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Genetic diversity of the pea root pathogen *Aphanomyces euteiches* in Europe

Carol Kälin[1] | Anna Berlin[1] | Agnese Kolodinska Brantestam[2] | Mukesh Dubey[1] | Anna-Kerstin Arvidsson[2] | Paul Riesinger[3] | Malin Elfstrand[1] | Magnus Karlsson[1]

[1]Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Uppsala, Sweden

[2]Nomad Foods Ltd, Findus Sverige AB, Bjuv, Sweden

[3]Novia University of Applied Sciences, Ekenäs, Finland

Correspondence
Carol Kälin, Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Uppsala, Sweden.
Email: carol.kalin@slu.se

Funding information
SLU Grogrund

Abstract

The oomycete pathogen *Aphanomyces euteiches* causes root rot in various legume species. In this study we focused on *A. euteiches* causing root rot in pea (*Pisum sativum*), thereby being responsible for severe yield losses in pea production. We aimed to understand the genetic diversity of *A. euteiches* in Europe, covering a north-to-south gradient spanning from Sweden, Norway and Finland to the UK, France and Italy. A collection of 85 European *A. euteiches* strains was obtained, all isolated from infected pea roots from commercial vining pea cultivation fields. The strains were genotyped using 22 simple-sequence repeat markers. Multilocus genotypes were compiled and the genetic diversity between individual strains and population structure between countries was analysed. The population comprising strains from Italy was genetically different and did not share ancestry with any other population. Also, strains originating from Finland and the eastern parts of Sweden were found to be significantly different from the other populations, while strains from the rest of Europe were more closely related. A subset of 10 *A. euteiches* strains from four countries was further phenotyped on two susceptible pea genotypes, as well as on one genotype with partial resistance towards *A. euteiches*. All strains were pathogenic on all pea genotypes, but with varying levels of disease severity. No correlation between the genetic relatedness of strains and virulence levels was found. In summary, our study identified three genetically distinct groups of *A. euteiches* in Europe along a north-to-south gradient, indicating local pathogen differentiation.

KEYWORDS
Aphanomyces root rot, genetic diversity, pathogenicity, *Pisum sativum*, virulence

1 | INTRODUCTION

The oomycete pathogen *Aphanomyces euteiches* is the causative agent of Aphanomyces root rot disease in a broad range of various legume host species, including pea (*Pisum sativum*). Pea is one of the most important legumes in the world and with the global trend towards a more sustainable food production and consumption, peas are becoming increasingly high in demand as a valuable source of plant-based protein (Ge et al., 2020). Aphanomyces root rot is the major constraint for increased pea production in Europe and can cause very high yield losses and negatively affect quality. Vining peas are harvested as immature seeds (green peas) and consumed as a vegetable. They are cultivated worldwide in areas with a temperate climate, and worldwide production reached 19.87 million tonnes in 2020 (FAO, 2021).

A. euteiches is a diploid, homothallic (self-fertile) species, although there are clear indications of occasional outcrossing (Quillévéré-Hamard et al., 2018). Through sexual reproduction, *A. euteiches* produces highly resilient oospores that serve as the primary inoculum and can remain dormant in the soil for several years (Hughes & Grau, 2013). Once a suitable host plant is present, the oospore germinates to form a sporangium that in turn produces actively swimming zoospores that are typically responsible for the infection of host root tissue. Common symptoms of infection include root browning and reduction of root volume and function. Late-stage symptoms include leaf chlorosis, wilting and in extreme cases plant death (Hughes & Grau, 2013; Wakelin et al., 2002).

Due to the soilborne nature of *A. euteiches*, management of root rot disease in pea is difficult and relies mainly on avoidance of highly infested fields and inoculum build-up. Forecasting methods include soil tests prior to sowing, using susceptible pea genotypes in field soil (Hughes & Grau, 2013). Crop rotation with nonhost crops is another widely used control measure. However, due to the long survival of resilient oospores in the soil, crop rotation periods should span at least 6–8 years to minimize the risk of root rot disease (Wu et al., 2018). These long intervals in crop rotation pose a considerable constraint to the total production of peas, especially for cultivation of vining peas where production sites must be close to processing factories to keep short time spans between harvest and processing in order to maintain good quality of the final product. Seed treatment with chemical or biological products may provide a limited protection towards the disease. Currently, there are no commercial pea varieties with complete resistance against Aphanomyces root rot, although pea genotypes carrying partial resistance have been identified (Desgroux et al., 2016; Hamon et al., 2011; Lavaud et al., 2015).

Previous population genetic studies from major pea production regions in the United States revealed high genetic diversity within fields but rather low diversity among populations, and no population structure at a regional level (Malvick & Percich, 1998a; Malvick et al., 2008). Le May et al. (2018) showed that North American *A. euteiches* strains isolated from pea could be divided into three different populations, while strains from cultivated pea in France formed a single population with no substructure. In contrast, a study based on codominant simple-sequence repeat (SSR) markers of French *A. euteiches* strains described two distinct genetic groups (Quillévéré-Hamard et al., 2018). Strains from the Bourgogne region showed higher levels of heterozygosity compared with strains from other parts of France.

Earlier studies, both in the United States and Europe, have investigated the link between genetic diversity of *A. euteiches* strains and host range and disease severity. No association between genotypic diversity and disease severity was detected using single-zoospore progeny of North American *A. euteiches* strains (Malvick & Percich, 1998b). Likewise, no relationship between race phenotype and genotype was detected in alfalfa-infecting *A. euteiches* strains (Malvick et al., 2008). Wicker et al. (2001) investigated pathogenic diversity among *A. euteiches* isolates from France and described four pathotypes based on their host range and aggressiveness. They further confirmed the existence of two virulence phenotypes for pea-infecting isolates with host range "pea" or "pea/alfalfa" (Malvick et al., 1998). Quillévéré-Hamard et al. (2018) reported on high diversity in aggressiveness between strains, especially in the Bourgogne population, but a weak relationship between genetic structure and aggressiveness.

A better understanding of the genetic diversity of *A. euteiches* on a European level is important for future efforts in breeding for disease resistance and for long-term deployment of management strategies. Therefore, this study aimed to investigate the genetic diversity among *A. euteiches* strains sampled across Europe with the emphasis on a north–south gradient, using codominant SSR markers. More specifically, we investigated (a) the genetic diversity and population structure of *A. euteiches* across Europe, and (b) the correlation between genetic variation and virulence on pea.

2 | MATERIALS AND METHODS

2.1 | Sampling and isolation of European *A. euteiches* strains

Strains of *A. euteiches* were collected from different European vining pea cultivation sites (Table S1). Soil samples were collected at 20–25 cm depth during October and November in 2012, 2014, 2018, 2019, and during May and June in 2020, and stored at 6°C in sealed plastic bags to retain humidity until culturing. In addition, roots of infected plants in production fields were sampled in 2018, in the beginning of May in Italy and in the beginning of June in France, at plant growth stage 35–60 according to the BBCH scale (Feller et al., 1995). *A. euteiches* was baited from each soil sample using the susceptible cultivar Linnea, as described by Olofsson (1967). All plant roots were washed in order to grade the characteristic colour and softness of roots caused by *A. euteiches*. After washing and microscopic investigation, root pieces of individual samples were placed on a filter under running water for 1 h and then moved to selective medium agar (Larsson & Olofsson, 1994). After 2–3 days, tips of hyphae growing out from the root pieces were cut and transferred to Petri plates with corn meal agar (CMA; BD Biosciences). Plates were incubated at 20°C for 10 days to initiate growth and then moved to 4°C for long-term storage in darkness. Strains were routinely transferred to new CMA plates twice a year. Ten previously genotyped *A. euteiches* strains from France (Moussart et al., 2007; Quillévéré-Hamard et al., 2018) were obtained on agar plates. For DNA extractions, strains were grown in glucose peptone broth (GPB; glucose 5 g/L, peptone 20 g/L) and incubated at room temperature and shaken at 120 rpm for 5–7 days. Mycelia were harvested by filtering through filter paper (grade 1003; Ahlstrom Munksjö) and immediately processed.

2.2 | DNA extraction and SSR amplification

Harvested mycelia were ground in 2 ml screw cap tubes with three 2 mm diameter glass beads per tube for 2 × 30 s at maximum speed, using a Precellys 24 Tissue Homogenizer (Bertin Technologies). Genomic DNA was extracted following a 3% hexadecyl-trimethyl-ammonium

bromide (CTAB) extraction protocol (Nygren et al., 2008) with an additional chloroform purification step. The DNA concentration and quality were measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific) and by 1% agarose gel electrophoresis.

PCR amplification of the 22 SSR markers was conducted using primers with 5′ FAM/HEX modifications (Table S2) following the protocol by Mieuzet et al. (2016) with minor modifications. Each PCR contained 1 U *Taq* DNA polymerase (Thermo Scientific), PCR buffer, 0.2 mM dNTPs, 1 μM fluorescence dye-tagged forward primers, 1 μM reverse primers and 20 ng genomic DNA template, made up to a total volume of 10 μl with sterile distilled water. The PCRs were run on a Veriti 96-well thermal cycler (Applied Biosystems) with an initial denaturation at 96°C for 5 min, followed by 20 cycles at 95°C for 60 s, an annealing step at 58°C for 60 s and extension at 72°C for 90 s. Some primer pairs were run with adjusted annealing temperatures (Table S2). PCR product concentrations were measured with a NanoDrop 1000 spectrophotometer, and the fragment size was verified through 2% agarose gel electrophoresis. Negative PCR amplifications were rerun to confirm null alleles. PCR products for two markers with different florescent dyes were pooled together with concentrations adjusted to 50 ng/μl for each product prior to being air-dried overnight at room temperature and sent to Macrogen Europe B.V. (Amsterdam, Netherlands) for fragment analysis on a 3730xl DNA analyser using standard parameters and HD400 as the internal standard.

2.3 | Allele scoring and primer quality assessment

Allele scoring was done using the GeneMarker software v. 3.0.1 (SoftGenetics LLC) using standard parameters (Fragment Plant, default data process). The 10 *A. euteiches* strains from France were used as an internal control to allow comparison with previous studies (Quillévéré-Hamard et al., 2018). Multilocus genotypes (MLGs) were obtained by combining data from the 22 loci for each sample. The Excel (Microsoft) plugin GenAlEx v. 6.503 (Peakall & Smouse, 2006, 2012) was used for the initial quality assessment of the data. The R package poppr v. 2.9.3 (Kamvar et al., 2014, 2015) was used to check marker performance and basic overall quality assessment. A genotype accumulation curve was created with loci being resampled 1000× without replacement and dropping monomorphic loci. A locus table including number of alleles for each locus and missing data percentage were calculated prior to clone-correcting the data set and calculating evenness. Of the 22 loci, four were monomorphic. Three monomorphic loci were excluded in further analyses, while the fourth showed an uneven distribution of missing values (null alleles present in strains from Finland and in two strains from Sweden) and was thus retained.

2.4 | Analyses of population structure of *A. euteiches* in Europe

Population genetic analysis was performed on strains grouped by country of isolation (see Figure 1b for overview). The number of unique MLGs was determined for each country. Genotypic diversity for each country was calculated as the number of MLGs divided by the number of samples in each country. Because the number of samples differed substantially between the different countries, the Simpson index (λ) was used to calculate the within-country genetic diversity (Simpson, 1949). Similarly, the adjusted index of association ($\bar{r}_d$) was used to describe linkage disequilibrium, as it is less sensitive to uneven sample sizes (Agapow & Burt, 2001). The $\bar{r}_d$ can only be calculated on groups including more than five individuals, and thus Norway and Italy were excluded from this analysis. The initial visualization of genetic diversity between samples using distance-based, covariance standardized principal coordinate analysis (PCoA) was done in GenAlEx v. 6.503. Minimum spanning networks (MSNs) based on Bruvo distance (Bruvo et al., 2004) were calculated using the bruvo.msn function in the R package poppr to visualize the relationship among strains. In addition, a neighbour-joining (NJ) tree was created using the bruvo.boot function with 1000 bootstrap resamplings. To estimate common ancestry between samples, the snmf function in the R package for Landscape and Ecological Association Studies (LEA) was used (Frichot & François, 2015). For this analysis, the number of genetic clusters (*K*) was ml set to range between 1 and 10 and the number of ancestral populations was selected via a cross-validation technique enabling an entropy criterion to choose the best *K* value (Alexander & Lange, 2011; Frichot et al., 2014). Missing data were complemented based on an ancestry coefficients estimation, taking into account ancestral genotype frequencies (Frichot & François, 2015).

2.5 | Assessment of *A. euteiches* virulence on pea

In the current work, we define pathogenicity of *A. euteiches* strains as the ability to cause disease (a qualitative measure) and virulence as the severity of disease symptoms (a quantitative measure) on pea. Three pea genotypes with different levels of susceptibility were used in pot experiments to assess pathogenicity and virulence of *A. euteiches* strains: Lumina (susceptible), Linnea (susceptible) and MN313 (partly susceptible). We used a phenotyping protocol under controlled conditions that is similar to assays used in commercial breeding programmes. Pea seeds were surface sterilized by washing in 70% ethanol for 1 min, rinsed with sterile water, and subsequently washed with 1% sodium hypochlorite for 5 min, followed by several washing steps with autoclaved water. Air-dried seeds were aseptically placed on 0.8% water agar and incubated at 25°C for 4 days in darkness. Strains of *A. euteiches* were grown on CMA plates for 2 weeks at 20°C in darkness prior to their use in infection. Square plastic pots (0.254 L) were filled with vermiculite (Sibelco) and a single 10 mm-diameter agar plug of *A. euteiches* inoculum was added directly into holes (c.4 cm depth and 1 cm diameter) made in the vermiculite. To prevent cross-contamination, tools used for the inoculation of *A. euteiches* were sterilized with 70% ethanol between strains. Furthermore, pots inoculated with different *A. euteiches* strains were kept on separate trays until scoring. Four-day-old, germinated pea seedlings were transferred

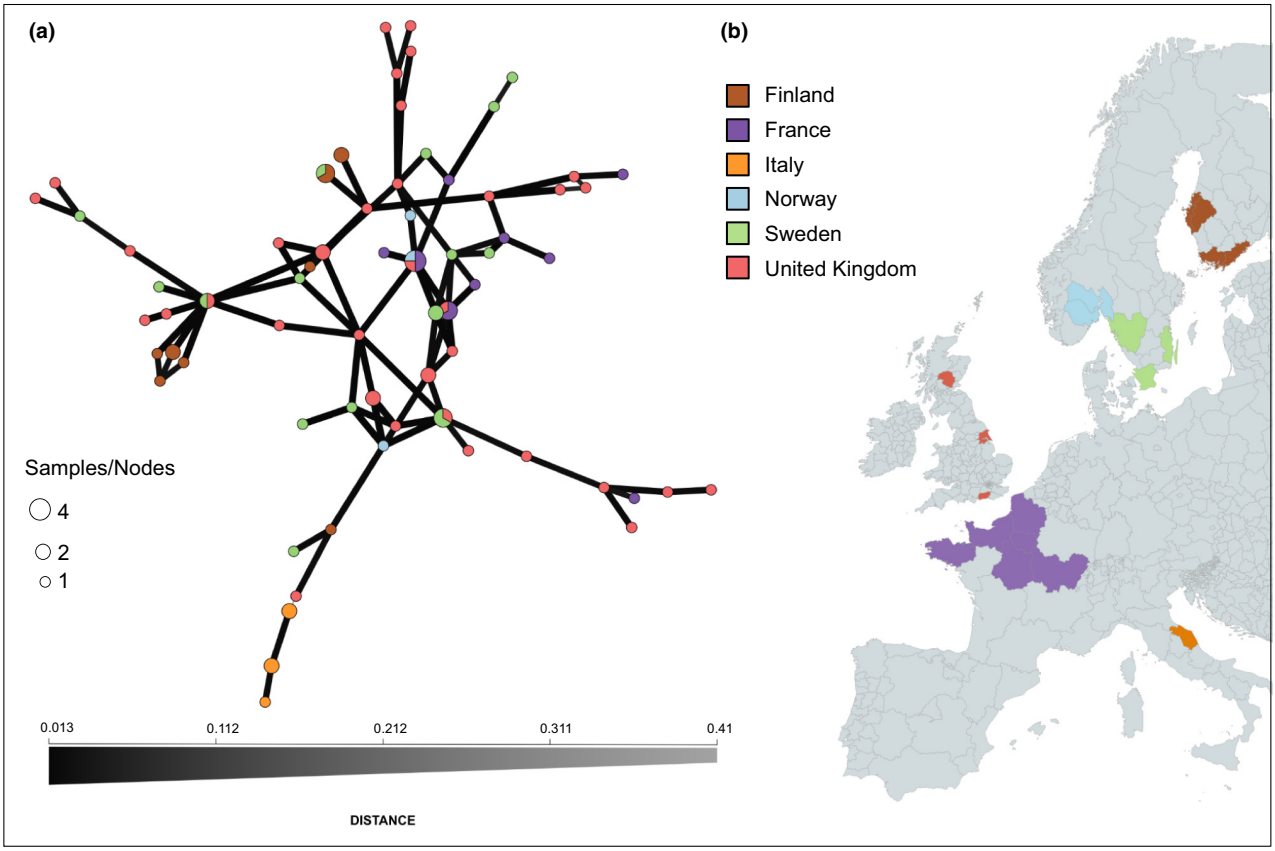

FIGURE 1 Genetic relationships and origin of European *Aphanomyces euteiches* strains. (a) Minimal spanning network based on Bruvo distance representing genetic distance between countries (colour) and number of strains (samples/node). Branch thickness represents genetic relatedness and shared multilocus genotypes between countries are indicated with split nodes. (b) Map showing *A. euteiches* strains originating from Finland (brown), France (purple), Italy (orange), Norway (blue), Sweden (green) and the UK (pink). The online tool MapChart was used for illustration (https://mapchart.net/europe-detailed.html). [Colour figure can be viewed at wileyonlinelibrary.com]

into the holes containing the inoculum, followed by incubation in a growth chamber (CMP6050; Conviron) at 22°C, 55% humidity and 150 μmol·m^{-2}·s^{-1} light intensity in a 12 h light/12 h dark cycle. The pots were kept on a tray filled with 2 cm of water to keep the vermiculite moist during the experiment. To account for unequal light or humidity conditions, the trays were randomly moved within the growth chamber at all watering occasions, every third day. The experiment was conducted with five pots (biological replicates) per treatment, and each pot contained four plants representing technical replicates. After 2 weeks of incubation, disease severity was assessed by washing the roots carefully in tap water and scoring them on a scale from 0 (completely healthy) to 100 (completely dead,) in steps of 10, by two different people.

2.6 | Statistical analysis

Disease scores were tested for normality followed by two-way analysis of variance (ANOVA) in R using the aov function (package stats v. 4.1.0; R Core Team, 2021) to assess the effects of scorer, cultivar and strain on disease scores. Cultivar × strain interactions were further analysed on their estimated marginal means using the emmip function (package emmeans v. 1.7.0, (Lenth, 2021) on the ANOVA output residuals. To analyse multiple pairwise comparisons, we further used the emmeans and pairs function on the fitted model with specified cultivar and isolate interactions.

3 | RESULTS

3.1 | Genetic diversity of *A. euteiches* strains

A total of 75 *A. euteiches* strains, originating from Sweden, Norway, Finland, the UK, France and Italy were isolated in pure culture (Table S1). Successful PCR amplification of 22 SSR marker loci was achieved for all 75 strains and 10 reference strains from France (Moussart et al., 2007; Quillévéré-Hamard et al., 2018), with the exception of markers Ae12, Ae45 and Ae63 where no amplification was found in the 11 strains from Finland and strains SE64 and SE65 from Sweden. These results were repeated twice and confirmed as true missing data. The number of identified alleles within loci varied from one (Ae04, Ae36, Ae63 and aph82) to four (Ae44; Table 1). Out of the four monomorphic loci, locus Ae63 had missing data exclusively in the Finnish population and two Swedish strains and was

TABLE 1 Characteristics of the simple-sequence repeat markers used in this study

Locus	No. of alleles	Missing data (%)	Evenness
Ae04[a]	1	0.0	n.a.
Ae12[a]	3	21.2	0.48
Ae13[a]	2	0.0	0.49
Ae17[a]	2	8.2	0.50
Ae32[a]	2	0.0	0.49
Ae34[a]	2	1.2	0.49
Ae36[a]	1	4.7	n.a.
Ae37[a]	3	0.0	0.56
Ae44[a]	4	1.2	0.49
Ae45[a]	3	15.3	0.43
Ae54[a]	3	7.1	0.84
Ae63[a]	1	15.3	n.a.
aph1[b]	4	1.2	0.64
aph4[b]	2	2.4	0.49
aph9[b]	4	1.2	0.44
aph20[b]	3	3.5	0.68
aph25[b]	2	4.7	0.49
aph32[b]	4	3.5	0.78
aph35[b]	3	3.5	0.77
aph50[b]	2	7.1	0.56
aph76[b]	2	1.2	0.49
aph82[b]	1	7.1	n.a.
Total	54	n.a.	mean 0.56

Abbreviation: n.a., not applicable.

[a]Locus described by Mieuzet et al. (2016).

[b]Locus described by Quillévéré-Hamard et al. (2018).

therefore kept in the data set for further analyses. Loci Ae04, Ae36 and aph82 were noninformative and excluded from the data in the genetic diversity and population structure analysis. Locus Ae54 had the most evenly distributed alleles (E_5 = 0.84), followed by aph32 and aph35 (Table 1). We observed no more than two alleles per locus and individual, indicating that the analysed strains were diploid. The genotype accumulation curve approached saturation and indicated that the number of markers included in this study was close to enough to differentiate the actual genetic differences in the sampled populations (Figure S1).

We found a total of 67 MLGs across all countries, with the highest proportion of MLGs in the strains collected in Norway, where the number of MLGs corresponded to the actual sample size (Table 2). However, in all other countries the number of MLGs was lower than the number of genotyped isolates, indicating the occurrence of clones in the respective populations. The genotypic diversity was high within all countries, with values of the Simpson diversity index (λ) ranging between 0.667 and −0.971. Values of λ revealed the largest diversity within the UK, which also comprised the highest number of genotyped strains (Table 2). Values of $\bar{r}_d$ were not significantly different from zero for any of the countries, indicating no linkage between alleles and thus no recombination (Table 2).

3.2 | Genetic structure of *A. euteiches* in Europe

MSNs showed that only five MLGs were shared between countries (Figure 1a). More specifically, Sweden and the UK shared two MLGs (MLG.17 and MLG.22) while MLG.61 occurred in both Sweden and Finland. The UK and France shared two MLGs (MLG.37 and MLG.38) where the latter was also present in Norway. Based on a PCoA, *A. euteiches* strains were divided into three main genetic clusters: one cluster containing all strains from Italy, one cluster containing all strains from Finland and two strains from Sweden (SE64 and SE65), while the third cluster contained all other strains (Figure S2). The NJ tree confirmed the separation between the Italian strains and the other European strains (Figure 2). These results were confirmed by LEA analysis on ancestral genotype frequencies that indicated two main genetic clusters, where the Italian strains belong to a different ancestral population from all other strains (K = 2; Figure S3). PCoA and the NJ tree did not identify clustering of strains according to regions within a country or by year (data not shown).

3.3 | Virulence of *A. euteiches* strains on pea

Ten strains of *A. euteiches* were selected for virulence assays on pea representing different geographic origins and the three genetic clusters identified in the PCoA: strains SE51, SE58 and SE64 from Sweden, strains FI2, FI37, and FI46 from Finland, strains IT30, IT32 and IT35 from Italy and the Rb84 reference strain from France (Moussart et al., 2007). Disease score values corresponded to the percentage of roots with disease symptoms (Figure 3a). As the disease severity was assessed by two different people, an initial ANOVA was performed that proved the scorer effect to be nonsignificant (p = 0.56). For the following analyses, a two-way ANOVA was performed with cultivar and strain as factors as well as their interaction effect. There were significant effects of strain ($p < 0.001$), cultivar ($p < 0.001$) and their interaction ($p < 0.001$) on disease severity (Table S5). When it comes to differences in susceptibility between pea genotypes, MN313 was less susceptible ($p \leq 0.045$) than both Lumina and Linnea to strains FI2, FI37 and IT30, less susceptible than Lumina to strains FI46 and IT32, and less susceptible than Linnea to IT35 and SE51 (Figure 3b, Table S6). Linnea was more susceptible ($p \leq 0.035$) than Lumina when infected with strains IT35 and SE58. All 10 *A. euteiches* strains were pathogenic on all pea genotypes, with significantly ($p < 0.001$) higher disease severity scores compared with the corresponding mock-treated controls (Figure 3b). There were also significant ($p \leq 0.044$) differences in virulence between *A. euteiches* strains, mainly involving a lower virulence of the Swedish strains compared with other strains (Figure 3b, Table S6).

TABLE 2 Population genotypic and genetic diversity based on 19 loci

Country	N^a	G/N^b	λ^c	$\bar{r}_d^d$	$p(\bar{r}_d)^d$
Norway	3	1	0.667	n.a.	n.a.
Sweden	17	0.882	0.933	0.004	0.594
UK	38	0.921	0.971	0.017	0.131
Italy	5	0.600	0.667	n.a.	n.a.
France	11	0.818	0.889	0.048	0.155
Finland	11	0.727	0.875	−0.126	0.997
Total/average	85	0.890	–	–	–

Abbreviation: n.a., not applicable.

[a]Number of samples.

[b]Number of genotypes divided by number of samples.

[c]Simpson index.

[d]$\bar{r}_d$ adjusted index of association and its *p* value.

4 | DISCUSSION

As a first study covering a geographic north-to-south gradient in collection of strains, the current work revealed higher genotypic diversity within *A. euteiches* populations than previously reported. We found three separate genetic groups of *A. euteiches* in Europe; strains from Italy and Finland (together with two strains from Sweden) form two separate groups, which in turn are genetically distinct from a larger group consisting of the remaining strains from Sweden, Norway, the UK and France. The *A. euteiches* strains from Italy are clearly genetically separated from the other European populations, based on all phylogenetic analyses. In combination with the lack of shared ancestry between these two main groups, this differentiation suggests very low migration rates of *A. euteiches* between Italy and the other sampling sites in north-western Europe. Climatic factors may probably act as drivers of local *A. euteiches* differentiation and selection. It has previously been shown that the ability to produce resilient oospores in certain *Phytophthora* species correlates with their establishment in northern latitudes at lower temperatures (Redondo et al., 2018). Alternatively, the genetic differentiation and lack of shared ancestry may be interpreted as the result of a recent introduction of non-European *A. euteiches* in Italy. However, testing this hypothesis requires sampling of *A. euteiches* from a worldwide distribution.

Due to the predominating homothallic reproductive mode of *A. euteiches* and its limited dispersal capacity (Grünwald & Hoheisel, 2006), it can be expected that the genetic diversity of *A. euteiches* is low within populations from limited geographic areas and increases with geographic distance. Here, we confirm the pattern with an overall low level of genetic diversity found in previous studies of French and North American *A. euteiches* populations (Grünwald & Hoheisel, 2006; Le May et al., 2018; Malvick et al., 1998; Mieuzet et al., 2016; Quillévéré-Hamard et al., 2018; Wicker et al., 2001). In the Grünwald and Hoheisel (2006) study, it was thought that a relatively higher diversity between populations was due to the limited spread of the soilborne pathogen. This explanation is valid for our data as well, given the proximity of both cultivation and processing sites of vining pea and limited choices of alternating fields in crop production, in combination with large geographic distances between production sites in different countries. Concurrently, indications of genetically differentiated groups have been reported from both the United States and France (Grünwald & Hoheisel, 2006; Malvick et al., 1998; Quillévéré-Hamard et al., 2018).

Within the large group of non-Italian *A. euteiches* strains, there are also indications of a genetic differentiation of strains from Finland and parts of Sweden compared with the remaining strains. This is supported both by PCoA and by the fact that three SSR markers failed to amplify PCR products from the Finnish strains. Notably, the two Swedish strains (SE64 and SE65) have missing data at the same loci as the Finnish strains and cluster together with the Finnish population in the PCoA. They were both sampled in the region of Kalmar, in south-eastern Sweden. The fact that the Swedish strains SE64 and SE65 are genetically similar to the Finnish strains suggests a movement of *A. euteiches* between these neighbouring countries. We also identified one MLG (MLG.61) that is shared between Sweden and Finland.

Additional support for international movement of *A. euteiches* is indicated by identical MLGs that are shared between France and the UK, as well as between Sweden and the UK. Given the limited long-range dispersal capacity of *A. euteiches*, it can be speculated that these movements are aided by human activities.

Within each country, the genotypic diversity was high, and only a few clones were identified. The nonsignificant values of $\bar{r}_d$ indicate no linkage between markers and limited clonal reproduction within each country. We acknowledge that the unequal sample sizes, that is, number of strains sampled per country and region, and the generally low number of *A. euteiches* strains limits the analytical power of a population genetics study, in particular within the populations. The not entirely saturated genotype accumulation curve indicates that we were not able to catch and describe the genetic diversity with the number and selection of markers used in this study. At the same time, our results indicate that in combination with the high genotypic diversity within each population, outcrossing is likely to occur in all populations, despite the more common selfing nature of the pathogen. One reason for this could be the pathogen's broad host range within the legume family. It allows for the possibility of outcrossing and genetic exchange between strains that have adapted to different legume species, which might be promoted by crop rotations including multiple host plants.

When phenotyping 10 *A. euteiches* strains, we observed a partial resistance of pea genotype MN313, which has been previously described by Wicker et al. (2001), and a generally high susceptibility of both Lumina and Linnea. As expected, all *A. euteiches* strains were able to infect and cause root rot disease on all tested pea genotypes, although the level of virulence differed between strains. This difference is partly correlated with geographic origin, as the Swedish strains display lower virulence than most other strains. However, this result requires confirmation with a larger data set and possibly different climatic conditions for phenotyping, taking into account that

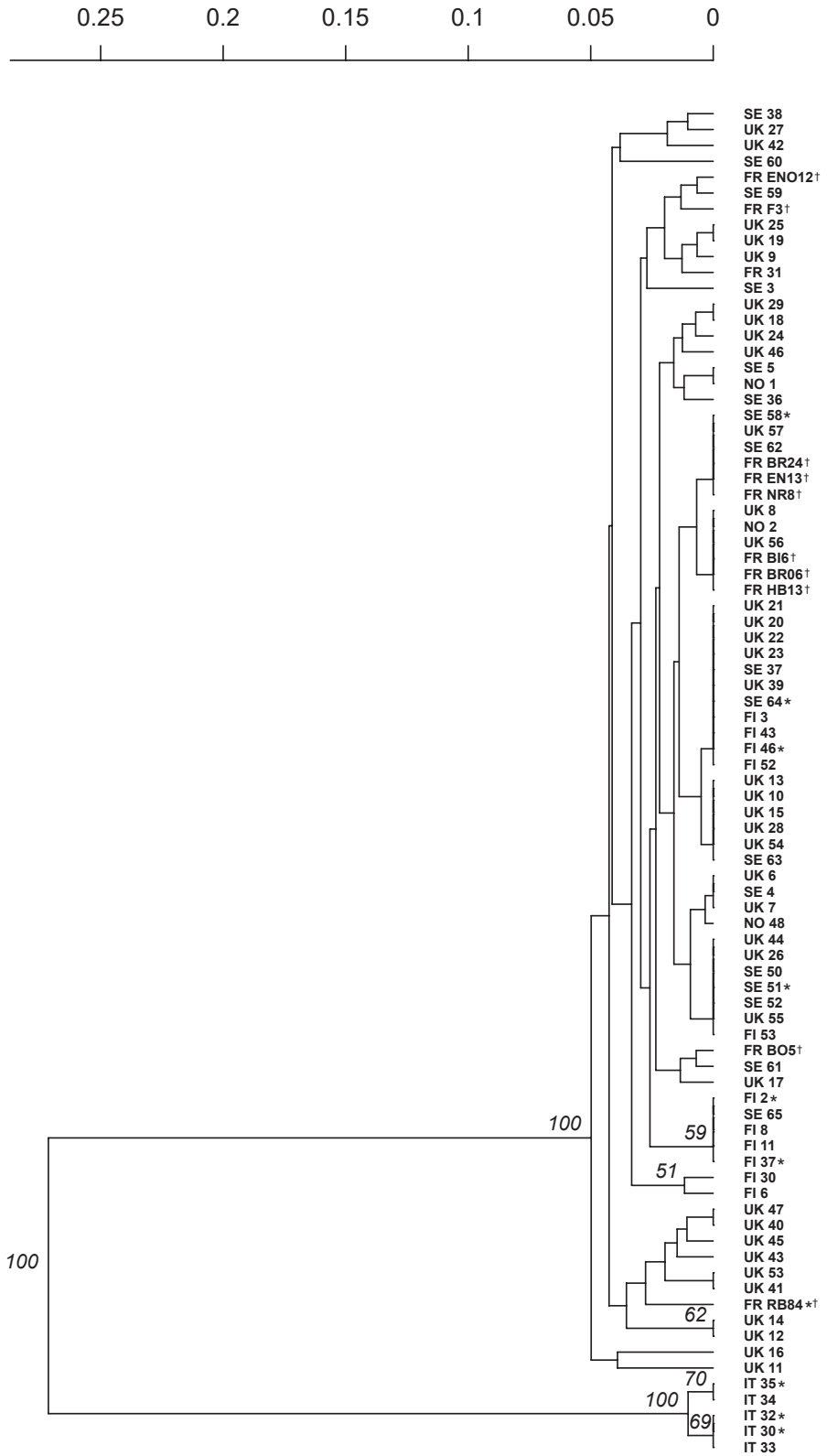

FIGURE 2 Neighbour-joining (NJ) tree showing genetic relatedness of *Aphanomyces euteiches* strains. An NJ tree comprising all 85 *A. euteiches* strains used in the study shows a clear separation of the Italian population from the other strains. The NJ tree was created with 1000 bootstrap resamplings and a cut-off value of 50. A two-letter country abbreviation was added to each strain ID. Strains marked with an asterisk (*) were phenotyped and the dagger symbol (†) marks the French reference strains.

disease severity caused by strains from latitudinal border regions might be climate-dependent. Differences in virulence are common between *A. euteiches* strains and have been observed by Malvick and Percich (1998a) as well as by Wicker et al. (2001), where virulence phenotypes were defined according to pathogenicity on different hosts, indicating host adaptation. Furthermore, there is no correlation between virulence and genetic structure in our data. This is shown by the fact that the reference strain Rb84 displayed a significantly higher virulence than the two Swedish strains (SE51 and SE58) that belong to the same genetic group. More support for this lack of phenotype–genotype correlation comes from the fact that even though the Italian strains were clearly genetically differentiated from

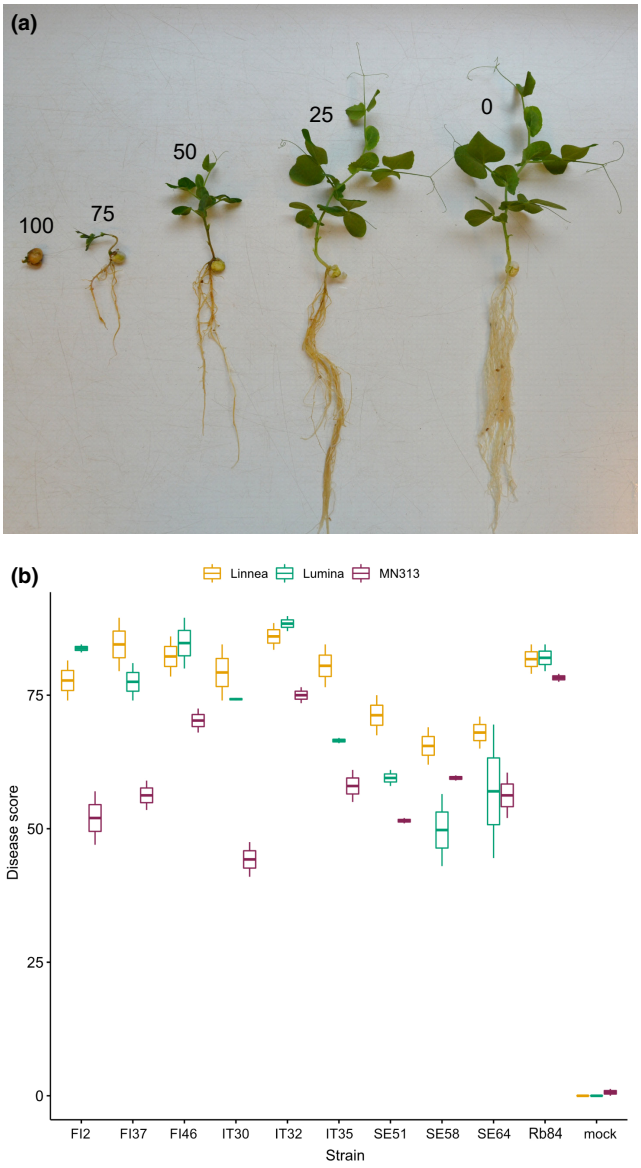

FIGURE 3 Assessment of *Aphanomyces euteiches* virulence on pea. (a) Five representative *Pisum sativum* plants show the range of disease severity caused upon infection with *A. euteiches*, 2 weeks postinfection. Disease severity was scored in steps of 10, with 0 being symptomless and 100 completely dead. (b) The boxplot shows the average disease score of 10 phenotyped *A. euteiches* strains (FI2, FI37, FI46, IT30, IT32, IT35, SE51, SE58, SE64 and Rb84) and the mock treatment (no infection) on three *P. sativum* cultivars Linnea (yellow), Lumina (blue) and MN313 (purple). [Colour figure can be viewed at wileyonlinelibrary.com]

the non-Italian strains, no consistent differences in virulence compared with strains from the other genetic groups were detected. This agrees with previous reports and is suggested to be partly due to the use of neutral markers, such as SSR, with limited genetic linkage to the loci encoding virulence factors (Quillévéré-Hamard et al., 2018).

Our results also highlight the importance of the experimental setup when performing virulence assays, and the risk of introducing biases in phenotypic assessment assays. The previously phenotyped French reference strain Rb84 was more virulent on the pea cultivar Lumina than on MN313 (Quillévéré-Hamard et al., 2018). However, in our virulence assay disease severity was high on all three pea genotypes upon infection with Rb84, with no significant differences between genotypes. One possible explanation for this difference might be related to the inoculum used in the different studies––an agar plug with mycelia in the current study compared with a zoospore solution used in the previous study. Our experimental approach further deviates in the method of allele amplification in PCRs. We tagged our forward primers directly with fluorophores instead of using an additional fluorescently labelled M13 primer. In the allele scoring, this resulted in a consistent base-pair shift when comparing with scored alleles of the 10 reference strains from Quillévéré-Hamard et al. (2018); however, this does not influence the overall results of the study.

In our study we found three genetically distinct groups of *A. euteiches* along a north-to-south gradient and signs of genetic differentiation between strains. Although no correlation between genotype and virulence was detected in the current work, the existence of genetic differentiation and a widespread capacity for occasional outcrossing among *A. euteiches* in Europe is a concern for future disease management strategies. Further, our results emphasize the need for complementing neutral genetic markers used in the current study with whole-genome sequencing and comparative genomics, in order to understand the genetic structure and virulence variation in *A. euteiches*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are included in the current article and its associated files.

ORCID

Carol Kälin https://orcid.org/0000-0002-8096-8331
Anna Berlin https://orcid.org/0000-0002-9518-5719
Mukesh Dubey https://orcid.org/0000-0001-7393-366X
Malin Elfstrand https://orcid.org/0000-0002-0214-5284
Magnus Karlsson https://orcid.org/0000-0001-6098-138X

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SUPPORTING INFORMATION

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