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# GENOTYPING AND IDENTIFICATION OF DISEASE RESISTANT BARLEY (*Hordeum vulgare* L.) LINES AND VARIETIES TO NET BLOTCH (*Pyrenophora teres* Drechsler)

Silviya V. VASILEVA*, Milena L. KOSTOVA and Marina P. MARCHEVA

Agricultural University, 12, Mendeleev, Blvd., 4000 Plovdiv, Bulgaria
*Corresponding author, S. V. Vasileva, E-mail: silviya.valentinova@gmail.com

***ABSTRACT.*** *The current research aimed to identify and genotype barley lines and varieties resistant to the fungal disease net blotch Pyrenophora teres Drechsler [anamorph Drechslera teres (Sacc.) Shoem]. Widely distributed Bulgarian and foreign elite barley genotypes were the object of the study. So far, all the varieties constituting the varietal structure of Bulgaria were valued as medium-sensitive (MS 4, 5) to very sensitive (VS 8, 9) according to their phenotypic reaction to net blotch. The genotypes used as resistance identifiers from the global gene banks, as a reference, showed sensitivity to local isolates. All barley forms of different origins, forming the trait collection were subjected to selection and genetic analysis. An ISSR marker system was used to genotype certain barley forms to their different phenotype reactions to Pyrenophora teres. We report quantitative trait loci (QTL) for potential net blotch resistance using combined data from ISSR analysis and phenotype observations. The obtained genetic map gives an idea of the identified polymorphisms' relation, which may further serve to establish their additional links with the behavior of the varieties with the examined plant pathogen. Moreover, the created map can also be used to identify associations of these markers with resistance to other pest levels, thereby allowing the identification of genomic regions contributing to horizontal (multiple) resistance, such as those of particular interest. Identification of ISSR markers associated with major QTLs which can be used for marker-assisted selection (MAS), and their validation in unrelated barley populations.*

***KEYWORDS:*** *barley, disease resistance, Drechslera teres, ISSR, Marker assisted selection, net blotch.*

## INTRODUCTION

Barley breeding programs in Bulgaria are mainly aimed at developing winter fees and malting varieties, combining maturity, resistance to abiotic stress, high productivity, and stable yields in the ever-changing environmental conditions. Dynamic market requirements, global climate changes on the one hand, and people's desire for an environmentally friendly and healthy lifestyle on the other, pose new challenges for breeders to create resistant varieties for economically important pathogens (Afanasenko et al. 2015, Ellwood et al. 2019, FAO https://www.fao.org/home/en).

Barley is attacked by many phytopathogens during its vegetation period. Foliar pathogens such as *Pyrenophora teres*, *Cochilobolus sativus*, *Septoria passerinii*, *Rhynchosporium secalis*, *Blumeria graminis,* etc. leads to serious yield losses (Neupane et al. 2015, O'Boyle et al. 2014, Tamang et al. 2015, 2021). *Pyrenophora teres* Drechsler [anamorph: *Drechslera teres* (Sacc.) Shoem.] has become increasingly widespread in Bulgaria (Nakova 2009, Vasileva et al. in print).

Symptoms are expressed in the formation of elliptical light brown spots with fine lines, in the form of a net (Akhavan et al. 2016, Ogrodowicz et al. 2017, Richards et al. 2016). When the lesions merge, the leaves begin to dry and soon necrotize (Oguz et al. 2017, Owino et al. 2013, Wang et al. 2015).

Epiphytosis have been reported in major barley production regions in countries such as the USA, Canada, Denmark, Norway, Australia, South Africa, etc. Infected plants produce small, low-quality seeds. The pathogen can cause 60-70% production losses. There are two forms: *Pyrenophora teres* f. *teres* (Net Form of Net Blotch - NFNB) and *Pyrenophora teres* f. *maculata* (Spot Form of Spot Blotch - SFNB) (Backes et al. 2021, Hickey et al. 2017, Islamovic et al. 2017, Wonneberger et al. 2017).

The aim of current research is genotyping and identification of disease resistant lines and barley varieties (*Hordeum vulgare* L.). Discovering the sources of resistance to net blotch (*Pyrenophora teres* Drechsler) and understanding their genetic determination are very important for the development of new barley breeding programs to create resistant varieties to this disease.

## MATERIALS AND METHODS

Plant material

37 widespread Bulgarian and foreign elite lines and varieties of barley have been studied (Table 1). All genotypes were included in a core collection in the Breeding Garden of the Department of Genetics and Plant Breeding at the Agricultural University – Plovdiv, Bulgaria during the study period. These genotypes were selected due to their different origins and different net blotch forms - phenotype reactions. “Resistant” and “Susceptible” controls were provided by National Small Grains Collection, USA (NSGC) (https://www.ars.usda.gov/) and NordGen, Norway (https://www.nordgen.org/en/).

Field trails

The field experiments were conducted during the period 2018-2021 on the territory of the experimental field at the Agricultural University. Characterizations of the plant net blotch disease reactions were made according to the scale of Tekauz (1984). The soil and climate conditions and the applied agrotechniques were traditional for the cultivation of barley in Bulgaria. No chemical control of fungal diseases was conducted in the breeding garden during the study period.

Greenhouse experiments

Seedlings of the studied genotypes were grown in potting soil in a controlled environment and inoculated with various isolates of Ptt (NFNB) and Ptm (SFNB) to confirm pathogenicity (Backes et al. 2021). After 7-10 days, seedlings were assessed for infection phenotype on the second and/or third leaf using a 1-10 or 1-9 reaction scale, respectively (Figure 1). Values of 1-5 (Ptt) and 1-3 (Ptm), were regarded as the resistant phenotype; higher values were classified as ‘susceptible’ (Tekauz et al. 1985).

Genotyping

Laboratory analyzes were performed at the Molecular Biology Laboratory of the Department of Genetics and Plant breeding. A Blood-Animal-Plant DNA Preparation - Colums Kit (Jena Bioscience, Jena, Germany) was used to obtain genomic DNA from the selected plant material. Subsequently, ISSR markers were applied to examine and genotype selection forms (Table 2). The isolated genomic DNA and PCR amplification products were visualized by electrophoretic separation of the products in 2% agarose gel.

Statistical analysis

Molecular and phenotypic data obtained were used to construct dendrograms using the statistical package SPSS for Windows. Identification of groups and preparation of genetic maps was performed using the program MapDisto.

Table 1. Scale evaluation of investigated specimens' phenotype reaction to *Pyrenophora teres* f. *teres* (Ptt) and *Pyrenophora teres* f. *maculata* (Ptm)

| Accession | Origin | Reaction to Ptt | Reaction to Ptm |
|---|---|---|---|
| Odysseus | Bulgaria | 3 | 7 |
| Hemus | Bulgaria | 5 | 7 |
| Jet | Ethiopia | 2 | 8 |
| Herta | Sweden | 6 | 8 |
| 102C2 | New Zeland | 2 | 6 |
| Betzes | Germany | 4 | 2 |
| Clho 475 | USA | 3 | 2 |
| Clho 646 | USA | 3 | 2 |
| Hector | Canada | 2 | 2 |
| Chevron | Sweden | 3 | 2 |
| Norbert | Canada | 2 | 5 |
| Nomini | UDA | 2 | 4 |
| 178 636 | Irak | 2 | 5 |
| Hertland | Canada | 2 | 4 |
| *H. bulbosum* | unknown | 2 | 2 |
| Aheloy | Bulgaria | 4 | 6 |
| Veslets | Bulgaria | 2 | 7 |
| Gorast | Bulgaria | 9 | 9 |
| Gratsioza | Czech Republik | 7 | 7 |
| Imeon | Bulgaria | 4 | 7 |
| Kazanova | France | 6 | 7 |
| Lardeya | Bulgaria | 6 | 4 |
| Neda | Bulgaria | 9 | 7 |
| Orpheus | Bulgaria | 7 | 8 |
| Pagane | Bulgaria | 8 | 8 |
| Sayra | Bulgaria | 7 | 7 |
| Typi | unknown | 6 | 5 |
| Flavius | Bulgaria | 8 | 5 |
| Yaspis | Bulgaria | 9 | 8 |
| Furki | unknown | 2 | 4 |
| Igri | Germany | 5 | 7 |
| Clho 223 | Agrentina | 6 | 9 |
| Himalaya | USA | 9 | 9 |
| Otis | USA | 4 | 8 |
| Steptoe | USA | 6 | 7 |
| OAC | unknown | 6 | 9 |
| Gerlah | France | 6 | 4 |


Figure 1. Tekauz's scale for determining the degree of attack by *P. teres*

Table 2. ISSR primers, used in the study for genotyping plant material

| PRIMER | SEQUENCE | BP | TA (°C) |
|---|---|---|---|
| ISSR_E4 | 5'-ACA-CAC-ACA-CAC-ACA-CYA-3' | 18 | 58 |
| ISSR_E5 | 5'-ACA-CAC-ACA-CAC-CYT-3' | 15 | 50 |
| ISSR_E6 | 5'-AGA-GAG-AGA-GAG-AGA-GC-3' | 17 | 58 |
| ISSR_E7 | 5'-CTC-TCT-CTC-TCT-CTC-TRC-3' | 18 | 58 |
| ISSR_E8 | 5'-DBD-ACA-CAC-ACA-CAC-AC-3' | 17 | 52 |
| ISSR_E10 | 5'-GAG-AGA-GAG-AGA-GAG-T-3' | 17 | 48 |
| ISSR_TOM3 | 5' CAC-ACA-CAC-ACA-CAR-G-3' | 16 | 50 |
| ISSR_TOM4 | 5'–RY-GAC-AGA-CAG-ACA-3' | 14 | 42 |
| ISSR_TOM7 | 5'-ACA-CAC-ACA-CAC-ACA-CYG-3' | 18 | 54 |
| ISSR_TOM8 | 5'-ACA-CAC-ACA-CAC-ACA-CG-3' | 17 | 50 |
| ISSR_TOM10 | 5'-GAG-AGA-GAG-AGA-GAG-AC-3' | 17 | 50 |

## RESULTS

The phenotypic response of each breeding line/variety to the disease is determined by the complex interaction between genotype and pathogen under specific environmental conditions. Observations on the occurrence and development of *Pyrenophora teres* were made of naturally infected plants under field conditions and artificial inoculation in laboratory conditions during the period 2018 – 2021. 37 barley lines and varieties were included in the phytopathological evaluation (Table 1). The highly productive varieties in Bulgaria - Neda and Gorast results of the breeding programs of the Department of Genetics and Plant Breeding (AU-Plovdiv). They were evaluated as very susceptible (range 7-9) to both forms of *P. teres*. Pagane and Yaspis (provided by the Institute of Agriculture in General Toshevo) were rated as "VS" to phytopathogens, as well. The varieties Odysseus, Hemus, Aheloy, Veslets, Imeon, Lardeya, Orpheus, and Sayra were provided by the Institute of Agriculture – Karnobat has a similar reaction to net blotch. Most of them are standards for malting qualities for the territory of Bulgaria. The results revealed that the breeding lines and varieties constituting the varietal structure of Bulgaria are valued as medium-sensitive (MS 4, 5) to very sensitive (VS 8, 9) (Table 1) according to the Tekauz’s scale (Figure 1).

Total genomic DNA was extracted for the study. As a result of the PCR reactions, several products were obtained, the combination of which created a clear DNA pattern specific to each genotype. Amplification with the primers ISSR_E6, ISSR_E7, ISSR_E10, and ISSR_TOM4 failed to detect polymorphisms between the genotypes examined (Figure 2). Amplification with the primers ISSR_E4, ISSR_E5, ISSR_E8, ISSR_TOM3, ISSR_TOM7, ISSR_TOM8, and ISSR_TOM10 resulted in the identification of polymorphisms between the inter-simple sequence repeats (ISSRs). The results revealed a significant number of polymorphic fragments (Figure 3). The results allowed reliable genetic differentiation of similar genotypes, which was one of the main objectives of this study. For the cultivars Otis and Gerlah, no products were obtained in the amplification experiments with the primers listed in Table 2 and were therefore excluded from the study.

The clustering of genotypes evaluated was based on the polymorphic markers identified, independent of changing environmental factors. The combination of genotype and phenotype data revealed the well-established distribution of genotypes according to their genetic distance, as reflected in the dendrogram in Figure 4.

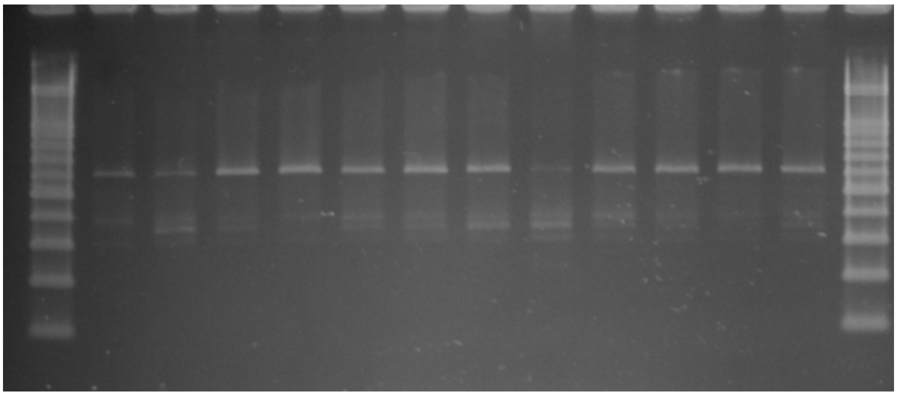

Figure 2. Amplification with primer ISSR_E10

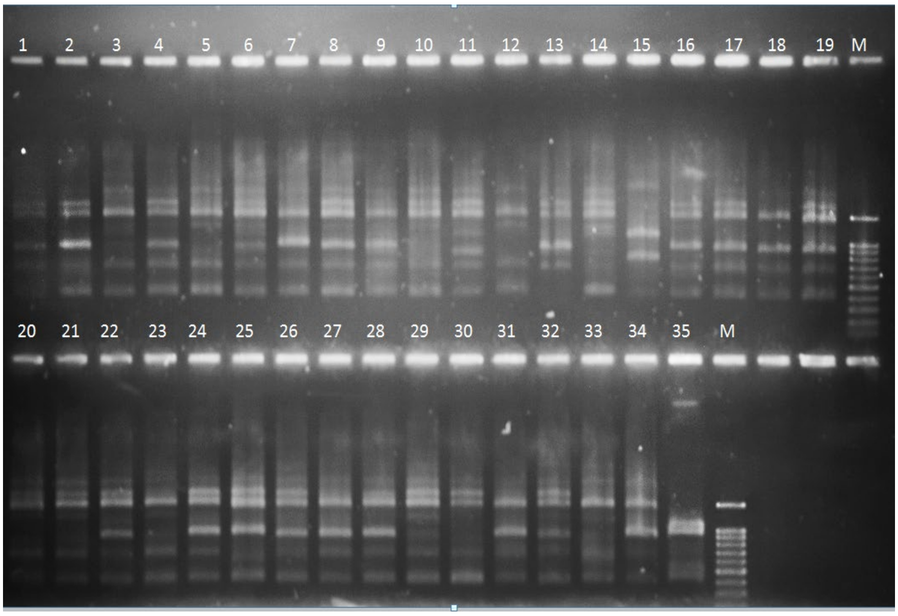


Figure 3. Amplification with primer ISSR_TOM3. Arrows indicate polymorphic bands M. Marker, 1. Odysseus, 2. Hemus, 3. Jet, 4. Herta, 5. 102C2, 6. Betzes, 7. Clho 475, 8. Clho 646, 9. Hector, 10. Chevron, 11. Norbert. 12. Nomini, 13. 178 636, 14. Heartland, 15. *H. bulbosum*, 16. Aheloy, 17. Veslets, 18. Gorast, 19. Gracioza, 20. Imeon, 21. Kasanova, 22. Lardeya, 23. Neda 24. Orpheus, 25. Pagane, 26. Sayra, 27. Typi, 28. Flavius, 29. Yaspis, 30. Furki, 31. Igri, 32. Clho 223, 33. Himalaya, 34. Otis, 35. Steptoe, M. Marker

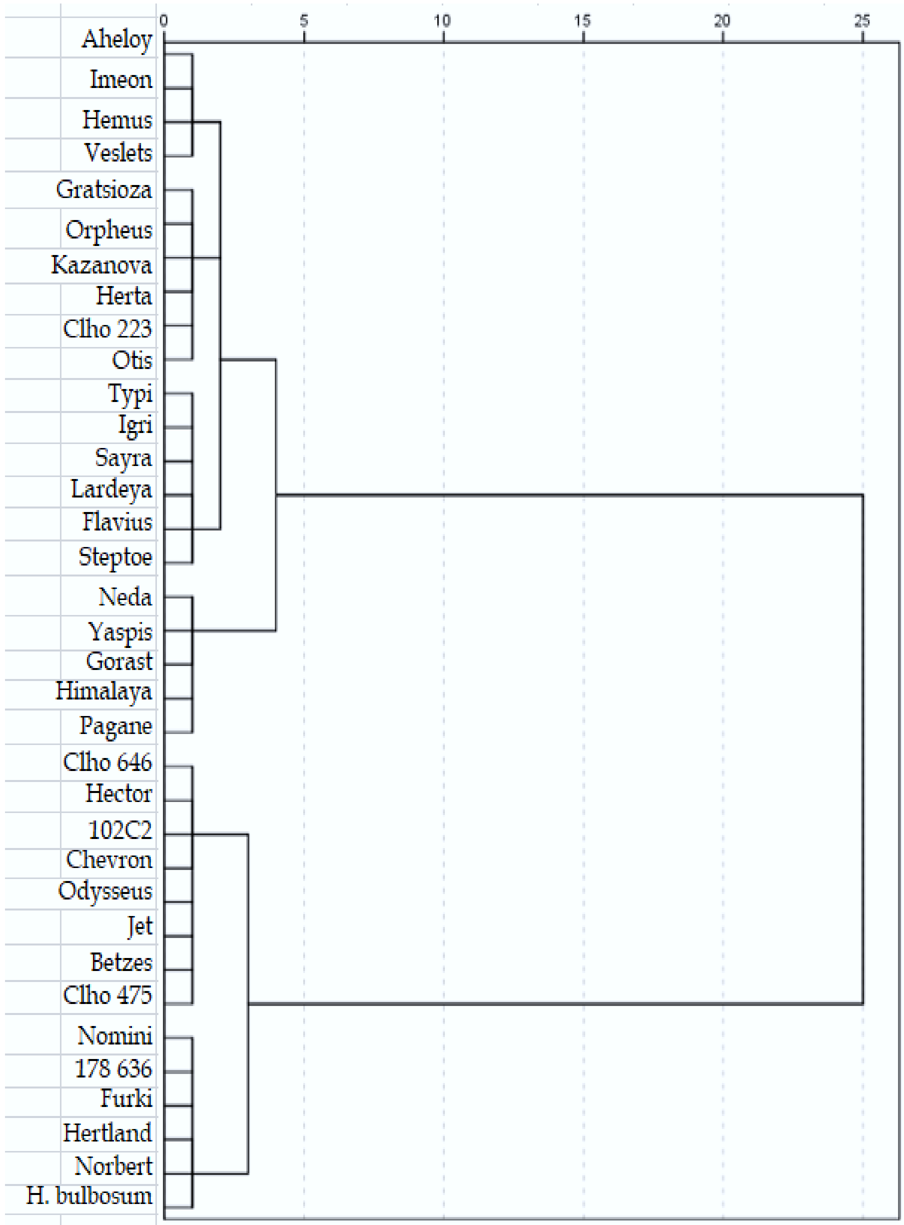


Figure 4. Dendrogram for the genetic distance of barley genotypes based on established DNA polymorphisms and phenotypic data

Two main clusters are observed. In the first cluster, two subgroups are formed. One of them is formed by the very sensitive varieties Neda, Yaspis, Gorast, Himalaya, and Pagane, with a range of 8-9 to Ptt and 7-9 to Ptm (Figure 5). The symptoms were observed from the early stages of their vegetation until maturity. The rest of the varieties that formed subgroups in the same cluster were mostly the varieties provided by the Institute for Agriculture – Karnobat, which had the common pedigree and phenotype reaction to net blotch. Among them, Kazanova, Herta, Gratsiosa, Chlo 223, Otis, Typi, Igri, Steptoe, and Flavius were spread. Genotypes Chlo 646, Hector, 102C2, Chevron, Odysseus, Jet, Betzes, Chlo 475, provided by Global Gene banks NSGC and NordGen formed the first subgroup of the second cluster. The second subcluster includes wild forms (Furki, *H. bulbosum*) and varieties (Nomini, Hertland, Norbert) which are used as a resistant control in the studies of O'Boyle et al. (2014), Tamang et al. (2019), etc. Those genotypes show a different range of susceptibility to local isolates (Figure 6).


Figure 5. Phenotype manifestation of very sensitive (VS) varieties to net blotch, form the main subgroup of the first cluster

After completing the experiments using polymorphic ISSR primers, the data obtained with their assistance was pooled and standardized. Analysis of the PCR products revealed that they were distributed in 2 cluster groups (Figure 7), covering 204.89cM for the first group and 73.46cM for the second one. The obtained genetic map gives an idea of the identified polymorphisms' relation, which may further serve to establish their additional links with the behavior of the varieties with the examined plant pathogen.


Figure 6. Phenotype manifestation in Hertland and Furki (persistent controls based on literature and studies by other authors)

## DISCUSSION

Genotyping and identification of disease-resistant barley lines and varieties are important genetic tools of modern breeding programs in Bulgaria. The application of marker-assisted selection has revealed great opportunities for rapid and cost-effective production of elite varieties. Genotypes used as resistance identifiers by other authors (Neupane et al. 2015, O'Boyle et al. 2014, Tamang et al. 2015) introduced from NSGC and NordGen have shown susceptibility to local isolates. Genetic analysis of a worldwide barley collection for resistance to the net form of net blotch disease has been presented revealing 254 significant marker-trait associations corresponding to 15 distinct QTL regions (Novakazi et al. 2019). The observation of the reactions between seedlings and adult plants to the pathogen could serve as an indicator for seedling screening and may be useful for a further selection of adult-plant resistance (Gupta et al. 2003, Grewal et al. 2008).

The development of molecular markers starting from restriction fragment length polymorphisms (RFLPs), (Botstein et al. 1980), via the application of PCR-based procedures, increases the possibilities of an efficient application of marker-assisted selection procedures in plant breeding.

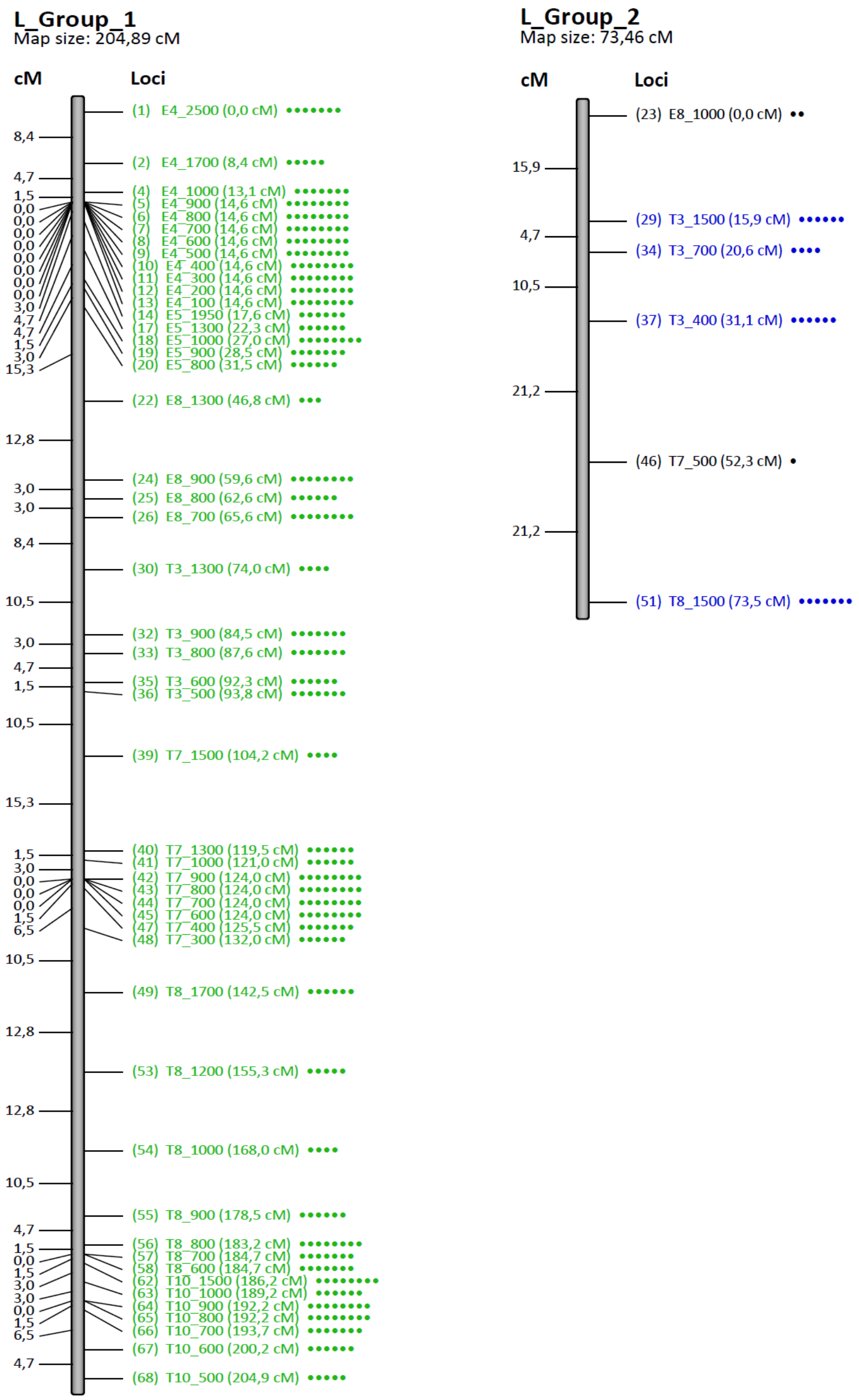


Figure 7. Map of the relative position of the identified polymorphic genomic loci

Molecular marker technology provides potential tools for mapping disease-resistance genes and the genotypic selection and promotes considerable resource-saving (Dracatos et al. 2020). The molecular genetic analyzes here demonstrate that the ISSR marker system can identify significant genetic diversity. The combination of genotypic and phenotypic markers results in a well-established distribution of genotypes according to their genetic distance and resistance to phytopathogens. In the present study, significant phenotypic diversity was observed in relation to the studied genotype's reaction to the investigated pathogen. Studies on net blotch resistance genes indicate a complex host-pathogen interaction controlled by both quantitative and major resistance genes. (Grewal et al. 2008). An ISSR marker system was used to genotype certain barley forms with respect to their resistance to *Pyrenophora teres* Drechsler. The authors reported molecular markers associated with qualitative and quantitative traits in barley associated with disease-resistant loci which represent a valuable starting point for marker-assisted selection (Sayed et al. 2004). Common core QTLs on chromosome 2H (R2=14-40%) and 7H (R2=24-80%) were found in RIL populations, suggesting core genes with broad resistance specificity. The major QTL on 7H has been shown to be a dominant susceptibility gene in both susceptible malting barley cultivars (Tamang et al. 2019). It's still challenging to better understand local isolate pathogenicity, epidemiology, and host-pathogen interactions, which are needed to breed more resistant cultivars (Clare et al. 2020).

## CONCLUSION

No resistant genotypes to Bulgarian isolates of the phytopathogen *Pyrenophora teres* Drechsler were found during the study. The varieties resulting from breeding programs in Bulgaria and those widespread in the country showed medium - to strong susceptibility to the pathogen from their early stages of development. The introduced genotypes used as a source of resistance to the studied pathogen such as Nomini, Norbert, etc. during their vegetation period (after tillering) showed disease symptoms, as well. The absence of resistant plants to the common isolates of *P. teres* may result, on the one hand, from the high pathogenicity of the Bulgarian isolates and, on the other hand, from the lack of expression of resistance genes.

Furthermore, the map generated can also be used to identify associations of these markers with resistance to other pests' levels, thereby enabling the identification of genomic regions contributing to horizontal (multiple) resistance, such as those of particular interest. Finding a balance between different breeding methods most appropriate to the needs and resources, such as molecular tools to map and screen-specific resistance genes or greenhouse seedling tests to select against specific virulence and multi-environmental field assays to track the resistance-environment interactions may be an optimal way to obtain profitable and long-lasting results and ensure durability.

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**** https://www.ars.usda.gov/ - United States Department of Agriculture