

EU2, a Fourth Evolutionary Lineage of *Phytophthora ramorum*¹

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Phytophthora ramorum is an aggressive Oomycete pathogen introduced into western North America and western Europe in the late twentieth century by the ornamental plant trade (Goss et al. 2011, Grünwald et al. 2012, Mascheretti et al. 2008, Prospero et al. 2007). The pathogen attacks a wide range of trees and shrubs, causing foliage blights and bleeding stem lesions both in nurseries and in the field (Rizzo et al. 2002, Werres et al. 2001). In North America, *P. ramorum* is known for causing sudden oak death, the dieback and mortality of millions of coast live oak (*Quercus agrifolia* Née) and tanoak (*Notholithocarpus densiflorus* (Hook. & Arn.) Manos, Cannon & S.H. Oh) trees along 1 500 km of near-coastal native forest in California and southwestern Oregon (Grünwald et al. 2008, Rizzo et al. 2002). The pathogen has also spread rapidly and widely across Europe within the nursery trade. From 2003 onwards, it was found attacking rhododendron and some broadleaf trees in the United Kingdom (Brasier et al. 2004) and subsequently native *Vaccinium* heathlands (P. Beales, Central Science Laboratory, personal communication). Since 2009, *P. ramorum* has caused sudden larch death, heavy dieback and mortality of plantation Japanese larch (*Larix kaempferi* (Lam.) Carrière) trees in western Britain and Northern Ireland, resulting in the felling of millions of trees (Brasier and Webber 2010, Webber et al. 2010).

Ivors et al. (2006) demonstrated three distinct genetic lineages in *P. ramorum*. These have since been informally designated NA1, NA2, and EU1 after their initial outbreak locations (Grünwald et al. 2009). NA1 and NA2 are confined to western North America, NA1 being predominant in the forests and found in most nurseries and NA2 so far confined to nurseries and adjacent waterways. Until recently, EU1 was the only lineage found in Europe. EU1 has also been found at a small number of nurseries in the Pacific Northwest. The recent appearance of the lineages is believed to involve independent introduction of NA1 and NA2 into North America and EU1 into Europe; with the appearance of EU1 in North America resulting from a secondary introduction from Europe (Goss et al. 2009a, Goss et al. 2011, Grünwald et al. 2012).

All three lineages are near clonal at their presumed centers of introduction, consistent with introduction bottlenecks (Goss et al. 2009b, Grünwald et al. 2008, Ivors et al. 2006, Vercauteren et al. 2010). Significant differences exist among them for important fitness characteristics, such as growth rate, colony stability, and aggressiveness (Brasier et al. 2006a, 2006b; Elliott et al. 2011). *Phytophthora ramorum* is heterothallic, and to date all NA1 and NA2 lineage isolates have been of A2 sexual compatibility type and EU1 isolates largely of A1 type (Brasier and Kirk 2004, Werres and Kaminski 2005). In Belgium, rare A2s of EU1 lineage have been observed, but these are probably products of somatic recombination from an A1 isolate (Vercauteren et al. 2011b). Gametangial formation between A1s and A2s is unusually sparse and gametangial meiosis often abnormal (Brasier and Kirk 2004, Boutet et al. 2010, Vercauteren et al. 2011a), and a coalescence analysis indicates the lineages may

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have diverged 150,000 to 500,000 years ago (Goss et al. 2009a). The lineages therefore appear to be partially reproductively isolated, adaptively different populations within *P. ramorum*.

In 2011, following the spread of *P. ramorum* onto larch in the United Kingdom, preliminary screening of larch isolates with SSR markers and *Cox II* sequencing led to evidence of a novel genotype on larch in Northern Ireland and western Scotland, potentially distinct from the three known lineages. The seven isolates were assigned to a new lineage: EU2. They came mostly from *Larix* but also from *Quercus*, *Rhododendron*, and *Vaccinium* (Van Poucke et al. 2012).

In a detailed SSR analysis with 18 primer pairs, all seven novel isolates had an identical SSR profile distinct from that of the EU1, NA1, and NA2 lineages. The differences were similar to those previously observed between the other lineages. No intra-EU2 lineage genotypic diversity was detected. Multilocus sequencing was carried out to determine the phylogenetic position of the EU2 lineage. Single sequences were obtained for all 11 loci except the heterozygous nuclear locus *Avh120*. For the five mitochondrial loci, the new EU2 lineage clustered with the three other *P. ramorum* lineages and separately from *P. hibernalis*. Within *P. ramorum*, the EU2 lineage was in most cases closest to the NA2 lineage and ancestral to it, though with weak support (<70 percent bootstrap). With the six nuclear loci, segregating sites were observed in all three known lineages as reported by Goss et al. (2009a). As only a single heterozygous site was observed in the ITS of the rDNA, this locus was not taken further into account. With the EU2 lineage, heterozygosity was observed in only one (*Avh120*) of the five remaining nuclear loci. The other three lineages were heterozygous at three (NA1) to five (EU1) loci. In phylogenetic trees, the unique EU2 lineage typically clustered with the three other *P. ramorum* lineages and separate from *P. lateralis* and *P. hibernalis*. Based on the β -*tub* and the *Cox I* loci, two PCR-RFLP tests were developed that effectively discriminate between all four lineages (Van Poucke et al. 2012). Both methods involve a restriction enzyme that cuts at least once in all lineages, so that the activity of the enzyme can be verified in all *P. ramorum* samples.

In sexual compatibility tests on carrot agarose medium between the EU2 isolates (of unknown mating type) and EU1, NA1, and NA2 tester isolates, gametangia were only produced in pairings with isolates of the NA1 or NA2 lineages and not with those of the EU1 lineage, establishing the EU2 isolates as A1 type. As previously observed with *P. ramorum* (Brasier and Kirk 2004), not all pairings (even between known A1s and A2s) were fertile, and gametangia were generally rare or very rare even in the fertile mixtures. The size and morphology of the gametangia produced in inter-lineage pairings involving EU2 isolates was similar to that in the control pairings and to that previously published for intra-specific pairings of *P. ramorum* (Brasier and Kirk 2004, Werres and Kaminski 2005).

The EU1 lineage has been present in Europe since at least 1993 and is now very widespread, occurring in most of western and central Europe (Anonymous 2011, Grünwald et al. 2012, Webber 2008, Werres et al. 2001). In contrast, the first recorded disease outbreak caused by EU2 is only in 2007 in Northern Ireland, and its known distribution is still restricted to Northern Ireland and to an area in southwest Scotland about 100 km away. The SSR profiles of the seven EU2 isolates examined were entirely uniform. The recent detection, limited geographical distribution, and genetic uniformity of EU2 suggest that it is a much more recent introduction than EU1. *Phytophthora ramorum* is particularly well suited to long distance spread via infested plant material, as has been demonstrated by its rapid spread on susceptible nursery stock, especially on *Rhododendron*, *Viburnum* and *Pieris* (e.g., Frankel 2008, Grünwald et al. 2012). Introduction via plant movement or plant trade is therefore the most likely mode of arrival of EU2 in the United Kingdom. Spread within the United Kingdom is also most likely to be associated with movement of infected plants, although other pathways such as movement of 'sporangial clouds' from heavy sporulation of EU2 on infected larch plantations, or movement between sites of spores attached to boots or machinery, cannot be ruled out. Brasier and Webber (2010) suggested that the epidemic on larch could simply reflect the intrinsic properties of *P. ramorum* or could be a result of its adaptation to larch. The fact that two lineages of *P. ramorum*, EU1 and EU2, are now involved suggests the epidemic is more likely to be an intrinsic property of the pathogen.

As with the other three lineages, the geographic origin of EU2 remains unknown. It has been suggested that the lineages might have separate geographic origins and could be at least equivalent to taxonomic subspecies (Brasier et al. 2006b). The coalescence analysis of Goss et al. (2009a) indicates that EU1, NA1, and NA2 have been evolutionarily divergent for a minimum of 100,000 years. Whether this is a result of their arising in different geographic locations or their becoming reproductively isolated within a single center of origin, has yet to be determined. A proper understanding of the evolution and behaviour of *P. ramorum* and its lineages may only come from studying its behaviour at its geographic source. Indeed the arrival of EU2 highlights an urgent need to identify the geographic origins of *P. ramorum* in order to understand the organism's natural ecology, the processes that have produced the lineages, and whether further lineages exist. Presently, studying the organism in the context of introduction and invasion, we may only be looking at half the picture.

All the isolates of the EU2 lineage examined here were of the A1 compatibility type, hence EU2 may be solely of A1 type, at least in its current introduced, as opposed to its endemic form. Since the other lineage present in Europe (EU1) is also of A1 type, the arrival of EU2 should not increase the theoretical risk for sexual recombination of *P. ramorum*, although inter-lineage somatic recombination is a possibility. Currently, findings of *P. ramorum* in the United Kingdom are subject to emergency European Union and United Kingdom plant health phytosanitary measures. Indeed, present evidence suggests EU2 may have arrived in the context of these ongoing emergency measures. The true significance of the arrival of EU2 for the future health of United Kingdom forests and plant heritage should become clearer when more information is available on its distribution across the British Isles and when the comparative behaviour of the EU2 and EU1 lineages is better understood, in particular their comparative aggressiveness and host range. For a full account of these observations see Van Poucke et al. (2012).

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