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AZF DELETIONS IN INFERTILE MEN FROM THE REPUBLIC OF MACEDONIA

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Abstract: Y chromosome deletions in the three azoospermia factor (AZF) regions constitute the most common genetic cause of spermatogenic failure. The aim of this study was to estimate the length and boundaries of the AZF deletions and to correlate the AZF deletions with the sperm concentrations, testicular histology, Y haplogroups and the ethnic origin of the men with deletions. PCR analysis of STS loci in the three AZF regions was used to characterize the deletions. Y haplogroup was predicted from a set of 17 Y short tandem repeats (STR) marker values. A total of nine men out of 218 infertile/subfertile men showed the presence of Y microdeletions. In eight patients the results were consistent with the presence of AZFc deletions, while in one patient a larger deletion involving both AZFb and AZFc regions was detected. In two patients, the deletion, initially diagnosed as AZFc, involved part of the distal part of the AZFb region and in one of them the deletion also extended into the region distal to the AZFc. The 3.5Mb AZFc deletion, due to homologous recombination between b2 and b4 amplicons, was detected in six men (66.7% of all Y deletions), thus being the most common type of AZF deletion among infertile men from the Republic of Macedonia. Patients with the 3.5 Mb AZFc deletion had azoospermia or severe oligozoospermia and variable histological results [Sertoly cell only syndrome (SCOS), maturity arrest (MA) and hypospermatogenesis (HSG)]. They were of different ethnic origin (Macedonian, Albanian and Romany) and belonged to different Y haplogroups (I1b, J2, E3b and G).

Key words: male infertility, azoospermia, oligozoospermia, AZF deletions, AZFc, AZFb+c, Y haplogroup.

Introduction

At least one in ten couples of reproductive age face difficulty conceiving a child despite an extensive period of unprotected sexual intercourse [1]. Male factor infertility is responsible for about 50% of these cases [2]. Inadequate or absent sperm production is a primary factor in a substantial percentage of these cases [1]. Y chromosome deletions constitute the most common genetic cause of such spermatogenic failure [3–6]. In 1976, Tiepolo and Zuffardi provided the first evidence that the long arm of the the Y chromosome is required for fertility in men, when they karyotyped 1170 men and found that six azoospermic men were missing most of the long arm of Y chromosome [3]. Subsequently, this cluster on Yq11 became known as the azoospermia factor or AZF. The use of polymerase chain reaction (PCR) of sequence tagged sites (STS) has made possible the detection of small, interstitial deletions invisible to karyotyping [7]. Later, the AZF region was subdivided into 25 deletion intervals (D1-D25) and the existence of three non-overlapping subregions, designated AZFa, AZFb and AZFc (Figure 1A), was proposed [5]. The frequency of AZF deletions in infertile men ranges in worldwide surveys from 5 to 20% [8,9]. Y microdeletions are found almost exclusively in patients with azoospermia or severe oligozoospermia [10]. The prevalence of Y microdeletions among the infertile males from the Republic of Macedonia was 6.4%, among patients with azoospermia 16.7% and among those with severe oligozoospermia 2.8% [11].

Men with AZFa, AZFb or large deletions involving two to three AZF regions are always associated with azoospermia and testis histology of Sertoly cell only syndrome (SCOS) or meiotic arrest (MA) [6, 12, 13]. Deletions involving the AZFc region account for up to 90% of all Yq deletions with phenotypes varying from azoospermia to severe oligozoospermia [4, 6, 14] and occasionally to milder oligozoospermia [15]. Although natural transmission of Y microdeletions has been reported, majority of the cases arise as a *de novo* event [16]. Recently, partial AZFc deletions, which remove a number of genes and transcription units, have also been described [17]. However whether and to what extend partial AZFc deletions affect spermatogenesis is still controversial [18]. Partial and polymorphic AZF deletions have been also reported in the AZFa [19] and AZFb regions [20].

Recent molecular analysis and sequencing of Yq have revealed eight large palindromic regions containing an array of different ampliconic sequences [21, 22]. They make up almost all of the AZFc sequence and 50% of the AZFb

sequence (Figure 1B). The AZFc region consists of 11 families of transcription units, most of which are exclusively expressed in testises. Distant homologous recombination between specific palindromic sequences is believed to be the mechanism for various Yq deletions (Figure 1C) [17, 19, 23]. The most common AZFc deletion is 3.5Mb in size and is caused by recombination involving b2 and b4 sequences [21].

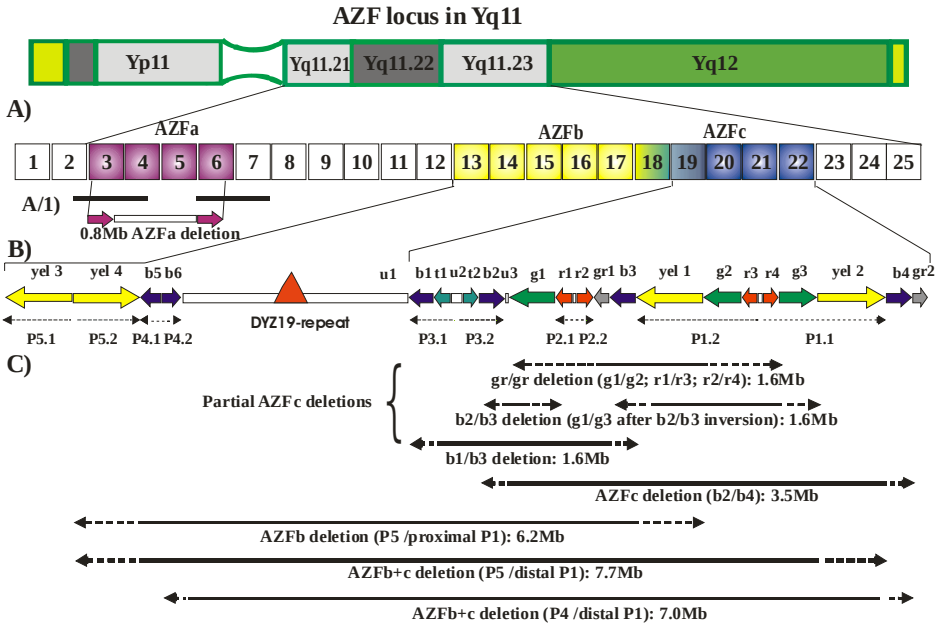


Figure 1. Schematic view of the AZF locus in Yq11. **A)** Deletion map of AZF locus: 25 intervals (D1-D25) and three AZF regions (AZFa, AZFb and AZFc). **A/1)** Complete AZFa deletion, caused by recombination of two homologous HERV15Yq1/q2 bloks. **B)** Structural organisation of the different amplicons in the AZFb and AZFc regions belonging to five palindromic structures (P1-P5). **C)** Partial and complete AZFc, AZFb and AZFb+c deletions caused by recombination between different amplicons.

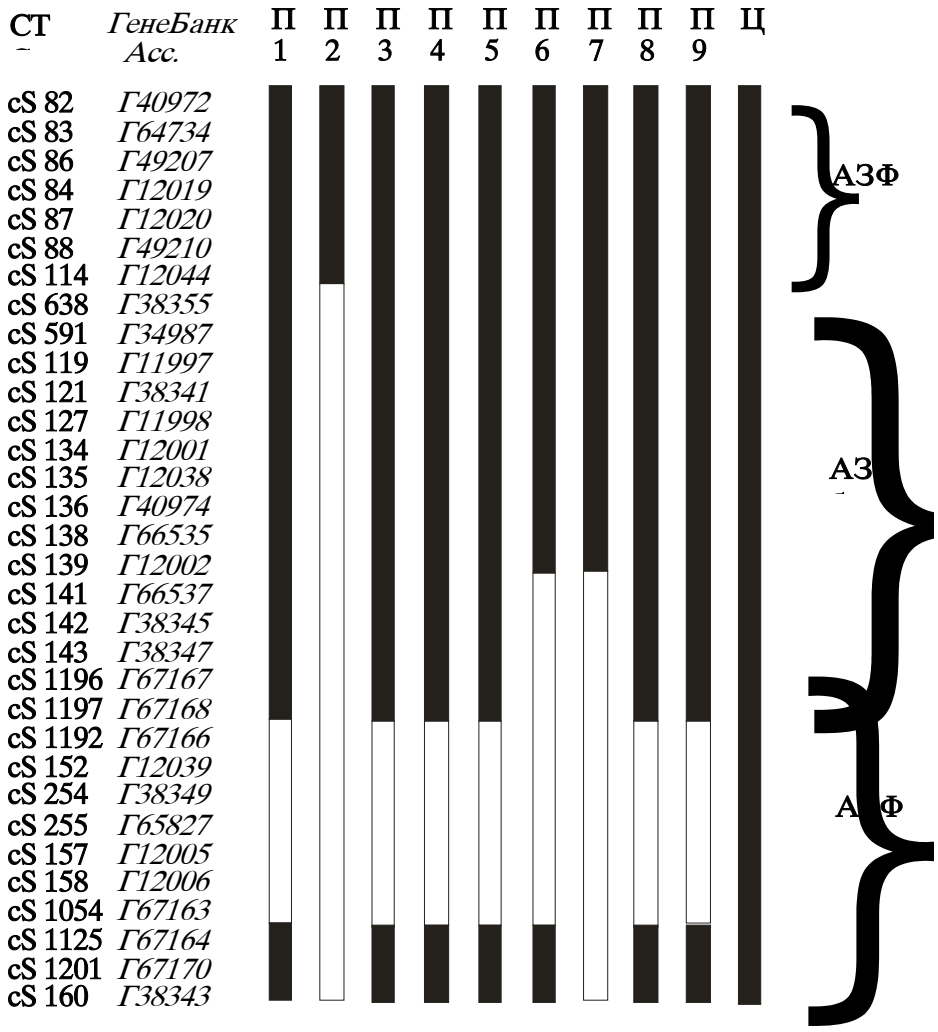
Слика 1. Шематски приказ на AZF локусот во Yq11. **A)** Делециона мапа на AZF локусот: 25 интервали (D1-D25) и три AZF региони (AZFa, AZFb и AZFc). **A/1)** Комплетна AZFa делеција, настаната како резултат на рекомбинација меѓу две хомологни HERV15Yq1/q секвенци. **B)** Структурна организација на различните ампликони во AZFb и AZFc регионите кои припаѓаат на пет палиндроми (P1-P5). **C)** Парцијални и комплетни AZFc, AZFb и AZFb+c делеции настанати со рекомбинација меѓу различни ампликони.

The aim of this study was to estimate the length and boundaries of the AZF deletions and to correlate the AZF deletions with the sperm concentrations, testicular histology, Y haplogroups and the ethnic origin of the men with deletions.

Material and methods

Two hundred and eighteen infertile/subfertile males, attending the Andrology Outpatient Clinic, at the Endocrinology and Metabolic Disorders Clinic, Faculty of Medicine in Skopje, were enrolled in the study. Semen analyses were performed according to the published guidelines [24]. Semen analysis showed azoospermia in 99 men, severe oligozoospermia ($< 5 \times 10^6$/ml) in 52, mild oligozoospermia ($> 5 \times 10^6$/ml) in 27, and normozoospermia in 40 men. Testicular biopsy was performed by open surgery; the testicular tissue sample was fixed in Bouin's solution, and the histological examination of spermatogenesis was performed on hematoxylin and eosin-stained sections.

DNA was isolated by standard Proteinase K/phenol-chloroform extraction/ ethanol precipitation method [25]. All patients were initially screened for the presence of deletions in the three AZF regions, following the guidelines for molecular diagnosis of Y microdeletions [26, 27]. The screening was performed by two multiplex PCR reactions analyzing six STS loci in the three AZF regions (sY84 and sY 86 in AZFa; sY 127 and sY 134 in AZFb; and sY254 and sY 255 in AZFc), as described previously [11]. Patients that showed presence of Y microdeletion were further analyzed by PCR amplification of additional 26 STS loci in the three AZF regions (sY82, sY83, sY87, sY88, sY114, sY 638, sY 591, sY 119, sY 121, sY135, sY 136, sY 138, sY 139, sY 141, sY142, sY143, sY1196, sY1197, sY1192, sY152, sY157, sY158, sY1054, sY1125, sY1201, sY160). The sequences of the oligonucleotide primers used for PCR analysis of the 26 STS were drawn from the GeneBank (accession numbers are given in Figure 2). The PCR was carried out in a total volume of 50 μl. The reaction mixture included 50–250 ng of each DNA sample, 1 × PCR buffer, 1.5 μmol/l $MgCl_2$, 200μmol/l deoxynucleotidetriphosphate (dNTP), 50 pmol of each primer pair and 1U Taq DNA polymerase. After an initial denaturation step at 95^0C for 5 min during which the Taq polymerase was added, cycle parameters were: 35 cycles of denaturation at 95^0C for 1 min, annealing at 56–62^0C for 1 min, and extension at 72^0C for 1 min and 30 sec. The programs were followed by the final extension step at 72^0C for 7 min. The PCR reaction products were then analyzed by electrophoresis on 1.5–2.5% agarose gels containing ethidium bromide (0.1 mg/ml) and visualized under UV light. The deletion of the loci was confirmed when a PCR product of expected size was not obtained after three PCR experiments. As a control, one sample of female, one sample of fertile male genomic DNA and one sample which contained all reaction components, but in which water instead of DNA was added (blank sample), were run with each set of primers.



Фигуре Schematic presentation of AZF deletions found in nine men. Shaded boxes indicate presence of STS; white boxes indicate absence of P1-P9, patients with AZF deletion; C, control - fertile

Слика 2. Шематски приказ на делециите откриени кај девет инфертилни мажи. Темните блокови покажуваат присуство на СТС белите блокови покажуваат отсуство на . , пациенти со АЗФделеци; , контрола - фертилен маж.

The Y haplogroup was predicted from a set of Y STR marker values using an Excel-based program, where an arbitrary number of STR markers is

input and a "fit score" for 10 haplogroups (E3a, E3b, G, I1a, I1b, I1c, J2, N3, R1a and R1b) is returned [28]. Seventeen Y STR markers (DYS456, DYS 389I, DYS390, DYS 389II, DYS458, DYS19, DYS 385a/b, DYS 393, DYS 391, DYS 439, DYS 635 (Y GATA C4), DYS 392, Y GATA H4, DYS 437, DYS 438, DYS 448), were analyzed by one multiplex PCR reaction, using the AmpFlSTR Y-filer™ PCR Amplification Kit (Applied Biosystems, Foster City, CA, USA) and individual Y STR alleles were typed using ABI-PRISM 310 Genetic Analyzer and GeneScan software (Applied Biosystems).

Results

A total of nine infertile/subfertile men with Y microdeletions were detected during the screening. In eight patients the results were consistent with the presence of AZFc deletions, namely the two STS in AZFc (sY254 and sY255) were lacking. One patient showed a larger deletion involving both AZFb and AZFc regions (sY127, sY134, sY254 and sY255 deleted).

Patients that showed presence of Y microdeletion were further analyzed by PCR amplification of additional 26 STS loci in the three AZF regions. Schematic presentation of the AZF deletions in the nine infertile men is shown in Figure 2. In order to estimate the length and boundaries of the AZFc deletions we performed PCR amplification of several STS markers (sY1197, sY1196, sY1192, sY1054, sY1125, sY1201) for detection of the most common AZFc deletion [21]. PCR analysis of these STSs showed absence of PCR products from the sY1192 and sY1054, and presence of PCR products from the sY1196, sY1197, sY1125 and sY1201 in six of the eight patients with AZFc deletions. Thus, six patients (66.7% of all the Y microdeletions) had the most common AZFc deletion of 3.5 Mb, which is a result of a homologous recombination between amplicons b2 and b4. All other tested STS loci were present in the six patients with the 3.5Mb AZFc deletion. In patient P6, diagnosed as having an AZFc deletion during the screening for Y microdeletions, the distal end of the deletion was identical to that of the 3.5Mb AZFc deletion (sY1054 absent; sY1125, sY1201 and sY160 present). In patient P7, also diagnosed as carrying AZFc deletion, the deletion extended distal from the AZFc region (sY1054, sY1125, sY1054 and sY160 absent). The proximal end of the Y deletion in patients P6 and P7 was identical and was determined within a region bounded by sY139, which was present and sY141 which was absent. Thus, the Y deletions in these two patients were not limited to the AZFc region, but they involved a part of the distal part of the AZFb region (STSs sY142, sY143 and sY141 lie in the distal part of the AZFb region). However, the length of these two deletions was smaller than the AZFb+c deletion determined in patient P2. The proximal end of the AZFb+c deletion was determined between sY114 and

sY638. The distal end of the AZFb+c deletion extended 3' from the distal end of the AZFc region (sY1054, sY1125, sY1054 and sY160 absent). Thus, the AZFb+c deletion in our patient differed from the AZFb+c deletions, extending from palindrome P5 to distal-P1 and from palindrome P4 to distal-P1 in which STSs sY1054, sY1125, sY1201 and sY160 were not deleted [23].

Sperm counts, testicular histology, Y haplogroup and the ethnic origin of the patients with Y microdeletions are given in Table 1. Eight patients were azoospermic and only one patient had sperm concentrations of $< 0.1 \times 10^6$/ml, consistent with the diagnosis of severe oligozoospermia. Testicular histology was available from six of the nine patients and showed SCOS in three azoospermic patients (two of them with the 3.5Mb AZFc deletion), maturity arrest in two azoospermic patients (one of them with the 3.5Mb AZFc deletion) and hypospermatogenesis (HSG) in one patient with severe oligozoospermia and 3.5Mb AZFc deletion.

Four different Y haplogroups were detected in men with Y microdeletions. The most common haplogroup was I1b (four patients), which is also the most common haplogroup among men from R. Macedonia (unpublished results). Three patients belonged to haplogroup J2 which is the most common haplogroup among Romanies and the third most common haplogroup among Macedonians (unpublished results). One patient belonged to haplogroup E3b, which is the second most common haplogroup among men from R.Macedonia (unpublished results), and one man belonged to haplogroup G. One patient was of Albanian, one of Romany and seven of Macedonian ethnic origin.

Table 1 – Табела 1

Sperm count, testicular histology, ethnic origin, AZF region deleted, size of the deletion and Y haplogroup in patients with Y microdeletions

Број на сперматозоиди, хистопатолошки наод на тестиси, етничко потекло, големина на делецијата и Y хаплогрупа кај пациентите со Y микроделеција

Patient	Sperm count ($x10^6$/ml)	Testicular histology	Ethnic origin	AZF region deleted	Approx. deletion size	Haplogroup
P 1	0	n.d.	Albanian	AZFc	3.5Mb	I1b
P 2	0	n.d.	Macedonian	AZFb+c	> 7.7Mb	I1b
P 3	< 0.1	HSG	Romany	AZFc	3.5Mb	J2
P 4	0	SCOS	Macedonian	AZFc	3.5Mb	I1b
P 5	0	SCOS	Macedonian	AZFc	3.5Mb	J2
P 6	0	MA	Macedonian	AZFc	> 3.5Mb	I1b
P 7	0	SCOS	Macedonian	AZFc	> 3.5Mb	J2
P 8	0	MA	Macedonian	AZFc	3.5Mb	E3b
P 9	0	n.d.	Macedonian	AZFc	3.5Mb	G

Discussion

The screening for the presence of Y microdeletions among infertile/subfertile men from R.Macedonia, using six STSs (two in each AZF region) identified nine patients with Y deletion, of whom eight with AZFc and only one with AZFb+c. Analysis of additional STSs showed that six patients had an identical deletion with proximal end within a 349 kb region bounded by STSs sY1192 and sY1197, and distal end within a 229 kb region bounded by STSs sY1054 and sY1125. Assuming the homologous recombination between amplicons b2 and b4, the size of this deletion was 3.5Mb [21]. This deletion was first described in 47 of the 48 studied individuals who lacked STS sY254, but possessed sY142 (proximal to AZFc) and sY160 (distal to AZFc) [21]. Two of the patients with a result of the initial screening consistent with AZFc deletion in fact had deletions that extended into the distal part of the AZFb region. The proximal end of the deletion in the two patients was identical and located between STS sY139 and sY141. The distal end of the deletion was same as the distal end of the 3.5Mb AZFc deletion in one and extended into the region distal to the AZFc in the other patient. Both patients were Macedonian and with a semen analysis showing azoospermia. They belonged to different Y haplogroups (I1a and J2) and showed different testicular histology results (SCOS and MA).

The 3.5Mb AZFc deletion was found only in patients with azoospermia and oligozoospermia. Patients with this deletion demonstrate variable testicular histology results: SCOS, MA and HSG. Others have also shown that patients with identical 3.5 Mb AZFc deletion have a variable, but always low, level of spermatozoal generation, as well as variable histological results [29]. Individuals with the 3.5Mb AZFc deletion were of different ethnic origin, four were Macedonian, one was Albanian and one was Romany. They also belonged to different haplogroups; I1b (two men), J2 (two men), E3B (one man) and G (one man). Thus, the 3.5Mb AZFc deletion occurs on different Y chromosomes and in men of different ethnic origin.

One of our patients had a deletion of both AZFb and AZFc regions. However this deletion differed from the common 7.7Mb AZFb+c deletion which extends from P5 to the distal arm of P1 and spare distal AZFc region. The AZFb+c deletion in our patient extended distal from the AZFc region, probably involving the heterochromatic region of the Y chromosome. The mechanism for the occurrence of the AZFb+c deletion, as well as for the two AZFc deletions, that are different from the 3.5Mb AZFc deletion and extend into the distal part of the AZFb region, cannot be predicted. Although homologous recombination between repetitive sequences is the most common mechanism for the occurrence of Y deletions, it was proposed that in addition to homology, factors such

as propensity for breaks could account for the P5, P1.1 and P1.2 deletion hotspots [23]. We did not detect any patient with a deletion of the AZFa or AZFb region or with a deletion involving all three AZF regions.

Conclusion

3.5Mb AZFc deletion due to homologous recombination between b2 and b4 amplicons is the most common type of Y microdeletion among infertile men from the Republic of Macedonia. Patients with this deletion have azoospermia or severe oligozoospermia, and variable histological results (SCOS, MA and HSG). They are of different ethnic origin (Macedonian, Albanian and Romany) and belong to different Y haplogroups (I1b, J2, E3b and G).

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Резиме

AZF ДЕЛЕЦИИ КАЈ ИНФЕРТИЛНИ МАЖИ ОД РЕПУБЛИКА МАКЕДОНИЈА

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Делеции во трите азооспермија фактор (AZF) региони на Y хромозомот претставуваат најчеста генетска причина за дефекти во сперматогенезата. Целта на ова истражување беше да се одредат должината и границите на AZF делециите и истите да се корелираат со концентрациите на сперматозоиди, хистопатолошкиот наод од тестисите, Y хаплогрупите и етничката припадност на мажите со делеции. AZF делециите беа карактеризирани со PCR анализа на повеќе „бележени места во секвенцата“ (sequence tagged sites – STS) локуси во трите AZF региони. Y хаплогрупата беше предвидена преку вредностите на алелите од 17 испитувани „кратки тандем повторувања“ (short tandem repeats – STR) маркери. Присуство на Y микроделеции беше најдено кај 9 од скринираните 218 инфертилни/субфертилни мажи. Кај осум пациенти резултатите од иницијалното скринирање укажуваа на присуство на AZFc делеција, додека кај еден пациент беше утврдено присуство на поголема делеција која ги опфаќа и AZFb и AZFc регионите. Кај двајца пациенти, беше утврдено дека AZFc делецијата всушност зафаќа и дел од проксималниот крај на AZFb регионот, а кај еден делецијата се протега и во регионот дистално од AZFc. AZFc делецијата од 3,5Mb, која е резултат на хомологна рекомбинација меѓу b2 и b4 ампликоните беше најдена кај шест мажи (66,7% од сите Y делеции). Така, таа е најчест тип на AZF делеција меѓу инфертилните мажи од Р. Македонија. Пациентите со 3.5Mb AZFc делеција се манифестираат со азооспермија и тешка олигозооспермија и со различни хистопатолошки наоди на тестисите [Sertoly cell only syndrome (SCOS), матурационен арест (MA) и хипосперматогенеза (HSG)]. Тие се со различно етничко потекло и припаѓаат на различни Y хаплогрупи.

Клучни зборови: машки инфертилитет, азооспермија, олигозооспермија, AZF делеции, AZFc, AZFb+c, Y хаплогрупа.

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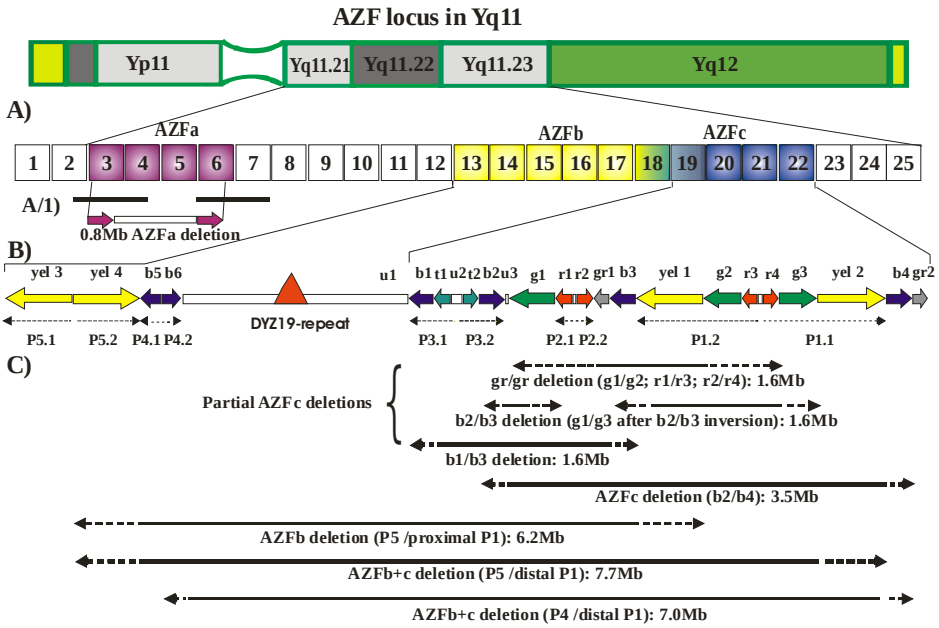

Figure 1. Schematic view of the AZF locus in Yq11. **A)** Deletion map of AZF locus: 25 intervals (D1-D25) and three AZF regions (AZFa, AZFb and AZFc). **A/1)** Complete AZFa deletion, caused by recombination of two homologous HERV15Yq1/q2 bloks. **B)** Structural organisation of the different amplicons in the AZFb and AZFc regions belonging to five palindromic structures (P1-P5). **C)** Partial and complete AZFc, AZFb and AZFb+c deletions caused by recombination between different amplicons.

Слика 1. Шематски приказ на AZF локусот во Yq11. **A)** Делециона мапа на AZF локусот: 25 интервали (D1-D25) и три AZF региони (AZFa, AZFb и AZFc). **A/1)** Комплетна AZFa делеција, настаната како резултат на рекомбинација меѓу две хомологни HERV15Yq1/q секвенци. **B)** Структурна организација на различните ампликони во AZFb и AZFc регионите кои припаѓаат на пет палиндроми (P1-P5). **C)** Парцијални и комплетни AZFc, AZFb и AZFb+c делеции настанати со рекомбинација меѓу различни ампликони.

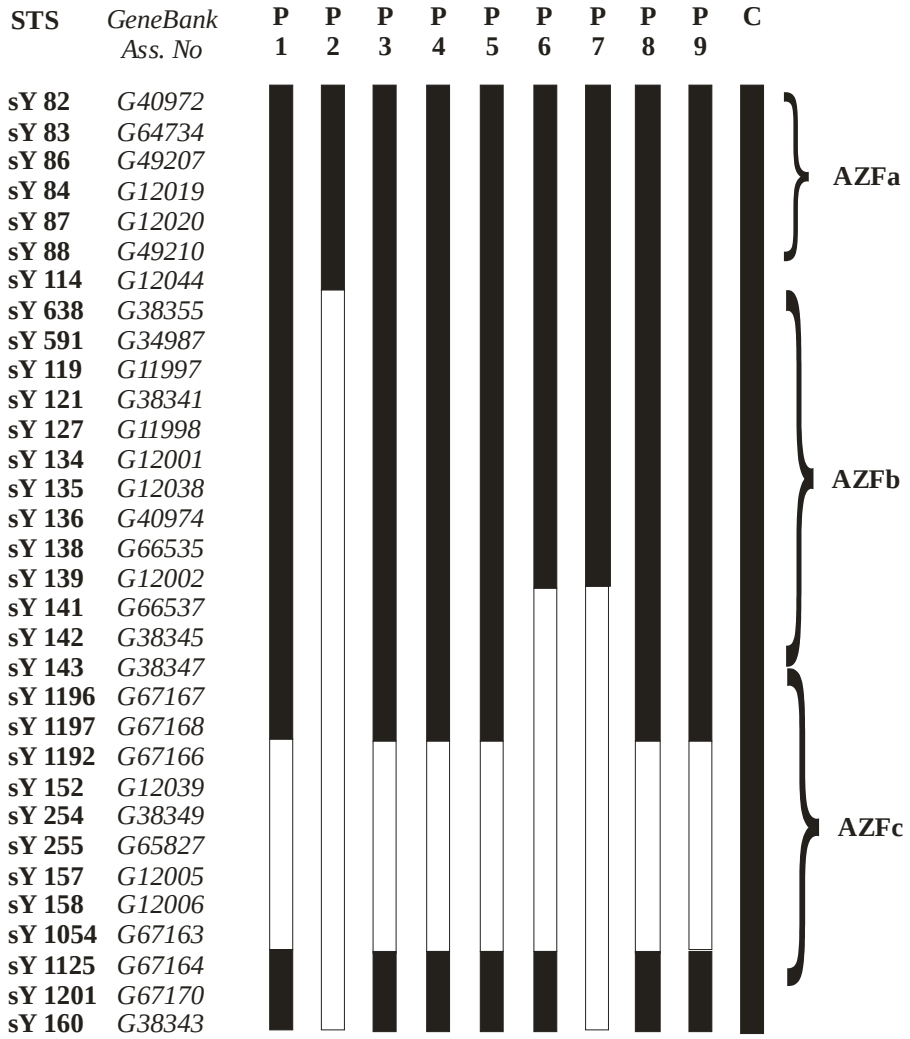

Figure 2. Schematic presentation of AZF deletions found in nine infertile men. Shaded boxes indicate presence of STS; white boxes indicate absence of STS. P1-P9, patients with AZF deletion; C, control - fertile men.

Слика 2. Шематски приказ на AZF делециите откриени кај девет инфертилни мажи. Темните блокови покажуваат присуство на STS; белите блокови покажуваат отсуство на STS. P1-P9, пациенти со AZF делеци; C, контрола - фертилен маж.

Figure 2. Schematic presentation of AZF deletions found in nine infertile men. Shaded boxes indicate presence of STS; white boxes indicate absence of STS. P1-P0, patients with AZF deletion; C, control – fertile men.

Figure 2. Schematic presentation of AZF deletions found in nine infertile men. Shaded boxes indicate presence of STS; white boxes indicate absence of STS. P1-P9, patients with AZF deletion; C, control – fertile men.