Molecular Human Reproduction Vol.12, No.8 pp. 519–523, 2006
Advance Access publication June 15, 2006 doi:10.1093/molehr/gal051

DAZ gene copies: evidence of Y chromosome evolution

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The *DAZ* gene, a contributing factor in infertility, lies on the human Y chromosome's *AZFc* region, whose deletion is a common cause of spermatogenic failure. Y chromosome binary polymorphisms on the non-recombining Y (NRY) region, believed to be a single occurrence on an evolutionary scale, were typed in a sample of fertile and infertile men with known *DAZ* backgrounds. The Y single-nucleotide polymorphisms (Y-SNPs) with low mutation rates are currently well characterized and permit the construction of a unique phylogeny of haplogroups. *DAZ* haplotypes were defined using single-nucleotide variant (SNV)/sequence tagged-site (STS) markers to distinguish between the four copies of the gene. The variation of 10 Y chromosome short tandem repeat (STRs) was used to determine the coalescence age of *DAZ* haplotypes in a comparable time frame similar to that of SNP haplogroups. An association between *DAZ* haplotypes and Y chromosome haplogroups was found, and our data show that the *DAZ* gene is not under selective constraints and its evolution depends only on the mutation rate. The same variants were common to fertile and infertile men, although partial *DAZ* deletions occurred only in infertile men, suggesting that those should only be used as a tool for infertility diagnosis when analysed in combination with haplogroup determinations.

Key words: *DAZ*/infertile/phylogeny/Y chromosome

Introduction

The human Y chromosome is strictly paternally inherited and, in most of its length, does not engage in pairing and crossing over during meiosis (Lahn and Page, 1997). Deletions in the Y chromosome *AZF*c region are associated with male infertility and occur *de novo* at a frequency of about 1/4000, being the most common known cause of spermatogenic failure (Reijo *et al*., 1995; Vogt *et al*., 1996). Within the *AZF*c region, there are six distinct families of massive repeat units (amplicons) organized in three palindromes (Kuroda-Kawaguchi *et al*., 2001; Skaletsky *et al*., 2003).

The *DAZ* gene family, located in the *AZF*c region, is organized into two clusters and contains a variable number of copies (Glaser *et al*., 1998; Saxena *et al*., 2000; Fernandes *et al*., 2004; Lin *et al*., 2005). Several mechanisms were suggested to explain the differences concerning the relative number of *DAZ* copies, such as intrachromosomal recombination events between amplicons in case of deletions (Repping *et al*., 2003; Vogt and Fernandes, 2003), gene conversion within palindromes to justify the duplications (Jobling and Tyler-Smith, 2003; Graves, 2004) and inversions like those originating in palindromic sequences (Kuroda-Kawaguchi *et al*., 2001; Repping *et al*., 2004). Gene conversion events, considered to be frequent when compared with base substitutions, might also influence the probability of rearrangements (Skaletsky *et al*., 2003), therefore favouring restoration of the original sequence and protecting the Y chromosome from the degeneration potentiated by haploidy (Hawley, 2003; Rozen *et al*., 2003; Graves, 2004).

The *DAZ* gene was derived from the autosomal homologue *DAZL* (*DAZ* gene-like) by transposition of the 3p24 chromosomal section to the Y chromosome about 40 million years ago (Reijo *et al*., 1995; Saxena *et al*., 1996; Seboun *et al*., 1997), subsequent to the splitting of the Old and New World monkeys (Seboun *et al*., 1997; Kumar and Hedges, 1998). Unlike *DAZL*, a gene that remains as a single copy, *DAZ* appears with a variable number of copies with 99.9% homology (Kuroda-Kawaguchi *et al*., 2001; Skaletsky *et al*., 2003). The variation of each *DAZ* gene copy seems to be essentially due to a tandem amplification of exon 7 (Saxena *et al*., 2000; Vogt and Fernandes, 2003). Deletions in *DAZ2*, *DAZ3* and *DAZ4* copies, found in fertile and infertile men, are described as familial variants inherited from father to son (Vogt *et al*., 1996; Saxena *et al*., 2000; Fernandes *et al*., 2002, 2004), although *DAZ1/DAZ2* deletions are restricted only to infertile men (Fernandes *et al*., 2002; Vogt and Fernandes, 2003; Ferlin *et al*., 2005). Comparisons between the two *DAZ* sequences available from Genebank show that only *DAZ1* exhibits a conserved structure, which suggests that it might be essential for human spermatogenesis (Vogt and Fernandes, 2003; Ferrás *et al*., 2004), although one case of a fertile man with a *DAZ1* deletion was recently described (Machev *et al*., 2004). *DAZ* haplotypes were previously defined using single-nucleotide variants (SNVs)/sequence tagged-site (STSs) to distinguish the four copies of the *DAZ* gene (Fernandes *et al*., 2002), and it has been shown to be a reliable method to identify each *DAZ* gene copy (Figure 1).

There are now more than 200 well-characterized non-recombining Y (NRY) biallelic markers, all having low mutation rates. These are assumed to be unique events in the evolutionary process, whose hierarchical structure allows the construction of a unique phylogeny of haplogroups (Y Chromosome Consortium, 2002). NRY markers are considered to be neutral, evolving only because of mutation rate (Jobling and Tyler-Smith, 2003). The largest fraction of extant European Y chromosome pool is composed of the I and R haplogroups thought

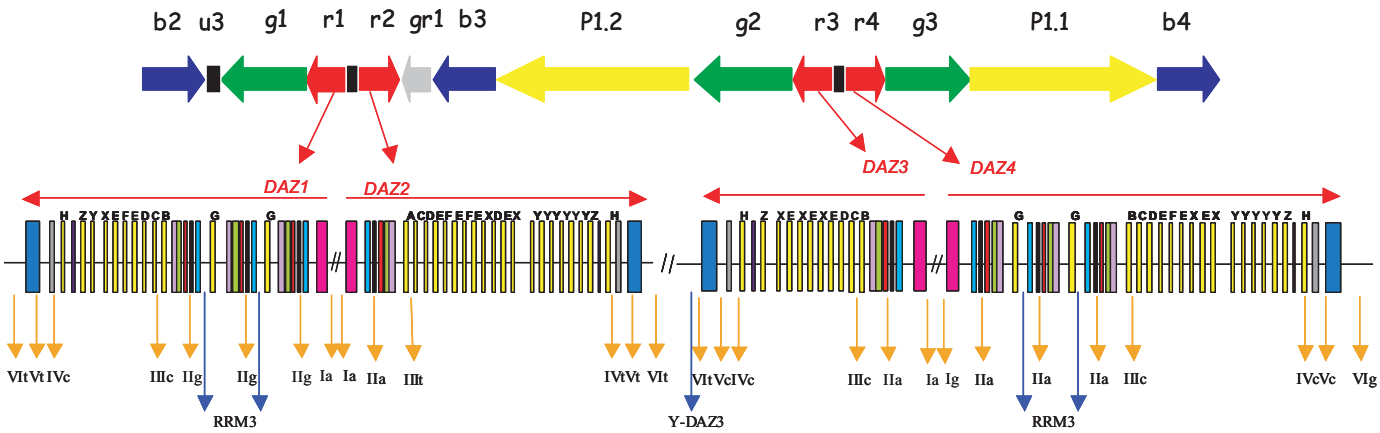


Figure 1. Localization of the *DAZ*-SNVs and STSs, by which the four *DAZ* gene copies can be distinguished, as previously described (Fernandes *et al*., 2002).

to be surviving lineages of Paleolithic origin. These haplogroups are believed to have expanded in the post-glacial period, after a severe bottleneck during the Last Glacial Maximum (20 000 years ago) (Semino *et al*., 2000). Haplogroups E and J, which represent approximately 20% of Europeans, were introduced in southern Europe from the Near East by immigrant farmers, during the Neolithic expansion ~10 000 years ago (Semino *et al*., 2004). A study suggested that a weak negative selection due to partial deletion of genes needed for spermatogenesis could act on some haplogroups, namely haplogroup D2 (Repping *et al*., 2003). Otherwise, the frequencies of matriarchal lineages defined by mitochondrial DNA and the patriarchal counterpart identified by Y chromosome haplogroups are similar in Europe (Richards *et al*., 2000; Semino *et al*., 2000), corroborating the theory of no selection on the Y chromosome structure.

Materials and methods

Samples

DNA samples from 97 infertile men presenting with varying clinical syndromes ranging from sertoli-cell-only syndrome (SO) to maturation arrest (MA) and hipospermatogenesis (HP) were analysed. All patients displayed a normal karyotype (46, XY), and no *AZF*-Yq11.2 microdeletions were detected by the use of the STS marker set previously established for *AZF*a, *AZF*b and *AZF*c microdeletions analysis (Vogt *et al*., 1996). The fertility status of the 91 fertile men was defined when fathering one or more children, with blood sampling taken at the nursery immediately after birth of the most recent child.

Additionally, 19 infertile men with complete *AZF*c deletions were analysed but not included in the group of infertile men.

All males involved in this study provided informed consent, following local ethical guidelines.

Y chromosome haplogroups

The STSs containing Y-SNPs were assayed as described (Underhill *et al*., 2000, 2001). Genotyping was performed using both native and engineered restriction fragment length polymorphism (RFLP) methods. The SNPs analysed are shown in their phylogenetic order defining the haplogroup status of each Y chromosome. The binary markers M4, M61, M147, LLY22g, M175 and P36 were assayed, but derived alleles were not observed. The haplogroup nomenclature and phylogeny used was the one proposed by the Y chromosome Consortium (2002).

SNV/STSs PCR for partial DAZ *deletions*

DAZ haplotypes, using SNVs/STSs to distinguish among the four copies of *DAZ* gene, were determined in all fertile and infertile samples through the analysis of six *DAZ*-single-nucleotide variants (SNVI–VI) and two *DAZ*-STS (*DAZ*-RRM3 and Y-*DAZ*3), as previously described (Fernandes *et al*., 2002).

Coalescence time of DAZ *haplotypes*

To define the coalescence time within each *DAZ* haplotype, we assayed 10 short tandem repeat (STR) (Powerplex Y system kit, Promega, Madison, WI, USA) in chromosomes belonging to the different *DAZ* types, using standard methodology. The coalescence time of STR variation was estimated as the average squared difference in the number of repeats between all current chromosomes and the founder haplotype (assumed to be modal), divided by $w = 6.9 \times 10^{-4}$ *per* 25 years (Zhivotovsky *et al*., 2004). The coalescence time was expressed in terms of thousands of years into the past (Kya), and the minimum sample size considered for coalescence age calculation was six individuals.

Statistical analysis

Frequency differences were calculated according to Fisher's exact test using the Arlequin software (Schneider *et al*., 2002), for fertile, infertile and also other samples from northern Portugal (no accession to the fertility state) (Gonçalves *et al*., 2005). The same procedure was used to compare the frequency of R haplogroup on fertile and infertile men, as well as those with complete *AZF*c deletion, and to compare the number of deletions within haplogroups in infertile men. Probability values of $P < 0.05$ were considered as statistically significant.

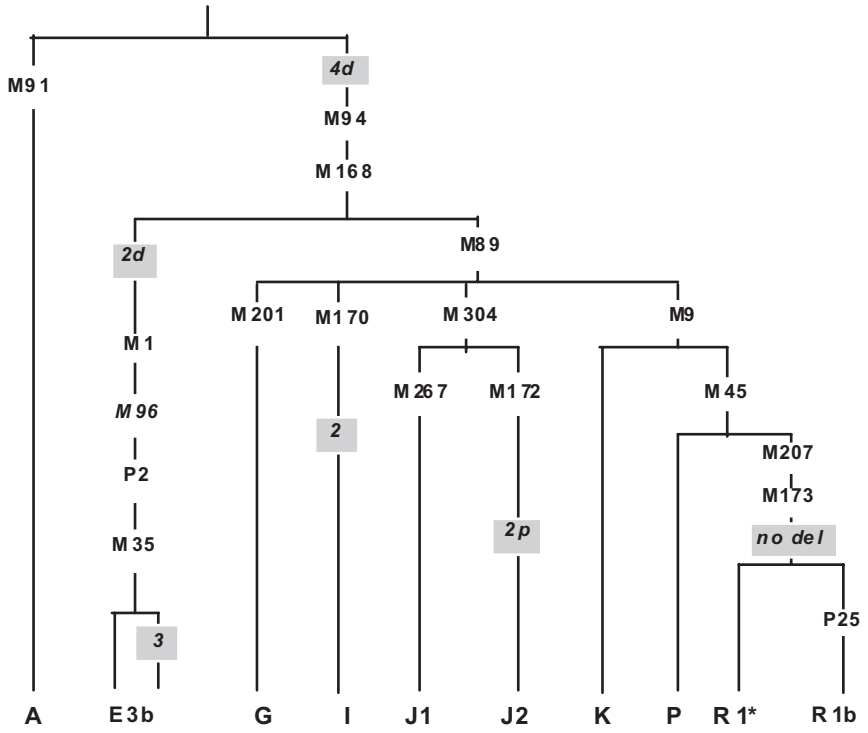
Results

The frequency distribution of Y chromosome haplogroups in fertile and infertile men is shown in Figure 2. Comparisons of haplogroup frequencies within fertile and infertile men showed no significant differences. Similar results were obtained when both groups were compared with the reference population from the same region of northern Portugal. Our data indicate a clear association of *DAZ* haplotypes and Y chromosome haplogroups (Table I) in accordance with previous studies, suggesting a possible association between *AZF*c partial deletions and Y chromosome variant haplogroups (Repping *et al*., 2003; Vogt and Fernandes, 2003; Machev *et al*., 2004; Repping *et al*., 2004; Vogt, 2005). As an example, the 2*4d pattern was exclusively found in haplogroup I, in fertile and infertile individuals. Moreover, the 4d pattern is common to the phylogenetically close haplogroups G, K, J1 and P, placing its occurrence at a time before divergence. All fertile individuals from haplogroups I and J2 display a characteristic *DAZ* haplotype.

The coalescence time for the more common *DAZ* haplotypes is shown in Figure 3 and is in accordance with that given for the associated haplogroups (Semino *et al*., 2000).

Of 19 individuals with complete *AZF*c deletion, the frequency of the R haplogroup was determined to be similar to that found in fertile and infertile men.

When we compared the number of deletions in infertile men within each haplogroup, significantly more deletions were found in haplogroup J1 than in haplogroup R ($P < 0.05$).



	n	Gene diversity										
Fertile	91	.613 (.055)		10	2	9	4	5	2	1	3	55
Infertile	97	.723 (.040)	1	14	4	13	6	6	2	1	3	47
Total	188		1	24	6	22	10	11	4	2	6	102

Figure 2. Phylogenetic tree of Y chromosome haplogroups. Haplogroup defining mutations assayed in this study are shown along the branches. The grey boxes show the relative position of *DAZ* mutations which leads to the *DAZ* haplotype.

Table I. Pattern of *DAZ* haplotype within each haplogroup for fertile (n = 91) and infertile men (n = 97)

Y chromosome haplogroup	Fertile men *DAZ* haplotype	*n*	Infertile men *DAZ* haplotype	*n*
R1b	No deletion	53	No deletion	43
	2d	1	2d	1
	4p	1	4p	1
			1*2	1
			1*2*4	1
R1*	No deletion	3	No deletion	2
			3*4	1
P	4d	1	4d	1
K	4d	2	4d	2
G	4d	2	4d	3
			2d*4d	1
I	2*4d	9	2*4d	10
			2*3*4	2
			1*2*3	1
J1	4d	4	4d	3
			1*2*4d	2
			2d*4d	1
J2	2p*4d	5	2p*4d	4
			4d	2
E3b	2d*3*4d	7	2d*3*4d	6
	2d*4d	3	2d*4d	7
			2*3*4d	1
A		0	3*4	1

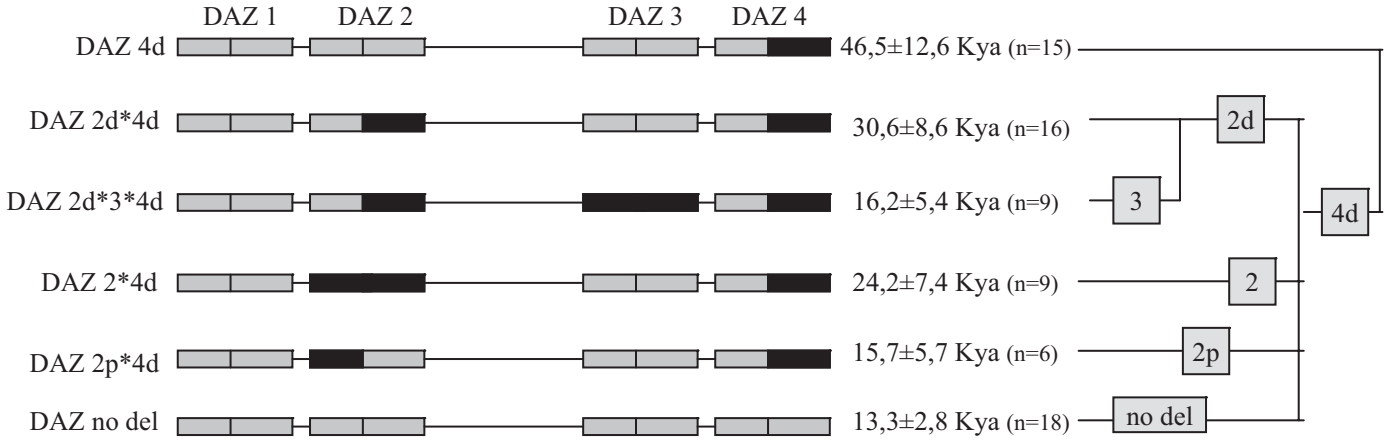


Figure 3. Estimated coalescence time for each *DAZ* haplotype using 10 Y chromosome STRs. Black boxes show the *DAZ* partial deletions.

Discussion

The concordance of *DAZ* haplotypes with the branching and inner variability is therefore an indicator of neutrally driven evolution on the basis of mutation rate alone. If *DAZ* haplotypes were to be affected by selective constraints, as a strong linkage to infertility or founder effect and/or bottleneck phenomena, their expected level of variability at the fast-evolving microsatellite (and consequently the coalescence time) would be smaller.

For the occurrence of specific *DAZ* partial deletions only in infertile men, two scenarios are possible: (i) they are connected to male infertility or (ii) they are within the normal variation range for the haplogroup but escaped the sampling. Care is needed when associating *DAZ* haplotypes with a causal factor of male infertility. For example, haplotype 2d*4d, present in infertile men from haplogroup G and J1, is in fact part of the normal variation found in both E3b case-study groups. Therefore, we suggest that a higher reliability for an association of *DAZ* haplotype-infertility demands the knowledge of their haplogroups. Nevertheless, there must be some weakness in the Y chromosome structure; if not, the number of deletions in infertile men from different haplogroups would be the same and no differences such as the ones existing between J1 and R haplogroups would have been observed.

On the contrary, the frequency of R haplogroup compared with those individuals with complete *AZF*c deletions was similar to both groups of fertile and infertile men. Therefore, the chromosome structure on the R haplogroup does not seem to protect against the deletion, despite palindromic sequences being more stable because of the reduced possibility of recombination (Kuroda-Kawaguchi *et al*., 2001; Fernandes *et al*., 2004).

Although not in the context of our study, it is possible that the distribution and frequencies of each *DAZ* haplotype may vary with geography and the demographic history of populations, as observed for the population perspective of the Y chromosome genetic system. The high frequency of the variant *DAZ* 'no deletions' can reflect the exclusive relation to haplogroup R, the most common in Europe (Semino *et al*., 2000; Jobling and Tyler-Smith, 2003). It therefore seems clear that the Y chromosome inversion described (Kuroda-Kawaguchi *et al*., 2001), based on the Genebank sequence of the subject with reference RPCI-11 (Vogt and Fernandes, 2003; Vogt, 2005), only exists in individuals belonging to the haplogroup R. Consequently, the *DAZ* haplotype 'no deletions' can no longer be regarded as a correct designation because it only represents a reference and not an ancestral. The oldest *DAZ* haplotype that represents the absence of the distal part of the *DAZ* gene copy 4 is the *DAZ* 4d haplotype. The distal part is shown to be present only in the sequence of the subject with reference RPCI-11 and not in the sequence of CTA/CTB men (Saxena *et al*., 2000; Vogt and Fernandes, 2003). The same might be observed in mitochondrial DNA lineages, where the Cambridge reference sequence (CRS) is not the ancestral but the reference obtained by the complete sequence (Anderson *et al*., 1981), revised in 1999 (Andrews *et al*., 1999).

In conclusion, we demonstrated that there is an association between the *DAZ* haplotype and the haplogroups of the Y chromosome. As *DAZ* partial deletions might be a polymorphic event associated with a specific haplogroup or an individual cause of infertility, those should only be analysed for infertility diagnosis when analysed in combination with haplogroup determinations. The possibility that a mutation defining a haplogroup could be more than only a single mutation—for instance, being associated with Y chromosome rearrangements—therefore remains open for debate.

Acknowledgements

We are grateful to David Shaproski and Christoph Roehrig for their help in preparing the final version of this manuscript. This study was partially supported by FCT (SFRH/BD/23616/05, 6664/01; POCTI/SAU-MMO/60709/04, 60555/04, 59997/04; UMIB).

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Submitted on February 7, 2006; resubmitted on May 8, 2006; accepted on May 11, 2006