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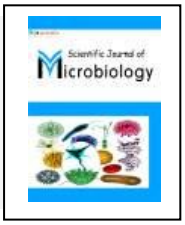

Original article

Evaluation of possibilities of genotyping of *Candida glabrata* clinical isolates with RAPD-PCR method and microsatellite analysis

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ABSTRACT

The aim of the study was to compare the discriminatory power of RAPD-PCR method using RSD10 primer and microsatellite $(GACA)_4$ analysis for genotyping clinical isolates of *C. glabrata*. Isolates were received from patients of two Polish hospitals: Children's Memorial Health Institute in Warsaw (n=17) and Medical University of Gdansk (n=37). Species identification was confirmed by two phenotypic methods: growth on chromagar plates (Biocorp) and Candida API ID 32C test (bioMérieux), as well as by PCR reaction. For both tested methods for genotyping, the size of amplified DNA fragments varied mainly between 200 and about 3000 bp. The sets of 9 and 8 different products of amplification were obtained in the case of microsatellite and RAPD-PCR method, respectively. The amplicon arrays were analyzed and dendrograms were constructed using a matrix generated by UPGMA (Unweighted Pair Group Method with Arithmetic Means) in MVSP program (Multi Variate Statistical Package) version 3.22. As a result 20 different genotypes were distinguished in the case of microsatellite analysis and the collection of strains was divided into 26 genotypes in the case of RAPD-PCR analysis with RSD10 primer. Additionally, an evident correlation between genotype classification and geographical origin of the

isolates was observed in the case of RADP-PCR method. The performed investigation revealed that RAPD-PCR amplification with RSD10 primer represents high discriminatory power in molecular differentiation of *C. glabrata* clinical isolates and can be recommended for at least preliminary analysis of possible route of transmission of this pathogen in hospitals and community.

1. Introduction

During the last two decades, the occurrence of fungal infections caused by *Candida* spp., has increased dramatically (Arendrup, 2010; Pfaller and Diekema, 2010). In most cases, *Candida* infections are derived from the individuals own endogenous reservoir when the host represents certain risk factors, such as immunosuppressive and cytotoxic therapies, treatment with broad spectrum antibiotics, AIDS, diabetes and drug abuse. *C. albicans* is still the most common fungal pathogen in humans, although recently a shift towards systemic infections by non-albicans (NAC) species has been reported. In a tertiary pediatric hospital in Poland the prevalence of non-albicans species increased from 12.5% in 2000 to 70% in 2010 (Dzierzanowska-Fangrat, 2013). Among NAC the yeast of the genus *C. glabrata* are the most common, currently they are responsible for approximately 26% of *Candida* bloodstream infections in the United States (Horn, 2009). Mortality rates up to 50% are commonly associated with *C. glabrata* infections and are higher than those associated with *C. albicans*. Moreover resistance to antifungal agents, used as a first-line antifungal therapy, especially fluconazole, is very common among *C. glabrata* clinical isolates (Fidel et al., 1999). The growing problem is also the transfer of virulent and drug-resistant strains within hospitals and in the community. Therefore, it is important to develop genotyping methods which could be used for analysis of spreading of these pathogens as well as correlation between specific genotype and drug susceptibility. Techniques such as macrorestriction analysis of genomic DNA followed by pulsed field gel electrophoresis (REA-PFGE) (Cormican et al., 1996), Amplified Fragment Length Polymorphism (AFLP) (Brost et al., 2003), PCR melting profile (MP-PCR) (Krawczyk et al., 2009) or techniques based on sequencing e.g. multilocus sequence typing (MLST) (Robles et al., 2004) guarantee high discriminatory power but nonetheless are expensive, time-consuming and require specialized equipment and well-trained staff. As a consequence, they are not achievable for most clinical laboratories. The aim of this study was to evaluate the possibilities of application of RAPD-PCR method using RSD10 primer (Samaranayake et al., 2003) and microsatellite $(GACA)_4$ (Meyer et al., 1997) analysis for genotyping clinical isolates of *C. glabrata*. Both analyzed methods can be performed in all laboratories that have access to basic equipment for PCR and agarose gel electrophoresis. Thus both of them could be applied for epidemiological studies of infections caused by *C. glabrata* especially in case of local epidemic in small geographical regions, hospitals or even particular wards.

2. Materials and methods

1.1. Fungal strains

Fifty-four (54) clinical isolates of yeasts preliminary classified as *C. glabrata* were received from various physiological sites: urine, feces, blood, fluid from the peritoneal cavity, stoma, bronchopulmonary lavage, swabs of the mouth, throat and anus from patients of two Polish hospitals: the Children's Memorial Health Institute in Warsaw (17 isolates, assigned on the figures with letter “w”) and the Medical University of Gadnsk (37 isolates, assigned on the figures with letter “g”).

1.2. Species identification

Species identification was confirmed by two phenotypic methods: growth on chromagar plates (Biocorp), *Candida* API ID 32C test (bioMérieux), as well as by PCR reaction. On chromagar plates *C. glabrata* isolates revealed a violet colour. Using the *Candida* API ID 32C test, a characteristic pattern of sugar assimilation was obtained, with only two positive fermentation results for glucose and trehalose (which is specific for this species). The molecular

identification was carried out according to the method developed by Brillowska and co-workers (2013), with slight modifications. DNA was extracted with a Genomic Mini AX Yeast Kit (A&A Biotechnology, Poland) according to the enclosed protocol. Individual PCR amplifications contained 1 μL of prepared DNA template and 19 μL of the following PCR master mixture: 10 μL of 2 x PCR Mix Plus High GC (A&A Biotechnology, Poland), (Taq DNA polymerase 0.1 U/μL, $MgCl_2$ 4 mM, dNTPs (dATP, dGTP, dCTP, dTTP), 0.5 mM of each, plus factors increasing the specificity of PCR reaction, red dye, loading buffer), 1 μL of each of primers solutions (10 μM) and 7 μL of distilled, nuclease-free water. The DNA sequences of the primers used were as follows: forward 5'-GAGTGGTATGACGAGCAATGGT-3' and reverse 5'-TGTATTGAAGATTCCCTCTCATATATC-3'. Amplification conditions were as follows: 4 min of initial denaturation at 94°C, 60 s of denaturation at 94°C, 30 s of annealing at 57°C, and 60 s of primer extension at 72°C repeated for 32 cycles and 10 min of final extension at 72°C. The PCR products (272 bp) were detected on 1.5% agarose gel with addition of ethidium bromide.

1.3. Genotyping

Two methods were used for molecular differentiation of analyzed population of *C. glabrata* clinical isolates. The RAPD-PCR reaction (Random Amplification of Polymorphic DNA PCR) uses short primers, which combine randomly with several fragments in the genomic DNA of the strain tested. The other method was based on a study of differences in location of short tandem repeats sequence $(GACA)_4$ within the genomes of strains tested.

The DNA sequences of the primers used for genotyping were as follows: $(GACA)_4$ 5'- GACAGACAGACAGACA-3' (Meyer et al., 1997) and RSD10 5'-CCGCAGCCA-3' (Shireen et al., 2011). PCR reaction mixtures contained: 10 μL of 2x PCR MixPlus High GC (A&A Biotechnology) 1 μl of primer $(GACA)_4$ or RSD10, 1.0 μL of DNA template solution and nuclease free water up to 20 μL.

In the case of all amplification reactions carried out for genotyping of the investigated group of isolates the conditions were as follows: 4 min of initial denaturation at 94^0C followed by 32 cycles of 94^0C for 1 min, 42^0C for 30 s and 720C for 90 s and final extension at 72^0C for 10 min. PCR products were separated on 2% agarose for 60 min at 110 V and compared with Gene Ruler 100 bp or 1 kb ladder (Thermo Scientific).

The obtained after electrophoresis amplicon arrays were analyzed and dendrograms were constructed using a matrix generated by UPGMA (Unweighted Pair Group Method with Arithmetic Means) in MVSP program (MultiVariate Statistical Package) version 3.22.

3. Results and discussion

The results of three mentioned methods: yeast growth on chromagar plate, *Candida* API ID 32C test and PCR, confirmed that received population represented only *C. glabrata* species, and both used for genotypic analysis revealed large molecular differentiation within this group of isolates. Both RSD10 and $(GACA)_4$ primers are at least partially complementary to multiple sites in the genomes of investigated isolates. As a result of PCR amplification with these primers several DNA fragments of different size are generated (Fig.1, A and B). Detailed visual analysis of received after electrophoresis profiles revealed that 8 different bands were amplified for microsatellite $(GACA)_4$ analysis and 9 fragments were generated with using RSD10 primer and RAPD-PCR method. In case of both applied for genotyping techniques the size of generated PCR products varied mainly between 200 and 3000 bp. The most frequently seen products of PCR amplification were the following: 350 and 900 bp for $(GACA)_4$ primer and 450, 1100 bp for RSD10 primer (Fig.1, A and B). Subsequent visual selection of genotypes would be difficult, therefore, the program MVSP 3.22 was used to assign the isolates to the genotype and to create dendrograms demonstrating the genetic relationship among the strains. Data were entered into the program on the basis of zero-one system (one- the band is present, zero- the band is absent). The generated with UPGMA program dendrograms revealed that RAPD-PCR method with using RSD10 primer allowed to divide the analyzed group of strains into 26 subgroups – genotypes, and 20 genotypes were distinguished using microsatellite $(GACA)_4$ analysis (Fig. 2, A and B).

Especially interesting results were obtained in the case of RAPD-PCR method. Only three out of 26 identified genotypes contained more than four strains. Two of them, counting respectively 5 and 6 strains, were composed only of isolates coming from the hospital in Gdansk. An evident correlation between genotype classification and geographical origin of the isolates was also observed in the case of most genotypes counting 2 – 3 strains, however the most numerous of the identified genotypes consisted of four strains from Children's Memorial Health Institute in Warsaw and three from Gdansk. The usefulness of RAPD-PCR using RSD10 primer for genotyping of human

isolates of *C. parapsilosis* was early confirmed by Dassanayake and Samaranayake (2000). The method was also successfully applied for molecular characterization of *C. albicans* by Samaranayake and coworkers (2003) as well as Issa and coworkers (2011), although from our best knowledge it was not tested for evaluating of genetic similarity of *C. galabrata* clinical isolates. Unfortunately, the known and important drawback of RAPD-PCR technique is its poor interlaboratory reproducibility thus it can be used in investigation into microorganisms' epidemiology only at a local level (Saghrouni et al., 2013).

Slightly less discriminatory power as well as correlation between geographical origin and genotype classification was achieved in the case of microsatellite analysis. In this case the three most numerous genotypes (counting six, six and thirteen isolated respectively) contained the strains from both institutions. There were also less unique banding patterns for individual isolates (n=6) in comparison to RAPD-PCR method (n=14) which also confirms the lower discriminatory power of microsatellite analysis. Relatedness of clinical isolates of *C. glabrata* have been previously analyzed with different microsatellite markers, including $(GACA)_4$. Generally, this method has been found as rapid, easy method of genotyping of yeast pathogens with good, but not optimal discriminatory power, which confirms our observation (Döğen et al., 2013).

4. Conclusion

Spreading of *C. glabrata* isolates with high virulence potential and resistance to most important antifungal agents is fast growing problem. Thus development of easy in handle techniques of molecular differentiation of these pathogens is highly desirable. The performed investigation revealed that RAPD-PCR amplification with RSD10 primer represents high discriminatory power in molecular differentiation of *C. glabrata* clinical isolates and can be recommended for analysis of possible route of transmission of this pathogen at a local level, mainly in hospitals, wards and in community in small geographical regions.

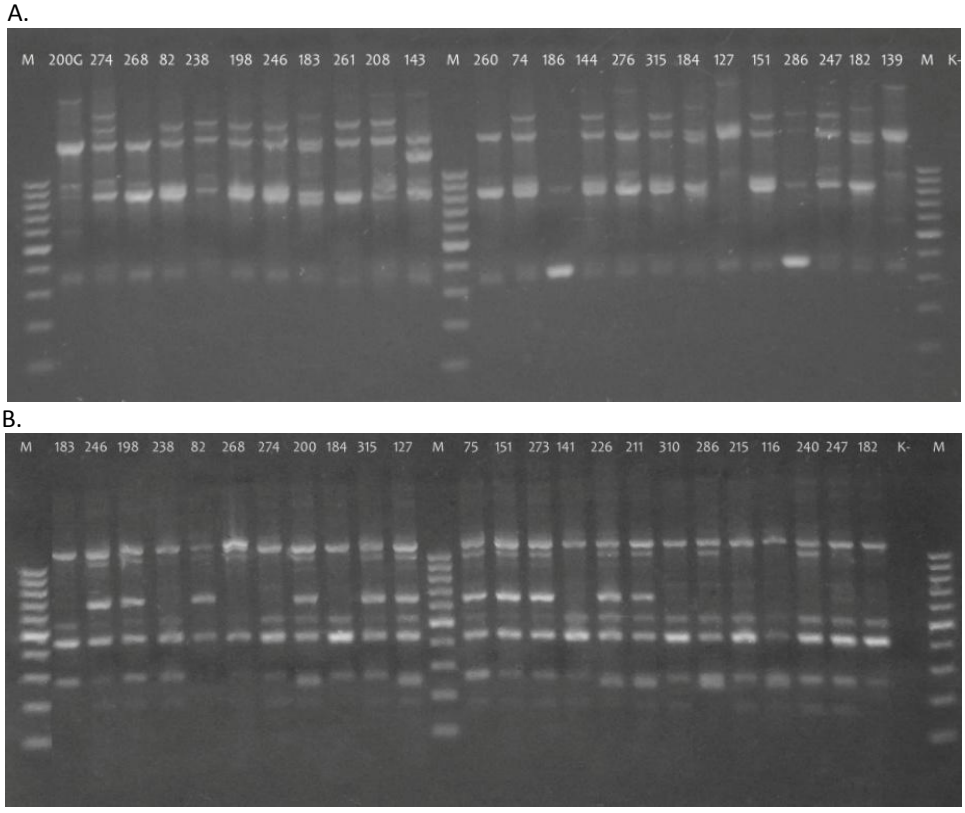
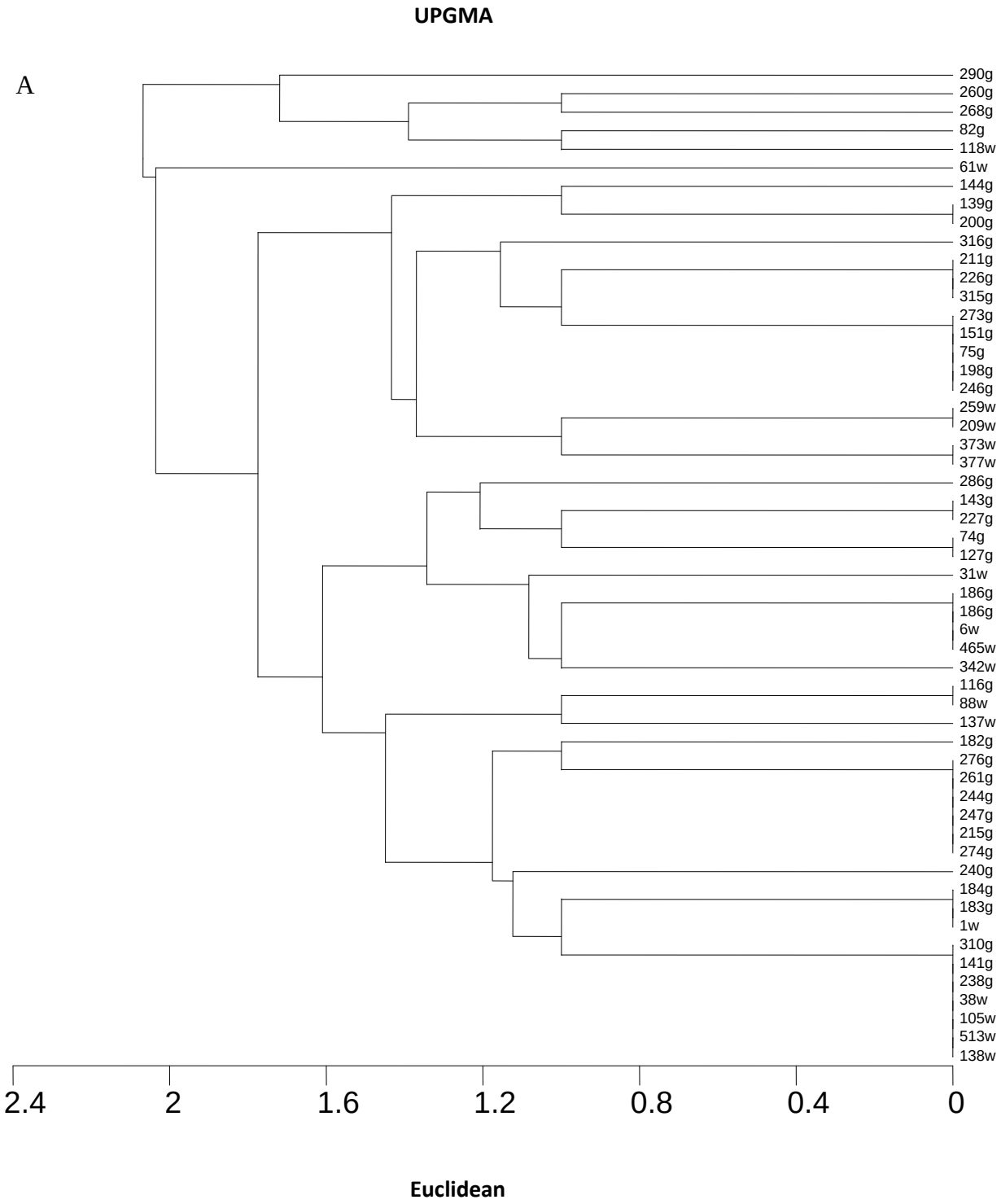


Fig. 1. The result of electrophoretic separation of *C. glabrata* DNA amplification products using primer $(GACA)_4$ (A), RSD10 (B) on 2% agarose gels, the time of separation 60 min, voltage 110 V. The lane M is molecular marker (100 bp).


UPGMA
A
290g
260g
268g
82g
118w
61w
144g
139g
200g
316g
211g
226g
315g
273g
151g
75g
198g
246g
259w
209w
373w
377w
286g
143g
227g
74g
127g
31w
186g
186g
6w
465w
342w
116g
88w
137w
182g
276g
261g
244g
247g
215g
274g
240g
184g
183g
1w
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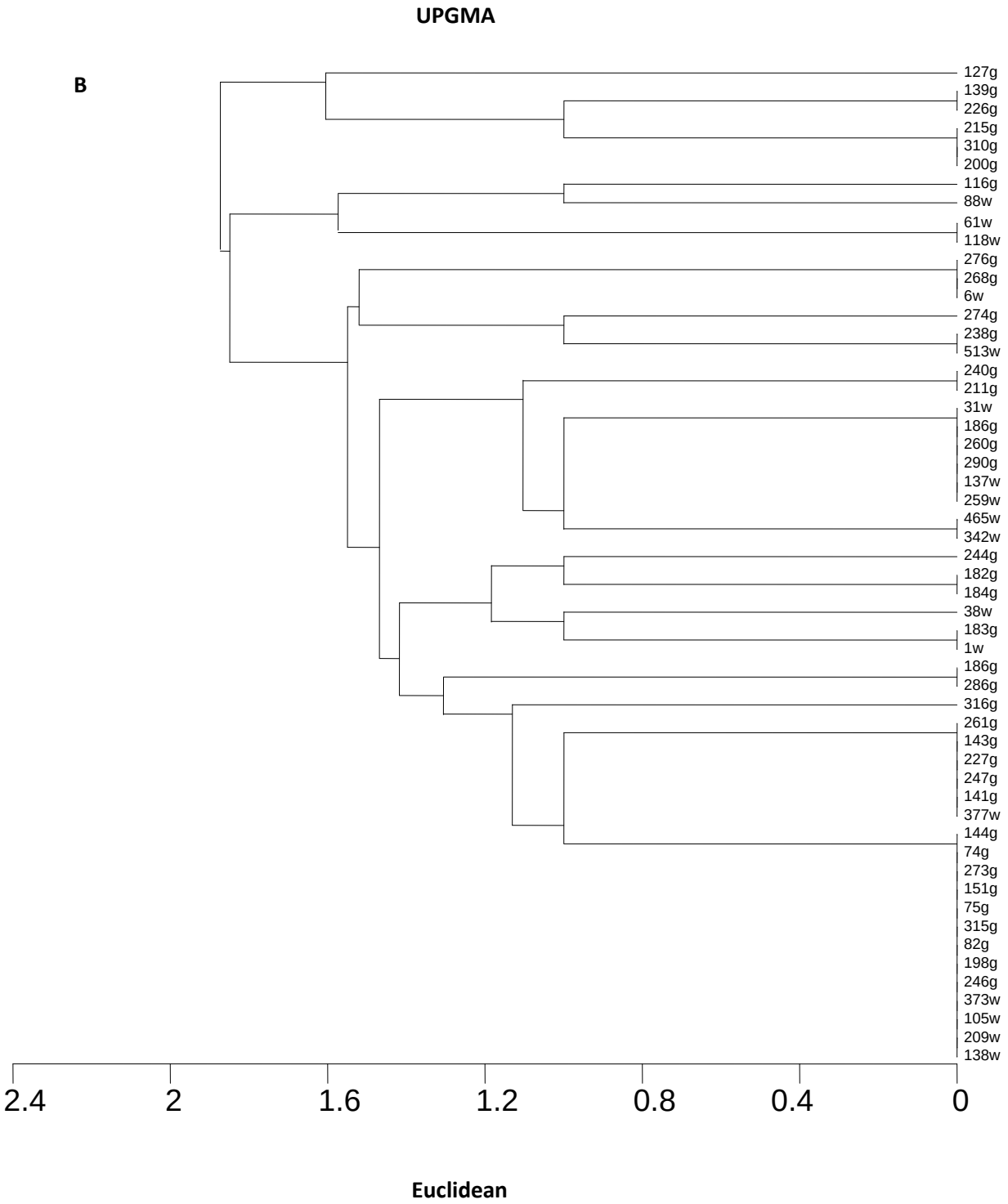

Fig. 2. Dendrograms demonstrating the genetic relationship among *C. glabrata* strains obtained with RSD10 and $(GACA)_4$ primers. Figure created with MVSP 3.22 program.

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