

PHYTOPHTHORA NEMOROSA, A NEW SPECIES CAUSING CANKERS AND LEAF BLIGHT OF FOREST TREES IN CALIFORNIA AND OREGON, U.S.A.

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ABSTRACT

Phytophthora nemorosa, a new species isolated from stem cankers on two species of Fagaceae and leaves of various hosts, is described. The new species resembles *P. ilicis* with homothallic, amphigynous antheridia and deciduous, semi-papillate sporangia, and has a related ITS-DNA sequence. Symptoms and host range are similar to *P. ramorum*, cause of Sudden Oak Death and leaf blight and shoot dieback diseases in California and Oregon forests, although *P. nemorosa* does not appear to cause wide-spread mortality of oak trees.

KEY WORDS

Phytophthora ramorum, *Phytophthora ilicis*, *Lithocarpus densiflorus*,
Quercus agrifolia, *Umbellularia californica*

INTRODUCTION

Phytophthora species are well known as pathogens of agricultural crops, and a few invasive and destructive forest species have been described, but the world's endemic *Phytophthora* flora is largely unexplored. Recent work with European oak forest soils (Jung et al. 2002, Hansen and Delatour 1999) and preliminary forest surveys in western North America (Hansen 2000), however, indicate that there is much to learn. In the course of the intensive survey effort and epidemiological research surrounding sudden oak death and *P. ramorum* in California and Oregon, we have encountered several *Phytophthora* species. One previously unknown species in particular was noteworthy for its aerial habit and frequency of isolation from plants also susceptible to *P. ramorum*. In this paper we describe this new

Phytophthora as *P. nemorosa*, a foliar and bole pathogen of various evergreen hardwood trees. It appears to be native to forests of western North America. Future papers will more fully describe its pathology and population biology. In previous accounts (Rizzo et al. 2002; Davidson et al. 2002), *P. nemorosa* was referred to as "P. ilicis-like."

METHODS

Phytophthora nemorosa was recovered from stem cankers and leaf lesions on corn meal agar amended with 10 ppm natamycin (Delvacid®), 200 ppm Na-ampicillin, 10ppm rifampicin, and 10 ppm dicloran (or 25 ppm Benlate) (PARP) or on a similar selective medium without dicloran or benomyl (CARP). Isolates of *Phytophthora ilicis* were recovered from infected English holly (*Ilex aquifolium*) leaves and stems by plating surface-disinfested (10% bleach 3 min., 3X rinse in sterile deionized water) pieces in PARP. Isolates were kept in sterile hemp seed water for long term storage.

Isolates were grown on Difco corn meal agar and V-8 agar amended with 30 ppm β -sitosterol (CMA β , V8S), and fresh potato dextrose agar (fPDA). Sporangia were produced by transferring discs from margins of mycelium grown on V8S into natural stream water. Oogonia, oospores and antheridia were observed on cultures grown on CMA. Specimens were stained and mounted in lacto-glycerol/cotton blue, and measured on a Zeiss Universal microscope with 40X neofluar objective and eyepiece micrometer.

Pathogenicity on holly was tested by transferring small blocks of PDA with mycelium to the wounded (five parallel scratches) underside of English holly leaves borne on detached branches. Leaves were incubated at 17-18 C with 12 hr photoperiod at 100% relative humidity for 10 days (Buddenhagen and Young 1957).

Isolates of *P. nemorosa* used for DNA extraction were grown in potato dextrose broth at 14 C for 10 days. Genomic DNA was isolated from 75 mg of lyophilized mycelium using the following modified CTAB extraction procedure. Lyophilized tissue was pulverized in a FastPrep® instrument (Bio101, Carlsbad, CA) for 10 s at 5,000 rpm. Pulverized tissue was incubated in 500-ml CTAB on dry ice for 2 min, then thawed at 75 C for 2 min. This freeze-thaw step was repeated twice, with the final thaw for 30 min. DNA was purified in phenol:chloroform:isoamyl alcohol (25:24:1), further cleaned by using the GeneClean® Turbo Nucleic Acid Purification kit (Qiagen Inc, Valencia, CA) according to the manufacturer's instructions, and eluted in 30 μ l ultra-pure water.

PCR was performed with primers ITS1 and ITS4 (White *et al.* 1990) as previously described (Bonants *et al.* 1997). Amplification products were sequenced with ITS1 and ITS4 as sequencing primers on an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Published sequences of other *Phytophthora* species were downloaded from GenBank, aligned using the multialignment program Sequencher 4.1.2 and then visually optimized. Phylogenetic analysis was performed using PAUP* version 4.0b10 (Swofford 2000). A neighbor-joining tree was generated using the Kimura 2-parameter distance option (Kimura 1980) and midpoint rooting. Support for the internal branches was obtained by bootstrap analysis from 1000 replications.

SPECIES DESCRIPTION

Phytophthora nemorosa E.M. Hansen and Reeser

Species homothallica, oosporas in cultura procreans; oogoniis in medio 33 μ m; antheridiis amphigynis. Sporangii semipapillatis, in medio 51 x 37 μ m. Sporangii saepe deciduis, cum pedicellulo brevi (<5-20 μ m).

Homothallic, with amphigynous antheridia. Oogonia average 33 μ m. Sporangia caducous, semi-papillate, average 51 μ m long by 37 μ m wide, with short pedicels (<5-20 μ m).

Type isolate: Isolate P-13, recovered from tanoak (*Lithocarpus densiflorus*) in Humboldt County CA is representative of the species and designated holotype. The type specimen (a dried agar culture) is deposited in the Oregon State University Mycological Herbarium (OSC # 104381), and a living culture is at ATCC (Accession # MYA-2948). Additional cultures are at CBS and University of California, Riverside. The ITS sequence is available on GenBank AY332651. Other isolates critically examined (Table 1, 2) include: 2052.1 from tanoak, Curry Co. OR; 2055.2 from tanoak, Curry Co. OR., GenBank AY332654; P7 from tanoak, Sonoma Co. CA., GenBank AY332652; and P11 from coast live oak (*Quercus agrifolia*), Contra Costa Co. CA., GenBank AY332653.

Etymology: *P. nemorosa* refers to the forest setting from which most isolates have been recovered.

Morphology: *P. nemorosa* is homothallic, producing terminal oogonia and oospores after 3 or 4 weeks in single culture on CMA β (Figure 1). Oogonia average 33 μ m (range 23 to 40 μ m). Antheridia are amphigynous, about 13 x 13 μ m (range 9-19 x 10-15) μ m. Oospores are slightly aplerotic, averaging 29 μ m (range 19 to 35 μ m) (Table 1). Cultures on CMA β

Table 1. Mean diameter of oogonia and oospores and length and width of antheridia for five isolates of *Phytophthora nemorosa*, (microns, +/- SD, n=10)

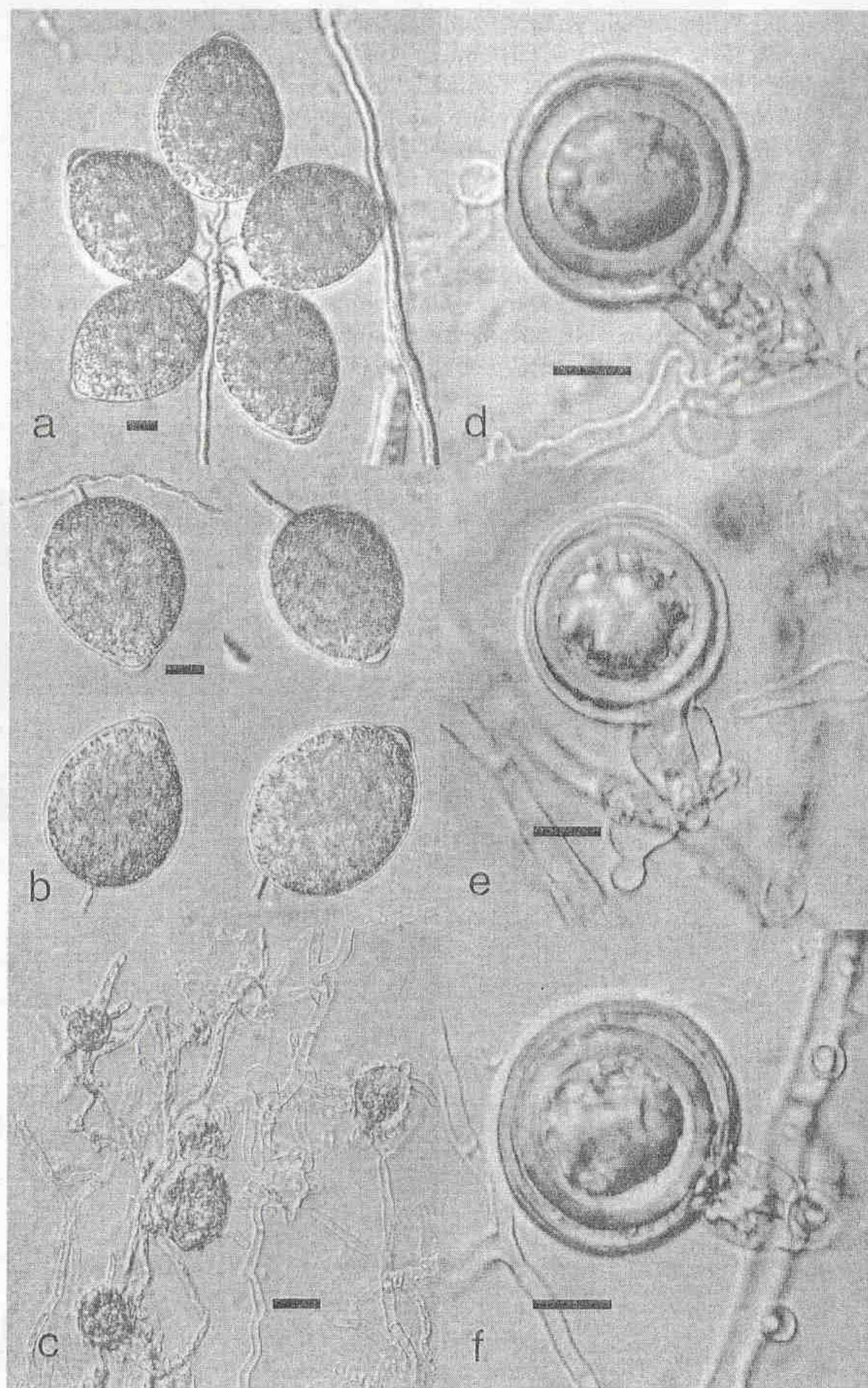
Isolate	Oogonium	Oospore	Antheridium
2052.1	32.5 +/- 2.4	29.7 +/- 2.7	12.5 +/- 2.2 x 13.0 +/- 0.8
2055.2	31.5 +/- 3.9	28.5 +/- 3.8	12.2 +/- 1.7 x 12.9 +/- 1.5
P7	34.1 +/- 5.2	29.1 +/- 4.9	14.4 +/- 1.5 x 13.4 +/- 1.1
P11	34.4 +/- 3.1	29.7 +/- 2.5	12.8 +/- 1.1 x 12.3 +/- 1.1
P-13 (type)	33.6 +/- 3.6	28.2 +/- 3.4	14.0 +/- 2.1 x 12.2 +/- 1.5

Table 2. Sporangial dimensions of five isolates of *Phytophthora nemorosa*, (microns, mean +/- SD, n=10)

Isolate	Length	Breadth	L:B Ratio	Pedicel	Apical plug
2052.1	56.3 +/- 4.4	44.6 +/- 4.4	1.3	7.4 +/- 1.6	9.7 +/- 1.3
2055.2	43.2 +/- 2.9	33.9 +/- 3.2	1.3	8.6 +/- 2.5	8.3 +/- 0.8
P7	50.5 +/- 6.2	37.2 +/- 7.6	1.3	10.3 +/- 6.1	9.0 +/- 1.4
P11	54.0 +/- 6.5	38.7 +/- 4.8	1.4	9.3 +/- 3.7	7.5 +/- 0.6
P-13 (type)	53.2 +/- 7.9	35.7 +/- 2.9	1.5	12.0 +/- 3.8	9.5 +/- 0.6

typically exhibit small, blistered hyphal swellings. Growth optimum is about 15°C, with little or no growth above 20°C. Sporangia formed in water are typically ovoid, scarcely semi-papillate, rarely bifurcate, appearing as terminal clusters on sympodially branched sporangiophores (Figure 1). Caducous sporangia average 51 µm in length (range 38 to 71 µm) and 37 µm in breadth (range 28 to 50 µm). L:B ratios range from 1.3 to 1.5 (average 1.4). Pedicel lengths average 10 µm (range <5-20µm), and apical plugs average 9 µm wide (Table 2).

Figure 1. *Phytophthora nemorosa*. a. sporangia formed in water, ovoid, semipapillate sporangia attached to sporangiophore showing close sympodia giving the appearance of terminal clusters, b. individual sporangia formed in water showing variable pedicel length, c. small hyphal swellings formed in CMAβ showing variable size and small blisters or finger-like projections, d., e., f., oogonia formed in CMAβ showing aplerotic oospores and amphigynous antheridia (Length of bar = 10µ.).



Host range and distribution: *P. nemorosa* has been isolated most frequently from necrotic leaf tips of myrtlewood or California bay (*Umbellularia californica*). It has also been isolated from leaves of manzanita (*Arctostaphylos* species) and coast redwood (*Sequoia sempervirens*). It has been recovered from bark cankers on tanoak (*Lithocarpus densiflorus*) and

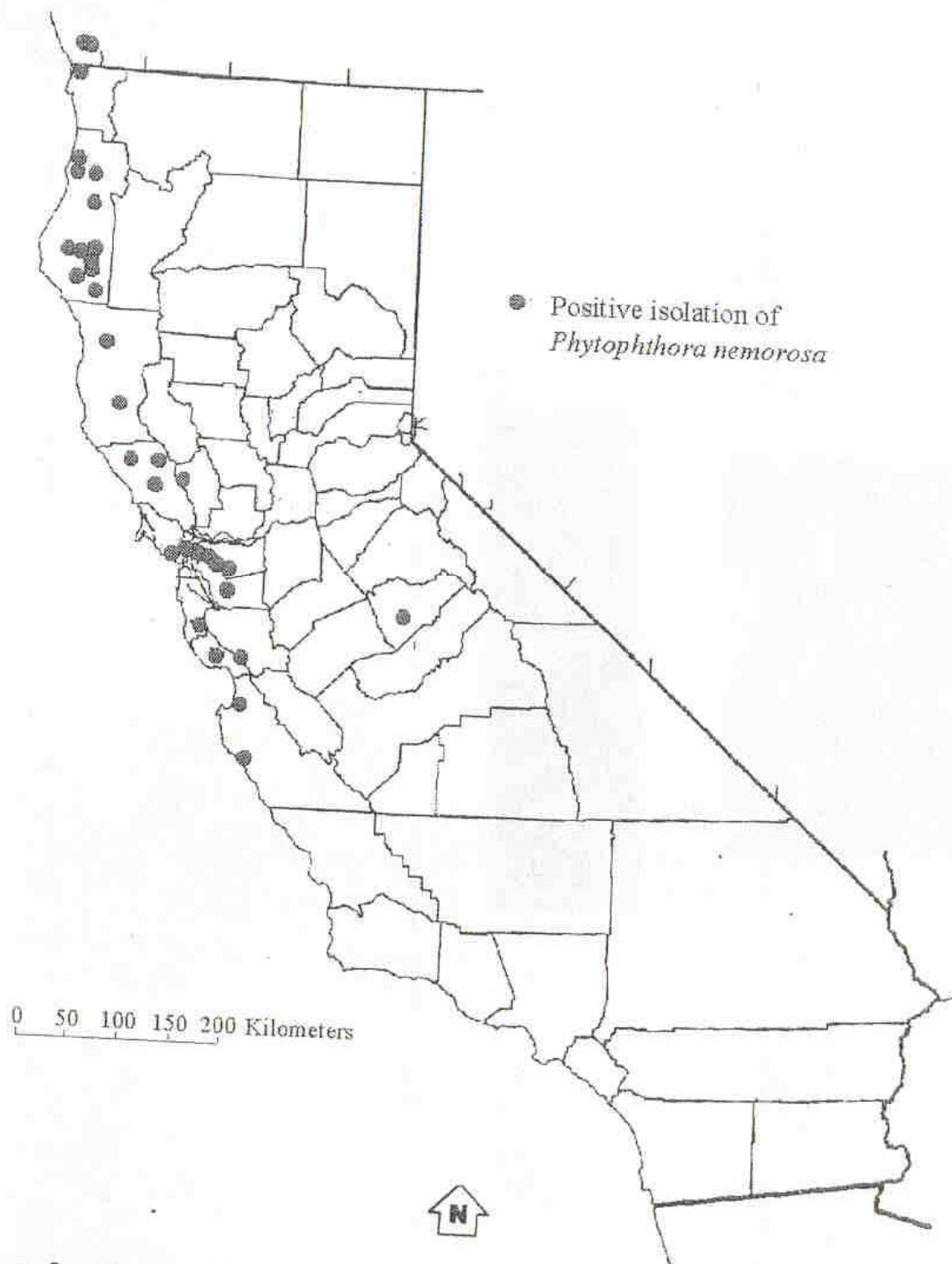


Figure 2. Geographic distribution of isolates of *P. nemorosa* from Oregon and California, U.S.A.

coast live oak (*Quercus agrifolia*) that are comparable in appearance to those caused by *P. ramorum*. Pathogenicity testing will be reported in a later paper, but work to date indicates that *P. nemorosa* is not pathogenic on English holly (*Ilex aquifolium*) as is *P. ilicis*, but does cause bark necrosis of tanoak in artificial inoculation (Davidson et al. 2002). *P. nemorosa* has been found in coastal counties from central California (Monterey Co.) to central Oregon (Coos Co.). It has also been isolated from one location in the Sierra Nevada Mountains (Figure 2).

Similar species: *P. nemorosa* and *P. ramorum* are remarkably similar in host range and symptomology. In culture, however, *P. nemorosa* grows more slowly, with a lower temperature optimum (15C) than *P. ramorum* (20C). Morphologically, *P. nemorosa* (homothallic and lacking chlamydospores), is readily distinguished from *P. ramorum* (Werres et al. 2001).

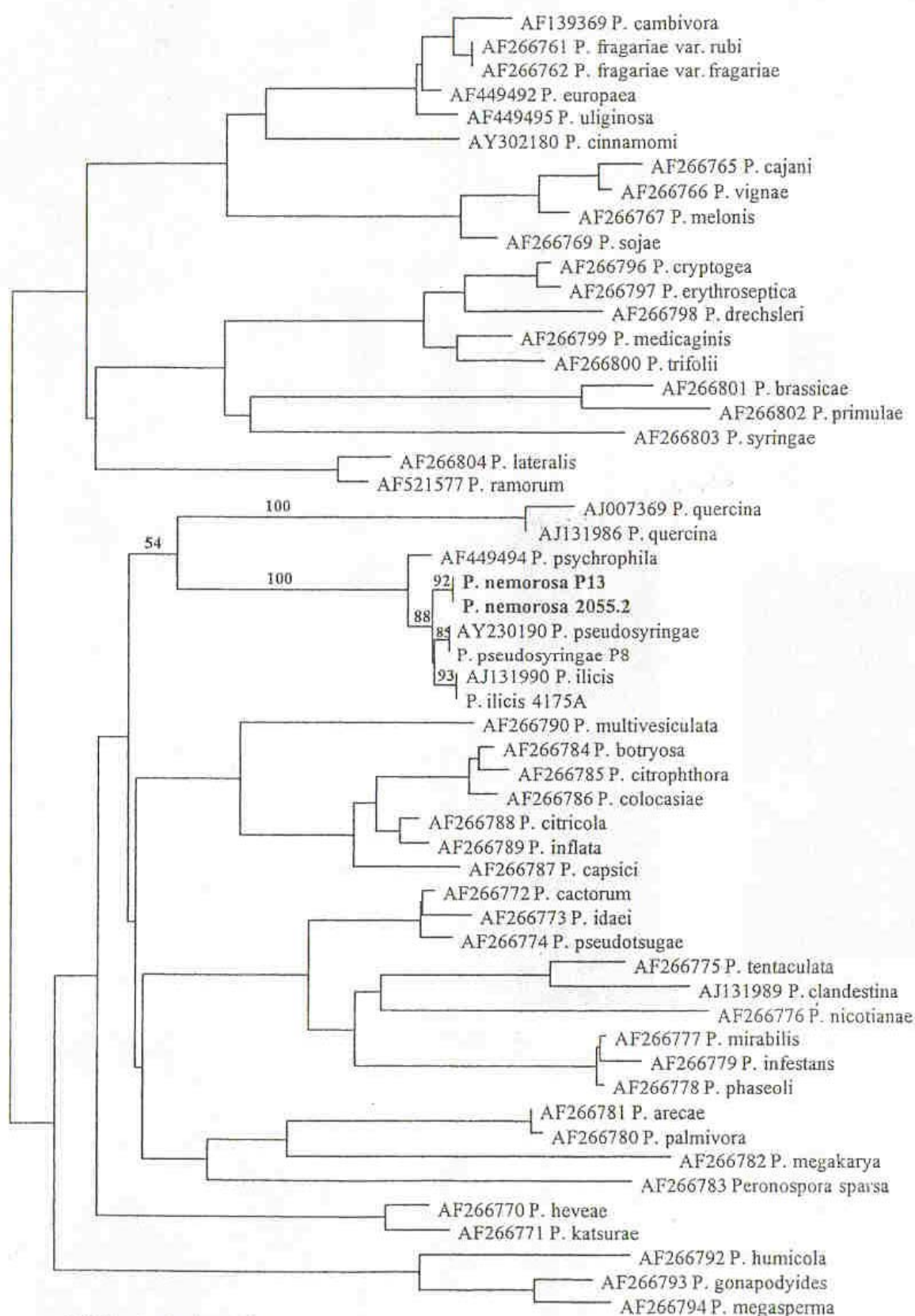
P. nemorosa falls in the artificial Group IV of Waterhouse (Waterhouse 1963, Erwin and Ribeiro 1996) based on gross morphology. Three species in this group are homothallic with amphigynous antheridia, and produce caducous, semipapillate sporangia. *P. ilicis* is most similar and is addressed below. *P. hibernalis* and *P. phaseoli* also share these features. The former is primarily a pathogen of citrus, and has longer sporangial pedicels (Erwin and Ribeiro 1996). The latter is mainly associated with lima bean and has smaller oogonia.

Phylogenetically, *P. nemorosa* falls in ITS DNA Clade 3 (Cooke et al. 2000), with *P. ilicis*, *P. psychrophila*, *P. pseudosyringae* (Jung et al. 2003), and *P. quercina* (Jung et al. 1999) (Figure 3). The latter two species have predominately paragynous antheridia. *P. nemorosa* is distinguished from *P. psychrophila* by its somewhat smaller oogonia (33 μ m vs 37 μ m), slightly rounder sporangia, and distinctly patterned colony growth on agar media where *P. psychrophila* grows without pattern. *P. ilicis* has smaller oogonia (27 μ m) than *P. nemorosa*, and is known only from *Ilex* species.

DISCUSSION

Phytophthora nemorosa is a newly recognized Oomycete species that causes lethal bole cankers on tanoak and coast live oak and foliar necrosis on myrtlewood or California bay, and other hosts. It is found in areas where *Phytophthora ramorum* is also active and it has not been possible to distinguish the diseases caused by these pathogens in the field, although the two organisms are not easily confused in culture. In the forest *P. nemorosa* is usually associated with single killed trees and has behavior suggestive of

an endemic pathogen, in contrast to the expanding patches of mortality associated with the exotic and invasive *P. ramorum*.



— 0.005 substitutions/site

Figure 3. ITS Phylogeny of *P. nemorosa* generated with PAUP* version 4.0b10 (Swofford 2000).

The ITS DNA sequence of *P. nemorosa* indicates close relationship to *P. ilicis* (a foliar pathogen of holly) (Figure 3), *P. psychrophila* (recently described from European oak forest soils; Jung et al. 2002), and *P. pseudosyringae* (Jung et al. 2003), and more distant relationship to *P. quercina*, all in ITS clade 3 (Cooke et al. 2000). The proliferation of newly recognized species in this clade is indicative of the taxonomic flux in the genus as a whole. This is in large part the result of increased attention to Phytophthoras in previously unexpected habitats, as well as to new molecular tools that aid diagnosis.

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