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Phytophthora ramorum

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Phytophthora ramorum

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Sydney E. Everhart, Javier F. Tabima, and Niklaus J. Grünwald

8.1 Introduction

8.1.1 Sudden Oak Death and Ramorum Blight

Phytophthora ramorum is a recently emerged plant pathogen and causal agent of one of the most destructive and devastating diseases currently affecting US horticulture and forests (Rizzo et al. 2002, 2005). This oomycete pathogen was discovered in Marin County, California, in the mid-1990s, causing sudden oak death on coast live oak (*Quercus agrifolia*) and tanoak (*Notholithocarpus densiflorus*) and simultaneously discovered in Europe causing foliar blight on *Rhododendron* and *Viburnum* (Rizzo et al. 2002; Werres et al. 2001). It is now known to affect more than 100 plant species, including economically important nursery and forest host species (Frankel 2008; Rizzo et al. 2005; Tooley et al. 2004; Tooley and Kyde 2007).

This pathogen has two distinct disease symptom classes (Grünwald et al. 2008). On some plant species, mostly woody ornamentals such as *Viburnum* and *Rhododendron*, symptoms are

nonlethal and show foliar or twig blight, which allow prolific production of aerial sporangia. In contrast, on coast live oak, tanoak, and Japanese larch (*Larix kaempferi*), infections can be lethal and include bleeding bole cankers (Fig. 8.1). Sudden oak death has resulted in about 80 % mortality of tanoaks in portions of the Los Padres National Forest in California, killing 119,000 tanoaks across approximately 3,200 ha (Rizzo et al. 2005). The high mortality of dominant oak species and foliar blight of understory shrubs has permanently altered natural forest ecosystems in the western US. Until recently, tree infections in Europe have been comparatively rare and primarily affected native beech and non-native oak species, however an outbreak in 2009 caused widespread mortality of Japanese larch in plantations in SW England. By 2010, an outbreak in Wales led to approximately half a million trees felled over 1,300 ha, and by 2013 the most recent surveys indicate the outbreak has expanded to an additional 1,800 ha of newly infected trees in south Wales (COMTF 2013).

Infested nursery plants offer a very effective means of dispersing the pathogen. This has happened twice in the United States with shipments of infected camellias from California that resulted in 1.6 million potentially infected plants detected in 175 infested sites in over 20 states (see California Oak Mortality Task Force web site <http://www.suddenoakdeath.org> for current status). State, national, and international quarantines have been imposed on all host plant species grown in affected areas, including eradication of affected

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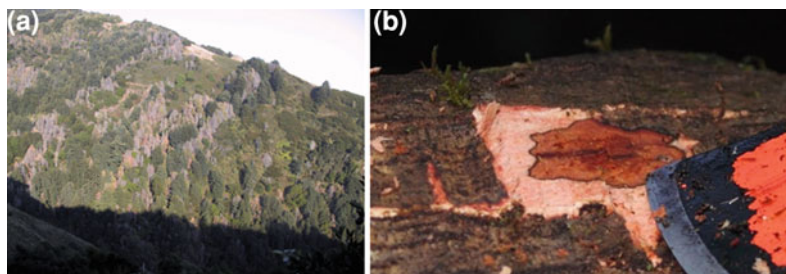


Fig. 8.1 **a** Sudden oak death epidemic on tanoak in Marin County, California (courtesy S. Frankel). **b** Sudden oak death symptoms showing necrosis found beneath

bleeding cankers produced on tanoak in native forests (courtesy S. Everhart)

nursery stock. Similarly, *P. ramorum* has been reported in 21 European countries, where emergency phytosanitary measures have been implemented since 2002 for member countries of the European Union (Walters et al. 2009). These trade regulations and phytosanitary measures can directly impact commercial nurseries and retailers. For example, horticultural nurseries across the US have lost millions of dollars from destruction of infected stock and suffer further losses from disrupted and lost markets. Furthermore, combining direct loss of nursery and ornamental crops, decrease in property values with dead/dying trees, and the costs of disease tracking and management, total economic losses are in the tens of millions of dollars (Cave et al. 2005; Frankel 2008). Risk analysis for the US has shown that if the pathogen spreads to forests on the East Coast as well as into new production systems on the West Coast, the economic impact of quarantine regulations on trade in conifer and hardwood products, logs, Christmas trees, and tree seedlings will be even greater (Cave et al. 2005; Frankel 2008). Rapid spread of disease since the mid-1990s and simultaneous discovery in Europe led to intensive research efforts to characterize the pathogen, which resulted in whole-genome sequencing only 3 years after formal description of the pathogen in 2001.

8.1.2 Taxonomy

Formally described in 2001, *P. ramorum* is a filamentous, diploid protozoan that is one of 117

currently recognized *Phytophthora* species commonly known as water molds. *Phytophthora* species are in the phylum Oomycota, which includes several notable plant pathogens, such as *Phytophthora infestans*, *P. sojae*, *P. cinnamomi*, *P. capsici*, and *Pythium* spp. This group is a member of the Stramenopiles and is most closely related to the golden-brown algae and diatoms. Current phylogenetic analysis of *Phytophthora* species based on seven nuclear loci places *P. ramorum* in clade 8c (Blair et al. 2008). The other close relatives of *P. ramorum* in clade 8c include *P. lateralis* that causes root rot of Port Orford-cedar (*Chamaecyparis lawsoniana*), *P. hibernalis* that primarily causes citrus brown rot and leaf/twig blight, and *P. foliorum* that causes leaf spot of *Rhododendron* spp. (Fig. 8.2). Although the origin of *P. ramorum* is unknown, the discovery of *P. lateralis* in Taiwan suggests that the origin of this clade may be in Eastern Asia (Brasier et al. 2010), but more direct evidence in support of this hypothesis is needed.

8.1.3 Life Cycle

In nature, the life cycle of *P. ramorum* currently includes only an asexual phase although a sexual phase is theoretically possible, where asexual spores include sporangia and chlamydospores and the sexual phase yields oospores. Asexual sporangia are produced from infected leaf tissue and germinate directly, producing a germ tube and appressorium to infect the host tissue, or indirectly by release of zoospores in the presence

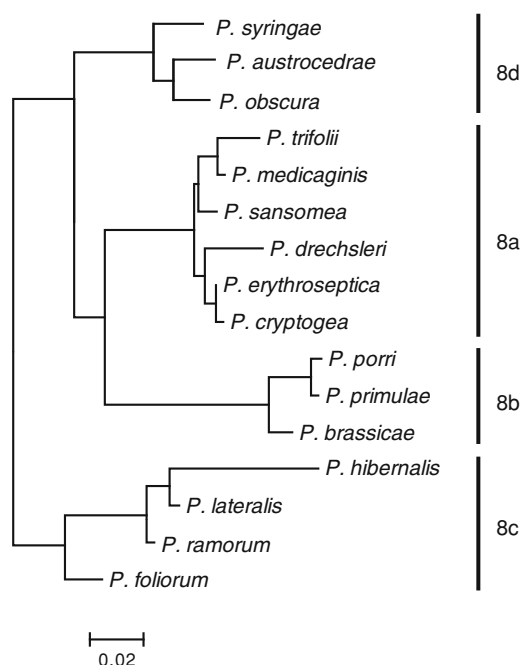


Fig. 8.2 Maximum likelihood phylogeny for *Phytophthora* clade 8 based on ITS sequence (adapted from Grünwald et al. 2012b). *Phytophthora ramorum* is a clade 8c taxon with *P. lateralis*, *P. hibernalis*, and *P. foliorum* as sister-taxa

of free water. *P. ramorum* readily produces thick-walled chlamydospores that can serve both as primary inoculum and provide a means of surviving adverse conditions such as drought. When favorable conditions return, the chlamydospore will germinate directly to infect the host or produce sporangia. *P. ramorum* is readily distinguished from other *Phytophthora* species through its formation of abundant, large chlamydospores (Werres et al. 2001). *P. ramorum* is heterothallic (out-crossing) and sexual mating in the laboratory, thus requires contact between individuals of compatible mating types, A1 and A2. Based on knowledge about other species, we expect that pheromones released by the opposite mating type induce formation of mating structures, oogonia and antheridia. Fertilization of oogonia by antheridia, followed by karyogamy, would lead to the development of oospores; however, this final process is not observed in nature. This species is currently considered solely clonal in its known range. Molecular evidence of sexual

recombination has not been detected and laboratory pairings among different mating types only leads to occasional formation of gametangia and, where meiosis has occurred, leads to aneuploid progeny of reduced fitness (Boutet et al. 2010, Vercauteren et al. 2011, Van Poucke et al. 2012). Oospores produced under controlled laboratory conditions appear aberrant and alleles do not segregate in a Mendelian fashion (Boutet et al. 2010; Brasier and Kirk 2004; Vercauteren et al. 2011).

8.1.4 Genetics

Like many *Phytophthora* species, *P. ramorum* is a diploid, clonal organism. There are four genetically and phenotypically distinct clonal lineages of *P. ramorum* (NA1, NA2, EU1, and EU2) that were named after the continent (North America or Europe) where each was first detected. Genetic variation that discriminates the four clonal lineages include AFLP, SSR microsatellites, and SNPs, whereas ITS and isozyme profiles are indistinct, consistent with the conspecific nature of the lineages. Phenotypic traits associated with fitness, such as growth rate, aggressiveness, and colony stability have been shown to vary with respect to lineage, where EU1 and NA2 are considered more aggressive than NA1, which also showed uniform and irregular growth types that are also called non-wild type (*nwt*) or senescent phenotypes (Braiser et al. 2006; Elliott et al. 2011).

In North America, three lineages are present, where NA1 genotypes are by far the most frequent and diverse in the US (Goss et al. 2009a, b; Ivors et al. 2006; Ivors et al. 2004; Mascheretti et al. 2008; Prospero et al. 2007, 2009). In Europe, the pathogen exists as the EU1 clonal lineage (Ivors et al. 2006; Vercauteren et al. 2010) and more recently, a fourth clonal lineage, EU2, was identified in Northern Ireland and subsequently in Western Scotland, causing epidemic disease on Japanese larch trees (*Larix kaempferi*; Van Poucke et al. 2012). Population genetic analyses using microsatellite markers have been used to characterize population

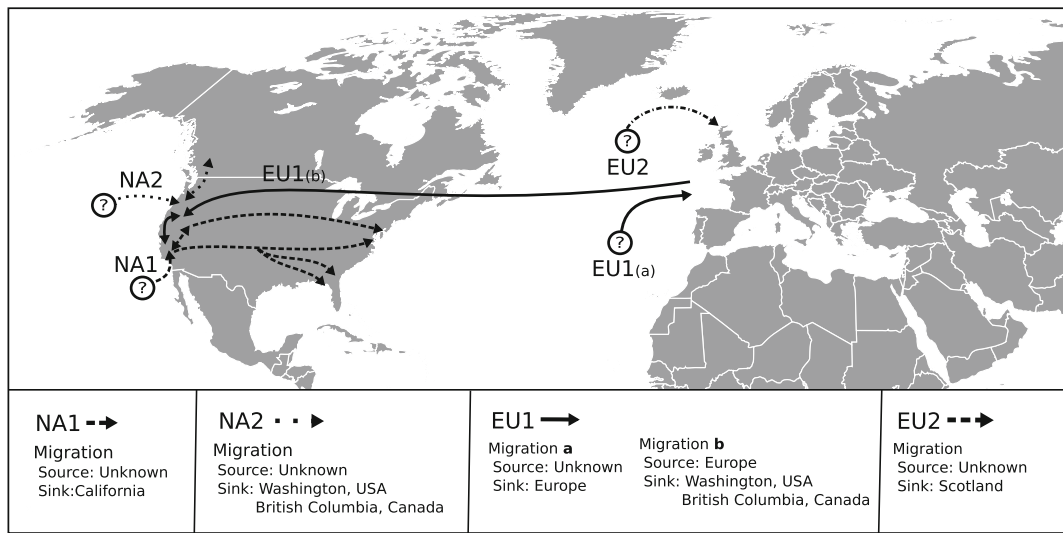


Fig. 8.3 Scenarios depicting repeated emergence and migration of the sudden oak death pathogen *P. ramorum* (after Grünwald et al. 2012). Five intercontinental migrations of *P. ramorum* are supported by population genetic and evolutionary studies. Shown are the most

likely scenarios for repeated introduction of the three known clonal lineages *NA1* and *NA2* into North America, *EU1* into North America and Europe, and *EU2* into Europe

structure and migration of the pathogen in North America and Europe (Fig. 8.3). Microsatellite genetic markers have been used to examine the population structure in the United States after a multi-state outbreak in 2004, with supporting evidence provided by trace-forward shipping records (Goss et al. 2009b), and also used to determine the direction and rate of migration within North America and from Europe by inferring migration using coalescent and Bayesian approaches (Goss et al. 2011). Finally, although the population structure of the *EU2* lineage has not been explored, the relatively lower amount of genetic variation as compared to the *EU1* lineage suggests that introduction has been more recent (Van Poucke et al. 2012).

8.2 Genome

8.2.1 Genome Structure

Whole-genome shotgun sequencing was performed on strain Pr-102 (ATCC MYA-2949). This strain was isolated in 2004 from a coast live

oak in Marin County, CA, and was selected because it had the same multilocus microsatellite genotype as the majority of strains genotyped previously (Ivors et al. 2004). Sequence data was generated from a combination of paired-end reads of small and medium insert plasmids (2–4 and 8 kb) and large insert fosmids (36 kb), with an estimated sequence depth of 7.7 fold coverage (Tyler et al. 2006). Over 1 million reads were assembled with the JGI assembler, Jazz, which formed 2,576 scaffolds ($N_{50} = 308$ kb) with total scaffold length of 66.7 Mb, and is available for download via the JGI website for *P. ramorum* (http://genome.jgi-psf.org/Phyral_1/Phyral_1.info.html).

The number of chromosomes in *P. ramorum* is unknown, but the genome structure of *P. ramorum* is similar to that of other *Phytophthora* spp., with a large portion of conserved, syntenic, gene-dense regions (Haas et al. 2009). Compared to *P. sojae* and *P. infestans*, there are 8,492 orthologous clusters in the *P. ramorum* genome that contains 9,664 genes that are orthologues or close paralogues, of which, 7,113 are strict orthologues (1:1:1), thus comprising a

core genome. This region of the genome encodes genes involved in cellular processes (i.e., DNA replication, transcription, and protein translation), with relatively fewer related to defense mechanisms (Haas et al. 2009). Unique regions of the genome, outside of the core genome, are thought to be related to host specificity and the lifestyle of the pathogen.

8.2.2 Repetitive DNA

The genomes of *Phytophthora* spp. are known to have highly repetitive regions, where the amount of repetitive DNA is correlated with the size of the genome. For example, *P. ramorum* has approximately 28 % repetitive DNA and a smaller genome as compared to *P. sojae* and *P. infestans* that have 39 and 74 % repetitive DNA, respectively (Haas et al. 2009). The amount of repetitive DNA is also unequally distributed throughout the genome, with a greater percentage of repeats found outside collinear blocks, thus suggesting that the genome size difference among species is largely due to repeat-driven genome expansion in these gene-sparse regions. Indeed, further investigations into the most likely mechanism of dramatic genome expansion in *P. infestans* showed these regions were enriched for transposons, where a striking 29 % of the genome was identified as corresponding to Gypsy-element type retrotransposons (Haas et al. 2009). The majority of these sequences show high similarity, suggesting a high rate of recent activity that has also been demonstrated experimentally (Haas et al. 2009). In addition, the most widespread of these retrotransposons shows similar GC content and codon usage with genes in *Phytophthora* species. For example, the codon usage of three dominant retrotransposons (*CopiaPr-1*, *GypsyPr-0*, and *GypsyPr-2*) showed a positive correlation with codon usage estimated from 10,000 open reading frames (ORFs) randomly selected from the *P. ramorum* genome. Codon usage is influenced by mutational bias and selection pressure, thus suggesting that genome invasion by these retrotransposons was

an ancient event, prior to the divergence of *P. ramorum*, *P. sojae*, and *P. infestans* (Jiang and Govers 2006).

Interestingly, gene families encoding host-defense related effector proteins (specifically the RxLR and CRN gene families) show correlation in the number of genes and size of the flanking intergenic regions, with retrotransposons flanking RxLR-type effectors significantly more frequently than average genes (Jiang et al. 2008; Haas et al. 2009). Further research examining the evolutionary dynamics of genes within these highly repetitive regions has shown that effector genes in the *P. ramorum* genome have the expected rapid birth–death rate expected from other well-studied genomes (Goss et al. 2009a; Grünwald and Goss 2009; Tyler et al. 2006). Evidence suggests that repetitive regions in both fungal and oomycete genomes provide evolutionary advantages including, for example, more rapid adaptation to new environments or conditions as well as rapid recombination and reshuffling of existing domains into genes with novel or revised functions (Raffaele and Kamoun 2012; Haas et al. 2009).

8.2.3 Comparison with Other Oomycete Genomes

To date, seven oomycete plant pathogen genomes have been sequenced and published, including *P. ramorum*, *P. infestans*, *P. sojae*, *P. capsici*, *Pythium ultimum*, *Albugo candida*, and *Hyaloperonospora arabidopsidis* (Table 8.1). Genome sizes range from 42.8 to 240 Mb, where *P. infestans* has the largest genome, roughly four times the size of *P. ramorum*. The number of predicted genes has substantially less variation across species, ranging from approximately 14.5–19 k genes, while most of the genome plasticity is found in the repetitive, gene-sparse regions of the genomes.

Notable features of the *P. ramorum* genome are evidenced in comparisons with other sequenced *Phytophthora* species. Pairwise comparison with *P. sojae* and *P. infestans* showed

Table 8.1 Comparison of features for Stramenopile genomes sequenced to date

Oomycete plant pathogens	Lifestyle	Mating system	Genome size (Mb)	Predicted genes	Repetitive sequence (%)	Effector proteins		Citation
						RxLR	CRN	
<i>Phytophthora ramorum</i> Sudden oak death and foliar blight	Hemibiotroph	Out-crossing	65	15,743	28	350	19	Tyler et al. (2006)
<i>Phytophthora sojae</i> Root/stem rot of soybean	Hemibiotroph	Selfing	95	19,027	39	350	100	Tyler et al. (2006)
<i>Phytophthora infestans</i> Late blight of potato	Hemibiotroph	Out-crossing	240	17,797	74	563	196	Haas et al. (2009)
<i>Phytophthora capsici</i> Phytophthora blight	Hemibiotroph	Out-crossing	64	17,123	19	357	29	Lamour et al. (2012)
<i>Pythium ultimum</i> Damping off	Necrotroph	Selfing (?)	42.8	15,323	7	0	26	Lévesque et al. (2010)
<i>Hyaloperonospora arabidopsidis</i> Downy mildew of <i>Arabidopsis</i>	Biotroph	Selfing	100	14,543	42	134	20	Baxter et al. (2010)
<i>Albugo candida</i> White rust	Biotroph	Selfing	45.3	15,824	17	26	6	Links et al. (2011)
Sister-taxa in the Stramenopiles								
<i>Thalassiosira pseudonana</i> Diatom	Marine phytoplankton	Unknown	34	11,242	2	0	0	Armbrust et al. (2004)
<i>Phaeodactylum tricornutum</i> Diatom	Marine phytoplankton	Unknown	27.4	10,402	5	0	0	Bowler et al. (2008)

approximately 37 Mb of the *P. ramorum* genome falls into collinear blocks (Haas et al. 2009). These collinear regions have higher gene density (median intergenic spacing 633 bp inside vs. 1.5 kb outside collinear blocks) and fewer repetitive elements (13 vs. 56 %). Despite several local rearrangements and non-orthologous genes, *P. ramorum* was found to have an expanded/diverse NPP1 gene family with 40 genes, whereas several fungal plant pathogens contain only 2–4 genes (Tyler et al. 2006). In contrast to RxLR and CRN proteins that function during the biotrophic phase of invasion, NPP1 proteins induce cell death and are part of the necrotrophic phase. *Phytophthora ramorum* is a hemibiotroph, which means that the initial infection process involves host invasion, followed by a switch to necrotrophy after establishment. This dual-form lifestyle was evidenced in the genome in the form of genes encoding cell-wall degrading enzymes (hydrolases and proteases) and may also be responsible for the diversity of the NPP1 gene family.

Phytophthora ramorum has more than 27 times the number of heterozygous single nucleotide polymorphic sites as *P. sojae*, which has only 499. This is likely attributed to their differing modes of reproduction, where *P. ramorum* is at least ancestrally out-crossing (heterothallic) and *P. sojae* is inbreeding (homothallic; Tyler et al. 2006; Goss et al. 2009a).

8.2.4 Evolutionary History and Clonality

Phytophthora ramorum emerged only recently as a plant pathogen in the Western hemisphere, but evolutionary analysis of the known clonal lineages indicates an ancient divergence from ancestrally sexual populations occurred approximately 150,000–500,000 years ago based on very crude assumptions given the lack of a molecular clock for oomycetes (Goss et al. 2009a). Both the European and US clonal lineages are gradually diverging from the ancestral, invasive NA1, NA2, or EU1 clones in a process of clonal divergence that is driven by the accumulation of random mutations and genetic drift, although selection

acting on adaptive mutations while not documented to date might also be active (Goss et al. 2009a, 2011; Vercauteren et al. 2010; Fig. 8.3). This pattern is apparent in all lineages as a result of using hypervariable loci for genotyping but is most prominent for NA1, possibly due to the larger population size (Goss et al. 2009a, 2011; Mascheretti et al. 2008, 2009; Prospero et al. 2007, 2009; Vercauteren et al. 2010). Despite the emergence of new genotypes in forest ecosystems, there is no clear evidence that selection is favoring any genotypes in established infestations, where dominant genotypes have remained unchanged over the last 10 years and minor genotypes emerge and disappear stochastically due to drift (Goss et al. 2009b). Changes in dominance of genotypes in new forest and nursery infestations have been documented and have been ascribed to founder effects during establishment, followed by genetic drift (Goss et al. 2009a; Mascheretti et al. 2009; Prospero et al. 2007).

8.2.5 Effectors

Phytophthora genomes have several classes of genes known as effectors that are involved in host-pathogen coevolution and provide signatures of selection (Kamoun 2006; Tyler 2009; Tyler et al. 2006). Effectors are small, secreted proteins produced by the pathogen that target host plant molecules and/or alter host plant processes. General types of effectors include cytoplasmic effectors, such as the RxLR and CRN (“crinkler”) gene families and apoplastic effectors that include several hydrolytic enzymes (proteases, lipases, glycosylases, enzyme inhibitors for host defenses, and necrotizing toxins). These families of proteins were discovered after availability of whole-genome sequences and since then have generated a large amount of scientific interest and research in effector biology.

The RxLR proteins, a superfamily of approximately 350 genes in the *P. ramorum* genome, is the largest class of host-translocated proteins and are typically found in areas of the genome that are highly repetitive. The amino acids motif RxLR (Arg-X-Leu-Arg) and in some

but not all cases dEER (Asp-Glu-Glu-Arg, with variability in the Asp) located in the N-terminal region of the proteins are highly conserved and expected signatures of this class of effectors (Tyler et al. 2006). Members of this gene class have been cloned and validated to be avirulence genes corresponding to host R genes, including *Avr1b-1* from *P. sojae* (Shan et al. 2004), *Avr3a* from *P. infestans* (Armstrong et al. 2005), and *ATR1* (Rehmany et al. 2005) and *ATR13* (Allen et al. 2004) from *H. arabidopsidis*. Figure 8.4 shows the species-specific birth and death process typically observed for effectors that are rapidly coevolving in an evolutionary arms race. While 35 effectors are conserved across *P. infestans*, *P. sojae*, and *P. ramorum*, individual clades show species-specific expansions that are thought to be the result of recombination and gene duplication.

Another group of important host-translocated effector proteins belong to the crinkler gene

superfamily (CRN), which received this name because these proteins were first shown to cause crinkling and necrosis of leaves when over-expressed in the plant *Nicotiana benthamiana* (Torto et al. 2003). This group contains more than 60 different gene families found in all species of oomycetes and are characterized by the presence of a LXFLAK amino acid domain (Leu-X-Phe-Leu-Ala-Lys; Kamoun 2007; Schornack et al. 2010). These genes encode modular proteins that are translocated into the cytoplasm and localize to the nucleus of the host cell by the way of an N-terminal signaling peptide and subsequently induce various responses in the host cell through a functional interaction with the C-terminal peptide (Schornack et al. 2010; Stam et al. 2013). The function of the CRN genes is not well defined, where some are known to induce cell death and function to translocate proteins into the cell that perturb host nuclear processes. CRN genes are abundant and diverse within the genus

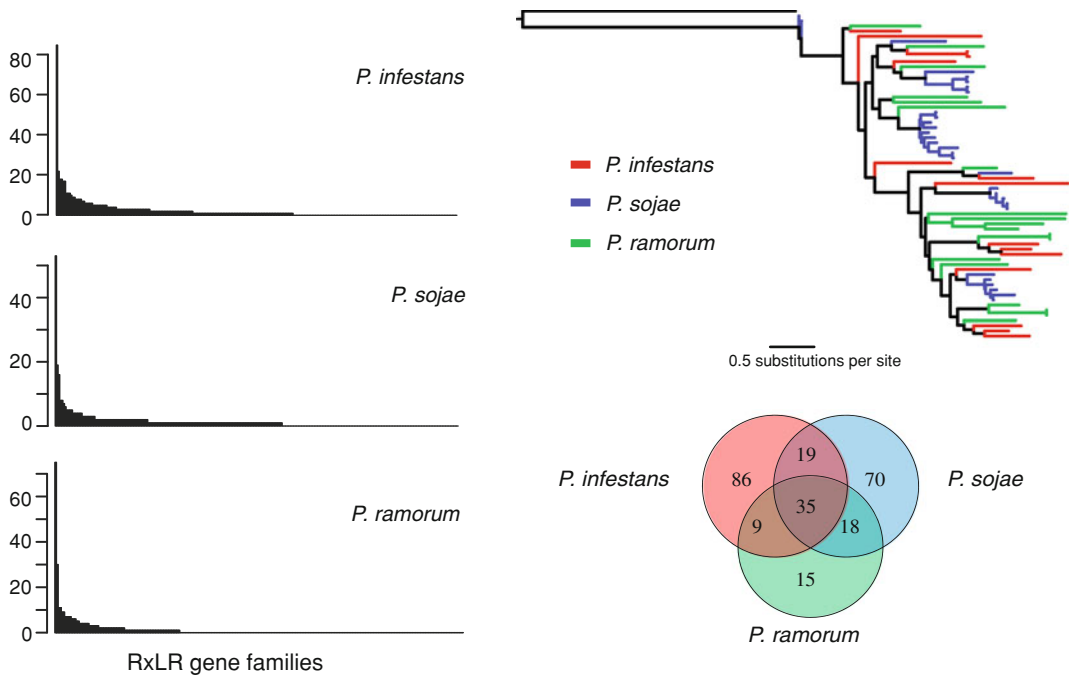


Fig. 8.4 RxLR family genes in *P. ramorum* (green) compared to *P. infestans* (red) and *P. sojae* (blue). The distribution of genes within RxLR gene families shows *P. ramorum* has a lower number of genes and RxLR families as compared to *P. infestans* and *P. sojae*. Phylogenetic analysis of the second most abundant RxLR

family (RxLR Family 2) shows a diverse relationship among genes belonging to each *Phytophthora* species. The number of unique and common families among the three species shows a core set of 35 genes that is shared and *P. ramorum* has the lowest proportion of unique RxLR gene families

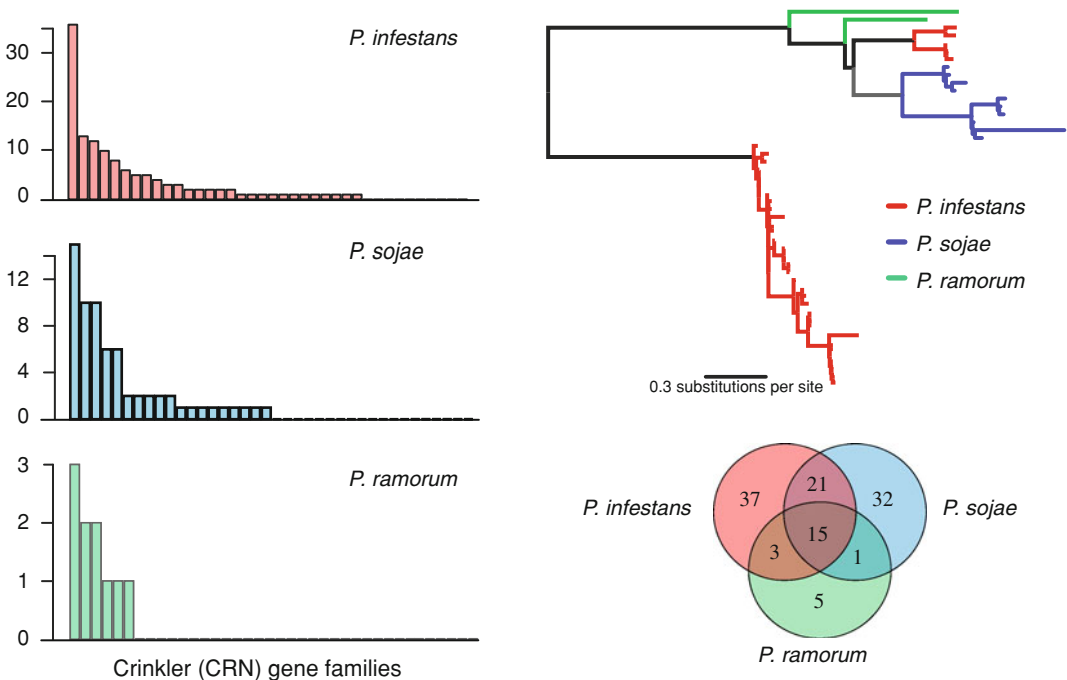


Fig. 8.5 Crinkler family genes in *P. ramorum* (green) compared to *P. infestans* (red) and *P. sojae* (blue). The distribution of genes within CRN gene families shows *P. ramorum* has fewer genes than *P. infestans* and *P. sojae*. Phylogenetic analysis of the second most abundant CRN family (LFLAKdom DWL DXZ CRN family) shows two

divergent sets of CRN family genes, where one branch of diverse CRN genes belong only to *P. infestans* and the other branch contains related genes found within all three species. There is a core set of 15 genes shared among these three species, where *P. ramorum* has only five genes not found in the other species

Phytophthora, with 76, 69, and 24 genes reported within *P. infestans*, *P. sojae*, and *P. ramorum*, respectively (Fig. 8.5). The origin of these genes, as shown in Fig. 8.5, likely first arose within a common ancestor and later independently diversified within each species. Interestingly, *P. infestans* shows a unique clade only observed in that species.

The necrosis inducing *Phytophthora* protein (NPP1) gene family is another group of effectors that have been described in *P. ramorum*. The NPP1 gene family was discovered in 2002 as a gene associated with triggering of plant defenses by *P. parasitica* (Fellbrich et al. 2002). These genes have been found in a variety of distantly related taxonomic groups, such as oomycetes, fungi, and bacteria, suggesting an ancient origin to this gene family. In *P. ramorum*, there are more NPP1 genes than in *P. infestans* and *P.*

sojae (Fig. 8.6). In contrast to RxLR and CRN proteins that function during the biotrophic phase of invasion, NPP1 proteins induce cell death and are part of the necrotrophic phase. Like the other effector gene classes NPP1 genes show an evolutionary signature of rapid birth–death differentiating species (Fig. 8.6).

8.2.6 Gene Expression

A striking amount of variation in colony morphology and virulence (lesion size) has been observed for *P. ramorum* in the NA1 lineage, even among isolates sharing the same multilocus genotype and lacking any known host adaptation (Huberli and Garbelotto 2011). One hypothesis to explain this is that *P. ramorum* is a generalist pathogen that responds to the host via epigenetic

NPP1 family genes

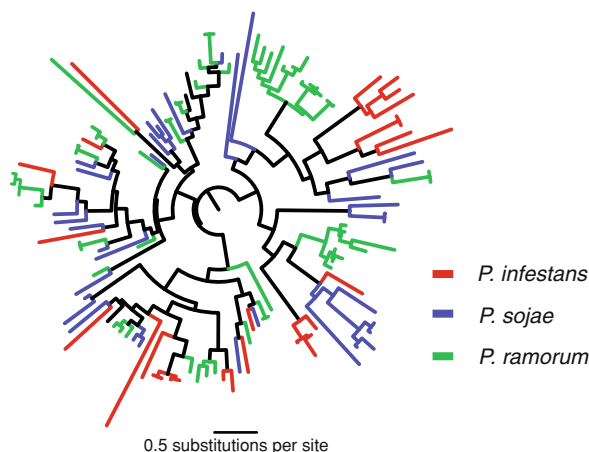
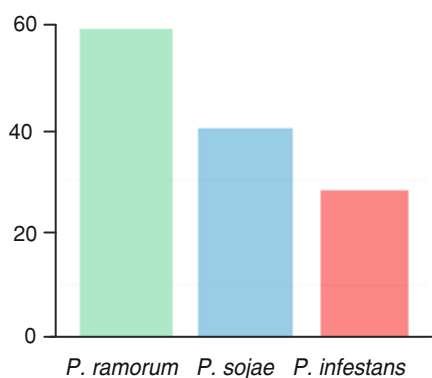


Fig. 8.6 NPP1 genes in *P. ramorum* compared to other *Phytophthora* species. *P. ramorum* (green) has more NPP1 genes than either *P. sojae* (blue) or *P. infestans* (red), with nearly twice as many NPP1 genes in *P.*

ramorum as *P. infestans*. Phylogenetic analysis of these genes shows a diverse relationship of NPP1 genes among the three *Phytophthora* species

regulation of gene expression, including virulence factors. To evaluate this, whole-genome expression was measured using a NimbleGen microarray designed with 61,963 probes for 15,488 gene models (approx. four probes per ORF), which was based on the published transcript of *P. ramorum* at the JGI website. Expression analysis of *P. ramorum* (NA1) following recovery from coast live oak, a dead-end host where infections are typically lethal and lead to little sporulation, was compared to the pathogen when recovered from bay laurel (*Umbellularia californica*), which is a transmissible host that yields prolific sporulation and is typically nonlethal to the host, showed significant difference in gene expression between isolates (Kasuga et al. 2012).

A total of 13 isolates were analyzed for whole-genome differential expression, where seven were from coast live oak and 6 were from bay laurel, with gene expression profiling performed on cultures grown in the lab, under dark conditions to reduce effects of circadian rhythm on gene expression. Results of gene expression profiling were first background corrected and normalized with a robust multi-array average (RMA) algorithm and then genes with low intensity were removed, thus resulting in a total

of 12,516 genes used for further analysis. Isolates recovered from coast live oak (dead-end host) showed reduced virulence when subsequently inoculated onto bay laurel (transmissible host) and many also showed a senescence phenotype (non-wildtype, *nwt*). This phenotypic profile is more often derived from isolates derived from dead-end hosts and among the seven isolates involved in transcription profiling, isolates with the senescent phenotype were hierarchically clustered in a different group from all other isolates. Furthermore, isolates derived from bay laurel showed up-regulation of five CRN genes, whereas isolates recovered from coast live oak showed over 454 up-regulated genes, with more than half (297) encoding transposable elements (transposon derepression). Epigenetic regulation of transposable elements is known in eukaryotes to function via RNA interference (RNAi), heterochromatin formation, and DNA/histone methylation (Whisson et al. 2012; Zeh et al. 2009). In *P. infestans*, these mechanisms have been explored, where RNA interference via small interfering RNAs (siRNA) and heterochromatin formation are currently seen as the predominant mechanisms (Ah-Fong et al. 2008; Judelson and Tani 2007; Van West et al. 2008).

8.3 Applications Resulting from the Genome

8.3.1 Molecular Markers

Various types of molecular markers have been developed as a result of the genome sequence that have been applied for identification and detection needs and also used to examine the population variation and disease epidemiology. Markers developed for identification and detection of *P. ramorum* were developed by several groups relying on different approaches and genic regions. For example, one assay relies on PCR amplification of the *coxI* and II genes for detection and discrimination of *P. ramorum* from two common, native *Phytophthora* spp., *P. nemorosa* and *P. syringae* (Martin et al. 2004). A more sensitive method of detection was developed using quantitative real-time PCR to amplify the ITS region (Tooley et al. 2004). Yet another group developed a nested approach for detection and discrimination using quantitative real-time PCR on the ITS region (Hayden et al. 2004, 2006). To determine the best methods for routine application by state and federal agencies, a large-scale comparison of protocols was performed and the best protocols emerging from this study are now used routinely for testing samples from nursery environments in federal and state laboratories (Martin et al. 2009).

8.3.2 Population Biology

Following genome sequencing of *P. ramorum* in 2004 (Tyler et al. 2006), multilocus analyses confirmed the presence of distinct clonal lineages, which are readily distinguished by all molecular marker systems employed to date including amplified fragment length polymorphisms (AFLP), simple sequence repeats (SSRs), and DNA sequences for mitochondrial or nuclear loci (Grünwald et al. 2009). Microsatellite markers (SSRs) were used most prominently to document sources of contaminated material and spread of the pathogen after its emergence.

Currently, the NA1 lineage is the most widespread in North America and microsatellite markers developed from the sequenced genome (also NA1) show more clonal variation (six of nine are polymorphic) than other North American lineages, thus enabling detailed population genetic analysis (Goss et al. 2009b). The NA1 clone was most likely introduced by importation of infected nursery host plants to a local nursery in Scotts Valley, CA in the late twentieth century from an unknown source population (Mascheretti et al. 2008, 2009; Fig. 8.3). However, it became clear that the emergence of the NA1 clonal lineage has distinct dynamics in forest and nursery environments. Forest populations are genetically indistinguishable at distances of over 50 km (Mascheretti et al. 2009), which is not plausible using only natural dispersal mechanisms of large airborne and splash-dispersed sporangia (Davidson et al. 2005; Mascheretti et al. 2008). Thus, the observed population structure was likely the result of human-assisted movement of the pathogen on plants or soil (including soil on shoes or vehicle tires; Mascheretti et al. 2009). From the initial introduction foci, *P. ramorum* spread rapidly to native tanoaks (*Notholithocarpus densiflorus*) and oaks (*Quercus* spp.) presumably through foliar infection of California bay laurel, a host on which the pathogen sporulates profusely, and led to the sudden oak death epidemic in forest ecosystems. In the decade since discovery, this clonal lineage has subsequently spread to the current limits of the sudden oak death epidemic up and down the California coast (Meentemeyer et al. 2004; Fig. 8.3). Interestingly, the infestation in southern Oregon was likely the result of an introduction from infected nursery stock because this population cannot be genetically linked to any one California forest population (Mascheretti et al. 2008; Prospero et al. 2007).

The NA2 clonal lineage was first observed in 2004 in California and Washington (Ivors et al. 2006). However, it appears that this lineage was introduced to either British Columbia or Washington from an unknown source (Goss et al. 2011; Fig. 8.3). Currently, it is only rarely found

in CA, more commonly found in WA and BC, and was only recently found in OR in 2012.

The EU1 clonal lineage was first detected in nurseries and established gardens in Germany and the Netherlands on ornamentals including *Rhododendron* and *Viburnum* (Werres et al. 2001). A few years later the pathogen was discovered in other areas of Europe. Currently, *P. ramorum* is present in many European countries, where it is mainly found in ornamental nurseries or gardens (Vercauteren et al. 2010). However, in 2009 there was an epidemic of ‘sudden larch death’ causing heavy dieback and death in plantations of Japanese larch (*Larix kaempferi*) in western Britain and Northern Ireland, leading to millions of trees being cut down. Neither the site nor the origin of the first introduction of *P. ramorum* in Europe has been determined.

The EU1 clonal lineage is also found in North America and appears to have been introduced into the Pacific Northwest (Grünwald and Goss 2011). A coalescent analysis with migration provided support for a unidirectional migration of the EU1 clonal lineages from Europe to North America (Goss et al. 2011; Grünwald and Goss 2011; Fig. 8.3). However, this effort could not establish whether the pathogen was introduced to British Columbia, Canada, or Washington, USA due to lack of power in the marker system and sample size used to infer migration routes. It has to be kept in mind that this pathogen cannot be sampled systematically like other pathogens as it is subject to eradication imposed by quarantine regulations thus making population genetic analysis more difficult. After its first introduction into the Pacific Northwest, EU1 has since migrated to California and Oregon (Grünwald et al. 2012a).

Another lineage, EU2, was recently described from Europe, first found in Northern Ireland in 2007 (Van Poucke et al. 2012). Thus far, it has only been recovered from a limited geographic region including Northern Ireland and Scotland, but has already been collected from several hosts (*Quercus robur*, *Larix kaempferi*, *Vaccinium myrtillus*, and *Rhododendron ponticum*). The EU2 mating type is the same as the EU1 lineage (A1 mating type) and genetic analysis (SSR and nuclear sequence) do not support the emergence

of this lineage as the result of recent sexual or somatic recombination between lineages (Van Poucke et al. 2012). The origin of this lineage and spread from Northern Ireland to or from Scotland are currently unknown (Fig. 8.3).

In summary, it appears that the four known clonal lineages are the result of five distinct intercontinental migrations including NA1 and NA2 into North America, EU1 and EU2 into Europe, and EU1 from Europe to North America (Grünwald et al. 2012a; Fig. 8.3).

8.4 Future Perspectives

Rapid advances in technologies such as genome (re)sequencing and genotyping by sequencing, combined with rapidly dropping costs of using these technologies provide a bright future for studying pathogens such as *P. ramorum*. In contrast to *P. infestans* and *P. sojae*, *P. ramorum* has a very wide host range. We currently do not understand what genomic signatures and features are responsible for host adaptation. The novel sequencing technologies will provide tools for addressing host adaptation in the genus *Phytophthora*. In fact, efforts are under way for sequencing the majority of species in the genus *Phytophthora*. It is hoped that these efforts will provide novel insights into host adaptation, effector biology, and evolutionary processes in this important genus. Also underway is the use of genotyping by sequencing for large-scale, partial genome sequencing using restriction enzyme digestion prior to sequencing. This approach enables discovery of tens of thousands of SNPs in many individuals at a fraction of the cost of whole-genome resequencing. Preliminary results have found approximately 40,000 SNPs that will be interrogated for use in molecular genetic tractability (Everhart and Grünwald unpublished data).

Traditional approaches of disease management based on host resistance breeding or chemical control will not work for management of sudden oak death. Resistance will not work because the host range is too large and because breeding is not economically feasible for timber

species of low value such as tanoak or for horticultural crops that include hundreds of cultivars in a single genus, such as *Rhododendron*. Given the fact that there is zero tolerance for infection of ornamentals in the nursery industry, use of fungicides is similarly not recommended as it can conceal latent infections. Use of fungicides is also cost prohibitive in natural forests. Novel means of control based on transgenic approaches should thus be explored as management alternatives.

Phytophthora ramorum has emerged globally at least four times over about two decades given the four distinct clonal lineages recognized to date. Despite recognition of this fact, we still lack knowledge of the geographic origin of these migrants. A concerted effort is needed to explore potential centers of origin of this pathogen. Knowledge of origins will provide tools for avoiding further migrations. The migrations of *P. ramorum* are driven by the movement of ornamental plants. Federal and state governments need to rethink the trade of ornamentals if further movements of exotic pathogens such as *P. ramorum* are to be avoided. Clearly, the repeated emergence of *P. ramorum* is a wake-up call that we should heed.

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