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The First Microsatellite-based Characterisation of *Blastocystis* sp. ST3 Isolates and Population Structure Analysis

Blastocystis sp. ST3 İzolatlarının Mikrosatelit Temelli İlk Karakterizasyonu ve Popülasyon Yapısının Analizi

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ABSTRACT

Objective: *Blastocystis* sp. is an intestinal Stramenopile that can infect both humans and animals. Genetic variability of *Blastocystis* has been investigated with a variety of molecular methods and different subtypes (ST) have been identified. The present study aimed to characterize microsatellite loci and population structure for *Blastocystis* sp. ST3, the most common ST in human faecal samples.

Methods: The genome of *Blastocystis* sp. ST3 in GenBank was analyzed for the presence of microsatellites containing at least eight repeat units. Specific primers were designed for each locus and polymorphisms were identified using bioinformatics tools. The population structure was determined, and microsatellite typing was conducted on 18 *Blastocystis* sp. ST3 genomic DNA samples from the routine laboratory at Aydın Adnan Menderes University Hospital.

Results: The whole-genome scan of *Blastocystis* sp. ST3 revealed 12 microsatellite loci with at least eight repeats. All loci were successfully amplified with the designed primers and eight of them were selected for genotyping. Microsatellite polymorphism analysis showed that each isolate had a unique profile (18 isolates, 18 different microsatellite types). Furthermore, the isolates were grouped into two distinct population clusters.

Conclusion: *Blastocystis* sp. ST3 isolates exhibited significant variability in their microsatellite repeats. The polymorphic microsatellite loci offer a novel approach to study the detailed genetic diversity and population structure of *Blastocystis* sp. ST 3.

Keywords: *Blastocystis* sp., genetic diversity, microsatellite, polymorphism

ÖZ

Amaç: *Blastocystis* sp. hem insanları hem de hayvanları enfekte edebilen intestinal yerleşimli bir Stramenopil türü olarak sınıflandırılmaktadır. Genetik çeşitliliği farklı moleküler yöntemlerle araştırılmış ve çok sayıda alt tipi (ST) tanımlanmıştır. Bu çalışmada insan dışkısı örneklerinde en yaygın saptanan *Blastocystis* sp. ST olan ST3'e özgü mikrosatelit lokusların karakterizasyonu ve popülasyon yapısının belirlenmesi amaçlanmıştır.

Yöntemler: *Blastocystis* sp. ST3'ün tüm genom dizisi taranarak en az sekiz tekrarlı mikrosatelit lokusları belirlenmiştir. Her bir lokusa spesifik primerler dizayn edilmiş ve polimorfizmler biyoinformatik araçlar ile analiz edilmiştir. Aydın Adnan Menderes Üniversitesi Hastanesi rutin laboratuvarından elde edilen 18 *Blastocystis* sp. ST3 genomik DNA örneği kullanılarak popülasyon yapısı belirlenmiş ve mikrosatelit tiplendirme gerçekleştirilmiştir.

Bulgular: *Blastocystis* sp. ST3 tüm genom taraması sonucu en az sekiz tekrarlı 12 mikrosatelit lokusu tespit edilmiştir. Dizayn edilen primerler ile lokusların tümü başarıyla amplifiye edilmiş ve bunlardan sekizi genotiplendirmede kullanılmak üzere seçilmiştir. *Blastocystis* sp. ST3 izolatları mikrosatelit polimorfizmlerine göre tiplendirildiğinde her bir izolat farklı bir profil (18 izolat, 18 farklı mikrosatelit tipi) göstermiştir. Ayrıca izolatların iki ayrı popülasyon grubunda toplandığı saptanmıştır.

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Türkiye PARAZİTOLOJİ Dergisi

Sonuç: *Blastocystis* sp. ST3 izolatlarının mikrosatelit tekrarları açısından birbirlerinden oldukça farklı bir yapıya sahip oldukları belirlenmiştir. Polimorfik mikrosatelit lokusları, *Blastocystis* sp. ST3 izolatlarının detaylı genotiplendirilmesine ve popülasyon yapılarının araştırılmasına olanak sağlayan yeni bir yaklaşım olarak görülmektedir.
Anahtar Kelimeler: *Blastocystis* sp., genetik çeşitlilik, mikrosatelit, polimorfizm

INTRODUCTION

Blastocystis is one of the most common protozoa in the human gastrointestinal system. Most researchers have agreed that *Blastocystis* transmission occurs via the faecal-oral route, primarily through the ingestion of cyst forms in water or food (1). Zoonotic transmission between a variety of animals and humans may also be possible (2). Many subtypes (STs) have been identified to date mostly relying on 18S rRNA coding gene polymorphisms, ST1-4 accounts for the great majority of human samples in many studies (3-5). The genetic diversity of *Blastocystis* is among the most interesting topics in recent years because it has a great contribution to host specificity, pathogenicity, experimental studies and also microbiota (5,6). Amplification of specific parts of *rRNA* genes and sequencing is the most common method for detecting genotypes. Moreover, Matrix-assisted laser desorption/ionisation mass spectrometry (MALDI MS) has been used for genotype determination (7).

Microsatellites are 2-9 bp length genetic markers that can be found abundantly in the genomes of eukaryotic organisms. They allow the identification of the causative agents precisely in populations of different genotypes because they contain high amounts of genetic diversity (8). Microsatellite markers are generally regarded as neutral alleles, which makes them ideal markers for determining the history of populations. In addition, up to today, MS markers have been used in several parasitic protozoa including *Leishmania tropica*, *Trypanosoma* sp., *Plasmodium* sp., and *Toxoplasma gondii* (9-12).

The development and application of alternative genotyping methods may contribute to the understanding of controversial issues in *Blastocystis* sp. pathogenicity such as pathogenicity. In the present study, we aimed to characterize the microsatellite loci for *Blastocystis sp.* ST3 for the first time in the literature and analyse the genetic structure of *Blastocystis* sp. ST3 isolates.

METHODS

Determination of Microsatellite Markers and Primer Design

The complete genomic sequence of a human *Blastocystis* sp. ST3 isolate, an assemblage of 917 partial sequences, was acquired from the National Center for Biotechnology Information database (Genbank, Acc. no: JZRK00000000). Microsatellites mining was carried out using the software Msatcommander 0.8.1 and http://insilico.ehu.es/mini_tools/microsatellites/website with the motif criteria, di-, tri-, tetra-, penta-, and hexanucleotide repeats of microsatellites at least 8 within the complete genome of *Blastocystis* sp. ST3 (13). Primers for microsatellite loci (n=2) were designed with Primer3, the online version, with default settings (14,15). For potential repetitive elements other than perfect repeats, the flanking regions were also analysed. In order to identify robust loci with an annealing temperature of at least 55 ºC, approximately 150-200 bp flanking each side of the repeat were included.

Blastocystis sp. ST3 Isolates

The ethical approval from a Local Ethical Committee in Aydın Adnan Menderes University Faculty of Medicine (no: 2015/10) was obtained. A total of 18 *Blastocystis sp.* ST3 isolates were used in the study. The isolates were previously acquired by culturing direct microscopy-positive faecal samples in the routine diagnostic laboratory at Aydın Adnan Menderes University Hospital. The cultures in 3 mL of Jones medium were subjected to genomic DNA isolation with DNAzol kit (Invitrogen, Life Technologies). *Blastocystis* sp. *SSU-rDNA* gene was partially amplified with the primers RD5 and BhRDr in a single round of polymerase chain reaction (PCR) (16). The amplicons were sequenced, and ST were detected using the *Blastocystis* sp. sequence typing database (pubmlst.org/*Blastocystis*) (17).

PCR Amplification and Genotyping

The optimal annealing temperature for the PCR amplification for each microsatellite locus was determined with gradient PCR. The fluorophores FAM and HEX were used to label the 5′ ends of the forward primer. The amplifications were performed in 30 µL of volume: 0.5 mM each of the primers, 1.5 mM $MgCl_2$, 2.5 mM dNTP, 1.0 U Taq polymerase, and 1-2 µL of template DNA. The reaction for each locus was set as follows: 95 °C for 5 min, 35 cycles (at 95 °C for 30 s, 55-60 °C for 30 s and 72 °C for 45 s), and a final extension at 72 °C for 7 min. The length of alleles was detected with an automatic sequencer.

Statistical Analysis

The allele sizes of microsatellites were exactly determined with Genemarker 2.6.3 (Soft Genetics LLC, USA). The calculation of genetic variation in microsatellite loci was performed with GenAlEx 6.5 (18). GENEPOP 3.3 software was used to detect genotypic linkage disequilibrium between pairwise loci (19). GenAlEx 6.5 program was used to detect the allele numbers (N_A), effective allele numbers (N_E), the frequencies of alleles, intra-population diversity of alleles, and pairwise comparisons of the isolates (18). The expected heterozygosity (H_E) of loci was calculated using the Arlequin 3.11 (20). In multiple loci, haplotype overlaps were determined with GenAlEx 6.5 (18).

Population Structure and Microsatellite Typing

Population structure was analysed with a Bayesian clustering method in STRUCTURE ver. 2.3 program (21). The admixture model with correlated allele frequency parameters was used to to detect the estimated number of genetic clusters (K). Ten runs were performed for each K value (ranging from 1 to 10) with 100.000 MCMC repetitions and a burn-in periods of 10,000. The ad hoc estimated likelihood of K (ΔK) was included in the determination of the most likely number of populations (K) based on the rate of change in the log probability of the data [Ln Pr (X/K)] (22). Structure Harvester version 0.6.94 was used to infer the most likely number of genetic clusters (K) with both the Evanno and Delta K methods (23). The first isolate was defined as MT1 and

the remaining different isolates (at least one different allele type) were annotated with a new MT number.

RESULTS

Microsatellite Variability and Population Structure

We detected 12 microsatellite loci with at least 8 repeats in *Blastocystis* sp. ST3 genome and of these 11 loci were polymorphic. All loci have three repeats, and most were characterized with $(GAT)_n$ and $(ATC)_n$ (Table 1).

There was no significant linkage disequilibrium when the loci were compared dually (p>0.05). The total number of alleles (N_A) for each locus changed from three to 11, the average was 6.55 alleles per locus (Table 1). The mean effective number of alleles (N_E) was 4.06 (range from 1.841 to 8.526). BHST3-142 loci (h=0.457) provided the minimum intra-population diversity, while BHST3-838 locus (h=0.883) provided the highest. The average expected heterozygosity value was determined as 0.740±0.146 (min: 0.399 and max: 0.935). Two distinct genetic groups were suggested with the analyses of microsatellite data by a structure clustering algorithm (K=2.297) (Figure 1).

Microsatellite Typing

We selected eight loci: BH142, BH217, BH278, BH302, BH399, BH476, BH806 and BH808 for MT typing of *Blastocystis* sp. ST3 isolates. These loci were selected relying on the reliability of microsatellite length, clarity in representations, and amplification/reproducibility. Microsatellite based typing of isolates revealed that each isolate had a unique MT type (Table 2).

DISCUSSION

Blastocystis sp. is a common enteric stramenophile with a high genetic diversity between isolates and an unclear role in the development of intestinal diseases and pathogenicity. The molecular typing of *Blastocystis* sp. isolates is valuable to study its population structure of parasites on a global scale. Genetically diverse isolates may influence the frequency of *Blastocystis* sp., for example, host specificity and adaptation to novel hosts (24). In addition, several studies reported a decreased bacterial diversity and changed intestinal microbiome in certain *Blastocystis* sp. ST (6). The development of novel genotyping methods is important for an improved understanding of the genotype distribution of *Blastocystis* sp. isolates and phenotypic characteristics. In the

Table 1. Characterization of 12 microsatellite markers for the *Blastocystis* sp. ST3

Locus	Primer sequence (5′-3′)	Size	N_A	Motif	N_E	h	H_E	GenBank accession number
BHST3-142	F: TACAGCTGTTCCACCCACTC	216-234	4	$(GAT)^{13}$	1.841	0.457	0.399	JZRK01000142
	R: CTCCCCTCTCCCCTAGTCAC							
BHST3-217	F: GGAGGGAGGTTCTTGGTCTC	232-349	6	$(ATC)^{10}$	3.767	0.735	0.778	JZRK01000217
	R: AGAGGGATGTGGTGGAGTTG							
BHST3-278	F: CCTCCTTGCGCTTCTGAC	385-409	6	$(AGC)^{11}$	3.375	0.704	0.745	JZRK01000278
	R: AGGAGGCGGAAAGGAGAAAG							
BHST3-302	F: GAGATGCGACGATTCTCCTC	298-325	6	$(ATC)^{11}$	3.600	0.722	0.765	JZRK01000302
	R: GATTCGCGTTCAATGGTTCT							
BHST3-366	F: ATGGTCAATTGGCTGATGGTG	141-201	9	$(ATC)^{12}$	4.154	0.759	0.804	JZRK01000366
	R: AGTTTCTGAGCCACATGGAGA							
BHST3-399	F: ATTCTTCGCACGATTCTTCG	364-370	3	$(GAT)^{8}$	2.160	0.537	0.569	JZRK01000399
	R: TCATGGAGGTGGTCAACAAA							
BHST3-476	F: AGAAGAAGCTCTTCGCGTTG	321-357	7	$(AAG)^{8}$	4.050	0.753	0.811	JZRK01000476
	R: CAGATCCGCGTAGGTCATTT							
BHST3-500	F: GTGGTTGAGGAGGAGGATGA	-	-	$(GAT)^{8}$				JZRK01000500
	R: GAGAGGAGGCGTCGATGATA							
BHST3-654	F: ATTGGTGATCGTGTTGGTGA	297-471	11	$(ATC)^{11}$	6.480	0.846	0.895	JZRK01000654
	R: TGGTGACGAGTTCGATGAAG							
BHST3-806	F: GACGTGGGTGAGGAGGATTA	367-385	6	$(GAT)^{9}$	4.263	0.765	0.811	JZRK01000806
	R: GTTGGAGAGTTCGGGGGTAT							
BHST3-808	F: TGACAGGTTGCTCCTTACCC	304-325	4	$(ATC)^{9}$	2.492	0.599	0.634	JZRK01000808
	R: TTGGAGTCGTTGGACATTGA							
BHST3-838	F: TAAATGTCGGAGGGAAGGTG	158-295	10	$(GAT)8(GTT)^{8}$	8.526	0.883	0.935	JZRK01000838
	R: GGGAGATGAGTGCATTGACA							
Mean			6.55		4.064	0.705	0.747	

N_A: Observed number of alleles, I: Shannon's information index, N_E: effective number of alleles; H_E: Expected heterozygosity

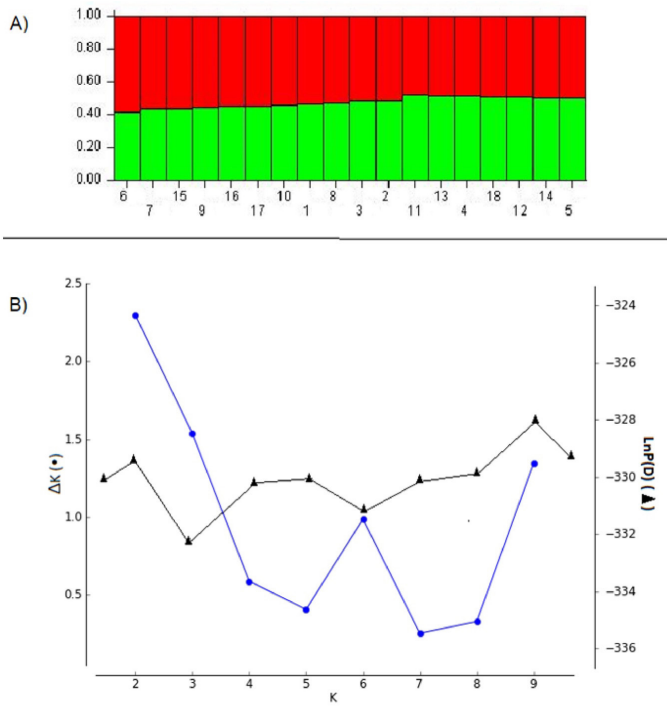

Figure 1. A) Bayesian clustering analysis (K=2) based on SSR data for the studied *Blastocystis* sp. ST3 (18 isolates), vertical black lines separate isolates, **B)** Scatter plot of the possible cluster numbers (K; horizontal axis) against the ad-hoc estimated likelihood of ΔK (vertical axis) by changing the likelihood rate (circle)

present study, we characterised the microsatellite markers for the most common *Blastocystis* sp. subtype in human faecal samples, namely ST3. The most significant finding from this study was that the microsatellite loci in *Blastocystis* sp. ST3 isolates were highly variable, with each of the 18 isolates exhibiting a distinct or unique microsatellite type. A great genetic difference between *Blastocystis* sp. ST was noted in the literature (25). The comparison of *Blastocystis* sp. ST1, ST4 and ST7 genomes found great divergence in terms of genome assembly size, number of protein-coding genes, guanine-cytosine content, average gene size (bp) and also number of introns (25,26). In addition to subtype level genetic difference in *Blastocystis* sp., the isolates were highly separated in terms of intra-subtype variation. A previous study investigated the intra-subtype genetic diversity and host specificity of *Blastocystis* sp. ST3 with multilocus sequence typing including SSU-rDNA and mitochondrion-like organelle sequences (27). The authors reported that human isolates were restricted to a single clade suggesting relatively high host specificity of ST3 human-to-human transmission. Another study reported that 11 human *Blastocystis* sp. ST3 isolates were distributed in nine sequence types with the same method. There were only three isolates that shared the same sequence type (28). However, the use of a single gene, most commonly SSU rRNA, detected a limited intra-subtype genetic diversity in *Blastocystis* sp. ST3 STs. For example, intra-subtype genetic polymorphisms were identified five in ST3 isolates in China and the similarity of SSU rRNA sequences was 99.8% in Iran (29,30).

In the present study, the population structure analysis of *Blastocystis* sp. ST3 isolates revealed two distinct groups. The genomic DNA samples in our study were limited to a specific location, the same hospital laboratory. Collecting samples

Table 2. The microsatellite types (MT) of *Blastocystis* sp. ST3 isolates

	Microsatellite loci								
Isolate	**142**	**217**	**278**	**302**	**399**	**476**	**806**	**808**	**MT**
1	3	5	2	3	2	5	6	3	MT1
2	3	2	4	5	1	1	4	3	MT2
3	3	1	6	1	1	5	4	2	MT3
4	3	2	5	2	1	5	6	2	MT4
5	5	6	4	2	1	1	6	3	MT5
6	3	3	3	6	2	1	5	4	MT6
7	4	6	3	1	2	6	3	3	MT7
8	3	3	3	3	2	4	1	2	MT8
9	3	4	5	3	2	3	2	2	MT9
10	1	2	3	3	1	5	6	1	MT10
11	3	2	5	3	1	2	2	2	MT11
12	3	5	3	3	2	3	2	2	MT12
13	3	2	3	3	2	1	6	2	MT13
14	3	2	5	4	2	5	4	3	MT14
15	1	2	3	2	3	2	5	3	MT15
16	2	4	1	3	2	5	6	2	MT16
17	3	5	3	2	2	2	3	3	MT17
18	3	2	5	5	2	5	6	3	MT18

from various localities, such as different cities or countries, will enable a detailed phylogenetic analysis of microsatellites in *Blastocystis* sp. ST3. The major contribution of the present study is the characterization of microsatellites in *Blastocystis* sp. ST3 and the presentation of the associated methodology. This characterization has important implications for future research and practices, particularly in areas such as zoonotic transmission, microbiota interactions, and pathogenesis. These are the widely discussed topics in the literature on *Blastocystis* (5). Microsatellite polymorphisms have previously been described as valuable genetic markers for investigating zoonotic transmission in both parasitic microorganisms. For instance, certain microsatellite alleles in *Cryptosporidium parvum* have been associated with human-adapted or zoonotic strains (31). Similarly, microsatellite markers have been used to compare *Plasmodium falciparum* isolates from patients with cerebral malaria and those with uncomplicated malaria, to determine if there is a possible link between genetic variation and pathogenicity (32).

CONCLUSION

In conclusion, the characterisation of microsatellite loci in *Blastocystis* sp. ST3 revealed different profiles or types showing high intra-subtype diversity in terms of microsatellite repeats. This novel genotyping approach may be used in molecular epidemiology and population structure screening studies.

*Ethics

Ethics Committee Approval: The ethical approval from a Local Ethical Committee in Aydın Adnan Menderes University Faculty of Medicine (no: 2015/10) was obtained.

Informed Consent: All participants were informed about the study, and their consents were obtained.

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Footnotes

*Authorship Contributions

Concept: S.E., H.E., B.B., Ö.G., Design: S.E., H.E., B.B., Ö.G., Data Collection or Processing: E.M., M.S., Analysis or Interpretation: S.E., E.M., M.S., Ö.G., Literature Search: S.E., E.M., H.E., Writing: S.E., E.M., H.E., Ö.G.

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