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High levels of genetic and genotypic diversity in field populations of the barley pathogen *Ramularia collo-cygni*

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Abstract

The ascomycete pathogen *Ramularia collo-cygni* causes Ramularia leaf spot (RLS) on barley. Although *R. collo-cygni* is considerd an emerging disease of barley, little is know about genetic diversity or population genetic structure of this pathogen. We applied a set of polymorphic AFLP

(Amplified Fragment Length Polymorphism) markers to investigate population genetic structure in two Northern European populations of *R. collo-cygni*. The distribution of AFLP alleles revealed low levels of population subdivision and high levels of genetic diversity at both locations. Our analyses included 87 isolates and of these 84 showed a unique genotype pattern. The genetic structure of populations in Scotland and Denmark is highly similar and we find no evidence of population sub-division. An analyses of molecular variance was used to show that 86% of the variance is attributable to within field genetic variance. In spite of the high levels of genetic and genotypic diversity in the *R. collo-cygni* populations, we find significant evidence of linkage disequilibrium among the AFLP alleles using a multilocus analysis. We propose that the high levels of genotypic diversity and the lack of population differentiation result from considerable levels of gene flow between populations most likely mediated by seed borne dispersal of inoculum.

Keywords

Ramularia collo-cygni, population genetics, AFLP, diversity index, genetic differentiation, linkage disequilibrium

Introduction

The ascomycte fungus *Ramularia collo-cygni* causes the disease Ramularia leaf spot (RLS) on barley (*Hordeum vulgare* L.). RLS is considered an emerging disease of barley with increasing importance to agriculture in Northern and Central Europe (Walters et al. 2008). Though *R. collo-cygni* was first described in 1893 (Cavara 1893), it was not until the late 1980's that widespread presence of the pathogen was reported in several countries in Europe, New Zealand, Argentina and Uruguay (Sachs 2006; Harvey 2002; Walters et al. 2008). RLS is estimated to cause losses up to 25% of the yield potential through a significant decrease of kernel size and quality (Greif 2002; Harvey 2002). *R. collo-cygni* has also been isolated from maize (*Zea mays* L. ssp. *mays*) (Stabentheiner et al. 2009) and from a number of uncultivated grasses such as *Agropyron repens* and *Lolium perenne* (P. Frei, pers comm.) suggesting a broader host range. It is so far not known whether isolates of *R. collo-cygni* generally are able to infect a broad range of host species or if host specialization (e.g. to barley) occurs within local populations.

It has been suggested that *R. collo-cygni* depends on the growth stage of its host for symptom development and that the disease therefore occurs mainly late in the growing season (Walters et al. 2008; Schützendübel et al. 2008). During infection *R. collo-cygni* undergoes a shift in lifestyle from asymptomatic endophytic growth to necrotrophic growth. The change in lifestyle has been hypothesized to be explained by ontogenesis of the host and the subsequent degradation of the antioxidative system in the leaves at the early stages of ripening in barley (Schützendübel et al. 2008).

The distribution and source of genetic diversity in populations of *R. collo-cygni* is so far poorly described. A teleomorph stage has so far not been well described. Asexual propagation occurs through the formation of conidiophores through stomatal openings and the subsequent dispersal of wind borne conidiospores (Walters et al. 2008; Stabentheiner et al, 2009).

To our knowledge there are only two studies of genetic differentiation in populations of *R. collo-cygni*. One study focuses on the frequency of an allele of cytochrome b conferring resistance to QoI fungicides (Matusinsky et al. 2011). The study demonstrates a clear correlation between the use of strobilurins and the frequency of the QoI resistance allele. In a recent study by Leisova-Svobodova et al (2012), AFLP markers were also applied to analyse genetic variability in populations of R. collo-cygni. In agreement with the findings reported here, the study reveals high variation within populations in spite of a linkage disequilibrium between AFLP alleles.

A further understanding of population dynamics, including levels of genetic diversity and reproductive mode of *R. collo-cygni* is important to develop improved and efficient management strategies for this emerging disease (McDonald and Linde, 2002).

The population genetic structure of an organism can be described by genotyping a set of individuals in one or several populations of interest using neutral molecular markers. When sequence information from multiple independent loci is not available, randomly amplified polymorphic loci such as AFLP (Amplified Fragment Length Polymorphism) markers can be applied. AFLP analyses allow the characterization and comparison of multiple loci scattered throughout the genome of the organism. We developed a set of AFLP markers for *R. collo-cygni* with the aim of describing population genetic structure in two European field populations of the pathogen. The objectives of this population genetic study were to 1) assess the level of genetic and genotypic diversity in four local Northern European population of *R. collo-cygni* where the pathogen is prevalent, 2) determine the extent of population differentiation and 3) estimate the extent of linage disequilibrium among AFLP alleles. The data reported here provide the first insight into population structure and reproductive mode of this fungal pathogen in two barley-producing regions.

Materials and Methods

Sample isolation and DNA extraction

Isolates of *R. collo-cygni* were collected from two locations in Denmark and two locations in Scotland during the 2008-growing season. In both countries the field sites, Tystofte and Sejet in Denmark and Bush and Lanark in Scotland, were trial sites either for the Danish Ministry of Food, Agriculture and Fisheries, Sejet Plantbreeding I/S or Scottish Agricultural College. Coordinates of the the Danish fields were 55:49:35N, 9:56:38E (Sejet Plantbreeding in Jutland) and 55:14:49N, 11:19:39E (Tystofte on Zealand). The distance between these locations is 108 km. Coordinates of the Scottish fields were 55:52:04 N, 3:12:38W (Bush, Edinburgh) and 55:40:28N, 3:46:33W (Lanark, Glasgow). The distance between these two sites is approximately 40 km. The sites in Denmark and Scotland are located on the same latitude however separated by the North Sea and a distance of 830 km (between Sejet and Lanark) (Fig. 1)

We collected leaves with RLS symptoms from plants at the stage of heading (approximately 55 on Zadoks scale) within an area of 400 m^2 at each site. The barley plants at Bush and Lanark originated from seeds from the same seed-lot while plants from Tystofte originated from seeds from multiple breeding companies. The barley plants at Sejet were from a seedlot originating from plants harvested at the location.

The collected leaves were washed in water and placed with the abaxial side upwards on potato dextrose agar (PDA) of pH 4.5. The infected leaves were incubated with a 16-hour light period at 22°C for two days. After incubation conidiospores emerging from conidiophores on leaf surfaces as conidia were picked with a sterile needle and transferred to new PDA plates. From each lesion one transfer was carried out. After two days the plates were controlled for germinating spores, and from each lesion single spores were transferred to new plates. This procedure was repeated four times to ensure that isolates were originating from single spores. A total of 87 isolates were obtained from the year 2008 from the four locations (Table 1).

After three weeks of growth on PDA medium a small amount of mycelia was transferred with a spatel to micro-centrifuge tubes. DNA was extracted using a modified CTAB method as described in Christiansen et al. (2006).

AFLP procedure

The AFLP electrophoresis method previously described by Vos et al. (1995) was used with the following modifications: Genomic DNA samples (250 ng) were digested with 5U of *MseI* and *PstI* enzymes in a total volume of 10µl for three hours at 37°C, followed by inactivation at 70°C for 15 minutes. DNA was ligated at 37°C for three hours in a 10 µl mixture consisting of 2.5 µM *MseI*, 0.25 µM *PstI* and 1U T4 DNA-ligase. Pre-amplification was carried out on 4µl ligated DNA using *Pst*I primer without selective nucleotide and *Mse*I-C as follows: two min. at 94°C and 20 cycles at 94°C for 30 sec., 56°C for 20 sec., 72°C for two min., followed 30 min. at 60°C. PCR with *Pst*I and *Mse*I primers with two selective nucleotides each were carried out on 3µl pre-amplified product as follows; two min at 95°C followed by nine cycles of 94°C for 20 sec., 65°C for 30 sec. (decreasing temperature by one degree per cycle), 72°C for two min. The last 22 cycles were carried out at 94°C for 20 sec.,

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56°C for 30 sec. and 72°C followed by a final extension at 72°C for two min. A total of 66 primer pair combinations from *Pst*I-2/*Mse*I-3 and *Pst*I-3/*Mse*I-3 were tested on seven isolates to identify those primers that produced clear and polymorphic bands (Table 2).

Data analysis and clone correction

PCR products were analysed on an AB3130*xl* Genetic Analyzer capillary machine and the obtained gel-images were visualized and analysed using the software Genemarker (Softgenetics®, PA, USA). Each individual band was subsequently scored manually using both gel-image function as well as peak height. In both cases default settings in Genemarker were applied for detection of bands with the recommended threshold intensity 50.

Bands between 90 and 650 base pairs were scored as either present "1" or absent "0". Only fragments that could be scored unambiguously were included in the AFLP analysis. To ensure reproducibility of the PCR and AFLP procedures DNA from four isolates not originating from the Danish or Scottish populations were repeated in all runs. Three of these control isolates originated from Foulum, Denmark (collected in 2007), and one from Baden Württemberg, Germany (collected in 2004). The dataset was clone corrected by excluding isolates with the same multilocus genotype.

Population genetic analyses

The clone corrected dataset was used for the further population genetic analyses. We defined sub-populations as the four local field populations, and populations as the pooled samples of isolates in either Denmark of Scotland. Population genetic parameters were calculated using the program GenAlEx 6.41 (Peakall and Smouse 2006). Average gene diversity (h) (Mueller and Wolfenbarger 1999) was calculated using the following formula:

$$h = (2N/(2N-1))*(\Sigma_i (1-p^2_i-q^2_i))/n,$$

where N is the number of individuals assessed, p and q are the frequencies of present and absent markers at the i'th locus and n is the total number of loci. Total gene diversity (H_t) within populations, genetic differentiation between populations (G_{st}) and genetic differentiation between sub-populations (D_{st}) (Nei 1973) were calculated as:

$$H_t = H_s + D_{st}$$

Where H_s is the average diversity within the studied sub-populations and D_{st} is gene differentiation amongst these (sub)-populations. Genetic differentiation (G_{st}) was calculated as:

$$G_{st} = D_{st}/H_t.$$

We further conducted an analysis of molecular variance (AMOVA) to determine the partitioning of variance. The AMOVA analysis was conducted in GenAlEx 6.41 using 999 permutations with the formula:

$$\Phi_{PT} = (V_{AP} + V_{AR})/(V_{WP} + V_{AP} + V_{AR}),$$

where V_{AP} is the variance among subpopulations, V_{AR} is the variance among countries and V_{WP} is the variance within subpopulations (Peakall et al. 1995). For the AMOVA and G_{st} analyses we calculated a *P*-value based on the null-hypothesis of no variation or differentiation between populations.

To quantify genetic distance (*D*) and identity (*I*) of populations we used Nei's parameters for *D* and *I* (Nei 1978), in which *I* is:

$$I = J_{xy}/((J_x J_y)0.5),$$

where J_x is the arithmetic means of probability of identity between two genes randomly chosen within population X, and J_y being the equivalent in population Y. $J_x J_y$ is then the probability of identity between two markers, one from each population chosen at random.

Finally, we generated a dendogram including all isolates to illustrate the distribution of genotypes according to their origin. The analyses were conducted using DendroUPGMA (http://genomes.urv.cat/UPGMA/) applying Pearson's coefficient with default settings (Garcia-Vallvè et al. 1999).

To address whether the association of AFLP alleles in *R. collo-cygni* are randomly associated as in a randomly recombining population we determined the extent of linkage disequilibrium between polymorphic ALFP markers. We applied a modified formula of the Index of Association (I_A) statistics; the r_d parameter which account for a large number of loci and therefore is appropriate for AFLP datasets (Agapow and Burt 2001). The Multilocus 1.3 software was used to calculate r_d values for each *R. collo-cygni* population and for a simulated dataset of a recombining population with random association of alleles. The resulting r_d values were compared to identify whether the parameter of *R. collo-cygni* population deviate significantly from the distribution of r_d values obtained from simulated recombining datasets.

Results

AFLP analyses

66 AFLP primer pairs were screened using seven *R. collo-cygni* isolates. Four of these isolates were subsequently used as controls to test for reproducibility in the further AFLP analyses. We selected five primer pairs showing clear and polymorphic band patterns in the size range 90 to 650 bp. AFLP genotypes were characterized for a total of 63 Danish isolates and 24 Scottish isolates (Table 1). The five primer combinations amplified between 43 and 66 fragments resulted in a total of 277 AFLP bands (Table 2). Among the 87 isolates nine bands were unique to single isolates.

The genotypic diversity reflected by the proportion of unique genotypes was very high and in the subpopulations from Tystofte, Bush and Lanark had a unique band pattern. In the subpopulation from Sejet we however identified three isolates with the same genotype. We assigned these identical genotypes as clones

Genetic diversity index within the North European populations

We used Mueller and Wolfenbarger (1999) genetic diversity index (h) to calculate the average gene diversity per locus and used this value to compare the diversity levels between different populations. Due to the unequal sample sizes we applied an unbiased measure of gene diversity accounting for the number of isolates analysed. The genetic diversity index for the total sample including both Danish and Scottish isolates was h=0.199 (Table 1). Dividing the populations we found that h was higher in the Danish sample (h=0.207) compared to the Scottish sample (h=0.166). Further comparing the four subpopulations (Sejet, Tystofte, Lanark and Bush) we found the lowest value of h in the Lanark subpopulation (0.139) and the highest genetic diversity (h=0.237) in the Tystofte subpopulation. We note that the highest proportion (78.7%) of polymorphic AFLP markers likewise was found in the Tystofte sub-population.

Genetic differentiation

We assessed the extent of population subdivision using Nei's parameter G_{st} (Nei 1973). Population differentiation measured as G_{st} between the pooled Danish and Scottish samples was 0.031 ($p = 0.01$). The extent of population differentiation between local subpopulations was in both Denmark and Scotland higher than the extent of differentiation between Denmark and Scotland. Between Sejet and

Tystofte G_{st} was computed to be 0.135 (p = 0.001) and between Bush and Lanark 0.042 (p = 0.096). The distribution of AFLP alleles thereby suggests that the extent of genetic variation in *R. collo-cygni* mainly is explained by high levels of variation within field populations and local differentiation between regional populations rather than differentiation on a larger geographical scale (Fig. 2).

We further analysed the spatial partitioning of genetic variation using an AMOVA analyses. The analyses showed that a large proportion, 86% ($p > 0.01$), of the total estimated variance is attributable to variation found within the field populations (Fig. 3). Only a smaller proportion, 14% ($p > 0.01$), is attributable to among-subpopulation variance component while there was no (0%) significant differentiation between the Danish and Scottish populations. The lack of geographical clustering is further illustrated by a dendogram reflecting the random relatedness between *R. collo-cygni* isolates based on their AFLP genotypes (Fig. 4).

Pairwise estimates of genetic identity (I) were calculated using the unbiased measure of Nei (Nei 1978a; Nei 1978b). The genetic identity between all pairs of subpopulations was >0.94 (Table 3). Though significance levels for differences in identity between locations or countries could not be calculated, Tystofte showed a lower extent of genetic identity to the other population samples.

Linkage disequilibrium

To address whether propagation of *R. collo-cygni* occurs through random mating we estimated the extent of linkage disequilibrium between AFLP markers. Complete linkage equilibrium ($r_d = 0$) occurs in a population with random mating between all individuals. Linkage disequilibrium between independent AFLP alleles is reflected by r_d values, which significantly deviate from the distribution of r_d values from a simulated recombining dataset. In the *R. collo-cygni* dataset we assessed r_d values in the pooled sample of Danish isolates and in the pooled sample of Scottish isolates and in both cases we found r_d values (0.031-0.024), which significantly deviated from values expected under random mating (p<0.001) in all four sub-populations. We also computed r_d values for the four individual subpopulations. In all four cases we also found support for linkage disequilibrium between AFLP alleles (p<0.001) (Table 1).

Discussion

We here present a set of AFLP markers intended for genotyping and population genetic analyses of the emerging barley pathogen *R. collo-cygni* and we report the first analyses of population structure in barley infecting strains. Our analyses show that there is no genetic structure in two *R. collo-cygni* populations from Northern European locations. On the other hand, the allele distribution in the two populations is highly similar although the populations are separated by hundreds of kilometers. The majority of genetic variation in *R. collo-cygni* is attributable to high levels of variation within local subpopulations rather than genetic differentiation between regional populations. The overall lack of population differentiation between the Danish and the Scottish populations suggests that *R. collo-cygni* either is dispersed over long distances or that the Danish and Scottish isolates were dispersed recently from the same source population. Gene flow and migration patterns have been extensively studied in the barley pathogen *Rhyncosporium secalis* (Zaffarano et al. 2009: Linde et al. 2009). *R. secalis* has been dispersed recently on a global scale, and human mediated transport of infected seeds has been suggested a main source of migrants. It is possible that *R. collo-cygni* in a similar way has been dispersed with barley through infected seeds. In support of this hypothesis, is the finding that the Tystofte sub-population showed a slightly distinct composition of alleles. The host population at Tystofte originated from a mixed seed lot of barley in contrast to the trials at Sejet, Lanark and Bush. The *R. collo-cygni* population at Tystofte showed a lower extent of genetic identity to the Sejet, Bush and Lanark sub-populations and had a higher level of genetic diversity than any of the three other populations. We speculate that the higher diversity reflects the origin of seeds in the barley host population. According to this hypothesis a main contributor to *R. collo-cygni* dispersal is seedborne inoculum analogue to the dispersal of *R. secalis* (Linde et al. 2009).

Whether wild grasses also provide a source of inoculum in barley fields is so far unknown, however the finding of *R. collo-cygni* isolates on non-cultivated grass hosts suggests that the spores may be contributed from alternative hosts (Walters et al. 2008). Future population genetic studies using co-dominant markers such as DNA sequence data or microsatellite markers will allow a more detailed characterization of individual isolates and a more profound comparison of *R. collo-cygni* populations isolated from different hosts and from seed and leaf material.

So far there the sexual stage of *R. collo-cygni* has not been reported however, this may not exclude that sexual recombination contribute to the propagation and population genetic structure of *R. collo-cygni*.

In other related Dothideomycete species, sexual recombination is known to occur frequently although the sexual fruiting bodies rarely are observed (Eriksen et al. 2001; Halama 2002). The high genotypic diversity observed in the *R. collo-cygni* populations suggests that sexual recombination contributes to the population dynamic of the species. We find only few identical genotypes indicative of clonal propagation of isolates. However, the multilocus analyses demonstrate a significant extent of linkage disequilibrium between AFLP alleles in the Danish and Scottish *R. collo-cygni* populations. A signature of linkage disequilibrium between independent markers is in agreement with non-random mating or clonal propagation, but may also result from natural selection of particular combinations of loci, or from genetic drift if the population size of the organism is small. With the *R. collo-cygni* dataset analysed here, we are not able to distinguish between these factors. The mating type genes of *R. collo-cygni* have so far not been described however insight into the mating type system and distribution of mating type alleles would provide further insight into mating behaviour and population dynamics of *R. collo-cygni*. The characterization and distribution of mating type idiomorphs in the related wheat pathogen *Mycosphaerella graminicola* and in the barley pathogen *Pyrenophora teres* have provided support for sexual recombination in these two species although the telomorph stages are rarely encountered (Zhan et al. 2002: Rau et al. 2005). In both species sexual recombination is a main contributor of genetic variance in field populations.

The population genetic study presented here has demonstrated a high level of genetic and genotypic diversity in *R. collo-cygni* in agreement with a population genetic study of Czech populations of *R. collo-cygni* (Leisova-Svobodova et al, 2012). Future studies should aim to characterize patterns of gene flow between geographical populations of *R. collo-cygni* and further assess patterns of reproductive mode. The sequencing and annotation of the *R. collo-cygni* genome is in progress (Havis et al. unpublished). The finished sequence will provide a valuable resource for the design of molecular markers for further population genetic and epidemiological studies.

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Tables:

Table 1. Result of the AFLP on *Ramularia collo-cygni* populations from Denmark and Scotland. Results are divided into each location (subpopulation), at population level and for all isolates together.

Population	No. of isolates[a]		Polymorhic loci[b]		Genetic diversity[c]		Linkage disequilibrium[d]	
	before	after	number	%	*h*	SE	*rD* value	P-value
Lanark	11	11	116	43,5	0,139	0,011	0,081	<0,001
Bush	13	13	147	55,1	0,162	0,011	0,140	<0,001
Scotland	24	24	163	61,1	0,166	0,011	0.024	<0,001
Tystofte	19	19	210	78,7	0,237	0,011	0,085	<0,001
Sejet	44	41	185	69,3	0,169	0,011	0,025	<0,001
Denmark	63	60	246	92,1	0,207	0,010	0.031	<0,001
Total	87	84	253	94,8	0,199	0,010		

[a]Number of isolates before and after clone correction.

[b]Number and percentage of polymorhic loci at subpopulation and population level.

[c]Nei's genetic diversity index calculated at the level of subpopulation and for the pooled population in both countries.

[d]Linkage disequilibrium calculated based on the formula of Agapow's (1980)

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Table 2. Primer combinations and numbers of bands amplified per primer combination.

Primer combination	No. of bands
Mse1-CAC + *Pst1*-AC	61
Mse1-CAT + *Pst1*-AGC	45
Mse1-CTC + *Pst1*-AC	66
Mse1-CTG + *Pst1*-CC	62
Mse1-CTT + *Pst1*-AGC	43
Total	277

*Table 3***.** Nei's genetic identity *I* (above diagonal) and genetic distance *D* (below diagonal) for the four subpopulations studied in the project.

Populations	Bush	Lanark	Sejet	Tystofte
Bush	…	0,985	0,989	0,965
Lanark	0,015	…	0,996	0,943
Sejet	0,011	0,004	…	0,948
Tystofte	0,035	0,057	0,052	…

Figure legends:

Fig. 1. Location of fields were isolates from where the four subpopulation were sampled in Scotland (A) and Denmark (B).

Fig. 2. Genetic differentiation (G_{st}) between the Danish and Scottish subpopulations and between the Danish and Scottish populations.

Fig. 3. Hierarchical analysis of genetic variation performed as Analysis of Molecular Variance (AMOVA). Results show that all variation can be found in each of the populations (Denmark or Scotland) and 14% of total variation is expressed at subpopulation level. Genetic differentiation between the Danish and Scottish subpopulation and between the Danish and Scottish populations

Fig. 4. Dendogram showing the relatedness between isolates based on Pearsons coefficient. Isolates marked with blue squares are from the Bush (Scotland) subpopulation, blue triangles from Lanark (Scotland), and isolates marked with red are from Denmark. Squares from Sejet and triangles from Tystofte. Scale bar indicates the similarity between the isolates.

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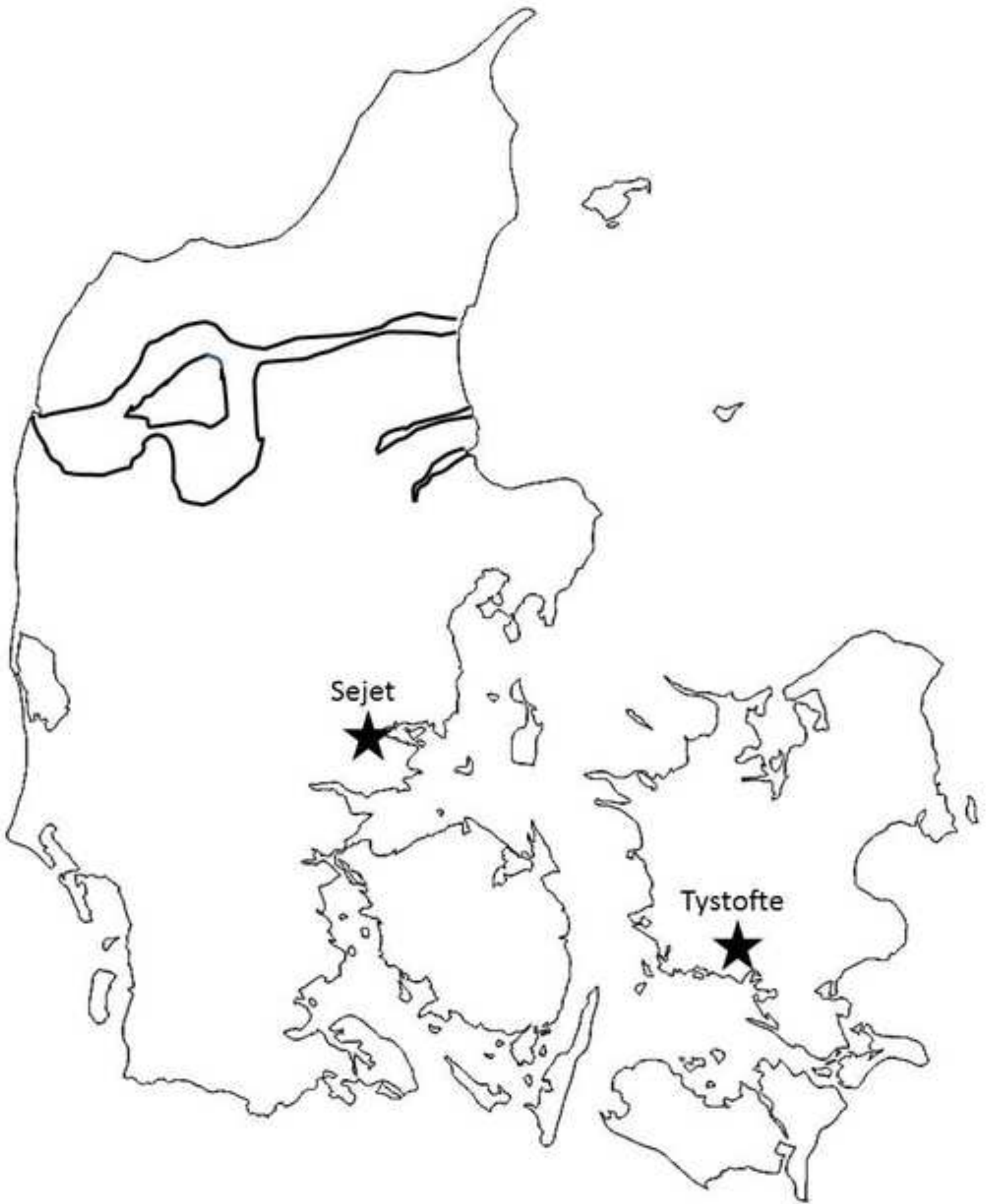

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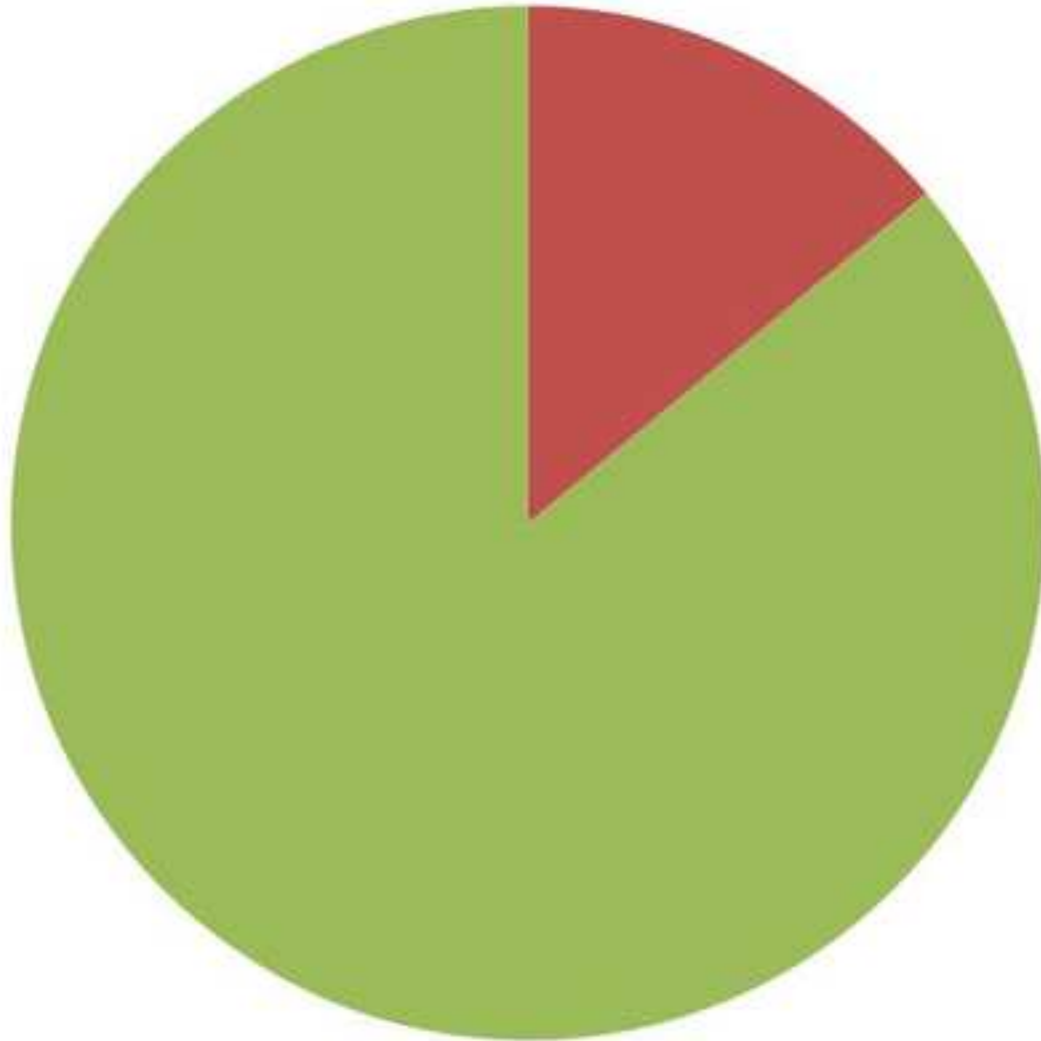

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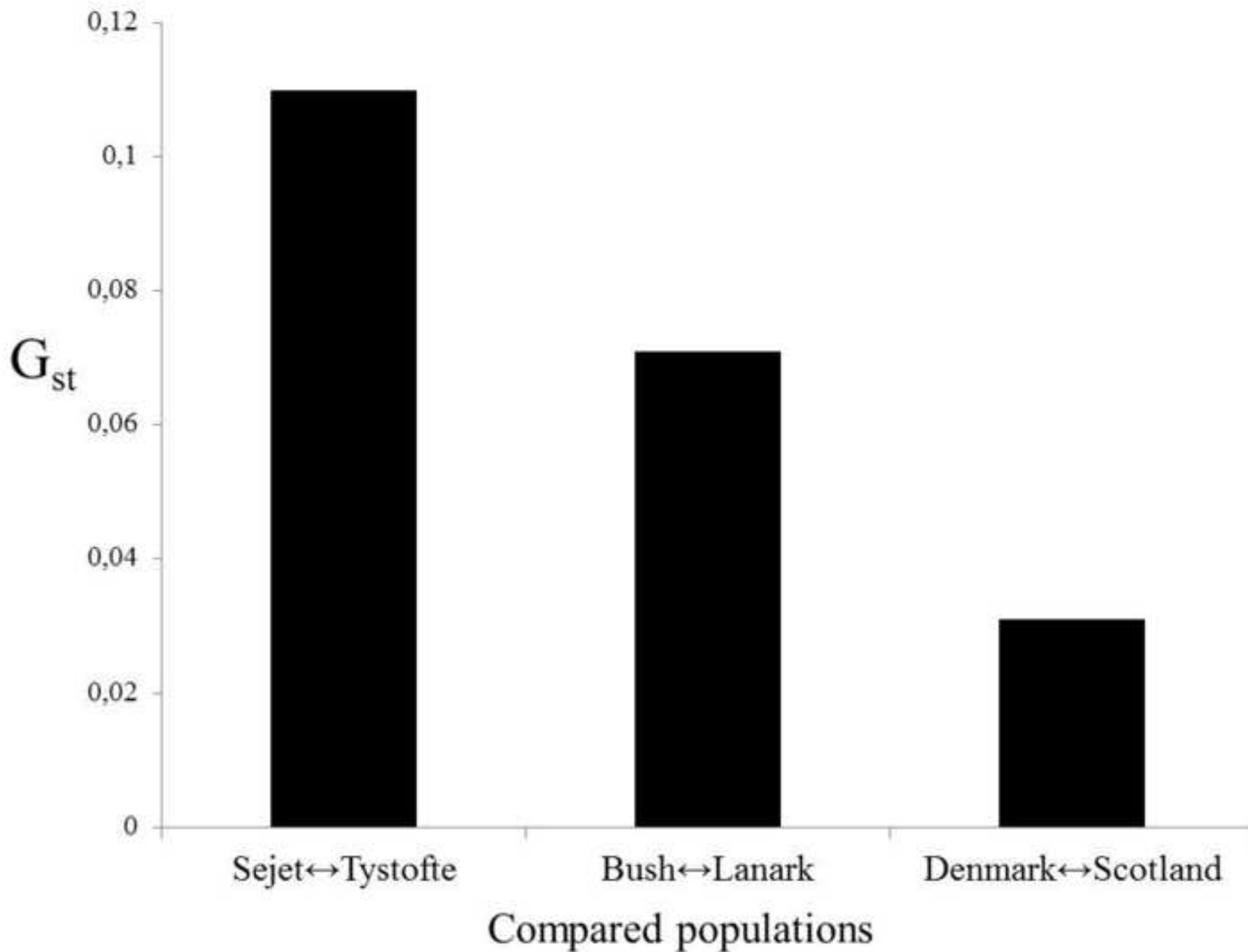

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