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ORIGINAL ARTICLE

Exploration of Nuclear DNA Markers for Population Structure Assessment in the Desmid *Micrasterias rotata* (Zygnematophyceae, Streptophyta)

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ABSTRACT

Freshwater green microalgae are diverse and widely distributed across the globe, yet the population structuring of these organisms is poorly understood. We assessed the degree of genetic diversity and differentiation of the desmid species, *Micrasterias rotata*. First, we compared the sequences of four nuclear regions (*actin, gapC1, gapC2,* and *oee1*) in 25 strains and selected the *gapC1* and *actin* regions as the most appropriate markers for population structure assessment in this species. Population genetic structure was subsequently analyzed, based on seven populations from the Czech Republic and Ireland. Hudson's Snn statistics indicated that nearest-neighbor sequences occurred significantly more frequently within geographical populations than within the wider panmictic population. Moreover, Irish populations consistently showed higher genetic diversity than the Czech samples. These results are in accordance with the unbalanced distribution of alleles in many land plant species; however, the large genetic diversity in *M. rotata* differs from levels of genetic diversity found in most land plants.

KNOWLEDGE of population genetic diversity within and among populations contributes to a better understanding of evolutionary processes such as gene flow, genetic drift, and speciation.

Despite their ubiquity and ecological importance, studies on population genetic structure in eukaryotic microorganisms are scarce. This is partly due to the uncovered cryptic diversity and associated uncertainty about species boundaries, hampering population-level studies of microbial eukaryotes (Gerstein and Moore 2011). Some authors proposed that the small organism size, large population sizes, and high dispersal potential of eukaryotic microorganisms would lead to high gene flow across large geographical scales, resulting in a ubiquitous species distribution in suitable environments (Fenchel and Finlay 2004; Finlay 2002). Large population sizes would be expected to prevent local extinction and result in undisturbed population diversity (Fenchel and Finlay 2004), leading to high local genetic diversity (Mes 2008). On the other hand, intensive gene flow would constantly erase genetic diversity among populations, leading to a relatively low global diversity and undifferentiated populations (Fenchel and Finlay 2004). Moreover, computer simulations indicate that organisms with partial clonal reproduction show lower genetic diversity within the populations (Vanoverbeke and De Meester 2010).

However, several recent studies challenge the assumption of low population differentiation in planktonic and benthic microorganisms (Bass et al. 2007; Casteleyn et al. 2010; Evans et al. 2009; Lebret et al. 2012; Medlin et al. 2000; Rengefors et al. 2012). These studies showed that high genetic differentiation correlates with geographical and/or ecological parameters. In freshwater systems, this pattern could be explained by two equivalent hypotheses. First, water bodies can be considered as islands in a sea of land, producing barriers to gene flow in the absence of desiccation-resistant propagules (Evans et al. 2009; Weisse 2008). Second, pronounced genetic differentiation among neighboring populations may result from founder effect followed by a rapid local adaptation (monopolization hypothesis; De Meester et al. 2002).

Recent population studies of free-living aquatic protists mainly focused on diatoms, one of the most abundant groups of eukaryotic microorganisms (Casteleyn et al. 2010; Evans et al. 2004, 2009; Godhe and Härnström 2010; Rynearson et al. 2006, 2009). These studies showed

spatial (Casteleyn et al. 2010; Evans et al. 2009) as well as temporal differentiation of populations, which may point to ecological constraints to gene flow (D'Alelio et al. 2009; Rynearson et al. 2006). Geographical and ecological population differentiation have also been shown in several other phototrophic microorganisms, such as prymnesiophytes (Iglesias-Rodríguez et al. 2006), raphidophytes (Kooistra et al. 2001), dictyochophytes (Riisberg and Edvardsen 2008) and dinoflagellates (Alpermann et al. 2009; Bolch et al. 2012; Lowe et al. 2012; Masseret et al. 2009). In addition, population studies of heterotrophic microorganisms, such as ciliates, suggested that ecological parameters such as salinity and temperature are important factors structuring microbial populations (Gächter and Weisse 2006; Lowe et al. 2005; Weisse et al. 2008).

Green algae are widespread and ecologically important in both aquatic and terrestrial environments. Aside from population genetic studies of marine green macroalgae (Benzie et al. 2000; Johansson et al. 2003; Leskinen et al. 2004; Zhao et al. 2011), only a single study showed a subtle level of genetic structure in terrestrial algal species (Doering and Piercey-Normore 2009). In addition, a few methodological papers on lichen photobionts have been published (Dal Grande et al. 2010; Fernández-Mendoza et al. 2011; Widmer et al. 2010), but none about freshwater green microalgae.

In this study, we assess population genetic diversity and structure in aquatic green microalgae using the common desmid species *Micrasterias rotata* Ralfs. This taxon forms a distinct species based on morphologic and genetic data (Neustupa et al. 2011; Škaloud et al. 2011). *Micrasterias rotata* occurs in a variety of habitats, from mesotrophic lakes to oligotrophic moors and bogs (Růžička 1981). These habitats have a scattered geographic distribution; thus potentially fitting an island-type model of evolution with restricted gene flow between populations. In addition, occasional sexual reproduction may produce higher genetic diversity when compared with strict asexuals. Conversely, the small size of the organism and assumed large populations may result in the absence of population structure. Furthermore, dispersal might be facilitated by the production of zygospores, the durable products of sexual reproduction (Růžička 1981).

The aim of this study was to investigate the degree at which desmid populations are structured. We tested different molecular markers by comparing variability in four nuclear DNA sequence regions in 25 *M. rotata* strains. Two markers (*gapC1* and *actin*) were selected to assess the population diversity and differentiation within and between seven populations of *M. rotata*.

MATERIALS AND METHODS

Origin of strains

A total of 44 *M. rotata* strains were obtained from culture collections (Sammlung von Conjugaten-Kulturen, Universität Hamburg [SVCK] and Culture Collection of Algae, Charles University in Prague [CAUP]), and by single-cell isolation from various natural habitats. The origins of the strains used for nuclear marker comparisons are listed in Table 1. For population structure analysis, we sequenced 3–5 strains from seven populations. The localities of the populations and number of successfully sequenced strains are listed in Table 2.

All strains were cultivated in MES-buffered DY IV liquid medium (http://botany.natur.cuni.cz/algo/caup-media.html#DY4) at 19 °C with illumination of 40 μmol/m^2/s provided by 18W cool fluorescent tubes (Philips TLD 18W/33, Royal Philips Electronics, Amsterdam, the Netherlands).

mRNA isolation and cDNA amplification

Total RNA from strain SVCK 287 was extracted with a Nucleospin kit RNA XS (Machery-Nagel GmbH & Co. KG, Düren, Germany). Subsequent precipitation and dilution were performed as described in Škaloud et al. (2013). cDNA was amplified from RNA using an Omniscript RT kit (Qiagen GmbH, Hilden, Germany).

Primer design

Conserved regions of nuclear housekeeping genes were obtained from the cDNA of the strain SVCK 287 using standard primers for *actin* (HG805306) (An et al. 1999; Cocquyt et al. 2010; Bhattacharya et al. 1993), oxygen evolving factor 1 (*oee1*) (HG805307) (Cocquyt et al. 2010), and cytosolic glyceraldehyde-3-phosphate dehydrogenase (*gapC*) (HG805308) (Petersen et al. 2003), and following the same PCR conditions as described in the above mentioned studies. The PCR products were checked on 1.8% TBE agarose gel. The bands of expected size were excised, dissolved in 30 μl of 1X TE buffer at 88 °C for 10 min, and purified with FastAP and Exo1 enzymes (Thermo Fisher Scientific, Waltham, MA). The resulting products were used for cycle sequencing with the initial PCR primers using an ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit, and following the manufacturer's instructions. Sequencing products were analyzed with an ABI 3100 Prism Genetic Analyzer (PE Applied Biosystems, Foster City, CA). The sequences were compared with published data to identify the intron positions. Finally, new primers were designed within the exon regions to obtain intron sequences. Newly designed primers (Table 3) were tested for optimal length and annealing temperature in the virtual environment of the Oligo Analyzer v 1.1.2 program (Kuulasmaa 2001).

DNA extraction, PCR reactions and sequencing

Total DNA was extracted as described in Neustupa et al. (2011). The PCR reactions were carried out in 20 μl volumes: 13.9 μl sterile Mili-Q water, 2 μl $MgCl_2$ (25 μM), 2 μl PCR Buffer 10X (Applied Biosystems), 0.4 dNTP (10 μM), 0.25 μl of each primer (Table 2), 0.2 μl AmpliTaq GOLD polymerase (5 U/μl), and 1 μl DNA (not quantified). Polymerase chain reaction conditions were set to an initial denaturation at 95 °C for 10 min followed by 40 cycles of

Table 1. Strain origin and accession numbers of samples used for nuclear DNA comparison

Strain	Sampling place	GPS coordinates	Accession numbers			
			actin	*gapC1*	*gapC2*	*oee1*
CAUP[a] K604	Pools by Cep, Czech Republic	N 48°55.394167′ E 14°50.399333′	HG805267	HG805353 HG805354	HG805382	HG805290
SVCK[b] 1	An unknown locality near Potsdam, Germany	–	HG805268 HG805269	HG805355 HG805356	HG805383 HG805384	
SVCK 26	Wildes Moor bei Husum, Germany	N 54°24.935167′ E 9°14.937′	HG805270	HG805357 HG805358		HG805291
SVCK 78	Bogs close to Korvanen, Finland	N 67°56.216667′ E 27°50.416667′		HG805359 HG805360	HG805385	HG805292
SVCK 93	Hammerfest, Norway	N 70°39.55′ E 23°41.116667′	HG805271	HG805361 HG805362		HG805293
SVCK 212	Timmer Moor near Hamburg, Germany	N 53°39.793667′ E 10°08.421′	HG805272	HG805363 HG805364		HG805294
SVCK 243	A bog near Sappel close to Millstatt, Kärnten, Austria	N 46°47.876667′ E 13°37.791′	HG805273 HG805274	HG805365 HG805366		HG805295
C8	A mountain fen near Nové Hamry, Czech Republic	N 50°21.841′ E 12°39.365′	HG805277 HG805278	HG805368	HG805388	HG805297
C12	Marienteich, Czech Republic	N 50°32.7255′ E 14°40.657333′	HG805279	HG805369	HG805389	HG805298
C13	A bog near Rod pond, Czech Republic	N 49°07.233167′ E 14°45.120667′	HG805280 HG805281	HG805370 HG805371	HG805390	HG805299
C8Hli2	The Lake Hlinír, Czech Republic	N 49°08.14818′ E 14°41.15064′	HG805282	HG805372 HG805373	HG805391 HG805392	HG805300
C3_Rod2	A bog near Rod pond, Czech Republic	N 49°07.276′ E 14°44.978′		HG805374	HG805393	HG805301
D4_Rod3	A bog near Rod pond, CzechRepublic	N 49°07.211′ E 14°45.017′		HG805375	HG805394	
F6Hut5	Pond Huťský rybník, Czech Republic	N 48°39.29466′ E 14°40.98138′	HG805283		HG805395 HG805396	HG805302
I6	Muckross Lake, Ireland	N 52°0.688833′ W 9°31.760667′		HG805376 HG805377		HG805303
Q6	A bog near E'tang Hardy, Aquitaine, France	N 43°43.143333′ W 1°22.157′	HG805284 HG805285	HG805378 HG805379		
C5_Ir_uzs	A moor near Lough Talt, Ireland	N 54°05.454′ W 8°56.935′	HG805286 HG805287			
D10_Ir_i12	Akreen More, Ireland	N 53°26.261′ W 9°54.874′	HG805288 HG805289	HG805380 HG805381	HG805397 HG805398	
F11_Ir_M1	A swampy bank of Croaghavehy ake, Ireland	N 54°43.97088′ W 8°31.01916′	HG805265 HG805266	HG805349 HG805350		
C2PdB1	The Upper Pond, Rybníčky u Podbořánek, Czech Republic	N 50°02.55906′ E 13°26.41572′	HG805236 HG805237	HG805314 HG805315		HG805304
C10Uhli7	The pond Uhlišťský rybník, Czech Republic	N 48°38.85546′ E 14°39.35658′	HG805248 HG805249	HG805330 HG805331		
E9Uhli7	The pond Uhlišťský rybník, Czech Republic	N 48°38.85546′ E 14°39.35658′	HG805250 HG805251	HG805332 HG805333	HG805399 HG805400	HG805305
B7Pre1	A moor near Přebuz, Czech Republic	N 50°23.10762′ E 12°35.90124′	HG805231 HG805232	HG805309 HG805310		
C7Pele3	Pelé, Czech Republic	N 48°57.48978′ E 14°57.8502′	HG805240	HG805320 HG805321	HG805401 HG805402	
C8_Ir_IR50	A small fen near Ellaghmore, Ireland	N 54°05.028′ W 9°03.228′	HG805252 HG805253	HG805345 HG805346		

[a]CAUP Culture Collection of Algae, Charles University in Prague.
[b]SVCK Sammlung von Conjugaten-Kulturen, University Hamburg.

denaturing at 95 °C for 1 min, annealing at 50/55 °C for 30 s, and elongation at 72 °C for 1.5 min, followed by final extension at 72 °C for 7 min. PCR for the *gapC* marker was performed in two separate reactions for different parts of the gene. Primer combinations and annealing temperatures for each gene are depicted in Table 3. PCR

Table 2. Sample sizes, locations, and ecological properties of sampling sites of the study populations

Population	GPS coordinates	pH	Conductivity	No of individuals sequenced for *actin*	No of individuals sequenced for *gapC1*
Pre1	N 50°23.02554′ E 12°35.81892′	5.3	56	3	3
PdB1	N 50°02.5599′ E 13°26.41584′	4.43	102	2	3
Pele3	N 48°57.45882′ E 14°57.82878′	5.44	45	3	3
Uhli7	N 48°38.74722′ E 14°39.37224′	5.66	37	4	4
IR50pl	N 54°05.028′ W 9°03.228′	5.4	79	2	3
IR50bubl	N 54°05.028′ W 9°03.228′	5.4	79	3	5
IRM1	N 54°43.97088′ W 8°31.01916′	5.0	68	4	3

products were precipitated with 3-M sodium acetate and ethanol, resuspended in 15 μl of redistilled water, and sequenced by Macrogen Inc. (Seoul, Korea) on an automatic 3730XL DNA sequencer using Big Dye Terminator cycling conditions.

Cloning

Intragenomic variability was assessed by cloning. PCR products were excised from 1.8% TBE agarose gel and purified with the Qiaquick Gel Extraction kit (Qiagen, Crawley, UK). The purified products were cloned using pGEM-T and pGEM-T Easy Vector Systems (Promega Corporation, Madison, WI). After cultivation, positive clones were diluted in 10 μl of redistilled water and denatured at 95 °C for 10 min. One microliter of the mixture was subsequently used for PCR amplification, with PCR conditions as above for each marker. PCR products were precipitated with 3 M sodium acetate and ethanol and sequenced at Macrogen Inc.

Sequence analyses

Electropherograms were checked and assembled using Seqassem v09/2004 (Hepperle 2004). Sequences of chimeric clones were manually detected and discarded from the analyses. Unique sequences were deposited in the European Nucleotide Archive with accession numbers as indicated in Table 1. Alleles used for population differentiation assessment were deposited under accession numbers HG805231–HG805266 for the *actin* region and HG805309–HG805352 for the *gapC1* region. Nucleotide sequence alignments were carried out with MEGA v.4 (Tamura et al. 2007) using the ClustalW algorithm (Thompson et al. 1994). Megablast searches (Zhang et al. 2000) against the nonredundant nucleotide database at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/) were used to check for the presence of related sequences.

Genetic diversity assessment

Levels of nucleotide variability were estimated as average per site pairwise nucleotide diversity (π, Nei 1987), haplotype (gene) diversity (H_d, Nei 1987), and the relationship between segregating sites and alleles sampled (θ_W, Watterson 1975), using the DnaSP v5.10.011 program (Librado and Rozas 2009). Haplotype networks were obtained using statistical parsimony analysis in TCS v1.21 (Clement et al. 2000), with fixed connection limit adjusted at 15 steps.

Table 3. Newly designed primers and their estimated melting and PCR annealing temperature

Marker	Primer name	Primer sequence	Estimated melting temperature (°C)	PCR annealing temperature (°C)
oee1	oxyF2	GGGTTCTGGCATCGCTAA	56	55
	oxyR2	CACTGTAAACAAGAAGGGAA	55	
gapC1	PWSETF1	CCTTGGTCTGAAACTGGAGT	60	55
	GIVErev	AATCCCAAATTTGTCATTAATA	54	
gapC2	gapC_F3	GATGACAACTGTGCATGCAGTG	66	55
	gapC_R4	CAGTTCTGGGAGCACCTTC	60	
actin	rot_ex_F1	ATGCTATCCTTCGTCTGGA	56	50
	ac_1516R	TAATCTTCATACTGTTGGG	52	

Population structure

Sequences from *gapC1* and *actin* were analyzed for three to five individuals from seven populations (Table 2). Population structuring was tested using Snn statistics (i.e., nearest-neighbor statistics) (Hudson 2000) implemented in DnaSP v 5.10.01 (Librado and Rozas 2009). This statistic indicates the frequency with which nearest-neighbor sequences are found in the same group. The associated *p*-values were obtained by computing 10,000 coalescent simulations based on observed data and assuming free recombination.

Isolation by distance (Mantel test) was inferred using the IBDWS Web Service v 3.23 (Jensen et al. 2005) with 10,000 randomization events, and genetic distances were calculated with traditional F_{ST} values.

Several neutrality tests were conducted to check whether the observed pattern of nucleotide variation is compatible with that expected under neutrality: Tajima's *D* (Tajima 1989), Fu and Li's *D** and *F** statistic, and Fu's F_S statistic. Evolutionary history of *actin* was inferred using the neighbor-joining method in MEGA 4.0 (Tamura et al. 2007) with bootstrapping based on 5,000 replicates. Evolutionary distances were computed using the Kimura two-parameter method (Tamura et al. 2004). Rate variation between sites was modeled with a gamma distribution (shape parameter = 1).

RESULTS

Exploration of molecular markers

The genetic diversity in the four nuclear regions (*oee1*, *actin*, *gapC1*, *gapC2*) was investigated among 25 *M. rotata* strains (Table 1). The *oee1* marker was successfully amplified in 15 strains. The sequenced region was 281 bp and did not contain any intron sequences (Table 4). All mutations except one were synonymous. Intragenomic variability was found at six sites. The *actin* gene was successfully amplified in 21 strains. The sequenced region was 366 bp and did not contain any intron sequences. All mutations were synonymous. Thirteen strains displayed intragenomic variability at 15 positions and subsequent cloning revealed the presence of two alleles in each cloned strain. The *gapC* gene was amplified in two nonoverlapping regions, designated here as *gapC1* and *gapC2*. The *gapC1* region was amplified in 23 strains. The sequenced region was 504 bp, and contained three introns of 39 bp, 152 bp, and 109 bp (Table 4). The majority of mutations were located in the intron region, and all exon-region substitutions were synonymous. Intragenomic variability was observed at 31 sites in 19 strains, and cloning of these strains revealed the presence of two alleles in each strain. To test the presence of additional alleles undetected by direct sequencing, two strains were cloned that had no detected intragenomic variability. Each strain contained only a single allele, demonstrating the absence of cryptic alleles in strains that showed no intragenomic variability. The *gapC2* region was amplified in 13 strains. The sequenced region was 404 bp, and contained two introns of 234 bp and 71 bp (Table 4). Intragenomic variability was found at 24 sites within the intron regions in six strains.

Genetic diversity analysis revealed nine different alleles in *actin*, 19 alleles in *gapC1*, and six alleles in *gapC2* (Table 4). Of these three markers, *gapC1* showed substantially higher values of H_d (haplotype diversity), π (nucleotide diversity), and θ_W (relation between segregating sites and number of alleles) than the other markers (Table 5). The *oee1* region was not analyzed further due to its low variability.

Only two different alleles were detected in each strain displaying intragenomic variability. Surprisingly, even that the *gapC1* and *gapC2* regions are part of a single gene, in three cases (CAUP K604, SVCK 78, C13) the intragenomic variability was detected in only one of these two regions examined.

Statistical parsimony analysis of the *actin* sequences revealed one abundant allele that was present in almost all investigated strains and five unique alleles (Fig. 1A). The haplotype networks of *gapC* regions were much more extended, containing a number of missing haplotypes (Fig. 1B, C). In the *gapC1* region, a cluster of two abundant alleles and distant clusters of less abundant alleles were discerned (Fig. 1B). A single abundant allele was also present in the *gapC2* region; however, haplotype network pattern cannot be generalized because only a low number of sequences were included (Fig. 1C).

Population structure

To investigate the extent of population divergence and structuring in *M. rotata*, variability in *actin* and *gapC1* sequences was assessed in seven selected populations for which three to five strains were sequenced. In total, we obtained 36 sequences of the *actin* coding region

Table 4. Properties of nuclear DNA regions analyzed in *Micrasterias rotata*

Molecular marker	No of individuals	No of sequences	No of sites	No of mutations	No of segregating sites	No of haplotypes	Coding sites	Noncoding sites	Coding	
									Nonsynonymous	Synonymous
oee1	15	15	281	6	0	–	281	0	1	5
actin	21	34	366	16	16	9	363	0	0	16
gapC1	23	42	504	31	31	19	204	300	0	8
gapC2	13	19	404	27	24	6	99	305	0	0

Table 5. Genetic diversity estimates of *actin*, *gapC1*, and *gapC2* sequences

Molecular marker	Haplotype diversity, H_d ± standard deviation	Nucleotide diversity, π ± standard deviation	Watterson estimator, θ_W (per sequence) ± variance (free recombination)
actin	0.699 ± 0.078	0.01169 ± 0.00148	3.913 ± 0.957
gapC1	0.928 ± 0.021	0.01792 ± 0.00157	7.204 ± 1.674
gapC2	0.725 ± 0.083	0.02546 ± 0.00226	6.867 ± 1.965

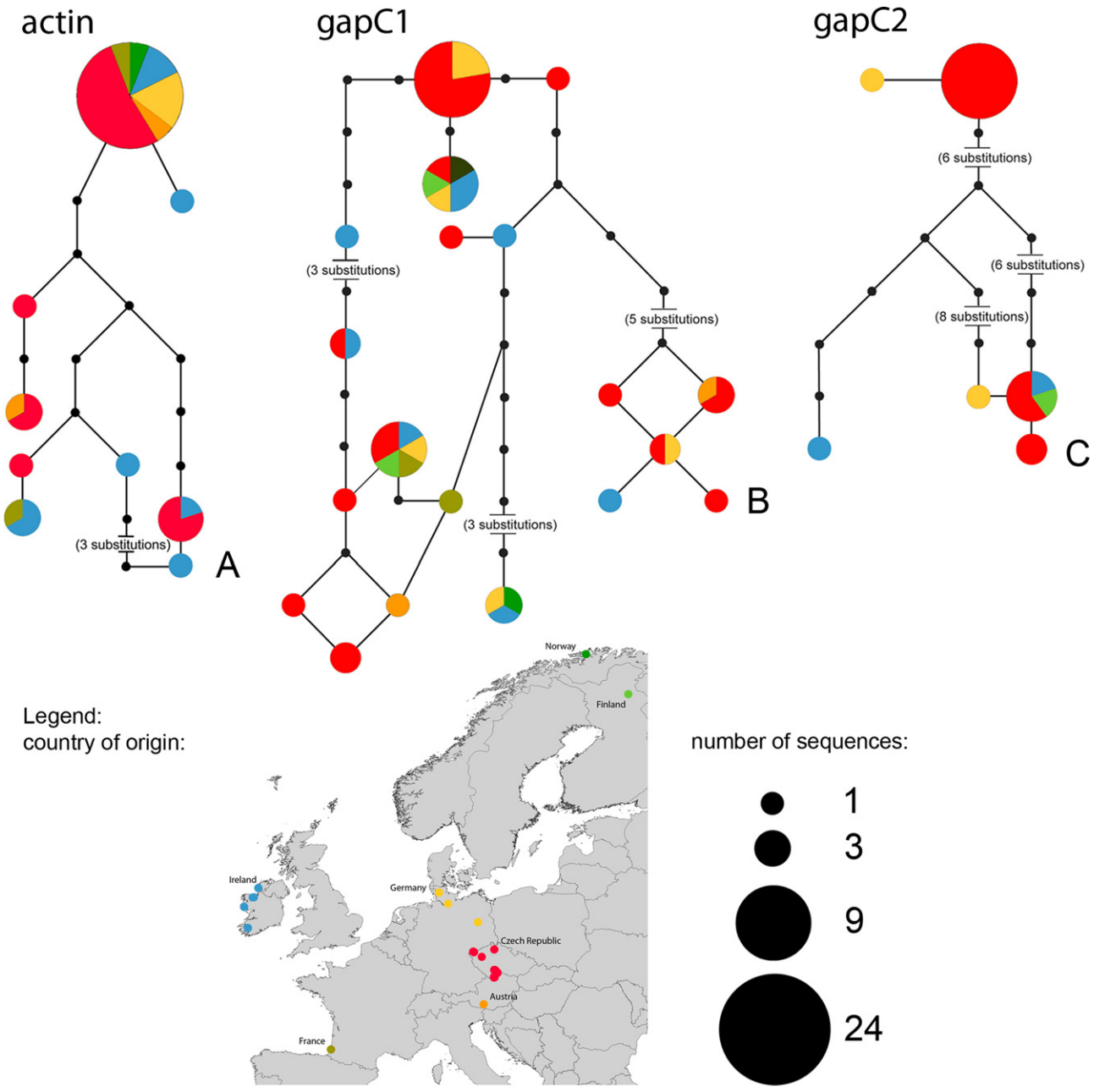

Figure 1 Statistical parsimony networks of analyzed sequences. The size of the circle depicts the number of sequences with the same allele. The color denotes country of origin. Dots correspond to one mutation change between alleles. **A**. *Actin* network, **B**. *GapC1* network, **C**. *GapC2* network.

(362 bp) and 44 sequences of the *gapC1* region (299 bp of intron, 204 bp of exon). Of these samples, 71.43% and 83.34% were heterozygous in the *actin* and *gapC1* regions, respectively. Markers had 11–31 single nucleotide polymorphisms (SNPs). Only synonymous SNPs were observed within the coding regions for both markers. The number of segregating sites and other estimates of DNA polymorphism were substantially higher for the *gapC1* region than for the *actin* gene (Table 6). Variability was actually higher than our estimations because a TT deletion, detected in six sequences, was excluded from the analysis.

At the haplotypic level, the sequences of *gapC1* revealed twice more alleles than the *actin* region. There was a considerable variation within populations both at the nucleotide and at the haplotypic level (Table 6). While the Czech populations consistently revealed only two or three alleles per population, Irish populations displayed higher intrapopulational variability, with almost every strain having a unique allele.

Population structuring was analyzed among seven different populations using Hudson's Snn, which was significant only for the *actin* region and supposed a panmictic occurrence of *M. rotata* (Snn around 0.5). However, when only two geographic regions were considered (Czech Republic and Ireland), nearest neighbors of actin sequences were found more frequently from strains at the same locality. The same trend was observed for the *gapC1* region, but the Snn value was lower than for the *actin* region (Table 7). Analysis of isolation by distance did not show a significant correlation between the genetic and geographic distance among sequences.

Table 6. Nucleotide polymorphism in population structure analysis, number of sequences (*N*), number of segregating sites (*S*), nucleotide diversity (π), number of haplotypes (*h*), haplotype diversity (H_d)

Population	Marker	*N*	*S*	π	*h*	H_d
Pre1	*actin*	5	2	0.00331	2	0.6
	gapC1	5	19	0.02076	5	1.0
PdB1	*actin*	4	6	0.01105	2	0.66667
	gapC1	6	17	0.01730	5	0.93333
Pele3	*actin*	5	5	0.00829	2	0.6
	gapC1	6	11	0.01304	3	0.73333
Uhli7	*actin*	7	6	0.00921	3	0.66667
	gapC1	8	25	0.02402	5	0.85714
IRM1	*actin*	6	8	0.01326	2	0.6
	gapC1	5	19	0.01796	5	1.0
IR50bubl	*actin*	6	9	0.01326	5	0.93333
	gapC1	9	20	0.01619	8	0.97222
IR50pl	*actin*	3	8	0.01473	3	1.0
	gapC1	5	19	0.02076	5	1.0
Czech populations	*actin*	21	10	0.00955	6	0.77619
	gapC1	25	29	0.01838	11	0.87000
Ireland populations	*actin*	15	11	0.01321	8	0.87619
	gapC1	19	22	0.01641	15	0.97076
Total	*actin*	36	11	0.01166	12	0.851
	gapC1	44	31	0.01754	23	0.93

Table 7. Genetic differentiation estimate of Hudson's Snn statistics for all seven populations and for two geographically separated localities

	Marker	Snn (Hudson 2000)
Among seven populations	*actin*	0.43984***
	gapC1	0.25648
Between the Czech and Ireland populations	*actin*	0.71160**
	gapC1	0.64235*

Asterisks denote a significancy based on 10,000 coalescent simulations: *$0.01 < p < 0.05$; **$0.001 < p < 0.01$; ***$p < 0.001$.

Neutrality tests (Tajima's *D*, Fu & Li's *D** and *F**) of the *gapC1* marker did not significantly deviate from zero. However, two neutrality test statistics of the *actin* gene (Fu & Li's *D** and *F**) were significantly different from zero, indicating non-neutral evolution (Table 8). The possibility that positive Fu & Li *F** values resulted from sampling across paralogs (Breen et al. 2009) was rejected by a neighbor-joining phylogenetic analysis that did not reveal two separated clusters of sequences (tree not shown). Moreover, the heterozygotes did not consist of sequences inferred in two separate clades, as would be the case with paralogs.

DISCUSSION

Comparison of nuclear DNA markers

Although desmids are considered to be haploid organisms and our tested genes were assumed to represent single-copy genes, most of the strains displayed intragenomic variability. Gene or genome duplication may explain this variability. Newly duplicated genes usually either accumulate mutations, becoming pseudogenes, or acquire new functions and become paralogs (Taylor and Raes 2004). Since the copies were similar in length, and only synonymous mutations were detected, we can exclude the presence of pseudogenes in our data. The existence of distinct paralogs is also unlikely. Paralogs can usually be recognized as distinct clades (Breen et al. 2009), which was not observed with our data. Moreover, cloning of strains lacking intragenomic variability did not reveal any hidden gene copies; these would be expected in all investigated strains in the presence of paralogs. Our observations may be explained by recent whole-genome duplications. Duplication of genomes, or polyploidy, is common in land plants (Adams and Wendel 2005), and may have occured in desmids, as well (Poulíčková et al. 2014). Studies investigating genome sizes in desmids are scarce. The single study focusing on chromosome numbers in *Micrasterias* species (Kasprik 1973) revealed an unexpected number of chromosomes in *M. rotata* (226–235) in comparison to other species (39–135). Moreover, a recently published study of DNA content variation clearly point to a much greater extent of chromosome variability in *M. rotata* (Poulíčková et al. 2014), showing that some strains could contain much less chromosomes than

Table 8. Estimates of neutrality tests for population studies

	Tajima's *D*		Fu & Li's *D**		Fu & Li's *F**		Fu's *Fs*	
	actin	*gapC1*	*Actin*	*gapC1*	*actin*	*gapC1*	*Actin*	*gapC1*
Total	1.83124	0.79213	1.44052*	0.81987	1.83727*	0.96208	−1.067	−4.122

*Significant results at $\alpha = 0.05$.

specified by Kasprik (1973). All these results point to possible polyploidization events during the evolution of *Micrasterias*. In addition, Kallio (1953) described the natural production of diploid forms in several desmids, especially *Micrasterias* species. Higher polyploids in natural populations are unlikely because they are considered to be nonviable (Kallio 1953); thus we can expect the occurrence of heterozygotes in natural populations. Therefore, the detection of strictly two copies in our isolates may concur with the presence of diploid heterozygotes in *Micrasterias* populations, and hence corroborate with our observations of intragenomic variability in *M. rotata*.

Our comparison of nuclear DNA regions showed the highest variability in *gapC* markers. Comparable numbers of segregating sites in *gapC1* and *gapC2* were not surprising as they represent two parts of the same gene. From a practical point of view, g*apC1* was more suitable for population genetic analysis as it showed higher PCR amplification success than the *gapC2* region. The *gapC* marker has previously been used for population studies of land plants (Banu et al. 2010; Breen et al. 2009; Shih et al. 2007; Szövényi et al. 2007) and fungi (Fernández-Mendoza et al. 2011). In comparing our results with these studies, the highest haplotype diversity for *gapC1* was found in *Micrasterias*. The *gapC* haplotype diversity (H_d) within angiosperms and bryophytes (*Populus* and *Sphagnum*) was comparable to H_d of *Micrasterias gapC2* marker and inferior to *gapC1*. However, the nucleotide diversity of *gapC* in *Micrasterias* was an order of magnitude higher than that in land plants (Banu et al. 2010; Breen et al. 2009; Szövényi et al. 2009). Even higher numbers of g*apC1* alleles in *M. rotata* are to be expected with increased sampling.

In our analysis, the *actin* marker displayed a lower number of segregating sites than *gapC*, but when we compared the proportion of segregating sites per unit of marker length, the difference was less pronounced. The *actin* marker has been frequently used in phylogenetic analyses of various algae (Bhattacharya et al. 1993, 1998; Hoef-Emden et al. 2005; Kroken and Taylor 2000; Sadowska-Des et al. 2013; Skaloud and Peksa 2010), and it was proved to be an eligible marker for ecological and population studies (Fernández-Mendoza et al. 2011; Nelsen and Gargas 2006; Peksa and Škaloud 2011). Nevertheless, in our case the longer sequenced region of *gapC1* marker favors its use for population studies. Moreover, *gapC1* contains introns, which are, unlike coding regions, considered to undergo neutral evolution, and therefore more suitable for population genetic analysis (Lowe et al. 2004).

Although we chose the *gapC1* marker as the most suitable for population studies, preliminary results revealed an unexpected variability among sequences, which may allow distinction between individuals. Such a highly variable marker is suitable for studies of natural mating systems, but a marker with a more moderate number of alleles are usually preferred for population structure analyses (Hartl and Clark 2007). Therefore, we decided to finally select two markers for subsequent population structure analyses, *gapC1* and the less variable *actin* marker.

Population structure analyses

Our results revealed that populations of *M. rotata* were geographically structured within Europe. Hudson's Snn statistic revealed significant occurrence of closely related alleles within geographical groups for the *gapC1* and *actin* markers. However, the *actin* marker provided better statistical estimates of genetic differentiation. The Hudson's Snn statistic is a very specific test using symmetric island model and assuming an infinite sites model of mutation, and it is considered to be the most powerful statistic to test differentiation between two or more geographic groups (Hudson 2000). We therefore trust that our results indicate significant genetic differentiation between the Irish and Czech populations.

We did not observe a pattern of isolation by distance. This could represent an artifact caused by a very high genetic diversity among the populations, hindering the pattern correlated with the geographic distance data. Many unique alleles in combination with low number of individuals per population obstruct the analysis of population structure with Mantel tests (IBD analysis). A comprehensive sampling on a broader scale will be needed to further analyze patterns of geographic population structure and isolation by distance.

Our investigations showed relatively high haplotypic diversity within populations, similar to recently published studies on aquatic protists (Lebret et al. 2012; Rengefors et al. 2012). However, high intrapopulational variability is usually connected with low genetic differentiation between populations and is often indicative of effective gene flow among populations. Our data does not support the general assumption of panmixia in microorganisms (Fenchel and Finlay 2003), but showed significant geographic structuring of populations. This is in concord with the studies on freshwater diatoms (Evans et al. 2009) and dinoflagellates (Rengefors et al. 2012), where the land has been shown to act as an effective barrier to the gene flow.

In addition to high intrapopulational diversity and genetic differentiation between the two geographically

separated *Micrasterias* populations, we detected a significant discrepancy in the degree of intrapopulational variability among the Irish and Czech populations. Higher levels of genetic drift and fixation of alleles are usually connected with bottlenecks and isolation of small populations. In the past 2 Myr, Ice Ages repeatedly decreased population sizes and connectivity among small refugial populations, which led to an accumulation of genetic variability during glacial periods (Hewitt 2004). Hence, the refugia can be characterized by higher genetic diversity and presence of rare alleles (Hoarau et al. 2007). In our analysis, both investigated markers consistently revealed a far higher number of alleles in the Irish populations compared to the Czech ones, which may indicate the existence of past refugia in Ireland. Moreover, the presence of refugia has already been suggested in southwestern Ireland for land plants (Sinclair et al. 1998; Szövényi et al. 2007) and macroalgae (Hoarau et al. 2007; Provan et al. 2005). Like most desmids, *M. rotata* is associated with peat bog habitats composed mainly of *Sphagnum* moss, which had one of the recently discovered refugia at the last glacial maximum at the southern coast of Ireland (Szövényi et al. 2007). In addition, Irish localities have higher species diversity of desmids (John and Williamson 2009) compared to the Czech Republic (Šťastný 2010). The natural state of some west Ireland localities (John and Williamson 2009) may sustain higher diversity at the population and species levels. The higher species and within-species diversity of desmids in Ireland may also be related to the higher density of lakes and bogs in the region. As has been suggested in diatoms, density of suitable habitats and associated degree of connectivity between these habitats is a good predictor of diversity (Vyverman et al. 2010). Another explanation for the lower haplotypic variability in the Czech populations may be found in different climate conditions between the Czech Republic and Ireland. The continental climate of the Czech Republic, characterized by longer and harsher winter season, and a warmer summer, is associated with higher freezing and desiccation stress, which may act as selection constraints, resulting in a lower number of alleles (Booy et al. 2000).

In summary, our observations provide evidence for the existence of geographical differentiation among the European populations of the desmid species *M. rotata*. Moreover, we found a disproportional distribution of alleles between the Czech and Irish populations, which can be explained by different evolutionary histories and/or adaptation to specific ecological conditions.

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