Population Biology of *Pseudoperonospora humuli* in Oregon and Washington

Hee Youn Chee, Mark E. Nelson, Gary G. Grove, Kenneth C. Eastwell, Stephen T. Kenny, and Robert E. Klein, Irrigated Agriculture Research and Extension Center, Washington State University, 24106 N. Bunn Rd., Prosser 99350

ABSTRACT

Chee, H. Y., Nelson, M. E., Grove, G. G., Eastwell, K. C., Kenny, S. T., and Klein, R. E. 2006. Population biology of *Pseudoperonospora humuli* in Oregon and Washington. Plant Dis. 90:1283-1286.

Pseudoperonospora humuli populations from Oregon and Washington were analyzed for genetic variation using random amplified polymorphic DNA (RAPD) and DNA amplification fingerprinting (DAF) markers. The genetic structure of the Oregon and Washington populations differed considerably. There was little genetic diversity in Washington, with only five RAPD and six DAF groups detected among 40 isolates tested. One genotype was predominant in Washington. In contrast, 18 RAPD and 34 DAF groups were found among the 40 isolates tested from Oregon. No unique band profile associated with host cultivar was observed. It is suggested that the distinct difference in population structure between the two geographic regions might be due to climatic differences resulting in a higher frequency of sexual reproduction of *P. humuli* in Oregon than in Washington.

Additional keywords: downy mildew, hop, *Humulus lupulus*

Cultivated hop (*Humulus lupulus* L.) is an herbaceous and dioecious perennial plant. Each spring, shoots emerge from the rootstock (crown) and, when grown under cultivation, are trained up strings attached to a trellis system 5 to 6 m high. Lateral branching ensues when the vines have grown about halfway up the trellis. By late June or early July, apical growth ceases and flowering occurs on the lateral branches. Only the female plants produce cones used in beer and ale production to provide bitterness and hop aroma/flavor. The commercial value of hop cones lies in the lupulin glands which contain resins and essential oils including alpha and beta acids. Washington, Oregon, and Idaho account for approximately 23% of the total world's hop acreage and 36% of the total worldwide alpha acid production (6).

Downy mildew, caused by *Pseudoperonospora humuli* (Miyabe & Takah.), is a

serious disease of hop in the Pacific Northwest. It is more severe in areas of high rainfall such as the Willamette Valley of Oregon than in arid climates such as the Yakima Valley of Washington (16). Perennation of *P. humuli* occurs primarily in the hop crown, from which systemically infected shoots (basal spikes) emerge in the spring and early summer (4). Zoosporangia from these basal spikes serve as the primary inocula, and the pathogen spreads to healthy leaves and shoots under appropriate environmental conditions. Free water and mild temperatures favor sporulation and distribution of zoosporangia, followed by release of zoospores and secondary infection (4,13). Oospores are formed in infected shoots and especially infected cones, but their role in the disease cycle has not been clearly established (4,13). Because they are abundant and have occasionally been reported to germinate in the laboratory, it was assumed that oospores are partly responsible for the seasonal production of primary spikes associated with perennation (1,7). However, repeated attempts to induce consistent germination of oospores in the laboratory or under field conditions have failed, and their role in the disease cycle is considered circumstantial (11).

Genetic markers generated by random amplified polymorphic DNA (RAPD) (17) have proven useful for studying genetics and phylogenetic relationships in a wide variety of fungi. A variation of the random-primer-polymerase chain reaction (PCR) technique called DNA amplified fingerprinting (DAF) has been successfully employed in the identification of closely related organisms including viruses, bacteria,

fungi, and plants (2). The objective of this research was to develop genetic markers suitable for characterizing the genetic structure of *P. humuli* populations, and utilize those techniques to compare diversity of the Oregon and Washington populations of this pathogen.

MATERIALS AND METHODS

Fungal isolates. Eighty hop basal spikes were collected from commercial fields and experimental plots in eight Oregon and six Washington hop yards. The 40 spikes from Oregon were collected from cultivars Nugget (20 isolates), Willamette (18 isolates), and Bullion (2 isolates). The 40 spikes from Washington were collected from cultivars Galena (13 isolates), Nugget (3 isolates), Cluster (18 isolates), and Chelan (6 isolates). The average distance between nearest neighboring sampled hop yards was 4.5 km (range 2.0 to 8.6 km) in Oregon and 9.0 km (range 5.8 to 13.4 km) in Washington. The greatest distance between sampled hop yards was 27 km in Oregon and 58 km in Washington.

Spikes were returned to the laboratory and sporulation of *P. humuli* induced by enclosing each individual spike in a plastic bag and incubating at 18°C for 12 to 18 h in darkness. Leaves exhibiting sporulation were harvested from each spike. Leaves that appeared contaminated with secondary microorganisms or otherwise degraded were not included in samples. Zoosporangia were harvested by vigorously shaking the infected leaves in distilled H₂O (dH₂O) for 30 s. Suspensions of zoosporangia were strained through several layers of cheesecloth, centrifuged at 1,200 × g for 5 min, and the supernatant discarded. Pellets containing zoosporangia were resuspended in dH₂O and centrifuged at 1,200 × g for 5 min. The pellet was resuspended in dH₂O and stored in a cryovial at -70°C for future use.

DNA extraction. DNA was extracted directly from zoosporangia using an extraction procedure modified from the cetyltrimethylammonium bromide (CTAB) procedure of Murray and Thompson (8). Suspensions of zoosporangia were centrifuged at 1,200 × g for 5 min, resuspended in 0.5 ml extraction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 100 mM EDTA), vortexed for 2 min with 450- to 600-μm-diameter glass beads, and transferred to a 1.5-ml microcentrifuge tube. After addition of 25 μl 20% sodium dodecyl sulfate (SDS), the mixture was gently

Corresponding author: M. E. Nelson
E-mail: mark_nelson@wsu.edu

Current address of H. Y. Chee: Biological Sciences, Konyang medical school, Nonsan-city, Chungnam, Korea. E-mail: hychee@konyang.ac.kr

Current address of R. E. Klein: California Pistachio Commission, Fresno, CA.
E-mail: bobk@pistachios.org

Washington State University Plant Pathology New Series Manuscript No. 0407.

Accepted for publication 27 February 2006.

DOI: 10.1094/PD-90-1283

© 2006 The American Phytopathological Society

shaken for 60 min at room temperature followed by serial addition of 75 µl of 5 M NaCl and 65 µl of 10% CTAB in 0.7 M NaCl followed by incubation at 65°C for 15 min. The mixture was divided into two 1.5-ml microcentrifuge tubes, mixed 1:1 with chloroform/isoamyl alcohol (24:1), followed by centrifugation at 11,000 × g at room temperature for 10 min. The aqueous phase was transferred to a 1.5-ml microcentrifuge tube, and nucleic acids were precipitated by addition of 0.6 volumes of cold isopropanol and incubation at –20°C for 30 min. Nucleic acids were pelleted by centrifugation at 11,000 × g at room temperature for 5 min. The pellet was rinsed twice with cold 70% ethanol (EtOH), dried in a transfer hood for 2 h, and resuspended in 100 µl of TE (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). Contaminating RNA was completely digested by addition of 1 µl of ribonuclease A (10 mg/ml; Sigma Chemical Co., St. Louis, MO) and incubation at 4°C overnight. The DNA was reprecipitated by the addition of 0.1 volume of 3 M sodium acetate, pH 5.2, and 2 volumes of 95% EtOH. After 20 min at –20°C, the nucleic acid was collected by centrifugation at 11,000 × g for 5 min, dried in a transfer chamber for 2 h, dissolved in 100 µl of TE, and quantified using the minigel method described by Sambrook et al. (12). The purity of the DNA sample was further checked by spectrophotometric absorbance (OD₂₆₀/OD₂₈₀).

RAPD analysis. A total of 120 decamer primers, including those listed in Table 1, were screened with 10 *P. humuli* isolates (five from Washington and five from Oregon) to select primers that exhibited good PCR amplification and consistent banding patterns following electrophoresis. RAPD amplification reactions (17) were performed in a 0.6-ml microcentrifuge tube containing a final volume of 12.5 µl of reaction mixture. The reaction mixture

consisted of: 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 200 µM each dNTP, 0.2 µM random primer (Op-eron Technology, Alameda, CA), 20 ng of genomic DNA, and 1 unit *Taq* polymerase (Promega, Madison, WI) overlaid with 50 µl of mineral oil (Sigma). The tubes were kept on ice during all manipulations. To confirm that observed bands were amplified DNA and not primer artifacts, genomic DNA was omitted for each primer as a control. Amplifications were conducted in a DNA thermocycler (Applied Biosystems, Foster City, CA) programmed for 5 min at 94°C followed by 45 cycles of 45 s at 94°C, 1 min at 36°C, and 2 min at 72°C, with final extension for 7 min at 72°C.

Amplification products were separated by electrophoresis on 1.5% agarose (Amersham, Madison, WI) gels for 90 min at 100 V in TBE buffer (0.09 M Tris, 0.09 M boric acid, and 0.002 M EDTA, pH 8.0). DNA bands were stained with ethidium bromide and photographed. Reproducibility of RAPD patterns was tested with two independent replicates of each PCR assay. Only clear and distinct bands were included in the analysis.

DAF. Seven arbitrary primers, seven or eight nucleotides in length, were screened with 10 isolates using a modification of the Caetano-Anolles (2) DAF technique. The 12-µl reaction mixture contained 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 200 µM dNTP, 6 mM MgCl₂, 10 ng template DNA, 3 µM primer, and 2 units *Taq* polymerase (Promega). Cycling parameters included an initial denaturation at 96°C for 4 min, followed by 30 cycles of 1 s at 96°C, 1 s at 36°C, and 5 s at 72°C. Amplified products were separated on 8% polyacrylamide gels (8.3 × 10.2 cm) at 90 V in TBE buffer. DNA bands were silver-stained, examined on a light box, and photographed.

Data analysis. Genetic variation among isolates was assessed by comparing the PCR amplified band profiles of all primer-isolate combinations. Reproducible amplified DNA fragments were scored for all isolates based on presence or absence. Very intense bands were referred to as major bands and less intense bands were referred to as minor bands. Intensity of bands was determined by visual observa-

tion. Variability was assessed within and among geographic regions, as well as host cultivar regardless of the geographic origin. A DNA fingerprinting similarity index (9) was used to quantify the genetic variation among *P. humuli* isolates collected from the two geographic regions.

Unique bands or band profiles associated with host cultivar or geographic region were evaluated by comparing all primer/isolate PCR products. Profiles for each primer were compared on the basis of the presence or absence of the RAPD and DAF products of identical length, and these data were used to calculate similarity coefficients. The SHAN program of NTSYS-PC Software (Numerical Taxonomy System, Version 1.8, Exeter Publishing Ltd., Setauket, NY) was utilized to compute the matrix of similarity coefficients among isolates (SIMQUAL function) (10). The range and average of similarity coefficients among isolates within a geographic region were used to assess genetic diversity. Differences in genetic polymorphisms between the two geographic regions were determined by measuring genotype diversity based on the Shannon-Weaver Index (H):

$$H = -\sum P_i \ln P_i$$

where P_i is the frequency of a given band profile based on RAPD and DAF data (5).

RESULTS

RAPD analysis. Discrete DNA fragments were amplified from genomic DNA using 10-base random primers (Fig. 1). Of the 120 primers screened, 73 primers (61%) produced discrete DNA fragments. Fifteen primers (Table 1) that showed consistent amplification and band profiles were selected for future studies. The selected oligonucleotide primers generated a total of 85 DNA amplification products in the 80 isolates studied. The number of bands generated by each primer varied from 4 to 10. The amplified fragments ranged from 400 to 1,500 bp. Among the 85 RAPD markers, 72 monomorphic and 13 polymorphic bands were observed (Table 1).

Primers differed in their ability to generate polymorphisms. Nine random primers gave nearly identical banding patterns for all isolates, and six primers generated

Table 1. Number of scored and polymorphic random amplified polymorphic DNA (RAPD) bands produced by *Pseudoperonospora humuli*, using selected primers

Random primer	Primer sequence (5'-3')	Total bands	Polymorphic bands
OPA01	CAGGCCCTTC	7	0
OPA03	ACTCAGCCAC	6	0
OPA08	GTGACGTAGG	10	4
OPA19	CAAACGTCGG	5	0
OPB04	GGACTGGAGT	5	1
OPB12	CCTTGACGCA	7	3
OPB18	CCACAGCAGT	4	0
OPC01	TTCGAGCCAG	6	2
OPC05	GATGACCGCC	5	2
OPC20	ACTTCGCCAC	6	0
OPD05	TGAGCGGACA	5	0
OPD07	TTGGCACGGG	5	1
OPE18	GGACTGCAGA	5	0
OPF09	CCAAGCTTCC	5	0
OPF10	GGAAGCTTGG	4	0
Total		85	13

Fig. 1. Agarose gel electrophoresis of random amplified polymorphic DNA (RAPD) products from DNA of *Pseudoperonospora humuli* isolates using primer OPB 12 (CCTTGACGCA).

polymorphism between isolates. Major bands with strong intensities were highly reproducible, but some bands had variable intensities on repeat runs. The cause of this variation was not determined. All primers produced bands common to all isolates. In addition, isolates from Oregon usually produced more bands than isolates from Washington. For example, OPC05 primer produced two or three additional major bands with Oregon isolates. Similarity between isolates and the number of unique genotypes was primer dependent. Variation in the specific pattern of one isolate compared with others was inconsistent in that it was observed with one primer but not with another. The relationships among isolates were more clearly represented by a similarity matrix. In construction of the matrix, it was assumed that corresponding bands arose by amplification of the same genetic locus. All isolate combinations within a population were assigned similarity values based on the assumption that corresponding bands represented amplification of the same genetic locus. The similarity value within populations appeared to

be different between the two geographic regions. Most isolates in Washington generated nearly identical banding patterns among isolates with similarity coefficients ranging from 97.6 to 100% with an average of 99.7%. In Oregon, the average similarity coefficient value among isolates was 95.6%, ranging from 90.6 to 100%.

Genotype diversity of Oregon isolates (2.857) based on the Shannon-Weaver Index was greater than that of Washington (0.348). While 18 genotypes (RAPD groups) were found in Oregon, only five were found in Washington. Thirty-six of 40 Washington isolates (90%) showed identical RAPD profiles, while the remaining four showed unique banding patterns. The Oregon isolates were not skewed to one genotype but more evenly distributed (Fig. 2). The dominant Washington genotype was also detected in Oregon (six isolates).

DAF PCR. In preliminary tests, seven primers were used to amplify genomic DNA from 10 isolates. Clear and reproducible polymorphic bands were obtained with DAF03 and DAF04 primers. Those

two primers were used to amplify genomic DNA of all 80 isolates. Typically, 5 to 10 major bands and 10 to 15 minor bands were produced by each primer. A total of 32 bands were scored of which 22 were polymorphic (Table 2). Most amplified DNA fragments were between 300 and 1,500 bp in size (Fig. 3).

Similarity coefficients based on DAF analysis indicated a largely uniform population in Washington and a highly variable population in Oregon. Similarity coefficients of Washington isolates ranged from 87.5 to 100%, with an average of 97.6%. Similarity coefficients of isolates from Oregon averaged 79.2%, ranging from 62.5 to 100%. Genotype diversity based on the Shannon-Weaver Index was 3.455 and 0.898 in Oregon and Washington, respectively. While 34 genotypes were found in Oregon, only six were found in Washington (Fig. 4). Thirty-four of the 40 Washington isolates showed 100% similarity between isolates. No banding patterns were found in both geographical regions. Bands or band profiles unique to the host cultivar were not found.

DISCUSSION

In this study, DNA polymorphisms generated by RAPD and DAF techniques were examined in an attempt to characterize the population structure of *P. humuli* in Oregon and Washington. RAPD analysis detected greater differences in genetic variability among isolates from Oregon than among isolates from Washington. Isolates from Washington were nearly clonal, with 90% belonging to a single genotype, while 18 genotypes were found among the 40 Oregon isolates. The DAF technique detected a large number of polymorphisms among isolates and was more sensitive than RAPD analysis in estimating the degree of genetic variation among isolates of *P. humuli* on the basis of similarity coefficients and genotype diversity. The premise of genetic homogeneity in the Washington population in contrast to relatively high diversity in Oregon was strongly supported by average similarity coefficients of 97.6 and 79.2% based on DAF markers. While 34 different DAF patterns were observed among the 40 Oregon isolates, only six were found from Washington, including one dominant type that comprised 85% of the 40 Washington isolates. Moreover, the DAF- and RAPD-based analyses both

Fig. 2. Incidence of random amplified polymorphic DNA (RAPD) groups in Oregon (□) and Washington (■). Values are percent population of each state. Group 1 was the only group detected in both states.

Fig. 3. DNA amplification fingerprinting (DAF) profiles of *Pseudoperonospora humuli* isolates from Oregon and Washington using the DAF03 primer (GTTACGCC).

Table 2. Number of scored and polymorphic DNA amplification fingerprinting (DAF) bands produced by *Pseudoperonospora humuli*, using selected primers

Random primer	Primer sequence (5'-3')	Total bands	Polymorphic bands
DAF03	GTTACGCC	17	12
DAF04	GAAACGCC	15	10
Total		32	22

Fig. 4. Incidence of DNA amplification fingerprinting (DAF) groups found in Oregon (□) and Washington (■). Values are percent population of each state. No banding patterns were found in both states.

Fig. 5. Mean monthly precipitation and average maximum temperature in the hop-growing regions of Oregon and Washington. The mean daily maximum temperatures for Salem, OR (●) and Yakima, WA (○) are based on 30-year and 40-year averages, as is the average total monthly precipitation (Salem, OR: □; Yakima, WA: ■). Source: National Climate Data Center (online).

revealed similar degrees of genotypic diversity when the two geographic regions are compared, namely, that there are few isolates displaying variation from Washington relative to the diversity observed in those from Oregon.

The distinct differences in *P. humuli* population structures between the two geographic regions suggest that sexual reproduction might be more important in the Oregon production area than in the Washington production area. Although the role of sexual reproduction of *P. humuli* in the disease cycle is not well understood, it has long been known that oospores can be found in infected hop tissue in most hop growing areas of the world, including both Oregon and Washington (1,13,14). Skotland (13), however, demonstrated that *P. humuli* overwinters as mycelium in infected hop rootstock and suggested that oospores in the soil are probably of little importance in the Yakima Valley of Washington. Oospores are rare in the Yakima Valley because the dry, hot summer weather inhibits secondary spread and aboveground infected tissue desiccates rapidly (15). Furthermore, Chee and Klein (3) demonstrated that oospore production

could be induced in the laboratory with *P. humuli* isolates collected from both Oregon and Washington but that high temperatures inhibited oospore formation. Figure 5 illustrates the climatic differences between the western Oregon (relatively cool and wet) and central Washington (relatively warm and dry) hop growing areas during the growing season. These reports suggest that oospore development is affected by local environmental factors. Favorable environmental factors such as high rainfall and low temperature combined with high disease incidence might induce relatively active sexual reproduction in the Willamette Valley of Oregon. Conversely, hot and dry weather, and resulting low disease incidence, might impede sexual reproduction in the Yakima Valley of Washington. In the present study, some isolates were found with identical RAPD and DAF markers in Oregon, suggesting that sexual reproduction might be followed by several rounds of asexual reproduction. The nearly clonal population structure in Washington suggests limited or no sexual reproduction.

This study compares the number of *P. humuli* genotypes measured by RAPD and DAF analysis that occur in two geographi-

cally defined areas with differing climates and disease incidence. This description provides the foundation for future research on the genetics, population structure, and epidemiology of this pathogen. While it is suggested that the greater number of genotypes detected in Oregon compared with Washington may be due to a greater incidence of sexual reproduction in Oregon, additional research is needed to better understand the role of sexual reproduction in these populations.

ACKNOWLEDGMENTS

Support for this research was provided by grants from Busch Agricultural Resources Inc., Hop Research Council, Miller Brewing Co., Oregon Hop Commission, and Washington Hop Commission.

LITERATURE CITED

- Bressan, E. N., and Nichols, R. A. 1933. Germination of the oospores of *Pseudoperonospora humuli*. Phytopathology 23:485-486.
- Caetano-Anolles, G. 1993. Amplifying DNA with arbitrary oligonucleotide primers. PCR Methods Applications 3:85-94.
- Chee, H. Y., and Klein, R. E. 1998. Laboratory Production of Oospores in *Pseudoperonospora humuli*. Korean J. Plant Pathol. 14:618-621.
- Coley-Smith, J. R. 1962. Overwintering of hop downy mildew *Pseudoperonospora humuli* (Miy. et Tak.) Wilson. Ann. Appl. Biol. 50:235-243.
- King, L. M., and Schaal, B. A. 1989. Ribosomal-DNA variation and distribution in *Rudbeckia missouriensis*. Evolution 43:1117-1119.
- MacKinnon, D. 2004. USA Hops 2004 Statistical Report. Hops Growers of America, Yakima, WA.
- Magie, R. O. 1942. The epidemiology and control of downy mildew on hops. NY State Agric. Exp. Stn. (Geneva) Tech. Bull. 267.
- Murray, M. G., and Thompson, W. F. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8:4321-4325.
- Nei, M., and Li, W. 1979. Mathematical model for studying genetic variations in terms of restriction endonuclease. Proc. Natl. Acad. Sci. USA 76:5269-5273.
- Rohlf, F. J. 1993. NTSYS-PC: Numerical Taxonomy System, Version 1.8. Exeter Publishing Ltd., Setauket, NY.
- Royle, D. J., and Kremheller, H. TH. 1981. Downy mildew of the hop. Pages 395-419 in: The Downy Mildews. D. M. Spencer, ed. Academic Press, New York.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, NY.
- Skotland, C. B. 1961. Infection of hop crowns and roots by *Pseudoperonospora humuli* and its relation to crown and root rot and overwintering of the pathogen. Phytopathology 51:241-244.
- Skotland, C. B. 1964. Life cycle of the hop downy mildew fungus. Hop downy mildew - A symposium. Modern Brewery Age. May 1964.
- Skotland, C. B., and Johnson, D. A. 1983. Control of downy mildew of hops. Plant Dis. 67:1183-1185.
- Skotland, C. B., and Romanko, R. R. 1964. Life history of the downy mildew fungus. Wash. Agric. Exp. Stn. Circ. 433.
- Williams, J. G., Kubelik, A. R., Livak, K. J., Rafalski, J. A., and Tingey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18:6531-6535.