Letters to the Editor

Are Sequence Family Variants Useful for Identifying Deletions in the Human Y Chromosome?

To the Editor:
We read with interest the report of a novel deletion of part of the azoospermia factor c (AZFc [MIM 415000]) region of the human Y chromosome (Fernandes et al. 2004). This article reported that the deletion is found only in branch N of the Y-chromosome genealogical tree, occurs through one mutational pathway, is $\sim$2.2 Mb in size, and has no effect on spermatogenesis. We, too, recently reported this deletion, which Fernandes et al. termed the “g1/g3” deletion and which we termed the “b2/b3” deletion (Repping et al. 2004). Our findings, however, differed from those of Fernandes et al. in several important particulars: (1) our screening of 1,563 men demonstrated that this deletion is not confined to branch N and that it has at least four independent origins; (2) our analysis revealed two mutational pathways, rather than one, that can generate the deletion, and we confirmed the existence of the inverted AZFc organizations that are the intermediate steps in these pathways; (3) on the basis of the reference sequence of the Y chromosome, we concluded that the size of the deletion is 1.8 Mb, rather than $\sim$2.2 Mb; (4) using interphase FISH, we confirmed the amplicon organization that was postulated in the deletion and also identified three instances of duplication subsequent to the deletion; and (5) because of the possibility of a compensatory factor on Y chromosomes in branch N and because of the limited number of deletions outside this branch, we concluded that a possible effect of this deletion on risk of spermatogenic failure cannot be excluded (Repping et al. 2004).

Beyond these differences, however, the characterizations of this and other partial deletions of AZFc (Repping et al. 2003) highlight a more important question. At issue is the relative utility of sequence family variants (Saxena et al. 2000), compared with that of plus/minus STSs, for identification and differentiation of deletions involving AZFc. AZFc is composed entirely of amplicons—repeat units 115–678 kb in length that only differ by $\sim$1 nt per 3,000 bp. These rare differences are called “sequence family variants” (SFVs). We previously relied on SFVs to map and sequence the AZFc region of one man’s Y chromosome (Kuroda-Kawaguchi et al. 2001). The report by Fernandes et al. (2004) emphasized the use of SFVs in identification of the novel deletion, whereas our analysis relied on plus/minus STSs for identification of the deletion, followed, in most instances, by confirmation with FISH.

Two observations led us to ask whether SFVs, as opposed to plus/minus STSs, offer the simpler and more robust means of detecting and distinguishing deletions in AZFc. First, figures 1 and 4 in the report by Fernandes et al. (2004) indicated that negative results at the plus/minus STS sY1192 or 50f2/C combined with positive

Table 1

<table>
<thead>
<tr>
<th>DELETION</th>
<th>sY142</th>
<th>sY1197</th>
<th>sY1191, sY1192, and/or 50f2/C</th>
<th>sY1291</th>
<th>sY1206</th>
<th>sY1201</th>
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<tr>
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<td>−</td>
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<td>−</td>
<td>−</td>
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<td>+</td>
<td>−</td>
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<td>+</td>
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</tr>
</tbody>
</table>

NOTE.—See Kuroda-Kawaguchi et al. (2001), Repping et al. (2003), Skaletsky et al. (2003), Fernandes et al. (2004), Repping et al. (2004), and GenBank for STSs.

* + = present; − = absent.

* Termed the “g1/g3” deletion by Fernandes et al. (2004).

* “Classical” AZFc.
Figure 1  Genealogical analysis of SFV patterns associated with b2/b3 and gr/gr deletions. In the SFV patterns, “C” indicates the cut variant described by Fernandes et al. (2004), “U” indicates the uncut variant, “B” indicates both variants, and + and − indicate the presence or absence, respectively, of the Y-DAZ3 variant. The order of SFVs is as shown in table 2 in the work of Fernandes et al. (2004): DAZ-SNV I, DAZ-SNV II, sY586 (DAZ-SNV III), DAZ-SNV IV, sY587 (DAZ-SNV V), DAZ-SNV VI, AZFc SFV 18 (assayed by Y-DAZ3), TTY4-SNV I, BPY2-SNV, GOLY-SNV I, and AZFc SFV 20 (AZFc-P1-SNV I) (Saxena et al. 2000; Kuroda-Kawaguchi et al. 2001 [Web table E]; Fernandes et al. 2002, 2004). The genealogical tree of extant human Y chromosomes and the branch designations are from the studies by Underhill et al. (2000) and the Y-Chromosome Consortium (2002). §, R1∗x is an abbreviation for R1∗(xR1a,R1/-USP9Y+3636). †, Termed “g1/g3” by Fernandes et al. (2004).
results at flanking STSs are sufficient to detect the deletion (table 1). Moreover, the b2/b3 deletion and other types of deletions involving AZFc can be distinguished by their plus/minus signatures, without the use of SFVs (table 1).

Second, table 2 in the report by Fernandes et al. (2004) showed that the SFV patterns of undeleted chromosomes vary considerably among different branches of the Y-chromosome genealogy and that the patterns also vary among individuals within branches. These observations suggested that the link between SFV patterns and particular types of deletions would likely not be consistent across the worldwide diversity of Y chromosomes.

The diversity of SFV patterns in undeleted chromosomes is not surprising, since AZFc is subject to large inversions, deletions, and duplications caused by ectopic homologous recombination between amplicons (Kuroda-Kawaguchi et al. 2001; Repping et al. 2003, 2004). Such events would rearrange the locations of particular variants and would blur the association between SFV patterns and particular types of deletions. The association would likely be further blurred by gene conversion, which frequently erases small sequence differences (i.e., SFVs) between amplicon copies on the Y chromosome (Rozen et al. 2003).

We experimentally investigated the consistency of SFV patterns in different types of deletions involving AZFc. First, using the SFVs employed by Fernandes et al. (2004), we typed 20 men reported elsewhere to have the b2/b3 deletion (Repping et al. 2004) (see GenBank Web site for SFV assays). These men represented branch N and three other branches of the Y-chromosome genealogy (fig. 1). Second, using the same SFVs, we typed 40 men reported elsewhere to have the gr/gr deletion, the other common partial AZFc deletion (Repping et al. 2003). These men represented 14 branches of the Y-chromosome genealogy (fig. 1).

The b2/b3 deletions outside branch N showed diverse SFV patterns, and the gr/gr deletions showed even greater diversity (fig. 1). This greater diversity was likely due to the larger number of independent gr/gr deletions studied. Two branches, F[xHK] and R1*x, contained numerous deletions and a high diversity of SFV patterns (fig. 1). In these branches, multiple independent deletion events probably account for the high diversity. By contrast, two other branches, D2b and N, contained numerous deletions but uniform SFV patterns. This uniformity is explained by the fact that all chromosomes in these branches descended from deleted founders (Repping et al. 2003, 2004; Fernandes et al. 2004). Thus, the chromosomes in each of these branches represent a single deletion event.

Our data also showed that the SFV patterns of b2/b3 and gr/gr deletions are not distinct from each other. For example, the b2/b3 pattern UUUCUU–CUUU (branch F*[xHK]) is more similar to the gr/gr pattern UUCUU+CBUB (branch F*[xHK], four differences [underlined]) than to the b2/b3 pattern UBBBCU–CCUC (branch N, six differences). In another example, the gr/gr pattern UBBBCU–UBUB (branch R1*x) is more similar to the b2/b3 pattern UBBBCU–CUUC (branch i, three differences) than to the gr/gr pattern BCCUCU+CBCC (branch R1*x, 10 differences).

In conclusion, the SFV patterns of b2/b3 and gr/gr deletions vary widely and are not clearly distinct. SFVs can offer insight only if one knows the common SFV organizations in the genealogical branches represented by the Y chromosomes being tested. However, SFV organizations across the Y-chromosome genealogical tree are largely unknown, and SFV patterns vary even among individuals in the same branch. Just as important is that a large number of two-step assays are needed for SFV typing and for determining the Y-chromosome branch. By contrast, six simple plus/minus STSs distinguish between the deletions involving AZFc (table 1). Thus, plus/minus STSs provide a straightforward means of identifying and distinguishing the deletions of part of AZFc, whereas, in most situations, SFVs do not.

Acknowledgments

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for STSs 50f2/C [accession number Y07728], sY142 [accession number G38345], sY1191 [accession number G73809], sY1192 [accession number G67166], sY1197 [accession number G67168], sY1201 [accession number G67170], sY1206 [accession number G67171], and sY1291 [accession number...
ber G72340] and for SFV assays DAZ-SNV I [accession number G73167], DAZ-SNV II [accession number G73166], sY586 [accession number G63907], DAZ-SNV IV [accession number G73168], sY587 [accession number G63908], DAZ-SNV VI [accession number G73169], Y-DAZ3 [accession number G73170], TTY4-SNV I [accession number BV012731], BPY2-SNV [accession number BV012732], GOLY-SNV I [accession number BV012733], and AZFc SFV 20 [AZFc-P1-SNV I] [accession number G73351]


Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for AZFc)

References


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Reply to Repping et al.

To the Editor:

We welcome the enormous contribution that Repping and colleagues have made to the elucidation of the DNA sequence and organization of the Y chromosome, but many questions remain unanswered after the sequencing of the Y chromosome of one man (Skaletsky et al. 2003). It was appreciated, almost a decade ago, that the structure of the AZFc region is particularly variable; six independent deletion events and four duplications that affect one short section of this region (50f2/C), together representing ~8% of normal men, were identified by Jobling et al. (1996), and this study could have detected only a small proportion of the total AZFc variation. Yet, it provides a useful benchmark for an assessment of our current understanding. We can now define the molecular basis of one of the deletions described in 1996, the haplogroup-12 “small” 50f2/C deletion (Fernandes et al. 2004; Repping et al. 2004b), and possibly a second (if the “small” 50f2/C deletion in haplogroup 2 [Jobling et al. 1996] corresponds to the b2/b3 deletion in YCC haplogroup F*) [xHK] or I [Repping et al. 2004b]), but the 50f2/C duplications all fall on haplotypic backgrounds different from those of the b2/b4 duplications (which include 50f2/C) described so far (Repping et al. 2003). Thus, researchers have still not accounted for at least 8 of 10 rearrangements reported in 1996. It seems that our current methods, whether based on SNVs/SFVs or on plus/minus STSs, allow us to describe only a small proportion of the variation present in this region.

Are plus/minus STSs, nevertheless, more useful than SNVs/SFVs for characterizing AZFc variation (Repping et al. 2004a [in this issue])? It is a matter of opinion. Even for the best-characterized variants, the gr/gr and g1/g3 (also known as “b2/b3”) deletions, it is unclear whether the independent deletions on different lineages...
represent true recurrent mutations—taking place at the same recombination site each time—or whether the recombination events have occurred in different locations within the amplicons on different occasions. In the latter case, conflation of different structures—which could have different gene contents—by plus/minus STSs would be a weakness of this classification scheme, and discrimination between them by SNVs, a strength (fig. 1 of Repping et al. [2004a] [in this issue]). It would, however, seem rash to rely on either of these two methods alone—FISH, used by Repping et al. (2003); Southern blotting, used by Fernandes et al. (2002, 2004); and quantitative PCR can all be helpful in defining the structures. But most important of all, this work highlights the importance of an evolutionary understanding of the Y chromosome, and we particularly welcome Repping et al.’s acceptance of this evolutionary approach.

Evolutionary interpretations must, however, be made with caution—we should avoid the “fallacy of the contemporary ancestor” (Jobling et al. 2004). Modern inverted Y chromosomes (see fig. 3 of Repping et al. [2004b]) are not the ancestors of haplogroup-N chromosomes, and their frequencies do not indicate which mutational pathway was followed. The best guide to the pre-N structure may be provided by haplogroup-O chromosomes, a sister clade to N in the current Y phylogeny (Jobling and Tyler-Smith 2003) and thus the closest known outgroup. The b2/b3 inversion has indeed been reported in haplogroup O (Repping et al. 2004b); if it was present in the common ancestor of the two lineages, the haplogroup-N deletion would result from a g1/g3 deletion following this b2/b3 inversion, rather than a b2/b3 deletion following a g1/g3 inversion. If so, the conclusions from the SNV-based study (Fernandes et al. 2004) would be more accurate than those from the plus/minus STS–based one (Repping et al. 2004b).

The present discussion can take place only because our methods for characterizing AZFc structures are pitifully inadequate. Rather than behaving like the proverbial group of blind men who encounter an elephant from different sides and insist on describing it from their own favorite partial perspectives, we should assume that all the inversions, duplications, and deletions that are permitted by the sequence will occur, limited only by the winnowing of natural selection. The resulting structures may differ, by many rounds of rearrangement, from the modern haplogroup-R GenBank sequence, but use of the SNP-based phylogeny (Jobling and Tyler-Smith 2003) may allow us to understand the relationship between these structures. It would be even better to develop radically improved ways of elucidating the entire structure so that we can obtain a reasonably complete view of this complex and evolutionarily labile region.

References


specific region of the human Y chromosome is a mosaic of

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Letters to the Editor

Problematic Use of Greenberg’s Linguistic
Classification of the Americas in Studies of
Native American Genetic Variation

To the Editor:
In recent years, there has been a burgeoning interest in
comparisons of genetic and linguistic variation across
human populations. This synthetic approach can be a
powerful tool for reconstructing human prehistory, but
only when the patterns of genetic and linguistic variation
are accurately represented (Szathmary 1993). If one or
both patterns are inaccurate, the resulting conclusions
about human prehistory or gene-language correlations
may be incorrect. Here, we present evidence that com-
parisons of genetic and linguistic variation in the Amer-
icas are problematic when they are based on Greenberg’s
(1987) classification of Native American languages, for
these very reasons.

Greenberg (1987) argued that all Native American
languages, except those of the “Na-Dene” and Eskimo-
Aleut groups, are similar and can be classified into a
single linguistic unit, which he called “Amerind.” His
tripartite classification (Amerind, Na-Dene, and Eskimo-
Aleut) was based on the method of multilateral com-
parison, which examines many languages simultaneously
to detect similarities in a small number of basic words
and grammatical elements (Greenberg 1987). Green-
berg (1987) also suggested that his three language group-
ings represent three separate migrations to the Americas,
and Greenberg et al. (1986) interpreted their synthesis
of the linguistic, dental, and genetic evidence as sup-
portive of this three-migration hypothesis.

Over the past 18 years, this three-migration model
has become entrenched in the genetics literature as the
hypothesis against which new genetic data are tested
(e.g., Torroni et al. 1993; Merriwether et al. 1995; Ze-
gura et al. 2004), and Greenberg’s linguistic classifica-
tion has been the primary scheme used in studies com-
paring genetic and linguistic variation in the Americas.
Of 100 studies of Native American genetic variation
published between 1987 and 2004, 61 cite Greenberg
(1987) or Greenberg et al. (1986), and at least 19 others
were influenced by his tripartite classification (15 studies
use the Amerind, Na-Dene, and Eskimo-Aleut group-
ings, and 4 others use the similar language groupings of
Greenberg’s student M. Ruhlen.

Whereas Greenberg’s classification has been widely
and uncritically used by human geneticists, it has been
rejected by virtually all historical linguists who study
Native American languages. There are many errors in
the data on which his classification is based (Goddard
1987; Adelaar 1989; Berman 1992; Kimball 1992; Poser
1992), and Greenberg’s criteria for determining lin-
guistic relationships are widely regarded as invalid.
His method of multilateral comparison assembled only
superficial similarities between languages, and Green-
berg did not distinguish similarities due to common an-
cestry (i.e., homology) from those due to other factors
(which other linguists do). Linguistic similarities can also
be due to factors such as chance, borrowing from neigh-
boring languages, and onomatopoeia, so proposals of
remote linguistic relationships are only plausible when
these other possible explanations have been eliminated
(Matisoff 1990; Mithun 1990; Goddard and Campbell
1994; Campbell 1997; Ringe 2000). Greenberg made
no attempt to eliminate such explanations, and the pu-
tative long-range similarities he amassed appear to be
mostly chance resemblances and the result of misana-
lysis—he compared many languages simultaneously
(which increases the probability of finding chance re-
semblances), examined arbitrary segments of words,
equated words with very different meanings (e.g., ex-
crement, night, and grass), failed to analyze the structure
of some words and falsely analyzed that of others, ne-
eglected regular sound correspondences between lan-
guages, and misinterpreted well-established findings
(Chafe 1987; Bright 1988; Campbell 1988, 1997; Golla
1988; Goddard 1990; Rankin 1992; McMahon and Mc-

Consequently, empirical studies have shown that “the
method of multilateral comparison fails every test; its
results are utterly unreliable. Multilateral comparison is
worse than useless: it is positively misleading, since the
patterns of ‘evidence’ that it adduces in support of pro-
posed linguistic relationships are in many cases math-
ematically indistinguishable from random patterns of
chance resemblances” (Ringe 1994, p. 28; cf. Ringe
2002). Because of these problems, Greenberg’s meth-
odology has proven incapable of distinguishing plausible
proposals of linguistic relationships from implausible
ones, such as Finnish-Amerind (Campbell 1988). Thus,
specialists in Native American linguistics insist that
Greenberg’s methodology was so flawed that it com-
pletely invalidates his conclusions about the unity of
Amerind, and Greenberg himself estimated that 80%–
Table 1
Populations and Language Classifications Used in AMOVAs

<table>
<thead>
<tr>
<th>POPULATION</th>
<th>LANGUAGE CLASSIFICATION</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheyenne/Arapaho</td>
<td>Amerind</td>
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<td>Algic</td>
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90% of linguists agreed with this assessment (Lewin 1988).

Given this, the use of Greenberg’s (1987) classification can confound attempts to understand the relationship between genetic and linguistic variation in the Americas. Many studies of Native American genetic variation continue to use this classification (e.g., Bortolini et al. 2002, 2003; Fernandez-Cobo et al. 2002; Lell et al. 2002; Gomez-Casado et al. 2003; Zegura et al. 2004). However, Hunley and Long (2004) recently showed that there is a poor fit between Greenberg’s classification and the patterns of Native American mtDNA variation. On the basis of their findings, we believe that Greenberg’s groupings should no longer be used in analyses of mtDNA variation.

To further evaluate how the use of this classification influences our understanding of the relationship between genetic and linguistic variation in the Americas, we examined how well different linguistic classifications “explain” the patterns of Native American Y-chromosome variation. Data were compiled on the Y-chromosome haplogroups of 523 Native Americans, representing 36 populations (table 1). We compared hierarchical analyses of molecular variance (AMOVAs), using Greenberg’s (1987) classification and a more conservative one (Campbell 1997) that is widely accepted by specialists in historical linguistics of Native American languages (Golla 2000; Hill and Hill 2000). The AMOVAs were based on population frequencies of the haplogroups known to be pre–European contact Native American lineages (Q-M19, Q-M3*, Q-M242*, and C-M130). All calculations were performed by Arlequin 2.000 (Schneider et al. 2000).

The AMOVAs show that differences among Green-
berg’s three groups could account for some genetic variance ($\Phi_{CT} = 0.319$; $P = .027$), but the more generally accepted linguistic classification (as given in Campbell [1997]) of the same populations (17 groups) explains a greater proportion of the total genetic variance ($\Phi_{CT} = 0.448$; $P < .001$). The magnitude of $\Phi_{CT}$ increases 40.4% when the accepted language classification is used, which indicates that it is important to consider language classifications other than that of Greenberg (1987) when evaluating the relationship between genes and language in the Americas. Other factors, such as geography, have likely influenced patterns of genetic variation more than language, but accepted language groupings should, nonetheless, be used when exploring these relationships.

Thus, in future studies comparing genetic and linguistic variation in the Americas, we recommend use of the consensus linguistic classification, as given in Campbell (1997), Goddard (1996), and Mithun (1999), rather than Greenberg’s tripartite classification (Greenberg et al. 1986; Greenberg 1987). In addition, since there is no legitimate reason to believe that “Amerind” is a unified group (linguistic or otherwise), it has been essentially abandoned in linguistics and should not be used in genetic analyses. Finally, because synthetic studies provide such important insights into human prehistory, we advocate continued collaboration between geneticists and linguists (and other anthropologists) to ensure accurate comparisons of genetic, linguistic, and cultural variation.

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The Phylogeography of Mitochondrial DNA Haplogroup L3g in Africa and the Atlantic Slave Trade

To the Editor:

From the 16th to the 19th century, ∼4 million slaves were transported from sub-Saharan Africa to Brazil. With the use of historical records, it is possible to estimate that ∼65% of them were Bantus from west-central Africa, ∼30% originated from western Africa, and ∼5% came from southeastern Africa (Klein 2002).

Salas et al. (2004) have compared the phylogeography of mtDNA haplogroups in Africa with available data on Brazilians and have concluded that their results agree with these historical estimates. However, they were careful to point out that the west-central African contribution to Brazil, signaled by a high frequency of haplogroups L1c and L3e (Bortolini et al. 1997; Alves-Silva et al. 2000; Bandelt et al. 2001), derives largely from an area (the Congo basin) that, thus far, has not been thoroughly analyzed for mtDNA variation. Also, Salas et al. (2002, 2004) called attention to the presence of the haplogroup L3g—which they had only encountered in eastern African populations—in three Brazilians (among the 92 African mtDNA haplotypes that were characterized), one Colombian, and one African American individual. On the basis of this observation, they proposed the occurrence of either direct slave trade from eastern Africa to America or hitherto undetected gene flow from eastern Africa into western or southeastern Africa and then into America.

Intrigued by this proposal, we tried to identify historical evidence of direct slave trade from eastern Africa to Brazil and, indeed, found some anecdotal reports but certainly not enough to explain the significant frequency of L3g seen among African mtDNA haplogroups in Brazil (proportion, 3.3%; 95% CI, 0.7%–9.2%). We then tried to identify other potential sources for the Brazilian L3g mtDNAs. While studying the mtDNA haplotypes of 10 individuals from Cameroon (described in Da Silva
et al. 1999), we identified 1 individual of undisclosed ethnic origin whose mtDNA unequivocally belonged to the L3g haplogroup (fig. 1). This finding stimulated us to search for further mtDNA data from Cameroon, and we came across an article by Destro-Bisol et al. (2004) in which they reported 4 instances of the L3g haplogroup among 53 Ewondo individuals. Moreover, the same authors described (on the Laboratory of Molecular Anthropology Web site) another 11 instances of the L3g haplogroup in several ethnic groups (Bakaka, Bassa, Ewondo, Daba, Fali, Podowko, and Mandara) from different geographical regions of Cameroon. The 14 mtDNA sequences from Cameroon belonged to only four different haplotypes.

We incorporated the four L3g lineages from Cameroon with those from eastern Africa and obtained the median-joining network shown in figure 1. One Brazilian haplotype was identical to the most common Cameroonese haplotype (seen in 8 of the 14 cases), whereas another was closely related. Both the third Brazilian and the single African American haplotype also clustered with Cameroonese sequences. A noteworthy feature of the network was that there did not appear to exist any clear segregation of the Cameroonese L3g haplotypes from the eastern African counterparts. This feature, plus the fact that the putative ancestral haplotype was seen in an individual from Sudan and that there is a much smaller haplotype diversity in Cameroon (0.602) as compared with eastern Africa (0.911, excluding the Hadza), suggests that the Cameroonese L3g lineages might have originated from eastern Africa by transcontinental gene flow, as put forward by Salas et al. (2004) in one of their two possible scenarios. At any rate, it appears that the L3g lineages seen in America probably have their immediate origin in Cameroon or in neighboring regions and not in eastern Africa.
Acknowledgments

We are grateful to Dr. Giovanni Destro-Bisol, Dr. Valentina Coia, and Dr. Gabriella Spedini from the Laboratory of Molecular Anthropology, Department of Animal and Human Biology, University of Rome “La Sapienza,” who very kindly gave us access to their data and allowed us to use their data in this letter. Our research was supported by the Conselho Nacional de Pesquisas of Brazil and the Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior.

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Reply to Bortolini et al.

To the Editor:

The availability of new mtDNA data from central Africa has allowed Bortolini et al. (2004 [in this issue]) to evaluate two alternative scenarios, formulated by Salas et al. (2004), regarding the source of the L3g mtDNAs carried from Africa to America by the Atlantic slave trade. Bortolini et al. proposed that the American L3g haplotypes have an Atlantic African provenance, rather than a direct eastern African origin, and that their most likely source was Cameroon or the neighboring regions.

On the basis of the extensive amount of new data that can be added to the L3g phylogeny (fig. 1), we are wholeheartedly in favor of this scenario. Of particular interest are three mtDNA sequences belonging to the L3g1 haplogroup that we observed in southwestern Africa (Angola and Cabinda), this region being the second most important source for the Atlantic slave trade (Thomas 1997), with an important demographic impact in Bra-
Figure 1 Median-joining network (Bandelt et al. 1999) of L3g mtDNAs. The circle sizes are proportional to the haplotype frequency in the sample. Positions are indicated as variants from the revised Cambridge reference sequence, minus 16,000; a letter next to the position indicates a transversion. Parallel mutations are underlined and diagnostic positions outside of the common mtDNA segment analyzed (from 16060 to 16362) are in brackets. We here define three sublineages of L3g: L3g1, characterized by 16051-16114-16189-16316; L3g2, characterized by 16093G-16287A; and L3g3, which lacks transition 16355 from the root. The histogram (bottom right) illustrates the frequency of L3g in different African regions and in America, and the map (top right) shows the diffusion pattern of L3g within Africa. A yellow dot indicates the presence of L3g in the population sample, whereas a black dot indicates its absence. Time estimates are computed as in Salas et al. 2002. Note that the Colombian mtDNA included in Bortolini et al.’s (2004 [in this issue]) network has been excluded from this phylogeny, whereas the Cameroonese data is included here. Eastern Africa: Tz = Tanzania, Hz = Hadza (Tanzania), Dk = Dakota (Tanzania), Sk = Sukuma (Tanzania), Iw = Iraqw (Tanzania), Ki = Kikuyu (Kenya), Tk = Turkana (Kenya), Ug = Uganda, Et = Ethiopia, Su = Sudan, Nu = Nubia (Sudan/Egypt). Northern Africa: Eg = Egypt, Mo = Berber (Morocco). Central Africa: Ew = Ewondo (Cameroon), Ba = Bakaka (Cameroon), Da = Daba (Cameroon), Fa = Falis (Cameroon), Ma = Mandara (Cameroon), Po = Podowkos (Cameroon), Ca = Cameroon, Fg = Fang (Gabon), Mk = Makina (Gabon), Gl = Galoa (Gabon). Southwestern Africa: Cb = Cabinda, An = Angola. America: Bz = Brazil, Hs = “Hispanic” (North America), Na = North America. Middle East: Sy = Syria, Is = Israel. TMRCA = time to the most common recent ancestor. Details of the L3g sequences will be supplied by the corresponding author on request.

Overall, L3g1 appears to reflect the contribution of southwestern Africa—and probably central Africa also—to America (66% of the American L3g sequences), whereas the American L3g2 mtDNAs might be of predominantly central African origin. Because L3g3 is present in central and southwestern Africa, it might be expected that L3g3 sequences will also be found in future surveys of American populations of recent African descent. Thus, Brazilian types (which occur within L3g1 and L3g2) can be of either central or southwestern African origin.

We can now briefly reconstruct a plausible history of L3g (fig. 1). Both the phylogeography and the time depth (TMRCA, 61,800 years ago ± 17,700 years) of L3g clearly testify to its eastern African origin. Indeed, the root type (16223-16293T-16311-16355-16362-16399) is found in Sudan, Uganda, and Tanzania, and L3g displays the highest divergence in Tanzania, Uganda, Kenya, and Ethiopia (with a strong founder event distinguishing the click-language isolate of the Hadza) (fig. 1). Diversity on the Atlantic coast of Africa is, by contrast, restricted to a few mtDNAs at the tips of the network. This may have been the result of interactions established after contact between southerly dispersing western and eastern Bantu speakers who spread from the Cameroon region and the Great Lakes, respectively.
Letters to the Editor

(Phillipson 1993). In the wake of this interaction, some L3g lineages may have been diffused towards the Atlantic west coast (Cameroon, Gabon, and Angola). The recent arrival of L3g on the Atlantic coast (during or subsequent to the initial Bantu dispersals) likely explains its low diversity in this region, in contrast with the high diversity in eastern Africa (e.g., a single L3g2 type accounts for most (~57%) of the central African L3g mtDNAs). Long-term networks established between central and southwestern Africa after the initial long, gradual, and intermittent western Bantu expansion (Vansina 1995) would have contributed to its subsequent diffusion. The Bantu expansion would also explain the distribution of other central African haplogroups (e.g., L1c) and the lack of strong genetic drift in southwestern Africa (which is detected in the southeast in some Bantu lineages [Salas et al. 2002]). More recently, these haplogroups would have been carried to America during the slave-trade period.

From this view, we can safely rule out the Atlantic coast of western Africa as an important source for American L3g, since this haplogroup has not been detected at present in a large sample (>1,200 mtDNAs) that includes individuals from, among other places, Cabo Verde, Senegal, Sierra Leone, and Nigeria. Some diffusion into northern Africa (Egypt, with signatures in Sudan and Nubia) as well as into the Middle East (Syria, Israel, and Palestine) has been detected, probably reflecting the haplogroup’s greater antiquity in eastern Africa.

In conclusion, we can now extend the putative area of origin of the American L3g to the Atlantic fringe that runs from Cameroon to Angola and can probably rule out a direct eastern African origin. The latter surmise also agrees with historical documentation. Important regions, however, remain uncharacterized, such as the Congo basin and the Central African Republic.

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Beckwith-Wiedemann Syndrome and IVF: A Case-Control Study

To the Editor:

A recent series of observations has suggested a link between in vitro fertilization (IVF) and imprinting disorders, such as Beckwith-Wiedemann syndrome (BWS [MIM 130650]) and Angelman syndrome (MIM 105830). BWS is a model imprinting disorder and is

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Table 1

<table>
<thead>
<tr>
<th>Clinical Feature</th>
<th>Finding in Patient</th>
</tr>
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<tbody>
<tr>
<td>Intracytoplasmic sperm injection</td>
<td>No</td>
</tr>
<tr>
<td>Frozen embryo</td>
<td>No</td>
</tr>
<tr>
<td>Day of transfer</td>
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</tr>
<tr>
<td>Sex</td>
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</tr>
<tr>
<td>Gestation (wk)</td>
<td>40</td>
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<td>Macrosomia</td>
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<td>Hypoglycemia</td>
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<tr>
<td>Macroglossia</td>
<td>Yes</td>
</tr>
<tr>
<td>Ear anomalies</td>
<td>Yes</td>
</tr>
<tr>
<td>Abdominal-wall defects</td>
<td>Exomphalos</td>
</tr>
<tr>
<td>Hemihypertrophy</td>
<td>No</td>
</tr>
<tr>
<td>Isolated loss of methylation at KVDMR1/LIT1</td>
<td>Yes, Not performed</td>
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</tbody>
</table>

characterized by prenatal and/or postnatal overgrowth, macroglossia, abdominal-wall defects, neonatal hypoglycemia, hemihypertrophy, ear abnormalities, and an increased risk of embryonal tumors (DeBaun et al. 2002). An analysis of BWS registries from three centers has shown the proportion of individuals with BWS conceived using IVF to be 3/65 (DeBaun et al. 2003), 6/149 (Maher et al. 2003), and 6/149 (Gicquel et al. 2003). These data suggest that ∼4% of individuals with BWS are conceived using IVF, a figure greater than the generally accepted usage of IVF in these centers. Further interpretation of these results has been limited because of a reliance by these studies on case records and questionnaire data to determine the method of conception in BWS cases, a lack of the use of appropriate controls, and a statistical significance that was either borderline (Gicquel et al. 2003; Maher et al. 2003) or not mentioned (DeBaun et al. 2003). A recent review of the epidemiology and molecular biology behind these and other related studies has highlighted the need for case-control studies in this area (Niemitz and Feinberg 2004).

We report here the results of what we believe is the first case-control study done to test the null hypothesis that there is no difference between the rate of IVF in BWS cases and that in non-BWS controls, in an Australian population.

The present study was possible because the State of Victoria, Australia, is serviced by a single clinical genetics service and laboratory providing molecular tests for BWS. This allowed complete ascertainment of children born in Victoria between 1983 and 2003 and diagnosed with BWS by a clinical geneticist. Only cases meeting the DeBaun criteria (DeBaun and Tucker 1998) were included in this study. Appropriate controls were obtained using data from the Victorian Perinatal Data Collection Unit, which registers all births of ≥19-wk gestation. For each BWS case, four live-born controls were randomly selected from babies born within 1 mo of that case, in which parity was 1 and the maternal age was within 1 year of the risk-set case. Manual record linkage was then used to determine if the BWS cases and the controls were recorded in the databases of the providers of IVF services in Victoria, with the use of maternal names and the dates of birth of mothers and babies. Ethics approval was obtained from all sites providing data. Statistical significance of differences in proportions between groups was assessed using Epi Info, with results expressed as odds ratios (ORs) and as Fisher’s-exact-test two-sided P values to account for cell sizes <5.

Among ∼1,316,500 live births in Victoria between 1983 and 2003 (2003 data were estimated, as they were known to be very similar to 2002 data), 37 cases of BWS were detected, giving an overall BWS prevalence of ∼1/35,580 live births for this period. The average maternal age for BWS cases was 27.0 years. Record linkage of the 37 BWS cases and 148 matched controls identified IVF as the method of conception in 4 BWS cases (10.81%) and in 1 control (0.67%), giving an OR of 17.8 (95% CI 1.8–432.9), and Fisher’s-exact-test two-sided P = .006. The clinical and molecular features of the four patients with BWS conceived using IVF are listed in table 1, and the reasons for the use of IVF were varied (two unexplained infertility, one egg donation, and one oligospermia). Our results indicate that if a child has BWS, the odds that the child was conceived using IVF is ∼18 times greater than that for a child without BWS, although the magnitude of this OR should be cautiously interpreted, given the wide CI. During the study period (1983–2003), 14,894 babies were born as a result of an IVF procedure (excluding gamete intrafallopian transfer). Using our population-based data, we can then estimate the absolute risk of having a live-born baby with BWS when IVF is used as the means of conception to be 4/14,894.
This study demonstrates that children conceived by IVF are significantly more likely to have BWS, compared with children conceived naturally. Our study design with a control group matched by maternal age has ensured that the rate of IVF procedures in the control (non-BWS) population is accurate for the entire study period, which encompasses a time from infrequent use of IVF (0.2% of pregnancies in 1983) to more frequent use (3% in 2003). We can quantify, for the first time, the risk of BWS in our IVF population as ~1/4,000, or 9 times greater than in the general population. The mechanisms underlying this increased risk remain unclear, but this study and previous studies (DeBaun et al. 2003; Gicquel et al. 2003; Maher et al. 2003) have shown that patients with BWS conceived by IVF consistently show isolated hypomethylation at the maternal KVDMR1/LIT1 locus at 11p15.5. By comparison, this molecular mechanism is observed in only 46% of our overall BWS population, with the remainder of BWS cases resulting from uniparental disomy of chromosome 11 (16%), biparental methylation of H19DMR (7%), or an unidentified mutation (31%). The preponderance of BWS cases conceived by IVF that show hypomethylation of maternal KVDMR1/LIT1 suggests that collection of in vitro cultures might disturb methylation in the oocyte or early embryo, predisposing to maternal allele demethylation.

The fact that the overall risk of BWS in children conceived using IVF remains low and that BWS is, in most cases, associated with a good long-term outcome makes it unlikely that this finding will deter couples from using IVF. Nor does it seem necessary to offer prenatal diagnosis for BWS to couples undergoing IVF. Questions remain, however, about potential effects of IVF on other regions of the genome that are subject to epigenetic regulation. In this context, the observation of a possible association between IVF and Angelman syndrome, another disorder resulting from hypomethylation of the maternal genome, is of some concern (Cox et al. 2002; Orstavik et al. 2003). Although long-term follow-up data of children conceived by IVF are generally reassuring, it remains possible that alterations in genomic imprinting might have other unrecognized health implications for children and adults who were conceived by IVF. Our data reinforce the need for long-term follow-up studies of children conceived by IVF.

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Electronic-Database Information
The URL for data presented herein is as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/omim/ (for BWS and Angelman syndrome)

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