-resistant isolates belonging to the old population have been reported (Goodwin *et al.*, 1996; McLeod *et al.*, 2001). The proportion of metalaxyl-resistant isolates fluctuates from year to year and within season (Gisi and Cohen, 1996). In the 1990s, resistance levels remained more or less stable in all European countries (Gisi and Cohen, 1996).

A previous study of genotypic and phenotypic diversity of *P. infestans* collected from Estonia in 2002 and 2003 (Runno-Paurson *et al.,* 2009) showed that all isolates in that collection belonged to the 'new population' (as identified via allozyme genotypes, mtDNA haplotypes and mating type). The results indicated that Estonian population of *P. infestans* is diverse, with potential for sexual recombination. Whereas in the previous study isolates were collected only from three regions (central, eastern and southern), more regions were included in the present study in order to provide more detail on the Estonian population of *P. infestans*.

The main objective of this study was to characterize the population of *P. infestans* in Estonia for metalaxyl sensitivity, virulence, mating type, RFLP fingerprint and mtDNA haplotype, to find answers to the following questions: (i) does the mating type ratio in Estonia suggest occurrence of sexual reproduction?; (ii) is phenotypic and genotypic variation at the same level as in other European countries as indicated by virulence and DNA-fingerprints?; (iii) are there any indications of oospore-derived epidemics? In addition, the impacts of time and regions were studied.

MATERIALS AND METHODS

Collection of isolates. Potato leaves infected by *P. infestans* were collected from Estonia during the period 2004-2007 from 25 sites (small-scale conventional, large-scale conventional productions and untreated experimental field plots) in six regions of Estonia (Table 1). Conventional producers were divided into two groups. On the small scale, farmers used seed potatoes of uncertain quality and did not follow the rotation rules. Chemical late blight treatments were applied occasionally, varying from no sprays to 1-4 sprays. On the large scale, farmers used high-quality certified seed potatoes, planted potatoes no more frequently than every 3rd year (with some exceptions) and applied fungicide 6-7 times per

Table 1. Origin and characteristics of *Phytophthora infestans* isolates collected from Estonia (2004-2007).

Region	Year	Number of sites	Number of isolates	Number of isolates tested for				
				Mating type	Metalaxyl resistance	Virulences	mtDNA haplotype	RG57 fingerprints
Northern	2004	1	17	17	17	17	4	4
	2005	1	10	10	10	10	5	4
Central	2004	1	22	22	20	22	4	4
	2005	1	25	23	23	25	5	5
	2007	1	19	15	15	19	0	0
Eastern	2004	1	19	18	18	19	4	4
	2005	1	18	17	17	18	5	5
	2006	2	45	45	44	45	0	0
	2007	1	23	21	21	23	0	0
South-eastern	2004	1	24	24	20	24	4	6
	2005	3	50	50	49	50	13	15
	2006	3	46	46	44	46	0	0
	2007	2	31	28	28	31	0	0
South-western	2004	1	13	14	13	13	4	4
	2006	1	13	13	13	13	0	0
	2007	1	16	16	16	16	0	0
Southern	2004	3	41	45	44	41	11	8
Total		25	432	424	412	432	59	59

Table 2. Metalaxyl resistance[a] among isolates of *Phytophthora infestans* in Estonia (2004-2007).

	Percentage of isolates			Isolates tested (n)
Year	S (%)	I (%)	R (%)	
2004	23.5	20.5	56.1	132
2005	15.2	41.4	43.4	99
2006	45.5	30.7	23.8	101
2007	78.8	6.3	15.0	80
Total	37.6	25.2	37.1	412

[a]S, metalaxyl-sensitive; I, intermediate metalaxyl-sensitive; R, metalaxyl-resistant.

season, while sometimes the fungicide was applied as often as 11 times/season. In experimental plots at Jõgeva Plant Breeding Institute (four sites) diverse cultivars and breeding lines were used, and the quality of seed potatoes was also diverse.

Isolates were collected at the beginning of outbreaks in all years. Ten to thirty blighted leaves, each with a single lesion (one per plant), were collected (Table 1). The plants were selected by randomising the distance from field edges, and from each plant the blighted leaf was also randomly chosen, excluding those that had several or no lesions.

Isolations were carried out as described in Runno-Paurson *et al.* (2009). All phenotypic tests were done in October-November of the year of isolation. Mitochondrial DNA haplotype analyses were done in November-December 2005.

All 432 isolates were tested for virulence; 424 were tested for mating type; 412 were tested for response to metalaxyl. Subgroups of 57 isolates were tested for mtDNA haplotype and for RG57 fingerprints (Table 1).

Phenotypic analyses. The resistance to metalaxyl of all 412 isolates was tested using a modification of the floating leaflet method (Hermansen *et al.,* 2000) as described in Runno-Paurson *et al.* (2009). The specific virulence of each isolate was determined by using Black's differential set of potato genotypes containing resistance genes R1-R11 (Malcolmson and Black, 1966) (provided by the Scottish Agricultural Science Agency). Laboratory procedures were as described in Runno-Paurson *et al.* (2009).

Mating types were determined according to Runno-Paurson *et al.* (2009). Tester isolates were the same as those described in Lehtinen *et al.* (2007).

Neutral marker assessment. A subset of 57 isolates was RFLP fingerprinted using Goodwin *et al.* (1992a) technique, as described by Runno-Paurson *et al.* (2009). The DNA fingerprints of the Estonian isolates were determined by comparing their patterns with those of three reference isolates (belonging to the US-1, US-8 and US-17 clonal lineages).

The mitochondrial DNA (mtDNA) haplotype of each of the 57 isolates in the subset was determined using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), a variation of the method by Griffith and Shaw (1998) as described by Runno-Paurson *et al.* (2009).

Data analysis. The isolates were described as a multilocus genotype consisting of DNA fingerprint bands, mtDNA haplotype and mating type. Genotypic diversity as well as race diversity was calculated with the normalized Shannon diversity index (Sheldon, 1969): $Hs=- \Sigma g_i \ln g_i / \ln N$, where g_i is the frequency of the ith multilocus genotype and N the sample size. The normalized index ranges from 0 (no diversity) to 1 (each isolate represents a unique genotype). Polymorphic bands of the RG57 fingerprints were scored as present (1) or absent (0).

Statistical analyses were performed with the SAS/STAT version 9.1 (SAS Institute, Cary, USA). Differences in the prevalence of the two mating types of *P. infestans* isolates between study sites and years were tested using a logistic analysis (GENMOD procedure in SAS) with a multinomial response variable (A1, A2, or both). Analogous logistic procedures were used to examine the differences in the resistance to metalaxyl (a multinomial response variable: resistant, intermediate or sensitive) between sites and years, and also between different haplotypes. Logistic analyses were also used to test for associations between each of the polymorphic RG57 bands and metalaxyl sensitivity, mating type and mtDNA haplotype. The dependence of specific virulence (percent of isolates that show virulence against particular R-genes) on years, sites, field types and R-genes was analyzed with type III ANOVA. "Site" was treated as a random variable and nested within "field type". Tukey HSD post-hoc tests ($\langle=0.05$) were applied to find specific differences between sites and R-genes. In all analyses, "year" was treated as a categorical variable. See Runno-Paurson *et al.* (2009) for more details.

RESULTS

Resistance to metalaxyl. In total, 412 isolates were screened for resistance to metalaxyl. Over the four years,

37% of isolates were resistant to metalaxyl, 25% were intermediate and 37% were classified as sensitive. Of the metalaxyl-resistant strains, 37% were A1 mating type, 40% were A2 mating type and 23% were self-fertile.

The proportion of metalaxyl-resistant isolates differed between sites ($\chi^2 = 42.20$, df = 11, $p < 0.001$) and years (($\chi^2 = 61.57$, df = 3, $p < 0.001$). The proportion of metalaxyl-resistant isolates ranged from 0 to 81% depending on site. In 2004, 56% of all isolates were resistant to metalaxyl, whereas in 2007, most isolates (Table 2) were sensitive to metalaxyl.

Metalaxyl-resistant strains were absent from only three fields (Enge 2007, Naha 2005 and Võnnu 2006 - where metalaxyl fungicide was not used). Significant differences between potato cropping systems were not observed ($\chi^2 = 0.05$, df = 1, $p = 0.82$). There was a strong association between metalaxyl resistance and sites where metalaxyl-containing fungicides had been applied ($\chi^2 = 9.24$, df = 1, $p = 0.0024$). Over four years, from crops known to have been sprayed with fungicide containing metalaxyl, 48% of isolates were resistant, while isolates collected from potatoes which had not been metalaxyl-treated, only 33% of isolates were resistant (Table 3).

Virulence. All known virulence factors (to overcome genes R1-R11) were found in this collection. We found significant differences in the frequencies of specific virulences among sites and years ($F_8 = 9.41$, $p < 0.001$) and virulences to specific R-genes also differed ($F_{10} = 234.31$, $p < 0.001$). No main effect of cropping systems was detected. Almost all isolates were virulent on differentials with genes R1, R3, R4, R7, R10 and R11. Least frequent was virulence against R9 (3%). Virulence against R5 was also infrequent (17%) (Fig. 1). Most of the isolates (>96%) were able to overcome four or more R-genes. Eighty-seven different pathotypes were detected (Table 4). The three most common pathotypes made up 39% (Table 4) of isolates in the sample. More than half of the races (>65%) were detected only once. The virulence complexity (average number of R-genes overcome) ranged from 4.8 for isolates from Laheotsa 2004 to 8.9 in isolates from Jõgeva 2005 (Table 5). The most common pathotypes were 1.3.4.7.10.11 and 1.2.3.4.7.10.11 (Table 4). The normalized Shannon diversity index was 0.54.

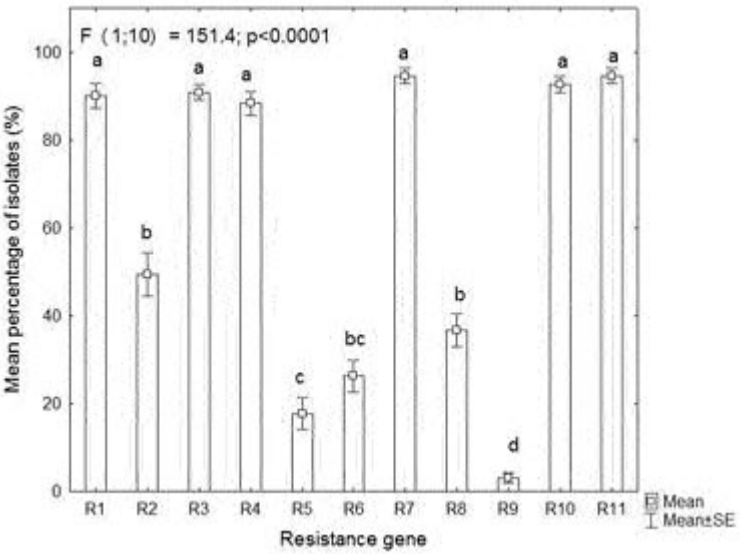

Fig. 1. Frequency of virulence to potato R-genes in the Estonian population of *Phytophthora infestans* in 2004-2007.

Mating type. Of the 424 isolates tested, 64% were of A1 mating type, 33% were A2 mating type and 3% were self-fertile. Both mating types were found at each sample site (25 sites). The frequency of A2 ranged from 3% to 71%. In 2004 and 2007, almost a half (44% and 48% respectively) of the isolates tested were A2 mating type. The proportion of the A2 mating type decreased from 44% in 2004 to 13% in 2006 and increased again to 48% in 2007. There were considerable differences between sites ($\chi^2 = 28.68$, df = 10, $p = 0.0014$) and years ($\chi^2 = 26.28$, df = 3, $p < 0.001$) in the occurrence of the two mating types. The 263 A1 isolates tested included 97 that were resistant to metalaxyl, 72 that were intermediate, and 94 that were sensitive. Among the 134 A2 mating type isolates, 53 were resistant, 28 were interme-

Table 3. Metalaxyl-resisistance[a] among isolates from fields with or without phenylamide fungicide treatments (years 2004-2007) and results of logistic analysis.

	Number (and percentage) of isolates		
Field management	S	I	R
Treated with metalaxyl n=106	30 (28.3)	25 (23.6)	51 (48.1)
Not metalaxyl-treated n=306	125 (40.8)	79 (25.8)	102 (33.3)

	Results of logistic analysis		
	DF	Chi-square	P
	1	9.24	0.0024

[a]S, metalaxyl-sensitive; I, intermediate-metalaxyl sensitive; R, metalaxyl-resistant.

Table 4. Number of isolates of different races among isolates of *Phytophthora infestans* from Estonia (2004-2007).

Race	Number of Virulence factors	Number of isolates	Race	Number of Virulence factors	Number of isolates
1.2.3.4.5.6.7.8.9.10.11	11	6	3.6.7.10.11	5	1
1.2.3.4.5.6.7.9.10.11	10	1	3.7.8.10.11	5	1
1.2.3.4.5.7.8.9.10.11	10	1	1.3.4.10.11	5	2
1.2.3.4.6.7.8.9.10.11	10	4	1.3.4.7.11	5	2
1.2.3.4.5.6.7.8.10.11	10	5	3.4.7.10.11	5	2
1.3.4.5.6.7.8.10.11	9	3	1.4.7.10.11	5	3
1.2.3.4.5.6.7.10.11	9	10	1.3.7.10.11	5	5
1.2.3.4.5.7.8.10.11	9	17	1.3.4.11	4	1
1.2.3.4.6.7.8.10.11	9	26	1.3.7.10	4	1
1.2.3.4.6.8.10.11	8	1	1.3.7.11	4	1
1.3.4.5.6.8.10.11	8	1	1.4.7.10	4	1
1.3.4.5.6.7.10.11	8	3	1.4.7.11	4	1
1.3.4.5.7.8.10.11	8	4	1.7.10.11	4	1
1.3.4.6.7.8.10.11	8	4	1.7.8.11	4	1
1.2.3.4.5.7.10.11	8	11	2.3.4.7	4	1
1.2.3.4.6.7.10.11	8	30	2.4.7.10	4	1
1.2.3.4.7.8.10.11	7	39	2.7.10.11	4	1
1.2.3.5.7.8.11	7	1	3.4.5.7	4	1
1.2.3.7.8.10.11	7	1	3.6.7.11	4	1
1.2.4.6.7.10.11	7	1	4.6.7.11	4	1
1.3.4.7.9.10.11	7	1	6.7.10.11	4	1
1.3.6.7.8.10.11	7	1	3.4.7.10	4	2
2.3.4.7.8.10.11	7	1	1.3.7	3	1
3.4.5.7.10.11	7	1	3.6.7	3	1
1.2.3.6.7.10.11	7	3	4.6.7	3	1
2.3.4.7.10.11	7	3	4.7.10	3	1
1.3.4.5.7.10.11	7	8	1.3.11	3	1
1.3.4.6.7.10.11	7	10	1.7.11	3	1
1.3.4.7.8.10.11	7	34	4.7.11	3	1
1.2.3.4.7.10.11	6	46	6.7.11	3	1
1.2.3.4.7.11	6	1	3.8.11	3	1
1.2.3.6.10.11	6	1	3.10.11	3	1
1.3.4.7.8.11	6	1	1.10.11	3	2
1.3.7.8.10.11	6	1	7.10.11	3	3
1.4.6.7.10.11	6	1	4.7	2	2
1.4.7.8.10.11	6	1	6.10	2	1
2.3.6.7.10.11	6	1	1.11	2	1
1.2.4.7.10.11	6	2	3.11	2	1
1.2.3.7.10.11	6	3	10.11	2	1
1.3.4.7.10.11	5	81	3.10	2	2
1.3.4.7.10	5	1	11	1	1
2.3.4.7.11	5	1	10	1	1
2.4.7.10.11	5	1	7	1	2
Total number of isolates					432
Total number of races					87

diate, and 53 were sensitive. The proportion of A2 mating type was higher among isolates from small-scale conventional fields (39%) than from large-scale conventional fields (26%) ($\chi^2 = 7.05$, df = 1, p = 0.0079).

RG57 analysis. Based on a worldwide collection of isolates, the RG57 probe can detect (presence/absence) a total of 25 restriction fragments/bands (Goodwin *et al.*, 1992a). In the subset of 57 isolates, nine of these bands were not detected (bands 4, 8, 11, 12, 15, 17, 19, 22 and 23), while five were present in all isolates (bands 13, 14, 20, 24 and 25). The remaining eleven bands (bands 1, 2, 3, 5, 6, 7, 9, 10, 16, 18, 21) were polymorphic. A total of 21 different fingerprints were detected. The four most common fingerprints I, XX, XXI and XXII (Table 6) represented more than half the isolates (67%). Fourteen unique fingerprints were detected at nine sites, but mostly among isolates collected in 2005.

Mitochondrial DNA haplotype. Of the four known mitochondrial DNA haplotypes, three were found. Among 57 isolates tested, 30 were Ia haplotype (51%), 24 were IIa haplotype (42%) and 3 were IIb haplotype (7%). For the 37 A1 isolates 20 were Ia, 14 were IIa and 3 were IIb. For the 20 A2 isolates 10 were Ia and 10 were IIa. There were differences in frequency of Ia and IIa between sites. Only IIa haplotype isolates were detected in Enge 2004, Kalsa 2005 and Kambja 2004.

Multilocus genotypes. Thirty-three multilocus genotypes were identified, of which 27 (81%) were detected only once, most of these collected in 2005 (Table 6). All belonged to the 'new' population of the pathogen that displaced the US-1 clonal lineage (A1 mating type, Ib mtDNA haplotype) (Spielman *et al.*, 1991; Lebreton and Andrivon, 1998). Genotypic diversity measured by the normalized Shannon index was 0.79. Three genotypes were common to Estonian samples: the most frequent, EE-1 was found 8 times, EE-2 was found 6 times.

Associations between markers. Most of the RG57 fingerprints were not associated with a single mtDNA haplotype (Table 9). There was no association between metalaxyl resistance and mtDNA haplotype ($\chi^2 = 1.66$, df = 3, p = 0.65). Of the 25 polymorphic RG57 bands, one polymorphic band (16) was significantly associated with metalaxyl tolerance ($\chi^2 = 8.28$, df = 2, p = 0.016). No associations were found between RG57 fingerprints, metalaxyl resistance or mating types.

DISCUSSION

Comparison of data from the current study with previous data (Runno-Paurson *et al.*, 2009) showed that the proportion of metalaxyl-resistant isolates of *P. infestans* remains in the same range as it was in Estonia in 2002-2003. Similar values have been found in recent years in other European countries (Hermansen *et al.*, 2000; Bakonyi *et al.*, 2002a, 2002b; Day *et al.*, 2004; Nagy *et al.*, 2006; Lehtinen *et al.*, 2007; Avendaño Córcoles, 2007).

The proportion of isolates with intermediate resistance to metalaxyl was high in the Estonian population during the period of 2002-2006; similar situation was

Table 5. Frequencies of specific compatibility (virulence) to potato R-genes in isolates of *Phytophthora infestans* from different locations and years in Estonia.

Sampling sites	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	Mean number of virulences/isolate	No of tested isolates
Antsla 2004	87	40	87	80	20	47	80	53	0	87	100	6.8	15
Enge 2004	100	38	100	100	15	15	100	38	0	100	100	7.1	13
Enge 2006	100	23	92	100	8	8	100	38	0	92	100	6.6	13
Enge 2007	81	69	94	81	13	19	94	50	6	75	88	6.7	16
Ingliste 2004	86	5	91	77	9	27	91	9	0	86	91	5.7	22
Ingliste 2006	92	48	96	100	36	16	96	48	8	96	96	7.3	25
Ingliste 2007	100	26	89	84	11	16	100	11	0	95	95	6.3	19
Jõgeva 2004	84	63	89	84	5	32	100	42	11	95	100	7.1	19
Jõgeva 2005	100	94	100	100	56	67	100	56	17	100	100	8.9	18
Jõgeva 2006	90	60	83	93	7	53	97	47	3	83	100	7.2	30
Jõgeva 2007	100	78	91	96	17	35	100	43	0	100	96	7.6	23
Jõusa 2006	100	27	100	100	20	7	100	20	7	100	100	6.8	15
Kalsa 2005	95	74	95	100	5	37	95	32	0	95	95	7.2	19
Kalsa 2006	100	53	93	100	7	20	100	47	0	100	100	7.2	15
Kambja 2004	100	60	100	73	13	0	100	20	0	100	100	6.7	15
Laheotsa 2004	53	29	82	59	6	24	82	0	0	71	71	4.8	17
Laheotsa 2005	100	90	100	100	70	20	100	30	20	100	100	8.3	10
Naha 2005	90	70	90	90	10	60	100	60	0	100	100	7.7	10
Naha 2006	81	13	81	88	6	6	94	31	0	94	88	5.8	16
Naha 2007	100	31	100	100	19	6	94	19	0	100	100	6.7	16
Paalimäe 2004	82	82	91	73	0	64	91	9	0	100	100	6.9	11
Võnnu 2004	63	29	79	71	4	38	92	33	0	79	83	5.7	24
Võnnu 2005	100	86	95	95	57	29	95	57	5	100	95	8.1	21
Võnnu 2006	93	47	80	87	7	27	93	60	0	100	100	6.9	15
Võnnu 2007	100	27	100	100	13	7	100	67	0	100	100	7.1	15
Total												6.9	432

noted in the UK by Cooke *et al.* (2003) and Day *et al.* (2004). However, by the year 2007 the proportion of isolates with intermediate resistance in the Estonian population had decreased to 6%.

The proportion of metalaxyl-resistant isolates in the Estonian population has fluctuated considerably throughout the recent years. From a level of about 20-35% in 2002 and 2003 (Runno and Koppel, 2006) it increased to 56% in 2004. This peak was followed by a decrease and among the isolates collected in 2006 and 2007, metalaxyl-resistant isolates were, again, in the minority (Table 2). Part of the reason for such a decrease (which was more pronounced in 2007) could be that in 2007 the weather conditions were not favourable for late blight development and the pathogen occurred quite late in the season, so farmers/growers did not treat crop so frequently and did not use metalaxyl fungicides as often as in other years. Although the proportion of metalaxyl-resistant isolates fluctuates from year to year it is still clear that this proportion has remained substantially high throughout the whole monitoring period. The most likely reason for the fluctuations is the changing intensity of the use of metalaxyl-containing fungicides. The situation is very similar in the Finnish population (Lehtinen *et al.,* 2007), where the extent of metalaxyl-resistant isolates has increased and the use of phenylamide fungicides is very common.

In the current study as might be expected, resistance was more often found in potato fields known to have been sprayed with phenylamide containing fungicides, 48% of isolates were resistant, whereas from fields which had not been phenylamide-treated, only 33% of isolates were resistant (Table 3). Metalaxyl was used mostly on large scale conventional fields.

Trials of late blight control with metalaxyl containing fungicides carried out in 2003-2005 (Runno and Koppel, 2006) at Jõgeva Plant Breeding Institute indicated that metalaxyl provided high protection under moderate pathogen pressure, but showed lowered efficacy only in conditions of extreme late blight pressure in 2004 when the foliage was already infected during the period when metalaxyl was used.

Results showed that in spite of the occurrence of resistant strains, the use of metalaxyl-containing fungicides is still effective. By following the application instructions for metalaxyl fungicides it is possible to restrict the selection for metalaxyl-resistant strains. Therefore metalaxyl could be used effectively for control of potato late blight a maximum of two times at the start of the fungicide treatments.

Comparing the current results with previous results in 2002-2003 (Runno-Paurson *et al.,* 2009), the frequency of virulence against R5 had increased from 5% to 17% and frequency against R9 had decreased from 14% to 3%.

The race 1.3.4.7.10.11 was the most common among Estonian isolates as it is in Finland, Norway, Denmark and Sweden (Hermansen *et al.,* 2000; Lehtinen *et al.*, 2007; Lehtinen *et al.,* 2008), in France and Switzerland (Knapova and Gisi, 2002), in Poland (Zimnoch-Guzowska, 1999) and in Austria (Avendaño Córcoles, 2007). Complex races are common in Estonian populations, a similar situation to those in Poland (Sliwka *et al.*, 2006) and Russia (Elansky *et al.,* 2001). The mean number of virulence genes per isolate increased from 6.3 in 2002-2003 to 6.9 in 2004-2007. A similar increase has also occurred in Finland and Norway (Lehtinen *et al.,* 2008).

In the current study the four most common pathotypes formed almost half (46%) of all isolates (Table 4), but in contrast to previous studies most of the pathotypes appeared only once, and the three most common pathotypes comprised only 16.8% of the population. Pathotype diversity calculated by the normalised Shannon diversity index also showed lower values (0.54) than in 2002-2003 when it was very high (0.92). The index value showed the same range as other European populations in Austria (0.56) (Avendaño Córcoles, 2007), Finland (0.35) and Norway (0.44) (Hermansen *et al.,* 2000).

The average percentage of A2 mating type in the current study was 33%, which is similar to results in previous studies in 2002-2003 (Runno-Paurson *et al.,* 2009). There have been reports from several European countries where the A1:A2 ratio is lower (Brurberg *et al.*, 1999; Hermansen *et al.*, 2000; Bakonyi *et al.*, 2002a, 2002b; Cooke *et al.,* 2006; Lehtinen, *et al.*, 2007). A higher proportion of the A2 mating type has been found in Austria, The Netherlands and Poland (Avendaño Córcoles, 2007; Zwankhuizen *et al.*, 2000; Sliwka *et al.*, 2006).

Both mating types were detected in all fields studied. It may indicate that blight epidemics are severe both on large conventional farms and in untreated experimental fields. This situation with continuous potato cropping in small-scale conventional fields increases the risk of oospore-derived infections and may cause more early attacks and consequent yield loss. The presence of both mating types in the same field indicates that oospores would be produced in potato foliage (Turkensteen *et al.*, 2000) and may change the epidemiology substantially. Sexual reproduction increases genetic diversity and leads to soil contamination with oospores, and associated early infection. Indeed, in 2004, symptoms indicated that the infection was probably caught from oospore-contaminated soil. Therefore more emphasis needs to be put on crop rotation (Turkensteen *et al.*, 2000).

In this study we noted differences between potato production systems. The proportion of A2 mating type was higher among isolates collected from experimental fields (40%) and small-scale conventional farm fields (39%) than from large-scale conventional fields (26%).

Table 6. Genotypes, phenotypes and locations of Estonian isolates of *Phytophthora infestans.*

Multilocus genotype	RG57 fingerprint[a]	RG57 genotype[b]	Mating type	mtDNA haplotype	Metalaxyl resistance[c]	Number of isolates	Number of sites	Year	Region[d]	Field type
EE-1	101010100100110100011 0011	I	A1	Ia	R, S	8	4	2004-05	C, N, S	S, L
EE-2	101010100100110100011 0011	I	A1	IIa	S, R, I	6	4	2004-05	E, SE, SW	E, L, S
EE-27	101010100100110100011 0011	I	A1	IIb	S	1	1	2005	C	L
EE-3	101010100100110100011 0011	I	A2	Ia	S, R	2	2	2004-05	C, S	L, S
EE-28	101010100100110100011 0011	I	A2	IIa	S, R	4	2	2004-05	E, S	E, S
EE-29	101010100100110000011 0011	XX	A1	Ia	I, R, S	4	3	2004-05	N, SE	L, S
EE-30	101010100100110000011 0011	XX	A1	IIa	R	2	1	2004	SE	S
EE-31	101010100100110000011 0011	XX	A2	Ia	S	1	1	2004	E	E
EE-32	101010100100110000011 0011	XX	A2	IIa	R	1	1	2004	C	L
EE-33	100010100100110100011 0011	XXI	A1	IIa	I	1	1	2005	SE	L
EE-34	100010100100110100011 0011	XXI	A1	IIb	R	1	1	2004	C	L
EE-35	100010100100110100011 0011	XXI	A2	IIa	S	2	2	2004-05	SE, SW	L
EE-36	101000100100110100011 0011	XXII	A1	IIa	I	1	1	2005	C	L
EE-37	101000100100110100011 0011	XXII	A2	Ia	I	4	1	2004	N	L
EE-38	101010101100110100011 0011	XXIII	A1	IIa	I	1	1	2004	C	L
EE-39	101010101100110100011 0011	XXIII	A2	Ia	I	1	1	2004	E	E
EE-40	101010101100110100011 0011	XXIII	A2	IIa	R	1	1	2004	E	E
EE-41	111010100100110000011 0011	XXIV	A1	Ia	R	1	1	2005	SE	S
EE-43	111010100100110100011 0011	XXV	A1	Ia	R	1	1	2005	SE	S
EE-44	111010100100110100011 0011	XXV	A1	IIb	S	1	1	2005	SE	S
EE-45	111000100100110100011 0011	XXVI	A1	Ia	I	1	1	2005	N	L
EE-46	110011000100110100011 0011	XXVII	A1	Ia	I	1	1	2005	SE	L
EE-47	101011101100110100011 0011	XXVIII	A1	Ia	R	1	1	2004	E	E
EE-42	101011100100110100011 0011	XXIX	A1	Ia	I	1	1	2005	E	E
EE-48	101010100100110100010 0011	XXX	A1	IIa	R	1	1	2005	SE	L
EE-49	101010000100110100011 0011	XXXI	A1	Ia	I	1	1	2005	SE	S
EE-50	100010100100110000011 0011	XXXII	A1	IIa	I	1	1	2004	C	L
EE-51	100010100000110100011 0011	XXXIII	A2	IIa	I	1	1	2005	SE	L
EE-53	100010001100110000011 0011	XXXIV	A1	Ia	S	1	1	2005	E	E
EE-54	100010001100110100011 0011	XXXV	A1	IIa	R	1	1	2005	E	E
EE-55	100010000000110100011 0011	XXXVI	A2	Ia	S	1	1	2004	S	S
EE-56	001010100100110100011 0011	XXXVII	A2	Ia	R	1	1	2004	S	S
EE-52	001000100100110100011 0011	XXXVIII	A2	IIa	R	1	1	2005	N	L

[a]RG57 fingerprint is denoted using '1' and '0' to indicate presence or absence, respectively, of bands 1-25 recognized by the RG57 probe (Goodwin et al., 1992).
[b]RG57 genotypes are numbered with Roman numerals consecutively according to Bakonyi et al. (2002b).
[c]S, sensitive; I, intermediate; R, resistant.
[d]Regions of Estonia: C, Central; E, East; N, North; SE, South-East; SW, South-West.
[e]L, large-scale conventional; E, experimental; S, small-scale conventional production.

Differences on this scale could signify higher genetic diversity among populations collected from experimental and small-scale productions, as there is a higher risk of sexual reproduction. However, our data may result from a sampling bias.

This study uncovered several new RG57 genotypes of *P. infestans* not previously reported. Among numerous RG57 fingerprints only three (I, XX and XXI) occurred in 2004 and again in 2005. Most of the 21 RG57 fingerprints are apparently unique to Estonia. Comparing the results of this study with other European studies, shows similarities in fingerprints from Russia, Finland, Norway, the Netherlands, Great Britain, and Belgium. The most common fingerprint in Estonia I, was identical with the Russian fingerprint MO-12 (Moscow region) (Elansky *et al.*, 2001), the Norwegian fingerprint N-27 (Brurberg *et al.*, 1999) and the British fingerprint RF060 (Day *et al.*, 2004) (Table 6). The fingerprint XX was identical with the Dutch fingerprint NL-86 (Zwankhuizen *et al.*, 2000), the fingerprint XXI with the Russian fingerprint MO-5 (Elansky *et al.*, 2001); XXIII was identical with the Russian fingerprint MO-17 (Elansky *et al.*, 2001) and the British fingerprint RF015 (Day *et al.*, 2004); XXXV was identical with the British fingerprint RF006 and the most common Northern Ireland fingerprint NI-1 (Day *et al.*, 2004, Cooke *et al.,* 2006).

The three mtDNA haplotypes (Ia, IIa and IIb) were found among our isolates. The Ib haplotype, associated with the old clonal *P. infestans* populations, was not found. Haplotype IIb was not found previously in Estonia. Interestingly, among isolates collected from metalaxyl-treated crops the extent of IIb haplotype, which is rare in Europe, was 23%. In the current and previous studies, both haplotypes were found in almost equal proportions. A high proportion of Ia haplotype (74%) was found in northern Estonia (Runno-Paurson, unpublished data). A high proportion of Ia haplotype has also been observed in Poland, England, Scotland, Wales,

The Netherlands and France (Lebreton and Andrivon, 1998; Cooke *et al.,* 2003; Lebecka *et al.,* 2007).

Our study indicates that the Estonian population of *P. infestans* is diverse, having a large number of multilocus genotypes. The high and stable frequency of A2 isolates and the occurrence of both mating types in the same field indicates a potential for sexual recombination and spread of fungicide resistance. More information is needed to clarify the role of oospores in the epidemiology of *P. infestans* in Estonia.

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