

Mitochondrial, Y-chromosomal and Autosomal Variation in Mbenzele Pygmies from the Central African Republic

Paolo Anagnostou^{1,2}, Valentina Coia¹, Gabriella Spedini¹ and Giovanni Destro-Bisol^{1,3}

¹ Department of Animal and Human Biology, University »La Sapienza«, Rome, Italy

² Department of Evolutionary and Experimental Biology, University of Bologna, Bologna, Italy

³ Italian Institute of Anthropology

ABSTRACT

In this paper, we carry out a combined analysis of autosomal (ten microsatellites and an Alu insertion), mitochondrial (HVR-1 sequence, 360 nucleotides) and Y-chromosomal (seven microsatellites) variation in the Mbenzele Pygmies from the Central African Republic. This study focuses on two important questions concerning the admixture and origin of African Pygmies. Ethnographic observations suggest a sex-biased gene flow between the Bantus and Pygmies, an issue which could be clarified through genetic analyses may shed light. A study of intrapopulation variation of mtDNA and Y-chromosome produces results in accordance with the hypothesized matrimonial behaviour. In fact, while shared mitochondrial haplotypes belonging to the L1c5 (or L1c1a1 clade) sub-haplogroup provides evidence of a Pygmy-to-Bantu female biased gene flow, a male biased gene flow from Bantu to Pygmies is supported by the distribution of the Y-chromosomes bearing M2 mutation. The second part of our study regards the question of the genetic relationships between Western and Eastern Pygmies. Our results favour the pre-Bantu hypothesis which suggests that the two Pygmy groups separated in ancient times (at least 18,000 years ago), whereas they do not support the recent divergence and differential admixture hypothesis which posits their separation as a consequence of the Bantu expansion (2,000–3,000 years ago).

Key words: Mbenzele Pygmies, mtDNA, Y-chromosome, autosomes, microsatellites

Introduction

There are two main reasons why African Pygmies have always attracted the interest of anthropologists. Firstly, they may be regarded as one of the few remaining hunter-gatherer groups that have yet to have complete acculturation¹. Secondly, they are characterized by a very small stature and other physical features, which probably reflect their adaptation to a tropical forest environment or retention of archaic traits².

African Pygmies have received particular attention from physical Anthropologists since the beginning of the nineteenth century³. Starting in the 60s, they have been the subject of several surveys of classical genetic polymorphisms. These studies revealed the genetic divergence of Pygmies from other African populations, and also suggested that Pygmies, together with San, are the most direct descendants of »proto-Africans«⁴.

More light has been cast on the genetic structure of this important population since the introduction of new techniques, which analyse genetic variability at DNA level. Chen et al. (1995)⁵ analysed restriction-site variation of mtDNA in several African populations. These authors observed that the mitochondrial DNA (mtDNA) sequences from the Biaka, Western Pygmies from the Central African Republic and the Eastern Mbuti Pygmies from Zaire, together with those from other sub-Saharan, belong to the most ancient of all human continent-specific haplogroups (haplogroup L). Watson et al. (1996, 1997)^{6,7} divided all African mtDNA lineages into 5 major clusters using a phylogenetic approach. Four clusters showed a starlike phylogeny indicative of a demographic expansion (L1a, L1b, L2 and L3) and coalesced between 19,000 and 77,000 years ago. The fifth (L1i) was structured in iso-

lated lineages coalescing 111,000 years ago – the likely outcome of remote expansion events across Africa. Most of the Biaka sequences (13 out of 17) were assigned to the L1i cluster, whereas almost all Mbuti lineages (12 out of 13) were equally divided between the L1a and L2 clusters. Using both the HVR-1 and 2, Batini et al. (2007)⁸ provided a new L1c phylogeny encompassing some Pygmy-specific clades (L1c1a, L1c4 and L1c5 respectively L1c1a2, L1c4 and L1c1a1a1 based on Behar et al. 2008⁹). Combining molecular data together with archaeological and paleoclimatological evidence, they hypothesized that the ancestors of Bantu and Western Pygmies separated between 60 and 30 kya. A subsequent study by Quintana-Murci et al. (2008)¹⁰ used a whole genome sequencing approach and refined the L1c phylogenetic structure, proposing that the ancestors of contemporary Pygmies started to diverge initially from an ancestral Central African population no more than 70 kya.

Calafell et al. (1998)¹¹ analysed variation at 45 microsatellite loci in numerous populations worldwide. Mbuti and Biaka Pygmies showed the highest heterozygosity, mean number of alleles and mean number of private alleles, which is a finding consistent with the idea that »Out of Africa« spread a subset of the variation generated in that continent into Asia and Europe. More recently, Watkins et al. (2003)¹² analysed 100 Alu insertions in a dataset containing numerous African populations and the two Pygmy groups were found to cluster separately, with an evident separation also at individual level. Further evidence of genetic differentiation among the two Pygmy groups has been reported by Tishkoff et al. (2009)¹³ using a broad panel of microsatellites and indels, but apparently undetected by an extensive genome-wide study of single nucleotide polymorphisms (Li et al., 2008)¹⁴.

Aims

In this paper, we reanalyse published data from mtDNA (HVR-1 sequences)^{8,15}, Y-chromosomal (seven microsatellites)¹⁶ and autosomal (ten microsatellites and an Alu insertion)^{17,18} variation in the Mbenzele, a Western pygmy group from the Central African Republic, to cast new light on two important questions concerning African Pygmies. Previously published studies of genetic variation in the Mbenzele Pygmies regarded ten autosomal microsatellites¹⁷, variation at protein coding loci¹⁹, Y-chromosome microsatellite and SNPs²⁰, and mitochondrial variation^{8,15}. Furthermore, the Mbenzele Pygmies have been studied in world-wide studies of genetic variation of entire mtDNA²¹ and X-chromosome sequencing²².

Ethnographic observations point towards the existence of an asymmetric gene flow between Bantus and Pygmies but, unfortunately, no direct genetic evidence is available to test this. According to Cavalli-Sforza (1986)⁴, there are five sources of gene flow between Bantu farmers and Pygmies: (i) admixture that occurred at the beginning of the contact between farmers and Pygmies, probably facilitated by social and economic inequalities between the two groups; (ii) marriages between a Pygmy

female and Bantu male favoured by the low bride price that the husband pay; (iii) children born from extramarital relationships between Pygmy females and Bantu males; (iv) adoption of children; (v) return to the Pygmy society of Pygmy women and of their children after the divorce from Bantu males. We test the congruence between the above observations and genetic variation of Western Pygmies by using polymorphisms of autosomal loci, mtDNA and of the non-recombining portion of the Y-chromosome.

The issue of evolutionary relationships between Eastern and Western Pygmies is an important test case to establish whether the sharing of complex traits with a probable adaptive meaning is necessarily related to a common evolution or it might result from a separate evolutionary history. The first option is sustained in the »Recent Divergence« hypothesis according to which Western Pygmies are the result of hybridization between the ancestors of present-day Eastern Pygmies and farmers who penetrated the equatorial belt in the course of the Bantu expansion, around 2–3 kiloyears ago (kya). The occupation of the rainforests and deforestation by Bantu farmers progressively reduced the Pygmies' habitat, eventually leading to a biological separation of Pygmy groups settled in the eastern and western sides of the tropical rainforest. The Eastern Pygmies remained relatively isolated from neighbouring populations, whereas Western Pygmies admixed with Bantus to a considerable extent²³. The combined effect of differential admixture and genetic drift could account for the genetic differences observed between the two Pygmy groups. We have named this view »recent divergence and differential admixture« (RDDA). The alternative explanation is that the separation between Eastern and Western Pygmies is independent from the Bantu expansion, and occurred before 2–3 kya. Hiernaux (1977)²⁴ suggested that the adaptation of Pygmy groups to the humid forest climate occurred through distinct evolutionary processes. This hypothesis seems to imply that the separation between Pygmies predates the Bantu expansion considerably. Unfortunately, it cannot be compared with the RDDA hypothesis as the author gave no indication of the time of population splits. The results of archaeological research in the tropical rainforest, [see Mercader (2002)²⁵ and references reported therein], make it possible to define a »pre-Bantu divergence« hypothesis in more detail. These studies indicate that the history of human occupation of African rainforests was more ancient and complex than generally thought. In fact, there is evidence of early human settlements dating back to the Early Stone Age (ESA) for the C.A.R., Congo, Uganda and Zambia and the Middle Stone Age (MSA) for an even wider area which extends from the Ivory Coast to the west and north, Kenya to the East and Zambia to the South. The roots of Pygmies should be, however, searched for in the Late Stone Age. In fact, in this period there was a considerable increase in site density relative to MSA and ESA throughout Central Africa. Of particular importance are the sites of the tropical forest of Ituri, since the last 18 kya, which suggests a presence of a hunter-gatherer community in the North-

east Congo Basin predating the arrival of farming in the same region by many millennia. Assuming that these sites are the remains of the nucleus from which present-day Eastern Pygmies evolved after the separation from the Pygmy branch, the 18 kya date could, therefore, be regarded as the minimum date for the divergence between the two Pygmy groups. We have named this view »pre-Bantu divergence« (PBD). The two competing hypotheses are tested both through the qualitative (median network of mtDNA) analysis of the association between a microsatellite (CD4) and an ALU linked polymorphism) and quantitatively by applying dating methods to mtDNA and autosomal results.

The Population

The Mbenzele belong to the western cluster of African Pygmies²⁶ which includes Pygmies from the Central African Republic, Congo Brazza and Southern Cameroon. The Mbenzele, like the Biaka Pygmies studied by Cavalli-Sforza^{4,14} and used in most studies cited above, belong to the Aka sub-group. Today, there are about 2,000 Mbenzele who are mostly settled in the southwestern region of the Central African Republic. Blood samples were collected from apparently healthy and unrelated individuals in the village of Mbelemboke (3.20N; 16.10E), in the southwestern region of the Central African Republic (see figure 1).

The height of the Mbenzele Pygmies (144.6±0.51 cm for females and 151±0.85 cm for males) is close to that of Biaka Pygmies (145.0±1.18 for females and 152.7±1.35 cm for males). By contrast, the Mbenzele are significantly different (t test, p=0.0001) from Mbuti Pygmies (136.0±0.72 cm for females and 144.4±0.54 cm for males) and C.A.R. Bantu farmers (154.4±0.72 for females and 161.2±1.28 for males) (Spedini and Capucci, unpublished data; see Danubio and Sanna²⁷ for a review).



Fig. 1. Geographic location of the Western Pygmies (Mbenzele and Biaka), Eastern Pygmies (Mbuti) and Ewondo Bantus.

Apart from the genetic studies mentioned above^{8,21,22,19}, the Mbenzele Pygmies have been characterized for protein loci²⁰ and in world-wide surveys of genetic variation of entire mtDNA²³ and X-chromosome sequencing²⁴.

Materials and Methods

Data analyses

The Arlequin software²⁸ was used to compute classical and molecular measures of intra-population diversity (see also Castri et al.²⁹). We performed a network analysis using the median algorithm of Bandelt et al. (1995)³⁰ in order to reconstruct mtDNA genealogy. This approach also indicates alternative events in the form of reticulations, which highlight alternative evolutionary pathways. To obtain estimates of divergence times between populations under a coalescent model, we used the method proposed by Nielsen and Wakeley (2001)³¹, assuming a finite-site model³². In calculations of divergence times, we used two different mutation rates (1.8x10⁻⁷ per site per year; 4.20x10⁻⁸ per site per year)³³, a generation time of 25 years and an initial effective size (Ne) of 5000 females. CD4 Alu haplotype frequencies were inferred by maximum likelihood using the Arlequin software²⁸. The dates of population splits were calculated using autosomal microsatellite data following the method of Goldstein et al (1995)³⁴. We used $\mu=3.73 \times 10^{-4}$ and $\mu=2.80 \times 10^{-4}$ and generation times of 20 and 25 years to estimate a range of values³⁵.

Results and Discussion

Variation of mtDNA and Y-chromosomal polymorphisms and sex biased gene flow

The matrimonial behaviour of African Pygmies as described by the above mentioned ethnographic observations expected to shape their gene pool in two ways. Firstly, female and male lineages of Pygmies should undergo different microevolutionary pressures: genetic drift should be the prevailing force for mtDNA evolution, while a higher effect of gene flow would be expected for Y-chromosome markers. Furthermore, as more specific effects, we would expect a male biased gene flow from Bantu to Pygmies and a female biased gene flow of Pygmy origin to Bantus.

Intra-population variation

The first expectation may be checked by comparing intrapopulation diversity of mtDNA and the Y-chromosome in the Mbenzele to those reported for other sub-Saharan populations. A comparison among the sub-Saharan populations analysed for both mtDNA and Y-chromosome is reported in Figure 2.

Concerning mtDNA, the Mbenzele show the lowest level of within-group diversity considering both haplotype diversity (h) and mean number of pairwise comparisons (k) (h=0.805±0.037; k=4.917±2.43). On the other hand, haplotype diversity (0.966±0.017) and the mean

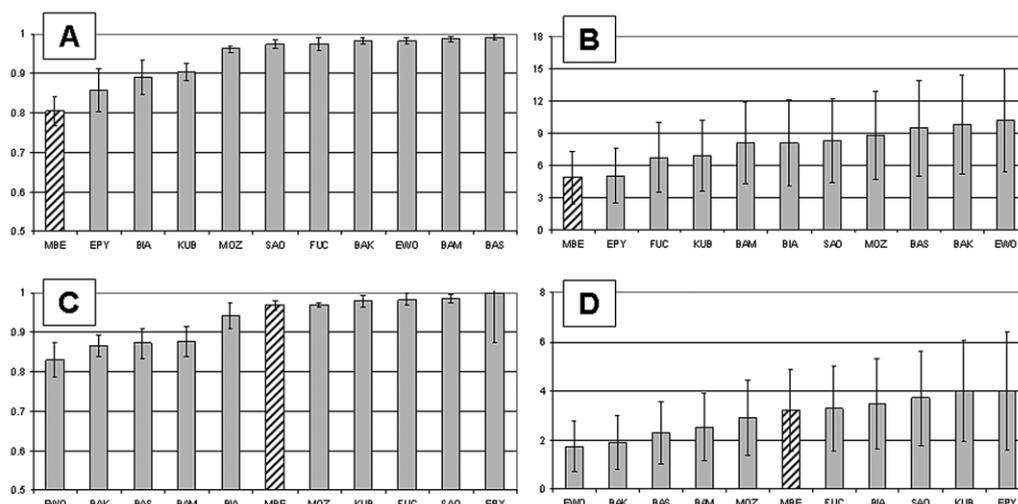


Fig. 2 Haplotype diversity (frames A and C) and mean number of pairwise comparisons (frames B and D) and relative standard errors in some sub-Saharan populations. Frames A and B refer to the hypervariable region-1 of mtDNA, whereas the frames C and D refer to the Y-chromosome microsatellite haplotypes built using loci DYS19, 389I, 390, 391, 392 and 393. Population abbreviations were obtained using the first three letters of each population: BAM, Bamileke [Cameroon: Destro-Bisol et al. (2004)¹⁵; Caglià et al. (2003)⁵⁰]; BAS, Bassa (Cameroon: unpublished data); BIA, Biaka Pygmies [Central African Republic: Vigilant et al. (1991)³³; Pritchard et al. (1999)³⁶]; EWO, Ewondo [Cameroon: Destro-Bisol et al. (2004)¹⁵; Caglià et al. (2003)⁵⁰]; FUC, Fulbe from Cameroon (Cameroon: unpublished data); KUB, Kung [Botswana: Vigilant et al. (1991)³³; Pritchard et al. (1999)³⁶]; MBE, Mbenzele Pygmies from C.A.R., [Central African Republic: Destro-Bisol et al. (2004)¹⁵]; EPY, Mbuti Pygmies [Zaire: Vigilant et al. (1991)³³; Pritchard et al. (1999)³⁶]; MOZ, Mozambicans [Salas et al. (2003)⁵¹; Pereira et al. (2001)⁵²]; SAO, Sao Tomeans [Equatorial Guinea: Mateu et al. (1997)⁵³; Corte Real et al. (2000)⁵⁴].

number of pairwise differences (3.246 ± 1.704) calculated for Y-chromosomes are in the mid of the distribution. Another independent Western Pygmy group, the Biaka shows a similar trend³⁶.

Analysis of shared haplogroups and haplotypes

Comparing the HVR-1 sequences to those reported for other sub-Saharan (see Destro-Bisol et al., 2004, for the dataset used for comparison), we found that 3 sequences are shared between our population and Bantus, i.e. the Ewondo who live in South Cameroon. One of these sequences belongs to the L1c1a sub-haplogroup (corresponding to the haplotype WP-M 162 in Destro-Bisol et al., (2004)¹⁵, while the remaining two sequences belong to the L1c5 sub-haplogroup (haplotypes WP-M 162 and WP-M 198). All these sequences reach a higher frequency in the Mbenzele than in the Ewondo (Destro-Bisol et al. 2004)¹⁵. There are four reasons which indicate that the finding of L1c5 sequences is evidence of Pygmy-to-Bantu gene flow. Firstly, all but one of the L1c5 haplotypes were found in the Western Pygmies. Secondly, the Western Pygmies display the highest L1c5 variation, considering both the haplotype diversity and the mean number of pairwise comparisons¹⁵. Thirdly, the L1c1a sequence most closely related to L1c5 is present at a particularly high frequency among the Western Pygmies. Of particular interest is the fact that Eastern Pygmies lack not only L1c5, but also L1c1a, the haplogroup from which L1c5 probably originated. Fourthly and finally, the absence of L1c5 in other sub-Saharan

populations of our large database makes it unlikely that its presence in Western Pygmies could be simply due to the maintenance of ancestral characteristics which have become diluted elsewhere by more recent demographic expansions.

The Bantu-to-Pygmy gene flow of paternally inherited characters is supported by the distribution of the haplogroup E1b1a. It carries the M2 mutation (also referred to as DYS271) which has been associated with the expansion of Bantu speaking populations from central western towards southern Africa^{37–39}. The estimated frequency of the haplogroup E1b1a among the Mbenzele Pygmies (0.609) is similar to that observed among the Biaka (0.650; recalculated from Cruciani et al., 2002³⁹). This suggests that the Bantu contribution to the male-specific gene pool of these two Western Pygmy populations is comparable. Concerning microsatellite haplotypes, it is worth noting that a percentage of 23.9 of Mbenzele haplotypes were shared by 35 percent of Biaka haplotypes (Pritchard et al., 1999)³⁶, which represents the highest level of haplotype sharing for the Mbenzele¹⁵. Five out of seven haplotypes shared between the Mbenzele and Biaka Pygmies belong to haplogroup E1b1a, commonly thought to be associated with the Bantu expansion⁴⁰.

Despite this feature of E1b1a, we cannot exclude that some of the observed haplotype sharing could be due to genetic admixture between Western Pygmies. However, it should be taken into account that recent autosomal data suggests that marginalization following Bantu ex-

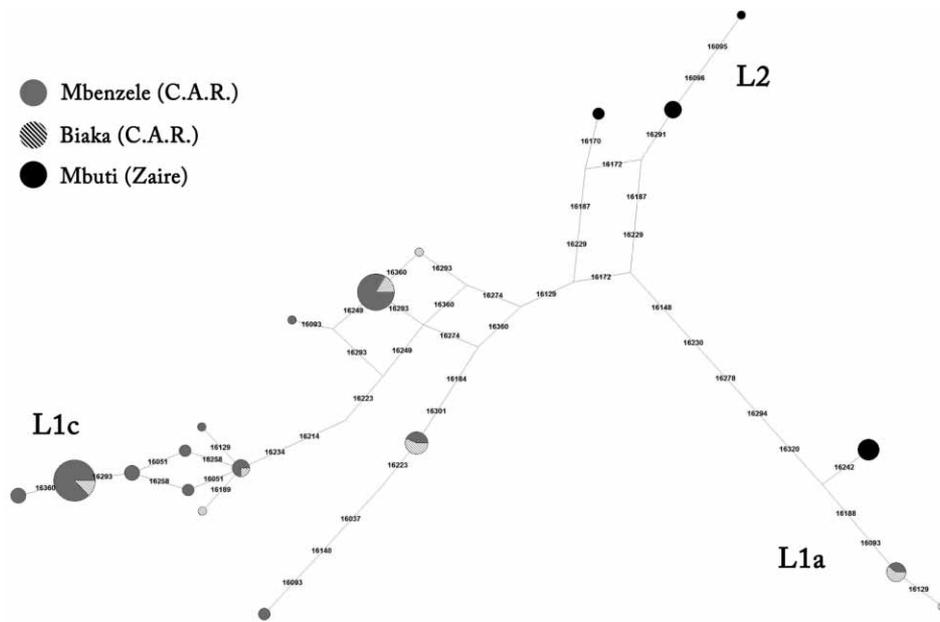


Fig. 3. Reduced median network (Bandelt et al. 1995³⁰) showing the relationships among mtDNA lineages of Biaka (17 individuals; Vigilant et al. 1991³³), Mbenzele (this study; 31 individuals) and Mbuti Pygmies (13 individuals; Vigilant et al. 1991³³). It was not possible to include seven further Mbuti sequences of Vigilant et al. (1991)³³ in the analysis due to the presence of missing positions. HVR1 sequences are represented by circles whose area is proportional to their population frequency. The Pygmy group to which sequences belong are indicated by means of different filling patterns (see legend inside the figure). The node marked by an asterisk corresponds to the sequence, which represents the »putative root« for the bulk of African mitochondrial haplotypes. Substitutions along the network refer to this sequence which differs from the CRS due to a transition at np. 16126, 16187, 16223, 16278, and 16311. Nucleotide position numbering follows Anderson et al. (1981)⁵⁵ (with link labels omitting the 16 prefix). Transversions are marked by an underlined capital letter and recurrent mutations by small letters (e.g. 51a or 93b). Cluster designation follows the classification of Salas et al. (2002)⁵¹. A filled square indicates the node at which the Neanderthal mitochondrial sequence (Krings et al. 1998)⁵⁶ branches off.

pansion led to a decreased mobility and intermarriages, thus lowering Pygmy to Pygmy gene flow (Verdu et al., 2009)⁴¹.

The Origin of African Pygmies

One inherent difficulty when estimating the time since separation between small sized populations is in the fact that their small long-term effective size enhances the confounding effect of genetic drift. In order to minimize this risk, we tested the RDDA and PBD hypotheses using different approaches and distinct genetic systems.

Mitochondrial DNA

As a preliminary step to the calculation of the time since separation between the two Pygmy groups, we built a reduced median network of HVR-1 sequences (from np 16024 to 16384) using the method devised by Bandelt et al. (1995)³⁰. In addition to a selection of Mbenzele sequences (belonging to 31 individuals), we considered a further 30 Pygmy individuals, 17 from Biaka (8 different sequences) of C.A.R. and 13 from Mbuti (5 different sequences)³³. The network shown in figure 3 is characterized by the presence of lineages belonging to three of the eight groups of sequences described by Watson et al.

(1997)⁷. Most of the Pygmy individuals (55 out of 61; 16 different sequences) belong to the L1a and L1c haplogroups. Most Mbenzele (30 out of 31 individuals analyzed: 10 different sequences) and Biaka (13/17:6 different sequences) are scattered across three main clusters that fall within the L1c haplogroup and derived sub-haplogroups L1c1a and L1c5⁸. These clusters are easily distinguishable from each other since at least four mutational steps separate them. Note that these sequences

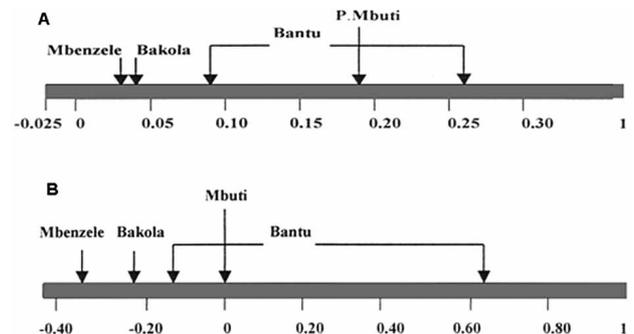


Fig. 4. Association between the CD4 alleles »6« (frame A) and »11« (frame B) and the ALU deletion on the short arm of chromosome 12, as measured by the »δ« parameter⁵⁷ in sub-Saharan populations. The data for comparison are from Tishkoff et al. (1996)⁵⁸.

belong to the L1i haplogroup according to the old nomenclature of Watson et al. (1997)⁷. According to the above-mentioned authors this haplogroup includes heterogeneous lineages which are phylogenetically and geographically isolated from the others and are probably the relics of ancient expansion (estimated at about 111,000 years ago) across Africa. Two Biaka (4 individuals), 1 Mbenzele (1 individual) and 1 Mbuti (6 individuals) lineages fall within L1a. According to Watson et al. (1997)⁷ the L1a group of sequences coalesces about 52,000 years ago and is found more often in Eastern, central and southern Africa. However, despite the fact that they can be found in the same cluster, the differentiation between Mbenzele and Biaka sequences and Mbuti sequences is again evident. In fact, sequences of C.A.R. and Zaire Pygmies differ in 4 nucleotide positions. The remaining 3 Mbuti lineages (7 individuals) fall into a cluster in the L2 group which dates back to 56,000 years ago and is widespread in the African continent (Watson et al. 1997)⁷. Despite their common origin, the genetic distance between Mbenzele and Biaka is considerable, and higher than most of those calculated for pairs of Bantu populations (data not shown). This is not surprising since the two Pygmy groups are very distinct and have probably undergone little, if any, genetic admixture on the female side. Thus, genetic drift may account for the observed differentiation and the comparison between the Mbenzele and Biaka may turn out to be useful in understanding how this microevolutionary force might increase genetic distances. It may be observed from the network that Mbenzele and Biaka mtDNA lineages cluster in three haplogroups with a low internal diversity but are separated from each other by a large number of mutational steps. Thus, most pairwise comparisons are between very diverse sequences. Consequently, genetic-distance values are higher than the ones obtained for pairs of populations with a smaller proportion of shared sequences but with less diverging sequences. By contrast, there is no haplotype sharing between Mbuti and the two groups from C.A.R., and almost all Mbuti lineages fall into clusters, which are very different from the Mbenzele and Biaka. Therefore, if the example of Mbenzele and Biaka is taken as a model of how genetic drift might lead closely related populations to diverge, it seems that the differentiation between the Mbuti and the two C.A.R. groups cannot be simply explained by genetic drift. Furthermore, one interesting conclusion can be drawn considering the differences between Pygmy mtDNA lineages by taking into consideration the phylogenetic analyses of Watson et al. (1997)⁷. While most of Mbuti mtDNA sequences belong to clusters (L1a and L2) which originated from expansion events, Pygmies from the C.A.R. show a relatively high frequency of more ancient and geographically restricted sequences.

In order to estimate the time since divergence between Western and Eastern Pygmies, we applied the method of Nielsen and Wakeley (2001)³¹ which allows us to obtain estimates of divergence times between populations under a coalescent model. To reduce the risk of

overestimating the time of split between populations, the parameter t (number of generations since population split) was calculated according to the formula $t=2NeT$ using the bounds of the 95% CI estimates of the parameters Ne (the effective population size) and T (the divergence time divided by twice the effective population size). The 95% CIs of both the splits between Biaka and Mbuti (18–1140 kya⁴²; 79–4888 kya³³) and between Mbenzele and Mbuti (25–828 kya⁴²; 107–3549 kya³³) are much larger than 3 kya. Therefore, applying a quantitative approach produces results which are congruent with the PBD and unable to support the RDDA hypothesis.

CD4/Alu association

According to the RDDA hypothesis, the Western Pygmies are a hybrid population between the Mbuti-like ancestors and Bantus. It follows that the gene frequency of Western Pygmies should fall into the range of those observed in the two parental populations. Gene frequencies of numerous polymorphisms at protein level in Western Pygmies fit those expected for a hybrid population between Eastern Pygmies and Bantu farmers^{43,44}. However, polymorphisms of autosomal loci have the intrinsic limit that an identical electrophoretic mobility or immuno-reactivity does not necessarily indicate identity by descent but only identity by state. We tested the compliance of gene frequencies of Eastern and Western Pygmies and Bantus by analyzing the association between the CD4 microsatellite and an Alu linked polymorphism⁴⁵. This test is a valid alternative to the use of protein loci since in we combined a phylogenetically stable locus (ALU) with another one which is more variable and subjected to a high mutation rate (CD4 microsatellite). The results do not support the RDDA hypothesis (Figure 4). In fact, the frequency of association between the ALU deletion and the most representative microsatellite alleles (composed by 6 and 11 repeat units) fall outside of the range of the values for the Eastern Pygmies and the Bantus. The same conclusion is achieved when considering another Western Pygmy population now being studied in our lab, the Bakola from Cameroon (Destro-Bisol et al. unpublished data).

Autosomal microsatellites

Goldstein et al. (1995)³⁴ in their microsatellite loci studies postulated that under a stepwise mutation model, the time periods since population splits are proportional to the squared difference in average allele lengths ($\delta\mu^2$) between populations. Applying this method to data published for African Pygmies^{17,46} we obtained a separation time of 29,115–48,382 years for Mbenzele and Biaka and 21,513–35,223 for Biaka and Mbuti. Given that we could use only six autosomal loci and some populations have been analysed for only a low number of chromosomes, the values are inevitably approximate. Furthermore, the used method assumes that gene flow is negligible and that genetic drift contributes less than mutation to population divergence. However, despite these caveats, we believe that our results merit discussion. Due to

its high density of vegetation, the large area which separates the Ituri Forest and the territories of Western Pygmies (~1500 km measured as air distance) is very difficult to cross without the help of an adequate technology, such as the iron metallurgy practised by Bantu peoples but unknown to African Pygmies⁴⁷. Therefore, the level of gene flow between the two Pygmy groups is expected to have been very low or absent. Concerning the problem of genetic drift, it is worth noting that no significant decrease in heterozygosity and number of alleles (these results, unpublished data on Apolipoprotein B 3' HVR in the Mbenzele, data from ref. 10 on Biaka and Mbuti Pygmies) is detected in Pygmies compared to Bantus or other worldwide populations. This suggests that no dramatic bottleneck shaped the genetic structure of autosomal loci in African Pygmy populations. Furthermore, when the data set used for the calculation of separation times was analysed by using the F_{ST} method, no genetic distance between the Biaka or Mbenzele and a non-Pygmy population exceeded those between C.A.R. and Mbuti Pygmies (data not shown). Therefore, the substantial genetic diversity between the two Pygmy groups is confirmed when we use a genetic distance method which considers genetic drift as the only agent which differentiates populations.

Final Remarks

In the first part of this paper, we carried out a test regarding the congruence between the ethnographic observations on the matrimonial behaviour of Western Pygmies and their genetic structure. Comparing the Mbenzele Pygmies to a collection of sub-Saharan populations analysed for mtDNA and Y-chromosome polymorphisms, we observed that their intrapopulation variation is at the lowest limit for the former genetic system, while it lies in the middle of the distribution for the latter. This is what we expect for a mating system in which genetic drift is the prevalent microevolutionary force for maternal lineages and gene flow for the Y-chromosome. Furthermore, the analysis of mtDNA shared haplotypes provides evidence of a Pygmy-to-Bantu gene flow, whereas the distribution of Y-chromosomal haplogroups indicates the existence of a flow in the opposite direction. All these results lead us to conclude that the genetic data is in line with the ethnographic expectations. Then, we compared

the RDDA and PBD hypotheses by using both qualitative and quantitative approaches. In the first case, the analysis of the association between the CD4 microsatellite and an Alu linked polymorphism do not support a model in which the Western Pygmies are a hybrid between Eastern Pygmies and Bantus, as implied by the RDDA hypothesis. Furthermore, the median network analysis of mtDNA variation suggests that the high genetic distances between the Mbuti and the two CAR groups cannot be simply explained by genetic drift. The quantitative analyses based on both mtDNA and autosomal data provide results which are congruent with the PBD hypothesis, since they suggest that the separation between Western and Eastern Pygmies occurred earlier than 18,000 years ago, but fail to support the RDDA hypothesis which posits the population split as a consequence of the Bantu expansion (2–3000 years ago). Our results on autosomal variation provide independent support to the hypothesis of an ancient separation between Eastern and Western Pygmies put forward by Destro-Bisol et al. (2004)¹⁵ on the basis of mtDNA variation which has been recently re-proposed by Patin et al. (2009)⁴⁸ using resequencing data from 24 independent noncoding genomic regions.

We intend to further develop this study by looking at variation among Pygmy populations, while taking into consideration highly informative loci that have yet to be studied in this regard (e.g. ALU loci and Y-chromosomal polymorphisms). Another important step will be to exploit the opportunities offered by this research project in terms of communication and promotion of Science, by an extensive evaluation of the bio-ethical implications and the didactic outputs⁴⁹.

Acknowledgements

This research was funded by the University of Rome »La Sapienza« and the Ministry of University and scientific research. We would like to thank the team of the Dispensary of Santé Intégrée, Catholic Mission of Mbeleboké Equipe du Dispensaire de Santé Intégrée, Mission Catholique du Mbeleboké (Central African Republic) and the blood donors, whose availability made this study possible. We dedicate this work to our dear friend and colleague Michele Belledi, who also collaborated on this project. He will be sadly missed.

REFERENCES

1. KENT S (Ed) *Ethnicity, Hunter-Gatherers and the 'Other': association or assimilation in Africa* (Smithsonian Institution Press, Washington and London, 2003). — 2. SHEA BT, BAILEY RC, *Am J Phys Anthropol*, 100 (1996) 311. — 3. CZEKANOWSKI J, *Forschunegn in Nil-Kongo-Zwischengebeit. Anthropologische Beobachtungen. Wissenschaftliche ergebnisse der Deutschen Zentral-Afrika-Expedition 1907-1908. Ethnographie-Anthropologie IV.* (Klinkhardt and Biermann, Leipzig, 1922). — 4. CAVALLI-SFORZA LL, *African Pygmies: an evaluation of the state of research.* In: CAVALLI-SFORZA LL (Ed) *African Pygmies* (Academic Press, Orlando, Florida, 1986). — 5. CHEN YS, TORRONI A, EXCOFFIER L, SANTACHIARA BENERECETTI AS, WALLACE DC, *Am J Hum Genet*, 57 (1995) 133. — 6. WATSON E, BAUER K, AMAN R, WEISS G, VON HAESLER A, PAABO S, *Am J Hum Genet*, 59 (1996) 437. — 7. WATSON E, FORSTER P, RICHARDS M, BANDEL T HJ, *Am J Hum Genet*, 61 (1997) 691. — 8. BATINI C, COIA V, BATTAGLIA C, ROCHA J, PILKINGTON MM, SPEDINI G, COMAS D, DESTRO-BISOL G, CALAFELL F, *Mol Phylogenet Evol*, 43 (2007) 635. — 9. BEHAR DM, VILLEMS R, SOODYALL H, BLUE-SMITH J, PEREIRA L, METSPALU E, SCOZZARI R, MAKKAN H, TZUR S, COMAS D, BERTRANPETIT J, QUINTANA-MURCI L, TYLER-SMITH C, WELLS RS, ROSSET S; GENOGRAPHIC CONSORTIUM, *Am J Hum Genet*, 82 (2008) 1130. — 10. QUINTANA-MURCI L, QUACH H, HARMANT C, LUCA F, MASSONNET B, PATIN

- E, SICA L, MOUGUIAMA-DAOUDA P, COMAS D, TZUR S, BALANOVSKY O, KIDD KK, KIDD JR, VAN DER VEEN L, HOMBERT JM, GESSAIN A, VERDU P, FROMENT A, BAHUCHET S, HEYER E, DAUSSET J, SALAS A, BEHAR DM, Proc Natl Acad Sci Usa, 105 (2008) 1596 — 11. CALAFELL F, SHUSTER A, SPEED WC, KIDD JR, KIDD KK, Eur J Hum Genet, 6 (1998) 38. — 12. WATKINS WS, ROGERS AR, OSTLER CT, WOODING S, BAMSHAD MJ, BRASSINGTON AM, CARROLL ML, NGUYEN SV, WALKER JA, PRASAD BV, REDDY PG, DAS PK, BATZER MA, JORDE LB, Genome Res, 13 (2003) 1607. — 13. TISHKOFF SA, FLOYD AR, FRIEDLAENDER FR, EHRET C, RANCIARO A, FROMENT A, HIRBO JB, AWOMOYI AA, BODO JM, DOUMBO O, IBRAHIM M, JUMA AT, KOTZE MJ, LEMA G, MOORE JH, MORTENSEN H, NYAMBO TB, OMAR SA, POWELL K, PRETORIUS GS, SMITH MW, THERA MA, WAMBEBE C, WEBER JL, WILLIAMS SM, Science, 324 (2009) 1035 — 14. LI JZ, ABSHER DM, TANG H, SOUTHWICK AM, CASTO AM, RAMACHANDRAN S, CANN HM, BARSH GS, FELDMAN M, CAVALLI-SFORZA LL, MYERS RM, Science, 319 (2008) 1100. — 15. DESTRO-BISOL G, COIA V, BOSCHI I, VERGINELLI F, CAGLIÀ A, PASCALI V, SPEDINI G, CALAFELL F, Am Nat, 163 (2004) 212. — 16. COIA V, CAGLIÀ A, ARREDI B, DONATI F, SANTOS FR, PANDYA A, TAGLIOLI L, PAOLI G, PASCALI V, DESTRO-BISOL G, TYLER SMITH C, Am J Hum Biol, 16 (2004) 57. — 17. DESTRO-BISOL G, BOSCHI I, CAGLIÀ A, TOFANELLI S, PASCALI V, PAOLI G, SPEDINI G, Am J Phys Anthropol, 112 (2000) 319. — 18. DESTRO-BISOL G, MAVIGLIA R, CAGLIÀ A, BOSCHI I, SPEDINI G, PASCALI V, CLARK AG, TISHKOFF S, Hum Genet, 104 (1999) 149. — 19. COIA V, SANSONETTI B, PAOLI G, TOFANELLI S, SPEDINI G, DESTRO-BISOL G, Am J Hum Biol, 14 (2002) 9. — 20. COIA V, CAGLIÀ A, ARREDI B, DONATI F, SANTOS FR, PANDYA A, TAGLIOLI L, PAOLI G, PASCALI V, DESTRO-BISOL G., TYLER SMITH C, Am J Hum Biol, 16 (2004) 57. — 21. INGMAN M, KAESSMANN H, PAABO S, GYLLENSTEN U, Nature, 408 (2000) 708. — 22. KAESSMANN H, HEISSIG F, VON HAESELER A, PAABO S, Nat Genet, 22 (1999) 78. — 23. CAVALLI-SFORZA LL, Anthropometric data. In: CAVALLI-SFORZA LL (Ed) African Pygmies (Academic Press, Orlando, Florida, 1986). — 24. HIERNAX J, Long-term biological effects of human migration from the African Savanna to the equatorial forest: a case study of human adaptation to hot and wet climate. In: HARRISON GA (Ed) Population structure and human variation (Cambridge University Press, Cambridge, 1977). — 25. MERCADER J, Evol Anthropol, 11 (2002) 117. — 26. MURDOCK GP, Africa: its people and their culture history (McGraw-Hill, New York, 1959). — 27. DANUBIO M, SANNA E, J Anthropol Sci, 86 (2008) 91. — 28. SCHNEIDER S, KUEFFER JM, ROESSLI D, EXCOFFIER L 1997. Arlequin ver1.1: a software for population genetic data analysis. — 29. CASTRÌ L, GARAGNANI P, USELI A, PETTENER P, LUISELLI D, J Anthropol Sci, 86 (2008) 189. — 30. BANDELT HJ, FORSTER P, SYKES BC, RICHARDS MB, Genetics, 141 (1995) 743. — 31. NIELSEN R, WAKELEY J, Genetics, 158 (2001) 885. — 32. HASEGAWA M, KISHINO H, YANO T, J Mol Evol, 22 (1985) 160. — 33. VIGILANT L, STONEKING M, HARPENDING H, HAWKES K, WILSON AC, Science, 253 (1991) 1503. — 34. GOLDSTEIN DB, RUIZ-LINARES A, CAVALLI-SFORZA LL, FELDMAN MW, Proc Natl Acad Sci USA, 92 (1995) 6723. — 35. CHIKHI L, DESTRO-BISOL G, BERTORELLE G, PASCALI V, BARBUJANI G, Proc Natl Acad Sci USA, 95 (1998) 9053. — 36. PRITCHARD JK, SEIELSTAD MT, PEREZ-LEZAUN A, FELDMAN MW, Mol Biol Evol, 16 (1999) 1791. — 37. UNDERHILL PA, PASSARINO G, LIN AA, SHEN P, MIRAZON M, LAHR R, FOLEY A, OEFNER PJ, CAVALLI-SFORZA LL, Ann Hum Genet, 65 (2002) 43. — 38. FRANCALACCI P, SANNA D, J Anthropol Sci, 86 (2008) 59. — 39. CRUCIANI F, SANTOLAMAZZA P, SHEN P, MACAULAY V, MORAL P, OLCKERS A, MODIANO D, HOLMES S, DESTRO-BISOL G, COIA V, WALLACE DC, OEFNER PJ, TORRONI A, CAVALLI-SFORZA LL, SCOZZARI R, UNDERHILL PA, Am J Hum Genet, 70 (2002) 1197. — 40. BERNIELL-LEE G, CALAFELL F, BOSCH E, HEYER E, SICA L, MOUGUIAMA-DAOUDA P, VAN DER VEEN L, HOMBERT JM, QUINTANA-MURCI L, COMAS D, Mol Biol Evol, 26(2009) 1581. — 41. VERDU P, AUSTERLITZ F, ESTOUP A, VITALIS R, GEORGES M, THÈRY S, FROMENT A, LE BOMIN S, GESSAIN A, HOMBERT JM, VAN DER VEEN L, QUINTANA-MURCI L, BAHUCHET S, HEYER E, Curr Biol, 24(19) (2009) 312. — 42. SAILLARD J, FORSTER P, LYNNERUP N, BANDELT HJ, NORBY S, Am J Hum Genet, 67 (2000) 718. — 43. CAVALLI-SFORZA LL, ZONTA LA, NUZZO F, BERNINI L, DE JONG WWW, MEERA-KHAN P, RAY AK, WENT LN, SINISCALCO M, NIJENHUIS LE, VAN LOGHEM E, MODIANO G, Am J Hum Genet, 21 (1969) 252. — 44. WJISMAN LL, Estimation of genetic admixture in Pygmies. In: CAVALLI-SFORZA LL (Ed) African Pygmies (Academic Press, Orlando, Florida, 1986). — 45. DESTRO-BISOL G, BATTAGLIA C, COIA V, BATINI C & SPEDINI G, J Anthropol Sci, 84 (2006) 161. — 46. PEREZ LEZAUN A, CALAFELL F, MATEU E, COMAS D, RUIZ PACHECO R, BERTRANPETIT J, Hum Genet, 99 (1997) 1. — 47. DE MARET P, NSUKA F, Hist Africa, 4 (1977) 43. — 48. PATIN E, LAVAL G, BARREIRO LB, SALAS A, SEMINO O, SANTACHIARA-BENERECETTI S, KIDD KK, KIDD JR, VAN DER VEEN L, HOMBERT JM, GESSAIN A, FROMENT A, BAHUCHET S, HEYER E, QUINTANA-MURCI L, PLoS Genet, 5 (2009) e1000448. — 49. DESTRO BISOL G, ANAGNOSTOU P, BATINI C, BATTAGLIA C, BERTONCINI S, BOATTINI A, CACIAGLI L, CALÓ MC, CAPELLI C, CAPOCASA M, CASTRÌ L, CIANI G, COIA V, CORRIAS L, CRIVELLARO F, GHIANI ME, LUISELLI D, MELA C, MELIS A, MONTANO V, PAOLI G, SANNA E, RUFO F, SAZZINI M, TAGLIOLI L, TOFANELLI S, USELI A, VONA G, PETTENER D, J Anthropol Sci, 86 (2008) 179 — 50. CAGLIÀ A, TOFANELLI S, COIA V, BOSCHI I, PESCARMONA M, SPEDINI G, PASCALI V, PAOLI G, DESTRO-BISOL G, Hum Biol, 75 (2003) 313. — 51. SALAS A, RICHARDS M, DE LA FE T, LAREU MV, SOBRINO B, SANCHEZ-DIZ P, MACAULAY V, CARRACEDO A, Am J Hum Genet, 71 (2002) 1082. — 52. PEREIRA L, MACAULAY V, TORRONI A, SCOZZARIR, PRATA MJ, AMORIM A, Ann Hum Genet, 65 (2001) 439. — 53. MATEU E, COMAS D, CALAFELL F, PEREZ-LEZAUN A, ABADE A, BERTRANPETIT J, Ann Hum Genet, 61 (1997) 507. — 54. CORTE-REAL F, CARVALHO M, ANDRADE L, ANJOS MJ, PESTONI C, LAREU MV, CARRACEDO A, VIEITA DN, VIDE MC, Progress in Forensic Genetic 8. (Elsevier Amsterdam, 2000) — 55. ANDERSON S, BANKIER AT, BARRELL BG, DE BRUIJN MH, COULSON AR, DROUIN J, EPERON IC, NIERLICH DP, ROE BA, SANGER F, SCHREIER PH, SMITH AJ, STADEN R, YOUNG IG, Nature, 290 (1981) 457. — 56. KRINGS M, STONE A, SCHMITZ RW, KRAINITZKI H, STONEKING M, PAABO S, Cell, 90 (1997) 19. — 57. BENGTSOON BO, THOMSON G, Tissue Antigens, 18 (1997) 356. — 58. TISHKOFF SA, DIETZSCH E, SPEED W, PAKSTIS AJ, KIDD JR, CHEUNG K, BONNE-TAMIR B, SANTACHIARA-BENERECETTI AS, MORAL P, KRINGS M, Science, 271(1996) 1380.

G. Destro-Bisol

Department of Animal and Human Biology, »La Sapienza« University, P le A. Moro 500185 Roma, Italia
e-mail: destrobisol@uniroma1.it

VARIJABILNOST MITOHONDRIJSKE DNA, Y KROMOSOMA I AUTOSOMALNE DNA MBENZELE PIGMEJA IZ SREDNJOAFRIČKE REPUBLIKE

S A Ž E T A K

U ovom smo radu iznijeli kombiniranu analizu varijabilnosti autosomalne DNA (deset mikrosatelita i Alu-insercija), mitohondrijske DNA (HVS I sekvence, 360 nukleotida) i Y kromosoma (7 mikrosatelita) Mbenzele Pigmeja iz Srednjoafričke republike. Ova studija usredotočila se na dva važna pitanja s obzirom na miješanje i porijeklo afričkih Pigmeja. Etnogeografska opažanja sugeriraju spolno pristran tok gena između Bantu populacije i Pigmeja, problem koji bi se mogao razjasniti genetičkom analizom. Istraživanje unutarpopulacijske varijacije mitohondrijske DNA i Y kromosoma dale su rezultate s obzirom na pretpostavljeno bračno ponašanje. Dok generalni mitohondrijski haplotip, koji pripada L1c5 podhaplogrupi, ukazuje na tok gena po ženskoj liniji od Pigmeja prema Bantu populaciji, muški tok gena pokazuje suprotan smjer, od Bantu prema Pigmejima po generalnoj podhaplogrupi s M2 mutacijom na Y kromosomu. Drugi dio studije odnosi se na pitanje genetičkih odnosa između zapadnih i istočnih Pigmeja. Naši rezultati ukazuju na pred-Bantu hipotezu, koja sugerira da su se dvije grupe Pigmeja odvojile u antička vremena (prije cca. 18,000 godina), te ne ukazuju na hipotezu nedavnog razilaženja i miješanja koja pretpostavlja njihovo razdvajanje kao posljedicu širenja Bantu plemena (prije 2,000–3,000 godina).