

Final Project Report

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Project title

Comparison of *Phytophthora ramorum* (Sudden oak death) populations from Europe and USA in support of pest risk assessment, management and policy

DEFRA project code

PHO 192

Contractor organisation and location

Forestry Commission Research Agency (FRA)

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Total DEFRA project costs

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Project end date

06/04/04

Executive summary (maximum 2 sides A4)

In a comparison, samples of EU and US isolates of *P. ramorum* exhibited significant differences in mean growth rate over a range of different growth environments: EU isolates grew faster than US isolates in most environments tested and exhibited limited growth rate variation. US isolates exhibited extensive growth rate variation. However, the EU and US groups also showed a broadly similar response across the different environments, indicating the two groups are conspecific.

EU isolates were significantly more aggressive than US isolates on bark of *Quercus rubra*, a susceptible host.

EU isolates were of a consistently of a uniform and characteristic 'wild type' colony morphology, consistent with their limited growth rate variation. US isolates, however, were either of a similar wild type or fell into a range of morphologically variable, often slow growing 'non wild type' colonies. Investigations involving single hyphal tip subcultures showed that EU isolates were rather stable and US isolates intrinsically developmentally unstable. The basis of this instability is unknown. A preliminary screening revealed no viral dsRNA in US 'non wild type' isolates.

The above differences are likely to reflect underlying genetic differences in genes governing fitness attributes of EU and US isolates i.e. be adaptive differences.

In standard sexual compatibility type (mating type) tests, using A1 and A2-type testers of other *Phytophthora* species, ie. interspecific sexual pairings, EU isolates were all of A1-type US isolates all of A2 type. Many isolates, however, failed to mate and the the *P. ramorum* mating reaction was shown to be weak and unpredictable compared with 'normal' A1 x A2 pairings between *Phytophthoras*. Also, using the same protocol, no gametangia could be obtained in pairings between the putative *P. ramorum* A1 and A2 compatibility types.

Attempts to induce A1 x A2 mating across polycarbonate membranes ie preventing physical contact between the two species but allowing sexual, compatibility substances to pass across, were unsuccessful, probably due to the intrinsically low fecundity of the interspecific pairings.

Tests of a wide range of media and culture conditions did not enhance the fecundity of the interspecific pairings.

A method was developed for producing gametangia of A1 x A2 isolates of *P. ramorum* for the first time *in vitro*. The method, now published (*Mycological Research* 108, 823-27, 2004) was communicated to other researchers and has been used successfully to identify compatibility types of *P. ramorum* isolates by research groups in North America and the UK .

Overall, it remains unclear whether *P. ramorum* is truly A1/A2 outcrossing, or indeed whether its sexual breeding system is functional. However, even if the sexual breeding system is non functional or only partially functional, there remains the possibility that somatic (non-sexual) recombination could occur between A1 and A2 *P. ramorum* isolates.

On the basis of the above observations it is proposed:

1. That EU and US isolates, though conspecific, comprise two separate phenotypic groups and should be considered distinct phenotypic populations.
2. That if recombination (sexual or somatic) were to occur between the EU and US populations, further additive allelic variation might be generated. ie there is a risk that recombination could lead to generation of further adaptive variation.
3. That it is not clear at present how these phenotypic differences between the EU and US populations came about. Two possible explanations (not mutually exclusive) are suggested.
4. That the EU and US populations might at some point need to be formally designated as separate subspecies of *P. ramorum*.

Scientific report (maximum 20 sides A4)**Background**

Phytophthora ramorum (the cause of sudden oak death in the USA) is a serious problem in central coastal regions of western USA, killing large numbers of tan oaks (*Lithocarpus densiflorus*) and certain *Quercus* species. Death is caused by bark bleeding lesions or bleeding cankers. Many other forest trees, shrubs and ornamental species are also affected and the pathogen is known to have a wide and growing host range. *P. ramorum* is the subject of Emergency EC measures which aim to prevent the spread of European isolates and the introduction of non-European isolates. This is primarily due to the potential threat the pathogen may pose to European tree species. The pathogen attacks the bark of some hosts but only the foliage and twigs of others. *P. ramorum* is now present in parts of Europe (e.g. The Netherlands, Germany, Belgium, Denmark, Sweden, Spain, Poland and the UK), and prior to November 2003 had only been found on certain ornamental genera in nurseries and in a few private gardens. In early November 2003 the disease was confirmed in a Southern red oak (*Quercus falcata*) within a commercial wooded garden in the south-east of England while the Netherlands reported a finding on a Northern red oak (*Quercus rubra*). Since then a number of trees were identified with bark bleeding cankers in woodlands in the south-west of England also having large amounts of infected understory rhododendron, especially *R. ponticum*. These trees included several *Fagus sylvatica* (beech), *Quercus cerris* (Turkey oak), *Aesculus hippocastanum* (horse chestnut) and *Nothofagus* (southern beech). *Castanea sativa* (sweet chestnut) and *Quercus ilex* (holm oak) have been found with infected foliage.

Preliminary studies by FR indicated behavioural differences between European and North American isolates of *P. ramorum*. Other research groups have revealed significant differences between them in neutral molecular markers. In view of these observations, to support the development of plant health policy the comparative risk to trees posed by European and North American isolates of *P. ramorum* - and the possible risk posed by genetic recombination between them - was investigated. Also considered was whether European and North American isolates represented components of a single cohesive species or discrete sub-populations. Population samples of European (EU) and North American (US) isolates were compared for their growth rates across different environments (G x E tests), phenotypic stability, host range, pathogenic aggressiveness and sexual behaviour.

G x E tests on samples of European (EU) and North American (US) *P. ramorum* isolates.*Growth rate tests.*

The comparative environmental responses of EU and US *P. ramorum* isolates were tested under a range of environmental or 'stress' conditions on Carrot agar (CA). Growth rate was the main continuous variable measured. Initially, two linear growth rate tests were conducted on Carrot agar (CA) at 20°C, near the growth optimum for the fungus. 30-39 isolates were used per sample. EU isolates grew significantly faster on average than US isolates and in some tests a total separation of the EU and US isolates occurred. e.g. In the second test 30 EU isolates averaged 3.62 ± 0.08 mm day⁻¹ and 39 US isolates 2.70 ± 0.33 mm day⁻¹. The range of the EU isolates was only 3.45 – 3.75 mm/day⁻¹ compared with the much wider range of 2.09 – 3.56 for the more variable US isolates (Fig. 1). In a third test at 20°C an EU sample grew faster on average than, and did not overlap with, the US sample even when the US sample comprised isolates freshly obtained from the field (courtesy Dr.

Dave Rizzo) and the EU isolates had been in culture 2 or more years. EU isolates were again also much less variable.

Similar tests were conducted at 12.5, 15 and 27°, with 35 EU and 39 US isolates. These supra- and sub-optimal temperatures are likely to put the pathogen under greater environmental stress, and so might reveal other underlying genetic differences between individual isolates, or between populations. The EU/US growth rate difference was maintained. i.e. The EU isolates always grew significantly faster, on average, than did the US isolates (Fig. 2). Again, in several tests a complete separation of the EU and the US isolates occurred. At the same time, the mean temperature growth curves for the EU and US samples were similar across the whole range of temperatures tested (12.5, 15, 20 and 27°). This indicates that although the EU and US samples differed markedly in growth rates, and therefore exhibit what are probably adaptive differences, they are most probably conspecific.

A series of growth rate tests were conducted with 35 EU and 39 US isolates involving other environmental stress factors at 20°C, including exposure to continuous light; and a range of optimal (1.5%) and supra-optimal (3, 4 and 5%) agar concentrations, resulting in decreased available water levels. Growth rates of all isolates decreased between 1.5 and 3% and then remained stable. The EU isolates again grew significantly faster on average than the US isolates under all light and agar conditions tested. There was no evidence of a differential response of EU and US isolates, again consistent with conspecificity of EU and US isolates. Growth tests were also conducted at 28-31°, to compare response of stress temperatures close to the organisms, upper temperature limit. Note of the isolates grew at 31°. When tested for growth at 30°, the apparent upper limit for growth of *P. ramorum*, only 37% (12/35) of EU isolates grew, whereas 80% (31/39) of US isolates did so. This indicates EU isolates have a slightly lower maximum temperature-limit for growth than do US isolates.

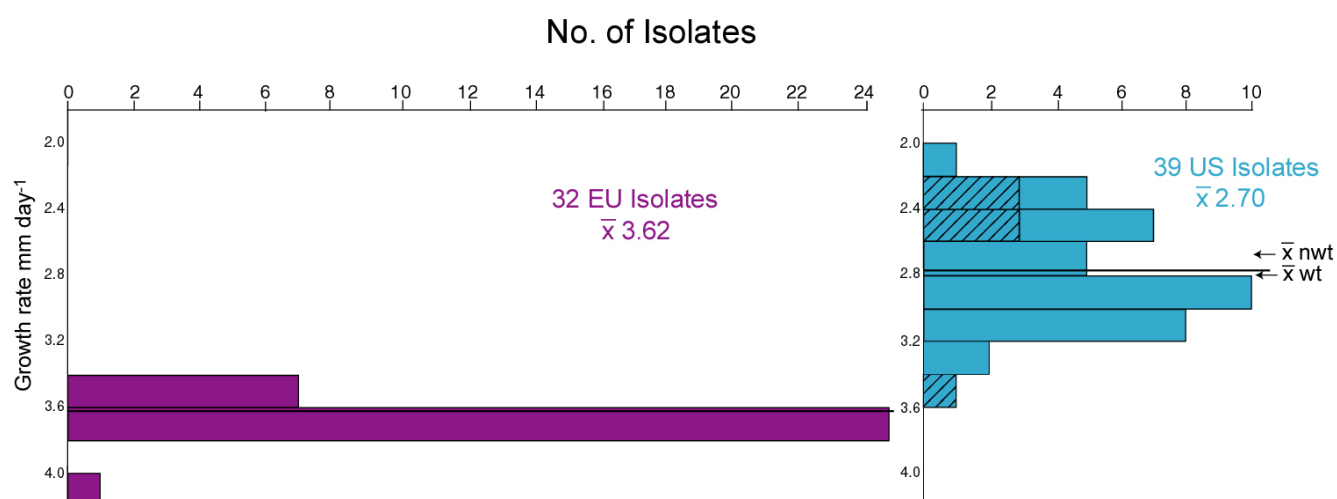


Figure 1. Growth rates of EU and US isolates.

Colony stability.

In all the above growth rate tests the variance of the EU samples was often very small (cf. Fig. 1), suggesting clonality for the many 'adaptive' genes governing growth rate; whereas that of the US samples was much larger, suggesting greater variability for these genes. Colony morphologies of the

isolates were examined in the three growth rate tests at 30°. EU isolates were of a uniform and characteristic 'wild type' colony morphology, consistent with their limited growth rate variation (Fig. 3). US isolates, however, were either of a similar wild type morphology or, unexpectedly, fell into a range of morphologically variable, often slow growing 'non wild type' colonies (Fig. 3).

To examine the intrinsic colony stability of EU and US isolates, especially in view of the unexpected colony variation shown by US isolates, routine mass subcultures and single hyphal tip subcultures were taken from selected isolates. These subcultures confirmed that EU isolates were intrinsically rather stable. In contrast, US isolates were intrinsically developmentally unstable, and furthermore, could change from the wild type form to an unstable non wild type form in culture. The basis of this developmental instability in US isolates is unknown. In a preliminary screening, selected 'non wild types' US isolates were examined for the possibility that they were virus infected. No viral dsRNA was detected.

In another test, a single *P. ramorum* isolate from Belgium, shown by S. Werres (pers. comm.) to be of A2 sexual compatibility type and not A1 sexual compatibility type (see 'mating type tests' below), was shown to be a typical EU isolate on the basis of its growth rate and colony type.

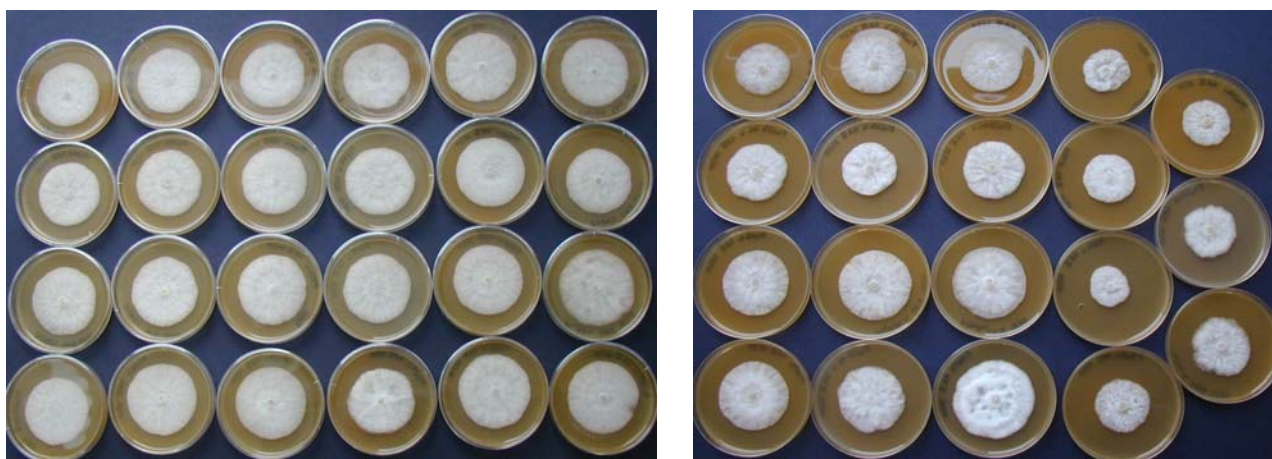


Fig. 2. Colonies of EU (left) and US (right) isolates of *P. ramorum*

Pathogenic aggressiveness on a susceptible host.

Three tests for comparative pathogenicity to bark on the susceptible tree host, *Quercus rubra* were conducted. Fresh cut stem ca. 1.1, 25cm were wound inoculated with from between 8-15 EU and 8-15 US isolates in a quarantine chamber and resulting lesion areas measured after 5-6 weeks. Samples of EU isolates were on average significantly more aggressive ($P < 0.01$ – $P < 0.001$) than US isolates in all three tests, though the ranges of the two groups overlapped in two of the three tests (Fig. 4). An EU sample was more aggressive on average even when the US isolates were freshly obtained from the field and the EU isolates had been in culture 2 or more years. These results therefore indicated further adaptive difference between the EU and the US isolates.

The potential host ranges of EU and US isolates were compared by wound inoculation of mature stems of ca 30 European and North American tree species. Comparisons were again based on average lesion sizes (ca 8-10 replicate inoculation points, with 2 EU and 2 US isolates) caused on the different tree species. The potential host ranges of the EU and US isolates were shown to be very similar, again consistent with the conspecificity of the EU and US groups.

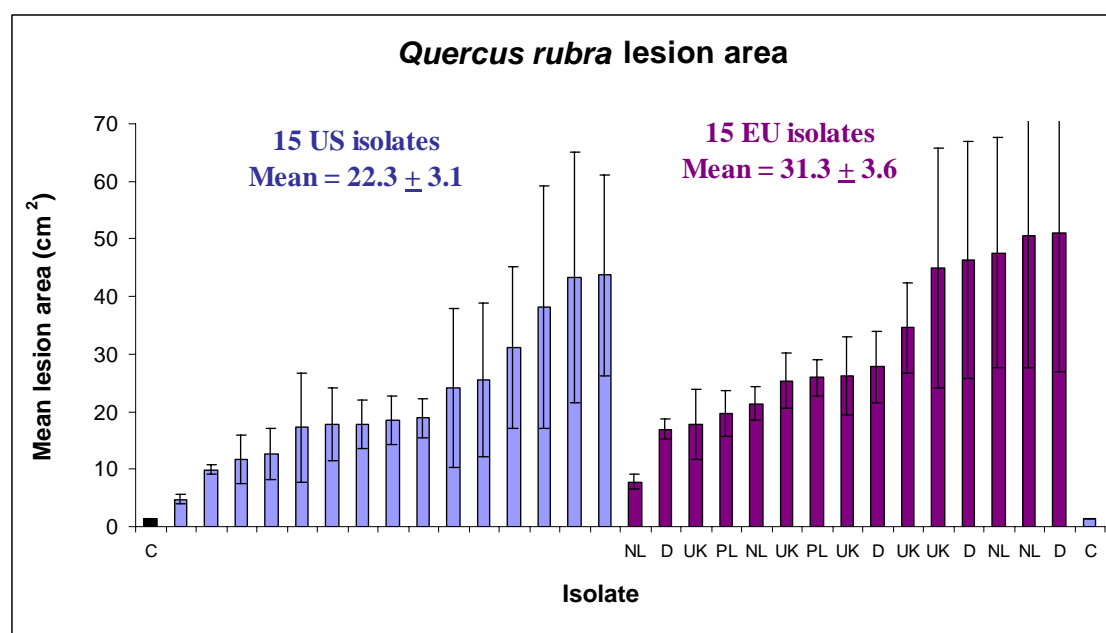


Fig. 3. Lesion sizes produced by EU and US isolates of *P. ramorum* at 20°C

Sexual compatibility (mating) types and breeding system.

Interspecific (between species) pairing tests.

To investigate whether *P. ramorum* is heterothallic/outcrossing, i.e. with 'A1' and 'A2' sexual compatibility types as in other heterothallic *Phytophthora* species, standard sexual compatibility type (mating type) tests were carried out on CA medium at 20°C. A1 and A2-type testers of two heterothallic *Phytophthora* species, *P. cambivora* and *P. drechsleri*, were paired with the varies EU and US *P. ramorum* isolates as 'unknowns'. Sixty EU isolates were shown to be all of A1-type (Table 1). Another 60 EU isolates failed to mate (the *P. ramorum* mating reaction is weak and unpredictable, see below). Thirty two US isolates (excluding European-type nursery isolates from the US Pacific north-west) were all of A2 type (Table 1). Another 6 US isolates also failed to mate. It is clear therefore that, unusually, the EU and US isolates differ with respect to their sexual compatibility or mating type. This result is consistent with the results being obtained by S. Werres, BBA Germany and other researchers.

However, it is important to note that production of gametangia (the sexual stage) in these interspecific pairings was highly abnormal. Instead of taking 2-3 days to form frequent to abundant gametangia, as in 'normal' A1 x A2 pairings with outcrossing *Phytophthoras*, gametangia were formed only after ca 40 days. Resulting gametangia were typically very sparse (rare to occasional in frequency). Many pairings were negative. Many pairings that were positive in one test failed (were negative) in sequential repeats.

Also, using the same standard pairing protocol on CA, no gametangia could be obtained in pairings between the presumptive, *P. ramorum* A1 and A2 compatibility types.

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Table 1. Pairing tests of *Phytophthora ramorum* isolates against *P. drechsleri* and *P. cambivora* A1 and A2 type testers. From Brasier & Kirk (2004).

Source	No. of isolates tested	No. of A1 type	No. of A2 type
Europe:			
UK ^a	80	31	—
Germany ^b	16	13	—
Netherlands ^b	6	6	—
Poland ^b	3	1	—
Spain ^b	4	—	—
France ^b	1	—	—
<i>Total</i>	120	60	—
USA:			
California ^c	27	—	22
Oregon ^d	11	—	10
<i>Total</i>	38	—	32

^a Isolates from *Rhododendron* and *Viburnum* spp. from 52 nurseries across 24 counties in the UK.

^b Isolates mainly from *Rhododendron* and *Viburnum* spp.

^c Isolates from native *Lithocarpus densiflorus*, *Quercus agrifolia*, *Umbellularia californica*, *Arbutus menzeisii*, *Vaccinium ovatum* and *Rhododendron* spp. from Marin, Sonoma, Napa and Santa Cruz Counties in California.

^d Isolates from native *Lithocarpus densiflorus* in the Brookings area of south-west Oregon

Polycarbonate membrane pairings

Interspecific pairings like those described above result in gametangia that may be hybrid, selfed or a mixture of both i.e. it cannot be guaranteed that the resulting gametangia are of *P. ramorum*. Both for taxonomic and for cytological studies, production of 'pure *P. ramorum*' gametangia is desirable. A series of experiments were therefore set up between A1 and A2 *P. ramorum* isolates and A2 or A1 *P. drechsleri* or *P. cambivora* isolates separated by polycarbonate membranes (pore size 0.2µm). Use of polycarbonate membranes prevents physical contact between the A1 and A2 isolates, but allows sexual compatibility substances (chemicals that break down self-incompatibility) to pass across. If successful, this could have resulted in the formation of 'pure' *P. ramorum* gametangia on one side of the membrane. However, although many membrane tests were conducted all were unsuccessful, probably due to the intrinsically low fecundity of the interspecific pairings (*P. ramorum* x *P. drechsleri* or *P. cambivora*) already described.

Attempts to enhance gametangial production: effects of media and culture conditions

The possibility of enhancing the fecundity of the interspecific pairing response by adjusting media and conditions was investigated. Six interspecific *P. ramorum* x *P. drechsleri* or *P. cambivora* A1 x A2 combinations were inoculated to a wide range of culture media including standard CA (as a control); 'strong' CA; CA + sunflower oil and CA + mixed vegetable oil to enhance sterol uptake (sterols stimulate sexual reproduction in *Phytophthora*); Red oak sapwood agar (ROSA) and viburnum sapwood agar (VSA), with a view to providing woody-host nutrients; CA incorporating 2%, 5% and 10% agar, with a view to reducing available free water; and CA overlain with a sterile cellophane membrane, with a view to providing a surface (leaf-like) effect and a higher oxygen environment for growth.

None of these tests resulted in any significant improvement in the rate of frequency of gametangial production in the interspecific pairings. In contrast, the intraspecific (same species) control pairings with A1 and A2 isolates of *P. drechsleri*, or with *P. cambivora*, resulted in rapid and abundant gametangial formation on most media.

Method for gametangial production between paired A1 x A2 P. ramorum isolate in vitro.

Abundant chlamydospores (and some sporangia) were produced by *P. ramorum* on all the above artificial media. This suggested that a diversion of energy resources into asexual reproduction might be one cause of the failure of *P. ramorum* to produce gametangia in intraspecific pairings and of the sparse gametangial production in the interspecific pairings. On CA at 20° chlamydospores usually appear in *P. ramorum* colonies after ca. 7 days. An attempt was therefore made to pre-empt the tendency to produce chlamydospores by bringing juvenile <2 day old *P. ramorum* A1 x A2 mycelia i.e. mycelia that might not yet be in 'chlamydospore-development mode', into close physical contact using a 'mycelial mixing' method (method now published by FR in *Mycological Research* 108, 823-27, 2004). The method also involved using very thinly poured CA medium.

The method was successful. When assessed after 10 days, gametangia were found in all A1 x A2 combinations tested (occasional to frequent) (Fig. x). Subsequently, gametangia continued to form in the extending mycelial growth beyond the original mixed inoculum points. Chlamydospores developed rapidly throughout. These were the first knowingly 'pure' *P. ramorum* gametangia to be produced in artificial culture. No gametangia formed in control A1 x A1 or A2 x A2 pairings using the same method.

In April 2003 this *in vitro* 'mycelial mixing' method for intraspecific pairings was communicated to other laboratories working on *P. ramorum*, for their further assessment. Similar results were obtained by three laboratories in North America UK (E. M. Hansen, N. Osterbauer, D. Huberli, personal communications) and by CSL and SORAD in the UK (P. Giltrap and C. Lane, and A. Schlenzig, personal communications). In all cases gametangial formation commenced relatively rapidly (ca. 3-10 days). At CSL, 97 European isolates (70 from the UK and 27 from other countries) were all successfully identified as A1 compatibility types with no negative results and, in very test, gametangia were produced in only 3-7 days (P. Giltrap and C. Lane, personal communication). However, though considerably faster than the interspecific pairing method, the inoculum-mixing method can be somewhat unpredictable: using previously fertile A1 x A2 *P. ramorum* isolate combinations, unexplained negative results sometimes occur.

Overall, it is still unclear whether *P. ramorum* is truly A1/A2 outcrossing, or indeed whether its sexual breeding system is functional. However, even if the sexual breeding system is non functional or only partially functional, there remains the possibility that somatic (non-sexual) recombination could occur between A1 and A2 *P. ramorum* isolates.

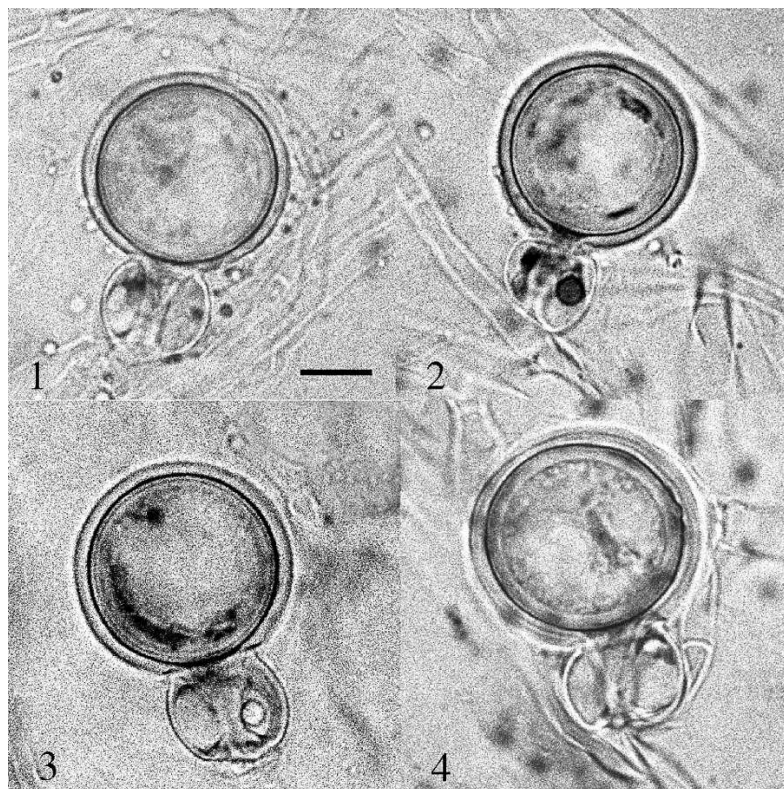


Fig. 4. Gametangia formed by paired A1 and A2 isolates of *P. ramorum* for the first time *in vitro*.

General conclusions re the evolutionary and comparative plant health risk status of EU and US isolates

In summary, the following preliminary conclusions can be drawn

- EU and US isolates of *P. ramorum* exhibit many similarities in continuous variable, such as overall growth-temperature interactions and host range, indicating conspecificity. Equally, there are significant and important differences between EU and US isolates for continuous variables such as growth rate, colony development and level of pathogenic aggressiveness. These characters are likely to reflect underlying genetic differences between EU and US isolates in genes governing fitness attributes i.e. be adaptive differences.
- These differences are further underlined by a difference in predominant sexual compatibility type, EU isolates being predominantly of A1 and US isolates of A2 type. (Also, by evidence of a range of small but significant differences in molecular polymorphism of adaptively neutral DNA demonstrated by a range of European and US laboratories).
- The EU and US isolates therefore comprise two separate phenotypic groups. It is suggested that for the present they should be considered as separate phenotypic populations. i.e. As distinct American and a European population types.
- The US population exhibits unusual phenotypic – and presumably genetic – instability. The European population comparative stability.
- The European phenotypic population exhibits both A1 and A2 sexual compatibility types (predominantly A1 but with a low frequency of A2s that are otherwise phenotypically and genetically similar to the A1s) i.e. If the sexual compatibility system is functional, the European population could potentially 'breed true'.
- If, as a result of introduction of European isolates into North America or *vice versa*, recombination (sexual or somatic) were to occur between the EU and US populations, further additive allelic variation might be generated. i.e. There is a risk that genetic recombination between EU and US population isolates could lead to generation of further adaptive variation, such as altered aggressiveness or possibly changes in host range.
- It is not clear at present how these phenotypic differences between the EU and US populations came about. i.e. What the origins, causes and evolutionary history of the differences has been. Two possibilities (not mutually exclusive) are suggested:
 1. That the two populations were originally phenotypically similar rather than distinct. That the differences they now exhibit reflect the different selection pressures the two populations have experienced since introduction into the EU or the US. e.g.:
 - Growth rate and cultural variation in the EU population is narrow and that in the EU population is more aggressive because this population has been subject to intense directional or stabilising selection when spreading on rhododendrons/viburnums in the specialised European nursery environment.
 - Growth rate and cultural variation in the US population is broad and the US population is less aggressive because it has been exposed to a more heterogeneous set of environments/selection pressures when spreading on many hosts and ecosystems in US forests.
 2. That the differences between the EU and US populations reflect prior adaptation before their introduction into the EU and US respectively. i.e. There may be two differently adapted 'source' populations of *P. ramorum* e.g. in different regions/ecosystems of China, or wherever *P. ramorum* originated (cf. *Mycological Research* 108, 1108-10, 2004).
 3. A combination of possibilities of (1) and (2).

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- If on further work possibility (2) appears likely to hold, we may need to reconceptualise the EU and US population types as two (partially reproductively isolated?) subspecies of *P. ramorum* to reflect their phenotypic and genetic differences. For example, they may need to be formally redescribed as *P. ramorum* subspecies *europaea* and *P. ramorum* subspecies *americana*.
- In view of the above it is recommended that for the present the EU and US population types be treated as separate entities for plant health and scientific research purposes. They could, for example, be termed '*P. ramorum* European type' and '*P. ramorum* American type' respectively.

Technology/Knowledge transfer activities

Scientific Journal Publications and PRAs

- Brasier, C.M. (2003). Sudden Oak Death: *Phytophthora ramorum* exhibits transatlantic differences. *Mycological Research* **107**, 258-259.
- Jones, D.R., Sansford, C.E., Brasier, C.M., Webber, J.F. (2000-2004). Pest Risk Analysis of *Phytophthora ramorum*. CSL/FRA, UK Defra/.FC websites.
- Brasier, C.M. and Kirk, S.A. (2003). Production of gametangia by *Phytophthora ramorum* *in vitro*. *Mycological Research* **108**, 823-827
- Brasier, C.M., Denman, S. A., Brown, A. V. and Webber, J.F. (2004). Sudden oak death (*Phytophthora ramorum*) discovered on trees in Britain. *Mycological Research* **108**, 1107-1110.
- Brasier, C. M. & Jung, T. (2005). Recent developments in *Phytophthora* diseases of trees and natural ecosystems in Europe. Proceedings of Third IUFRO conference on *Phytophthora* in forest trees, Freising, Germany 2004 (In press).
- Brasier, C. M. Kirk, S. A. and Rose, J.R. (2005). Adaptive differences between European and American populations of *Phytophthora ramorum*. Proceedings of Third IUFRO conference on *Phytophthora* in forest trees, Freising, Germany 2004 (In press).

Presentations to Scientific and Public Meetings

- Brasier, C.M., Rose, J., Kirk, S.A. and Webber, J.F. (2002). Pathogenicity of *Phytophthora ramorum* isolates from North America and Europe to bark of European *Fagaceae*, American *Quercus rubra* and other forest trees. Abstract, Sudden Oak Death Science Symposium, Monterey, California, 15-18 December 2002. <http://danr.ucop.edu/ihrmp/sodsymp/paper>

Presentation to Defra interested parties, York, October 2003

Presentation to Defra Chief Scientist, London, December 2003

Presentation to 1st EU RAPRA project meeting , Forest Research Agency, February 2004

Other technology transfer

Electronic circulation of method for producing oospores of *P. ramorum in vitro* to other *P. ramorum* laboratories in UK, Europe and North America. April 2003.