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RESEARCH ARTICLE

Correlation of isozyme profiles with genomic sequences of *Phytophthora ramorum* and its *P. sojae* orthologues

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Abstract A correct interpretation of isozyme patterns can be seriously hampered by the lack of supporting genetic data. The availability of the complete genome sequence of *Phytophthora ramorum*, enabled us to correlate isozyme profiles with the gene models predicted for these enzymes. Thirty-nine *P. ramorum* strains were characterised employing isozyme analysis on malate dehydrogenase (MDH), NADP-dependent malic enzyme (MDHP), 6-phosphogluconate dehydrogenase (PGD), glucosephosphate isomerase (GPI) and lactate dehydrogenase (LDH) comprising nine putative loci. One isozyme band was enzymatically stained for PGD whereas multiple bands were detected for GPI, MDH, MDHP and LDH. All putative loci were monomorphic except for *Ldh*-2. Genome mining revealed that the assembled genome sequences of *P. ramorum* and *P. sojae* each contain one *Gpi* and one *Pgd* gene model. For MDH, two gene models were identified, encoding a cytosolic and mitochondrial type, respectively. Also for MDHP *P. ramorum* has two gene models that are both duplicated in *P. sojae*. Both species contain six *Ldh* gene models, including pseudogenes. The *Ldh* gene models are clustered and located in regions that show a high level of conserved synteny. This study demonstrates that insight into the gene models encoding isozymes helps to interpret isozyme profiles in *Phytophthora*.

Keywords Gene duplication · Conserved synteny · Endosymbiosis · Sudden oak death

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Introduction

Isozymes, separated by electrophoresis under native conditions, are well defined by their mobility and identity. Isozyme analysis is a robust method to study the population structure of plant pathogenic *Phytophthora* species, to delineate species in the *Phytophthora* genus and, by using dimeric enzymes, to discover sexual progeny and even hybrids between species. Hitherto, the interpretation of the isozyme banding patterns was hampered by the lack of knowledge of the genes encoding the various enzymes. The release of the genome sequence of two *Phytophthora* species enabled us to correlate isozyme profiles with the gene models predicted for these enzymes.

Phytophthora ramorum Werres, de Cock & Man in 't Veld is certainly among the most destructive plant pathogens discovered in the last decade (Werres et al. 2001). It is now generally assumed that *P. ramorum* is an invasive species from hitherto unknown origin which was almost simultaneously introduced in Europe and North America in the 1990s of the previous century. The two populations were considered to be separate introductions: in Europe mainly mating type A1 was present whereas in North America mating type A2 was found (Werres et al. 2001; Rizzo et al. 2002). Meanwhile, both mating types have been identified on both continents (Hansen et al. 2003; Werres and De Merlier 2003; Werres and Kaminski 2005). Transatlantic differences were also revealed by sequence analysis of the

mitochondrial encoded *CoxI* gene (Kroon et al. 2004b) and by AFLP analysis (Ivors et al. 2004). It was inferred from AFLP analysis that the North American population was largely dominated by a single clonal lineage, whereas the European population consisted of an array of closely related AFLP lineages (Ivors et al. 2004). In contrast, the morphology of the North American isolates showed more variation than the European isolates (Brasier 2003). The pathogenicity of North American and European strains is rather similar although differences in aggressiveness and host range were observed (Brasier and Kirk 2004; Tooley et al. 2004).

Phytophthora ramorum was initially described as a pathogen on *Rhododendron* and *Viburnum* in Europe (Werres et al. 2001), but in North America it was identified as the causal agent of sudden oak death, a disease responsible for the deaths of hundreds of thousands of oaks (*Quercus agrifolia*, *Q. kelloggii*, *Q. parvula* var. *shrevei*) and tanoaks (*Lithocarpus densiflorus*) (Rizzo et al. 2002). In the course of time, *P. ramorum* has been associated with disease symptoms on many hosts and the list is still expanding (Ivors et al. 2006). The discovery of *P. ramorum* raised great concern among phytopathologists and politicians in Europe due to the possible threat that this species could impose on natural ecosystems in Europe. As a result the European Commission has issued emergency phytosanitary measures to prevent further introduction and dispersal of *P. ramorum* in Europe (EU Commission Decision no 2002/757/EC). The measures are aimed to avoid the encounter of the two mating types which could theoretically produce new pathotypes. Whether *P. ramorum* has a functional sexual system is yet unknown. The mating behaviour is unusual compared to that of other heterothallic species that produce gametangia in intraspecific pairing within a few days. Many tester strains from other species (*P. cambivora*, *P. cinnamomi*, *P. drechsleri*, *P. palmivora*) were not able to induce sexual structures in *P. ramorum* (Werres et al. 2001). Mating experiments on artificial media using North American and European strains of *P. ramorum* were negative (Brasier and Kirk 2004). Only inoculation of living *Rhododendron* stem sections with both mating types, and mixing juvenile, pre-chlamydospore producing mycelia produced sexual structures successfully (Werres and Zielke 2003; Brasier and Kirk 2004). It is tempting to speculate that hybridization events in the evolutionary history of *P. ramorum* may be the cause of this abnormal mating behaviour, since hybrids are notorious for their fertility problems (Brasier et al. 1999; Man in 't Veld et al. 2007). Alloploidy resulting in fixed heterozygosity is considered to be a hallmark of hybridisation. Isozyme analysis of a limited number of isolates already revealed fixed heterozygosity at two isozyme loci in *P. ramorum* (Man in 't Veld et al. 2002b). Although hybridization has been shown to exist in the genus *Phytophthora* (Man in 't Veld et al. 1998; Brasier et al. 1999; Man in 't Veld et al. 2007), the observed heterozygosity might theoretically also be the result of gene duplication.

Isozyme analysis is often used to characterize and define species and to study the population structure (Oudemans and Coffey 1991a, b; Man in 't Veld et al. 2002a, 2007; Man in 't Veld 2007). Isozyme markers have the advantage over DNA markers that they are suitable to detect crossings: when two different isozyme alleles are present at the same locus, and the encoded enzyme is dimeric, the gene products can combine in three different ways, resulting in two different homodimeres and one heterodimer, the latter with intermediate physicochemical properties. Characteristic three-banded patterns can be observed upon gel electrophoresis and enzymatic staining (Richardson et al. 1986). The value of isozyme genotyping increases when isozyme profiles can be linked to genome sequences, gene models and expressed sequence tags (ESTs) encoding the encountered enzymatic activity, because such a combined analysis helps to better interprete the isozyme patterns. Multiple isozyme bands can be the result of various types of post-translational modifications (Seet et al. 2006) or different (although related) catalytic activities. Alternatively, isozymes can be encoded by slightly different members of a multigene family, or by unrelated genes whose products target the same substrate and have similar catalytic activities.

The initial aim of this study was to use isozyme genotyping to investigate the population structure of *P. ramorum* in North America and Europe. It appeared, however, that the isozyme polymorphism was extremely low. To be able to interpret the banding pattern and to correlate the isozyme profiles with the alleles and the number of gene copies present in the genome we mined the genomes of *P. ramorum* and *Phytophthora sojae* (Tyler et al. 2006) and investigated the genes encoding the isozymes.

Materials and methods

Strains and mycelium cultivation

The *Phytophthora* species and strains used in this study are listed in Table 1. All strains were grown in 50 ml of tryptone soy broth medium (TSB) in 250-ml Erlenmeyer flasks on a rotary shaker at 40 rpm in the dark. To start the culture three mycelial plugs (Ø 5 mm) were added per flask. The plugs were cut from actively growing colony margins of 3-day-old cultures on V8 agar. After incubating the cultures at 23°C for 7 days, the mycelium was collected by filtration, dried between filter paper and stored at −80°C.

Table 1 Isolates of *Phytophthora ramorum*, *Phytophthora hibernalis* and *Phytophthora lateralis* used in this study, their hosts, origin, isozym profiles and mating type

Isolate number	Host	Origin	Isozym loci								Mating type
			Mdh-2	*Mdhp*-1	*Mdhp*-2	*Ldh*-1	*Ldh*-2	*Ldh*-3	*Pgd*	*Gpi*	
P. ramorum											
CBS 101327[a]	*Rhododendron*	Netherlands	AA	BB	n.a	AA	ABC	ABDE	BB	AC	A1
CBS 101328	*Rhododendron*	Netherlands	AA	BB	n.a	AA	AB	ABDE	BB	AC	A1
CBS 101329	*Rhododendron*	Netherlands	AA	BB	n.a.	AA	AB	ABDE	BB	AC	A1
CBS 101330	*Viburnum*	Netherlands	AA	BB	n.a.	AA	AB	ABDE	BB	AC	A1
CBS 101331	*Rhododendron*	Netherlands	AA	BB	n.a.	AA	AB	ABDE	BB	AC	A1
CBS 101332	*Rhododendron*	Netherlands	AA	BB	n.a.	AA	AB	ABDE	BB	AC	A1
CBS 101548	*Rhododendron*	Germany	AA	BB	AA	AA	ABC	ABDE	BB	AC	A1
CBS 101549	Recycling water	Germany	AA	BB	AA	AA	ABC	ABDE	BB	AC	A1
CBS 101550	*Rhododendron catawbiense*	Germany	AA	BB	AA	AA	ABC	ABDE	BB	AC	A1
CBS 101551	*Rhododendron catawbiense*	Germany	AA	BB	AA	AA	ABC	ABDE	BB	AC	A1
CBS 101552	Recycling water	Germany	AA	BB	AA	AA	AB	ABDE	BB	AC	A1
CBS 101553	*Rhododendron catawbiense*	Germany	AA	BB	AA	AA	AB	ABDE	BB	AC	A1
CBS 109279	*Rhododendron*	Germany	AA	BB	n.a.	AA	AB	ABDE	BB	AC	A1
CBS 109278	*Viburnum bodnantense*	Germany	AA	BB	n.a.	AA	AB	ABDE	BB	AC	A1
PD 20021945[b]	*Viburnum tinus*	England	AA	BB	AA	AA	ABC	ABDE	BB	AC	A1
PD 20022965	*Rhododendron*	France	AA	BB	AA	AA	AB	ABDE	BB	AC	A1
PD 20023399-2	*Viburnum bodnantense*	Belgium	AA	BB	AA	AA	AB	ABDE	BB	AC	A1
PD 20023443-1	*Rhododendron*	Spain	AA	BB	AA	AA	AB	ABDE	BB	AC	A1
PD 20023443-2	*Rhododendron*	Spain	AA	BB	AA	AA	AB	ABDE	BB	AC	A1
PD 20035548	*Quercus rubra*	Netherlands	AA	BB	n.a.	AA	AB	ABDE	BB	AC	A1
P1403[c]	*Vaccinium ovatum*	USA	AA	BB	AA	AA	ABC	ABDE	BB	AC	A2
P1404	*Lithocarpus densifloris*	USA	AA	BB	AA	AA	ABC	ABDE	BB	AC	A2
P1420 sz	*Quercus agrifolia*	USA	AA	BB	AA	AA	ABC	ABDE	BB	AC	A2
P1422	*Quercus parvula*	USA	AA	BB	AA	AA	ABC	ABDE	BB	AC	A2
P1424 sz	*Quercus agrifolia*	USA	AA	BB	n.a.	AA	AB	ABDE	BB	AC	A2
P1428	*Quercus agrifolia*	USA	AA	BB	AA	AA	ABC	ABDE	BB	AC	A2
P1429	*Umbellularia californica*	USA	AA	BB	AA	AA	ABC	ABDE	BB	AC	A2
P1496	*Umbellularia californica*	USA	AA	BB	AA	AA	ABC	ABDE	BB	AC	A2
P1497	*Quercus agrifolia*	USA	AA	BB	AA	AA	ABC	ABDE	BB	AC	A2
P1498	*Quercus agrifolia*	USA	AA	BB	AA	AA	ABC	ABDE	BB	AC	A2
P1499	*Quercus agrifolia*	USA	AA	BB	n.a.	AA	AB	ABDE	BB	AC	A2
USA 0.4[d]	*Quercus* sp.	USA	AA	BB	n.a.	AA	AB	ABDE	BB	AC	A2
USA 0.13	*Quercus* sp.	USA	AA	BB	n.a.	AA	AB	ABDE	BB	AC	A2
USA 13	*Lithocarpus densifloris*	USA	AA	BB	AA	AA	AB	ABDE	BB	AC	A2
USA 16 (P1348)	*Quercus agrifolia*	USA	AA	BB	AA	AA	AB	ABDE	BB	AC	A2
USA 217 (P1349)	*Rhododendron*	USA	AA	BB	AA	AA	AB	ABDE	BB	AC	A2
USA 240	*Vaccinium*	USA	AA	BB	AA	AA	ABC	ABDE	BB	AC	A2
USA 351 (P1370)	*Arbutus*	USA	AA	BB	AA	AA	AB	ABDE	BB	AC	A2
USA 354 P1371)	*Umbellularia californica*	USA	AA	BB	AA	AA	ABC	ABDE	BB	AC	A2
P. hibernalis											
CBS 522.77	*Aquilegia vulgaris*	New Zealand	CC	AA	n.a.	n.a.	BB	DD	AA	BB	H[e]
CBS 953.87	*Citrus* sp.	USA	CC	AA	AA	n.a.	BB	DD	AA	BB	H
CBS 270.31	*Citrus sinensis*	Portugal	CC	AA	n.a.	n.a.	BB	DD	AA	BB	H

Table 1 continued

Isolate number	Host	Origin	Isozym loci								Mating type
			Mdh-2	*Mdhp*-1	*Mdhp*-2	*Ldh*-1	*Ldh*-2	*Ldh*-3	*Pgd*	*Gpi*	
P. lateralis											
CBS 102608	*Chamaecyperus lawsoniana*	USA	BB	AA	n.a.	AA	AA	CD	BB	AC	H
CBS 168.42	*Chamaecyperus lawsoniana*	USA	BB	AA	n.a.	n.a.	AA	DD	BB	AC	H

n.a.: no activity

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[c] Forest Research Agency, Alice Holt Lodge, Farnhem, Surrey, UK

[d] Department of Environmental Science, University of California, Berkeley, USA

[e] Homothallic

Protein extraction

Frozen mycelium was thawed at 4°C for 3 h before protein extraction. Routinely, about 0.5 g of mycelium was ground for 3 min in a chilled mortar with sand and 70 µl of extraction buffer [0.1 M Tris–HCl (pH 7.0), 1 mM dithiothreitol, 50 mM ethylene diamine tetra-acetic acid (EDTA), 10% poly vinyl pyrrolidone (PVP) w/v, 50 µg/ml soybean trypsin inhibitor, 0.1 mM phenyl methyl sulfonyl fluoride (PMSF) and 5% glycerol (v/v)]. The homogenate was centrifuged for 10 min at 14,000 rpm (4°C) in Eppendorf tubes. The supernatant was collected and stored at −80°C.

Electrophoresis and enzyme staining

The supernatants were separated on native polyacrylamide gels at 4°C, using the automated PhastSystem of Pharmacia (Roosendaal, The Netherlands). For detection of NADP-dependent malic enzyme (MDHP, EC 1.1.1.40) and 6-phosphogluconate dehydrogenase (PGD, EC 1.1.1.44) a 10–15% gradient polyacrylamide gel was used, for malate dehydrogenase (MDH, EC 1.1.1.37) and lactate dehydrogenase (LDH, EC 1.1.1.28) a 8–25% gradient polyacrylamide gel and for glucose phosphate isomerase (GPI, EC 5.3.1.9) a 12.5% homogeneous polyacrylamide gel. All gels were prepared in a Tris–acetate buffer (0.11 M; pH 6.4). The running buffer, contained in 2% agarose gel strips, consisted of 0.25 M Tris and 0.88 M L-alanine (pH 8.8). For each assay 0.7 µl extract was applied to the gel. Supernatants assayed for MDH activity had to be diluted 5–10 times to avoid smearing. After electrophoresis, gels were immersed immediately in freshly prepared staining solutions in the dark at 37°C.

For MDH activity gels were stained in 25 ml solution containing 0.2 M Tris–HCl pH 8.0, 440 mg L-malic acid (di-sodium salt), 12.5 mg β-nicotineamide adenine dinucleotide (NAD), 7.5 mg nitro blue tetrazolium (NBT) (Sigma N6876, Zwijndrecht, The Netherlands), 1 mg phenazine methosulfate (PMS) (Sigma P9625, Zwijndrecht, The Netherlands), for MDHP activity in 25 ml staining solution containing 0.2 M Tris–HCl pH 8.0, 440 mg L-malic acid (di-sodium salt), 12.5 mg nicotinamide adenine dinucleotide phosphate (NADP), 7.5 mg NBT, 1 mg PMS, for LDH in 23 ml 0.1 M Tris–HCl pH 7.5, 2 ml lactate solution (5.3 ml lactic acid (98%) + 24.5 ml 1 M Na_2CO_3 + 10.2 ml distilled water), 12.5 mg NAD, 7.5 mg NBT, 1 mg PMS, for PGD in 25 ml 0.1 M Tris–HCl pH 8.0, 10 mg 6-phosphogluconic acid (tri-sodium salt) 50 mg of $MgCl_2$, 10 mg NADP, 7.5 mg NBT and 1 mg PMS and for GPI in 25 ml of 0.1 M Tris–HCl pH 8.0, 20 mg of fructose-6-phosphate, 50 mg of $MgCl_2$, 10 units of glucose-6-phosphate dehydrogenase (Sigma G5760), 12.5 mg of NAD, 7.5 mg of NBT and 1 mg of PMS.

For the five isozymes tested, clearly interpretable bands were scored in alphabetical order as individual alleles according to their relative mobility from slow to fast. Since *Phytophthora* species are diploid, two identical letters were assigned to one band, thus reflecting homozygosity. When more zones of activity were present on the gel, they were assigned to individual putative loci and numbered according to their relative mobility with the slowest migrating band numbered as 1. All tested isozyme loci with the exception of *Ldh*-1 and *Ldh*-3, encode dimeric enzymes (Man in 't Veld et al. 2002a). A three-banded pattern was interpreted as the product of two different alleles encoding a dimeric enzyme. A four-banded pattern was interpreted as the product of four different alleles encoding a monomeric enzyme. When referring to enzyme activity capital letter codes are used (e.g. MDH). Isozyme alleles and putative loci are indicated in italics (*Mdh*) and genes, gene models, or genome sequences are italized and underlined (*Mdh*).

Database analysis

Genes encoding the analysed isozymes (MDH, MDHP, LDH, PGD and GPI) were identified in the genomes of *P. ramorum* and *P. sojae* using the genome sequence data

from the US Department of Energy Joint Genome Institute (DOE-JGI; http://genome.jgi-psf.org/) (Tyler et al. 2006). The database contains a functional assignment based on automatic analysis. Searches were performed by using the available options and extended with Blast analysis of entries in the NCBI database. ESTs of *P. sojae* and *P. infestans* were retrieved from http://www.pfgd.org/ (Gajendran et al. 2006). Subcellular localization of the proteins was predicted by programs available at the psort www server (http://psort.hgc.jp/)(Nakai and Horton 1999). Multiple alignments were constructed with ClustallX (version 1.83) with default parameters. Distance-based trees were generated using the bootstrap option and Njplot.

Results

Isozyme polymorphism in *P. ramorum* is low

Isozyme patterns were determined in a set of 39 *P. ramorum* isolates that originate from a variety of host plants and were collected from North America and Europe (Table 1). In addition, we included strains from closely related *Phytophthora* species: two *P. lateralis* strains and three *P. hibernalis* strains. Five different isozymes were analyzed: malate dehydrogenase (MDH), NADP-dependent malic enzyme (MDHP), 6-phosphogluconate dehydrogenase (PGD), glucosephosphate isomerase (GPI) and lactate dehydrogenase (LDH), which are all known to give distinct patterns in various *Phytophthora* species (Oudemans and Coffey 1991a, b).

Isozyme analysis with MDH generated two zones of activity in *P. ramorum*, assigned to two putative loci *Mdh*-1 and *Mdh*-2 (Fig. 1a; Table 1). Due to smearing, however, the interpretation of the *Mdh*-1 locus was seriously hampered. A similar problem was encountered previously with other *Phytophthora* species (Man in 't Veld et al. 2002a), and therefore this locus was not analysed further. In contrast, the *Mdh*-2 locus in *P. ramorum* consistently exhibited a sharp monomorphic band. Single bands were also observed in *P. hibernalis* and *P. lateralis* but in all three species the mobility was different (Table 1).

Isozyme analysis for MDHP consistently generated one identical strong band for all *P. ramorum* strains tested. This band differed in mobility from the single band that is characteristic for both *P. hibernalis* and *P. lateralis* (Table 1). In addition, for most *P. ramorum* strains a faint band was observed that was initially interpreted as a staining artifact (see below). One strong band for all strains was obtained for PGD. The PGD band in *P. ramorum* migrated with the same mobility as the single band in *P. lateralis* but differed in mobility from the single band in *P. hibernalis* (Table 1). GPI consistently generated two bands with similar mobility in both *P. ramorum* and *P. lateralis*, whereas *P. hibernalis* exhibited one single band (Table 1).

Lactate dehydrogenase isozymes exhibited a complex pattern: three zones of activity were observed and these were assigned to three putative loci *Ldh*-1, *Ldh*-2 and *Ldh*-3 (Fig. 2; Table 1). Two of these (*Ldh*-1 and *Ldh*-3) contain monomeric isozymes whereas *Ldh*-2 is known to represent dimeric isozymes (Man in 't Veld et al. 2002a). At the *Ldh*-2 putative locus most *P. ramorum* strains showed a three-banded pattern possibly representing two different alleles, but some had a five-banded pattern that could be the result of a more complex genomic situation (Table 1). Strains with three or five-banded patterns could not be correlated to either the geographic location where the strains were collected nor to the host origin. At the *Ldh*-3 putative locus all

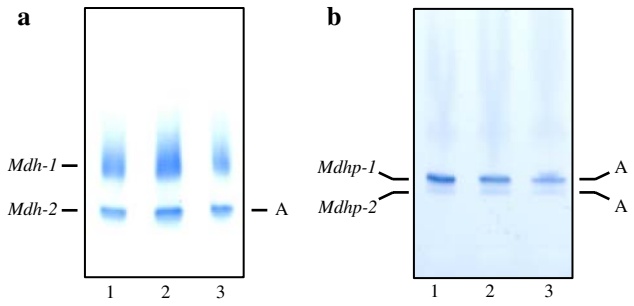

Fig. 1 Malate dehydrogenase and malic enzyme isozyme patterns. Representative isozyme patterns of malate dehydrogenase (MDH) or malic enzyme (MDHP) generated for *P. ramorum* and visualized by enzymatic staining. Putative loci and alleles are indicated (*Mdh*-1 and *Mdh*-2^A in **a** and *Mdhp*-1^A and *Mdhp*-2^A in **b**). **a** MDH patterns of USA 0.13 (*lane 1*), CBS 101332 (*2*), USA 0.4 (*3*). **b** MDHP isozyme patterns of strain P1496 (*1*), P1429 (*2*) and P1498 (*3*)

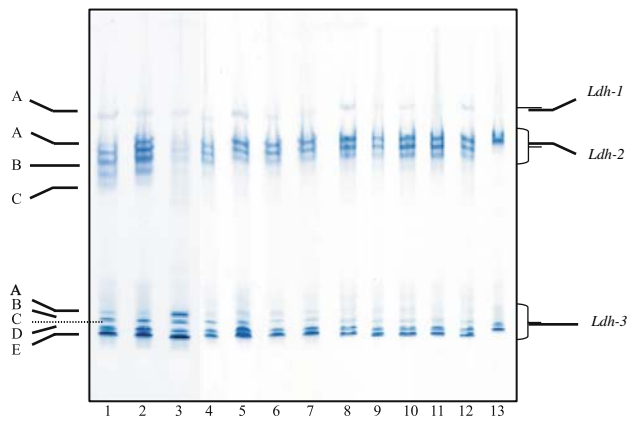

Fig. 2 LDH isozyme patterns. Isozyme patterns of lactate dehydrogenase (LDH) generated by *P. ramorum* (*1–12*) and *P. lateralis* (*13*). Putative loci and alleles are indicated [*Ldh*-1^A (monomeric), *Ldh*-2^A, *Ldh*-2^B, *Ldh*-2^C (dimeric), *Ldh*-3^A, *Ldh*-3^B, *Ldh*-3^C, *Ldh*-3^D, *Ldh*-3^E (monomeric)]. LDH patterns of the following strains are shown: P1498 (*lane 1*), P1422 (*2*), P1420 (*3*), USA 15 (*4*), CBS 101332 (*5*,*8*, and *12*), USA 0.13 (*6*), USA o.4 (*7*), PD 20023443-2 (*9*), PD 20023443-1 (*10*), PD 20023399-2 (*11*), CBS 102608 (*13*)

P. ramorum strains generated four bands with identical mobility. The LDH patterns generated in *P. hibernalis* and *P. lateralis* differed from those in *P. ramorum*, and from each other.

Two *Mdh* gene models for two *Mdh* loci

Surveying the *P. ramorum* genome revealed two gene models encoding MDH (Table 2). They share only 29% homology and most likely represent the two *Mdh* loci found by isozyme analysis. *Phytophthora sojae* has two highly homologous orthologues (92.8 and 90.5%, respectively) and for each of the two, ESTs were found. The low level of homology between the two *Mdh* paralogues is probably due to their evolutionary origin: gene model Pr_72140 has high homology with mitochondrial type MDH, whereas gene model Pr_71856 has high overall homology to soluble MDH. The latter was predicted to encode a cytosolic protein and this is supported by the phylogenetic tree shown in Fig. 3. Obviously, the existence of two *Mdh* loci in *Phytophthora* is not due to locus duplication, as hypothesised by Watts and Watts (1968), but is most likely the result of endosymbiosis. There is evidence that after the endosymbiotic event genes were transferred from the organellar genomes to the nucleus (Paquin et al. 1997; Krampis et al. 2006). The prokaryotic-like *Mdh* gene in *P. ramorum* (Ps_72140) has apparently undergone the same transfer, because no *Mdh* genes are found on the mitochondrial genome (Martin et al. 2007). The teleost fish *Sphyraena idiastes* has one mitochondrial and two cytosolic forms of *Mdh*, the latter being the result of gene duplication (Lin et al. 2002). Also *Trypanosoma brucei* has three *Mdh* genes but in this organism the third copy encodes a glycosomal type MDH (Aranda et al. 2006).

The detection of two *Mdh* gene models is consistent with the isozyme analysis that implicated two putative loci in *P. ramorum* (this study) and in several other *Phytophthora* species (Oudemans and Coffey 1991a, b; Man in 't Veld 2007). Their low homology (29%) explains why the two gene products do not dimerize (Fig. 1). Based on the current information we cannot determine which of the two gene models corresponds to *Mdh*-1 or *Mdh*-2.

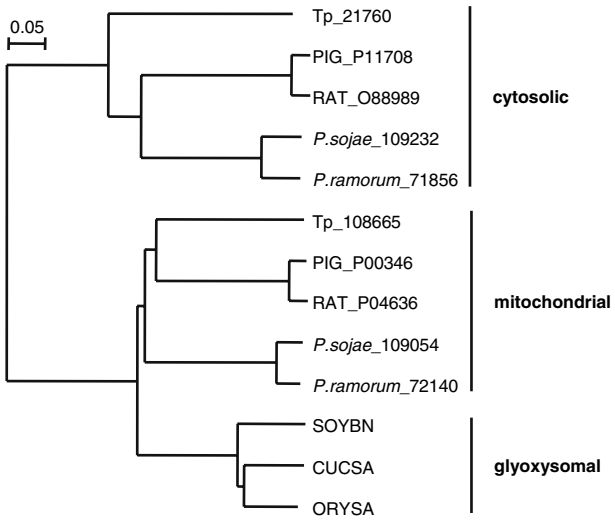

Fig. 3 Phylogram of MDH. The MDH protein sequences were used to construct a phylogenetic tree using ClustalX and depicted by njplot. MDH protein sequences for *P. ramorum* (Pr_72140), *P. sojae* (Ps_109054) and *Thalassiosira pseudonana* (Tp_21760 and Tp_108665, respectively a cytosolic and a mitochondrial type) were retrieved from http://genome.jgi-psf.org/. The following MDH protein sequences were retrieved from GenBank: PIG (*Sus scrofa*; accession numbers P11708 and P00346); RAT (*Rattus norvegicus*; O88989 and P04636); CUCSA (*Cucumis sativus*; P46488); ORYSA (*Oryza sativa*; Q42972); SOYBN (*Glycine max*; P37228). The two MDHs from pig and rat represent cytosolic and mitochondrial types, respectively. The plant MDHs are glyoxysomal types

Phytophthora has multiple *Mdhp* genes

In the genome of *P. ramorum* two *Mdhp* gene models were identified: Pr_74544 and Pr_52827. The latter is incomplete; it lacks the 5′ end of the open reading frame and encodes only 261 of the approximately 600 AA predicted to comprise the full length protein. In *P. sojae*, both genes appear to be duplicated, resulting in four *Mdhp* gene models (Table 2). Ps_136971, one of the two orthologues of

Table 2 Gene models identified in *P. ramorum* and *P. sojae* for the enzyme activities assayed

Enzyme activity	*P. ramorum*	*P. sojae*
GPI	Pr_71134	Ps_162909
LDH	Pr_71422; Pr_71423[a]; Pr_71424; Pr_76507[a]; Pr_46278[b]; Pr_76504	Ps_109081; Ps_109083[a]; Ps_109084; Ps_109085; Ps_109086; Ps_137917
MDH	Pr_72140; Pr_71856	Ps_109054; Ps_109232
MDHP	Pr_74544; Pr_52827[b]	Ps_131026; Ps_136640 Ps_136968; Ps_136971[b]
PGD	Pr_71783	Ps_108585

[a] Pseudogenes

[b] Incomplete sequences, due to assembly artifacts, sequence errors or missing sequence data

Pr_52827 is also incomplete (455 AA) whereas its paralogue, Ps_136968, is full length (599 AA). Three of the four *P. sojae* *Mdhp* genes (except Ps_136968), have corresponding ESTs and are thus active genes. BLAST analysis revealed that Pr_74544 and its *P. sojae* orthologues, have homology with mitochondrial located MDHP proteins and, are indeed predicted to be transported to the mitochondria based on their mitochondrial targeting sequence. Also Ps_136968, the full-length ortholog of Pr_52827, was predicted to be localized in mitochondria, although, intriguingly, in BLAST analysis it had highest homology to several chloroplast-precursor MDHPs. The sequence homology between the two paralogues of *P. ramorum* is low (~40%). A phylogenetic tree shows two branches representing a prokaryotic and a eukaryotic clade, respectively (Fig. 4). The prokaryotic clade contains small MDHP representatives and fusion proteins whereas eukaryotic types are larger enzymes.

The presence of two *Mdhp* gene models is not consistent with the single strong band detected by isozyme analysis. For three out of four homologues in *P. sojae* there are ESTs and it is therefore likely that both genes in *P. ramorum* are active genes. In view of this it must be noted that a second,

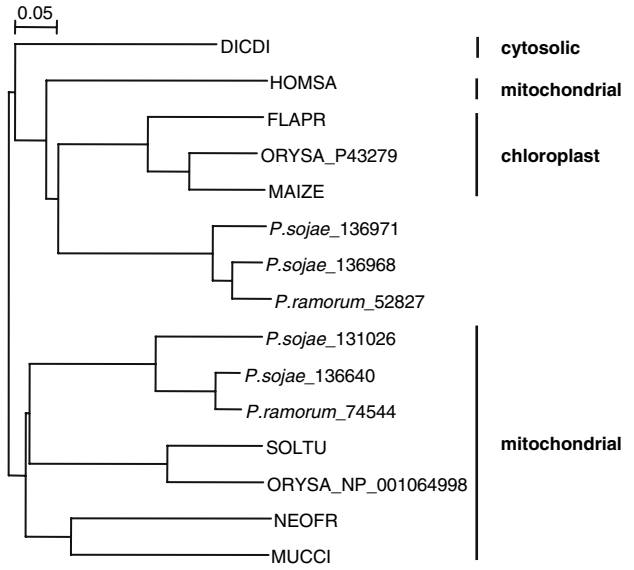

Fig. 4 Phylogenetic tree of MDHP proteins. The MDHP protein sequences were aligned with characteristic MDHP proteins from other organisms using ClustalX and depicted by njplot. MDHP protein sequences for *P. ramorum* (Pr_74544 and Pr_52827), and *P. sojae* (Ps_131026, Ps_136640, Ps_136968 and Ps_136971) were retrieved from http://genome.jgi-psf.org/. Other sequences were retrieved from Genbank: MAIZE (*Zea mays*; accession number P16243); ORYSA (*Oryza sativa*, P43279 and NP_001064998); FLAPR (*Flaveria pringlei*, P36444); HOMSA (*Homo sapiens*, AAA36197); DICDI (*Dictyostelium discoideum*, AAQ95658.1); NEOFR (*Neocallimastix frontalis*, AAC49572); SOLTU (*Solanum tuberosum*, P37225); MUCCI (*Mucor circinelloides*, AAO26053.1). Subcellular location is indicated as described at NCBI or as predicted by psort

previously not considered faint isozyme band with slightly higher mobility was observed in *P. ramorum* strains (Fig. 1b). This band was initially interpreted as a staining artefact since the simultaneous presence of two *Mdhp* isozyme bands has no precedent in 15 other *Phytophthora* species (Oudemans and Coffey 1991a, b). However, upon reconsideration, we favour the view that this faint band should be considered as the product of a second *Mdhp* gene model. The low sequence homology between the two paralogues (~40%) most likely prevents the formation of dimers resulting from the two genes. The absence of a second distinguishable *Mdhp* band in 15 species could either mean that a second gene is not present in those species, or that there is comigration of gene products, a phenomenon that was previously observed for two different *Mdhp* dimers in *P. nicotianae* (Man in 't Veld et al. 1998). Alternatively, the visibility of weak bands may have been seriously hampered by diffusion. Diffusion increases with increasing mobility, especially on starch gels as used by Oudemans and Coffey (1991a, b). In this study, gradient gels were used that diminished the diffusion problem. Occasionally two *Mdhp* bands, even equally strong, have been observed on gradient gels in other species e.g. *P. cactorum*, *P. citricola* and *P. syringae* (unpublished results).

One *Pgd* gene model explains one *Pgd* locus

We used a *Pgd* DNA sequence of *P. infestans* (Andersson and Roger 2002) to screen the *Phytophthora* genomes. In both, *P. ramorum* and *P. sojae* one *Pgd* gene model was identified (Table 2) and one *P. sojae* EST was found. The similarity between the *Phytophthora* PGD proteins is high, above 98%. One *Pgd* gene model in *P. ramorum* explains the one *Pgd* locus found by isozyme analysis (this study). However, in several *Phytophthora* species PGD isozyme profiling revealed two zones of activity and those were assigned to two putative loci. This was the case, e.g., in, *P. capsici*, *P. citrophthora*, *P. megakarya*, *P. nicotianae* and *P. palmivora* (Oudemans and Coffey 1991a). Since no intermediate bands were observed the genes at those two loci seem to have low sequence homology thus preventing dimerization. Possibly the second gene was acquired by endosymbiosis in an ancestral species but subsequently lost in *P. ramorum* and *P. sojae*. The species known to have two putative *Pgd* loci are in different clades from those comprising *P. sojae* or *P. ramorum* (Kroon et al. 2004a).

One *Gpi* gene model suggests one *Gpi* locus

BLAST analysis revealed the presence of only one *Gpi* gene model in *P. ramorum* (Table 2). It is highly homologous to a *Gpi* gene cloned from *P. infestans* (95% similar). As in *P. infestans* (Ospina-Giraldo and Jones 2003), the

gene has no introns, it encodes a protein of 557 AA and is located adjacent to a gene encoding a protein with putative *S*-adenosylmethionine-dependent methyltransferase activity (Pr_93719). Also in *P. sojae*, one gene model was identified (Ps_162909) and, based on EST data, this gene is expressed. The neighbouring genes resemble those found in *P. infestans* and *P. ramorum* indicating conserved synteny between the three *Phytophthora* species.

The identification of only a single *Gpi* gene model in *P. ramorum* points to the existence of only one putative isozyme locus with two different alleles, one of them being a null-allele (Richardson et al. 1986). The two banded GPI isozyme pattern that we find in *P. ramorum* (this study) is comparable with GPI profiles observed in several other *Phytophthora* species (Tooley et al. 1985; Oudemans and Coffey 1991a; Goodwin et al. 1995; Ospina-Giraldo and Jones 2003). *Gpi* genes have been cloned from a number of *Phytophthora* species and strains, and in some *P. infestans* strains, multiple alleles were identified (Ospina-Giraldo and Jones 2003) and cloned (M.D. Ospina-Giraldo, personal communication). For a long time GPI isozyme analysis in combination with peptidase (PEP) isozyme analysis was a preferred method for characterizing *P. infestans* strains and populations (Tooley et al. 1985; Spielman et al. 1991; Goodwin et al. 1995). In those studies at least six different genotypes were revealed (Tooley et al. 1985; Bakonyi et al. 2002).

Ldh genes belong to a multigene family and are clustered

Mining the genome sequences revealed multiple *Ldh* gene models in *P. ramorum* and *P. sojae* (Table 2). In all cases the encoded proteins belong to the D-lactate dehydrogenase family (EC 1.1.1.28). With the exception of one gene model in *P. ramorum* (Pr_46278), the *Ldh* genes are positioned in clusters spanning 25 kb in each of the two species (Fig. 5). The order and the orientation of the *Ldh* orthologues and the neighbouring genes is conserved in the two species (Fig. 5). This kind of conserved synteny has been described for several other gene families that are clustered in *Phytophthora* (Costanzo et al. 2006; Jiang et al. 2006a, b; Meijer and Govers 2006; Meijer et al. 2006). Two of the six *Ldh* paralogues identified in *P. ramorum*, are most likely pseudogenes (Pr_76507 and Pr_71423) and one is not full length. Pr_46278 is located at the start of a small scaffold and the sequence assembly is not complete. This gene model is actually the only one that, in the present assembly, is not located in the cluster. This could be due to assembly artefacts although genome rearrangement or transposition cannot be excluded. One *P. sojae Ldh* gene model is truncated and probably a pseudogene (Ps_109083). The other five encode proteins of 333 AA, consistent with the lengths of many LDHs deposited in Genbank. Nearly all *P. sojae Ldh* genes are expressed (not shown). *Phytophthora Ldh* genes are most related to bacterial *Ldh* genes and to a lower extent to those from higher eukaryotes.

Considering the high sequence homology between the different *Ldh* genes and the similarity in length, complex LDH isozyme patterns are not surprising. However, the monomeric or dimeric nature of a particular enzyme cannot be deduced from the DNA sequence and therefore it is not possible to assign particular *Ldh* gene models to the putative loci *Ldh*1, *Ldh*-2 or *Ldh*-3. Three of the *Ldh* gene models could be responsible for the five banded *Ldh*-2 pattern consisting of dimeric enzymes but with the same reasoning four gene models are needed to explain the four banded *Ldh*3 pattern consisting of monomeric enzymes and one

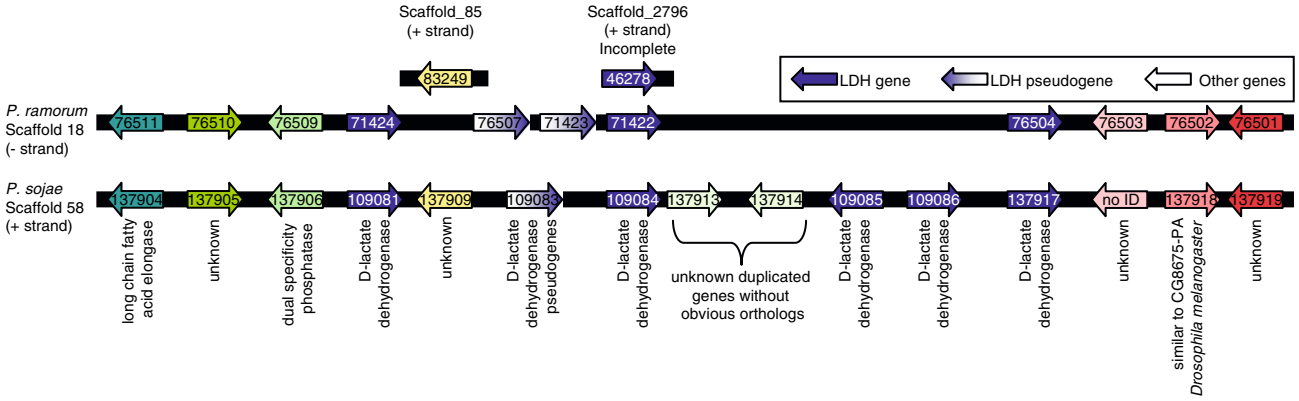

Fig. 5 Schematic representation of genomic regions in *P. ramorum* and *P. sojae* with LDH gene clusters. Depicted are regions with conserved synteny on scaffold 18 of *P. ramorum* and scaffold 58 of *P. sojae*. Orthologous gene models are aligned. The orthologue of *P. sojae* gene model Ps_137909 is located on a different scaffold in *P. ramorum* (Pr_83249 on scaffold_85). One *P. ramorum Ldh* gene model, Pr_46278 which is the closest homologue of Ps_109084 and nearly identical to Pr_71422, is not part of the gene cluster on scaffold_18 but located on a different scaffold (scaffold_2796). The gene models and scaffolds are not drawn on scale

additional gene model for the monomeric band at the *Ldh*-1 putative locus. Since two of the six gene models in *P. ramorum* are likely pseudogenes it is difficult to explain how the *Ldh* gene family can be responsible for the complex isozyme pattern. The different monomeric bands representing *Ldh*-1 or *Ldh*-3 could be the result of post-translational modifications but also staining artefacts cannot be excluded. So-called "nothing" dehydrogenases, for example representing alcohol dehydrogenase activity, are known to appear upon LDH staining as artefacts (Richardson et al. 1986). Several of the *P. ramorum* strains showed a three-banded *Ldh*-2 pattern consistent with two different alleles, whereas a five-banded pattern is consistent with three different alleles (assuming that the middle band is a superposition of the homodimeric BB band and the heterodimeric AC band, Fig. 2). Possibly, expression of one of the alleles ($Ldh\text{-}2^{C}$) sometimes fails for obscure reasons; these bands are often weak suggesting low expression. In conclusion, the available *Ldh* gene models in *P. ramorum* could not be linked unambiguously to a specific isozyme locus.

Incidentally, *P. brassicae* (Man in 't Veld et al. 2002a) and *P. syringae* (unpublished results) showed a five banded *Ldh*-2 pattern similar to the patterns found in this study in some of the *P. ramorum* strains. This suggests a considerably high level of conservation of the *Ldh* genes between species and probably also conserved synteny as seen in *P. ramorum* and *P. sojae*. One should be cautious however, with interpreting five-banded isozyme patterns. The five-banded *Gpi* isozyme patterns observed in some *P. infestans* strains upon staining for GPI activity appear to be caused by trisomy. This was demonstrated in sexual progeny in which segregation of the *Gpi* isozyme patterns was analysed (Goodwin et al. 1992) and is consistent with the existence of three different sequences (different alleles) of the *P. infestans Gpi* gene (Ospina-Giraldo and Jones 2003). Trisomy is not uncommon in *P. infestans* and it complicates genetic analyses in this species (Van der Lee et al. 2004).

Discussion

Isozyme genotyping of *P. ramorum* strains originating from Europe and North America revealed a remarkable genetic homogeneity at eight isozyme loci, confirming that they are indeed conspecific. Although some variation was detected at the *Ldh*-2 locus throughout the population the selected isozymes were not suitable to detect the level of variation as revealed by AFLP and microsatellite markers (Ivors et al. 2004, 2006). With these isozymes it is, however, possible to delineate *P. ramorum* from the closely related species *P. lateralis* and *P. hibernalis*. These species have different alleles at both the *Mdh*-2 locus and the *Mdhp* locus, but share alleles at the three putative loci, *Ldh*-1 (*P. lateralis*), *Ldh*-2 and *Ldh*-3. These isozyme patterns confirm the differences between these species as revealed by ITS sequence analysis (Ivors et al. 2004).

For several of the isozymes (GPI, MDH, MDHP and PGD) an unambiguous correlation was found with the gene models deduced from the genome sequence of *P. ramorum*. Six *Ldh* gene models were identified in *P. ramorum*. Even though the existence of a small *Ldh* gene family likely explains the complex isozyme patterns found upon LDH staining it is not possible to assign particular *Ldh* gene models to specific isozyme bands.

For various studies dealing with population analysis of *Phytophthora* isozyme profiling has proven to be a very valuable tool (Tooley et al. 1985; Oudemans and Coffey 1991a; Man in 't Veld et al. 2002a; Ospina-Giraldo and Jones 2003). The same is true for deciphering the hybrid nature of emerging *Phytophthora* species (Man in 't Veld et al. 1998, 2007). In this study, we investigated correlations between isozyme patterns and genome sequences in *P. ramorum*, and made attempts to link phenotypes with gene models. A serious limitation of linking genomic sequences to isozyme bands is encountered when multiple zones of activity are found, assigned to multiple putative loci (e.g. LDH, MDH and MDHP). In these cases, particular genomic sequences cannot be assigned to a specific isozyme locus and additional experiments are needed to clarify this.

The high level of colinearity that is described for *P. ramorum* and *P. sojae* (Tyler et al. 2006) is also observed with other *Phytophthora* species, at least for a range of large random genomic regions that have been investigated so far (Jiang et al. 2006b; Meijer et al. unpublished). Although the analyses presented here will contribute to improve the interpretation of isozyme patterns in a much wider range of *Phytophthora* species than just *P. ramorum*, one has to remain cautious with interpreting isozyme patterns in one *Phytophthora* species based on gene models found in another. The two *P. ramorum Mdhp* genes, for example, are duplicated in *P. sojae* and this could easily result in a more complex isozyme patterns involving superposition of different gene products which are difficult to explain by two gene models. Another example of gene duplication is the elongation factor EF-1α gene. Based on southern blot analysis, this gene was reported to be single copy in *P. infestans* (Van't Klooster et al. 2000). However, in both *P. ramorum*, and *P. sojae* (and also *P. infestans*, unpublished results) two EF-1α gene models are found.

In conclusion, the present study demonstrated that the number of gene models found in *P. ramorum* for GPI, MDH, MDHP and PGD fitted well with the observed isozyme loci (1, 2, 2, and 1, respectively), For LDH, however,

several highly homologous gene models were found but it was not possible to explain all observed LDH isozyme variation. In addition, the investigations have shown that multiple sequences, encoding a particular isozyme, originate from different sources: gene duplication (LDH, EF-1a), endosymbiosis related events (MDH and MDHP) or even both (MDHP in *P. sojae*) but no indication was found for recent hybridization events. A limitation to this research is the lack of genomic sequences of multiple *Phytophthora* isolates. If those become available, it may be possible to correlate DNA polymorphisms with isozyme variation.

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