Initiation of Genetic Demographic Monitoring of Bonobos (*Pan paniscus*) at Iyema, Lomako Forest, DRC

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Abstract: Research on wild apes is not only fundamental for elucidating human origins but for their conservation as well. Despite their relative size, apes are difficult to observe in the wild prior to habituation, limiting our ability to accurately assess demography and kin relations. Non-invasive genetic sampling provides an indirect source of this information. Here, we present findings of a pilot genetic survey of a wild community of bonobos that are in the initial stages of being studied. Fifty-three fecal samples were collected over eight days near the Iyema field site in the Lomako Forest, DRC. DNA was first extracted and quantified using a qPCR assay. Samples with a sufficient amount of DNA were genotyped at 11 microsatellite loci and sexed using an amelogenin assay. Thirty-three of 53 samples yielded a sufficient amount of DNA for complete genotyping. We identified 19 individuals, including six males and 13 females. Mean allelic richness across all loci was 5.7 and expected heterozygosity was 0.69. Estimates of population size indicate between 26 and 66 individuals are present in the study area, but more than one community may be present. These results contribute to our ongoing efforts to study and monitor the bonobos at Iyema to better understand their demography, behavior, and conservation. Our study also highlights the utility of genetic analyses in pilot and survey research.

Key Words: Non-invasive sampling, microsatellites, genetic capture-recapture, community size estimation, conservation genetics

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Introduction

Bonobos (*Pan paniscus*) are classified as Endangered and are threatened by hunting and habitat loss (Fruth *et al.* 2008). Despite over forty years of research in the wild, we are still limited in our understanding of this species; especially compared to chimpanzees (*P. troglodytes*). Political instability and the difficulties of accessing bonobo habitat have both contributed to this knowledge gap. What we do know of wild bonobos is largely based on long-term data from three research areas: Lomako (Badrian and Badrian 1984; White 1992; Van Krunkelsven *et al.* 1999), LuiKotale (Hohmann and Fruth 2003), and Wamba (Kano 1992) (Fig. 1). While continued work at these established sites is important, the development of new, sustainable field sites is necessary for bonobo research and conservation. Of particular interest from new sites are demographic data, which can be difficult to obtain in non-provisioned bonobos due to their arboreality and fission-fusion social structure (Kano 1992). Non-invasive genetic sampling can help us overcome these challenges. Genetic data have already been used on wild bonobos to inform behavioral observations (for example, Gerloff *et al.* 1999; Hohmann *et al.* 1999; Surbeck *et al.* 2011), understand population structure (Kawamoto *et al.* 2013), and infer male philopatry and female dispersal (Hashimoto *et al.* 1996; Eriksson *et al.* 2004, 2006; Kawamoto *et al.* 2013). Continued use of genetic data is necessary for longitudinal monitoring to track population size trends and assess the potential effects of anthropogenic activity on genetic health. Genetic studies of wild bonobos are thus essential to their conservation and to understanding the species.

We conducted a non-invasive genetic analysis as part of our ongoing effort to reinitiate studies of, characterize, and habituate the bonobos at Iyema, in the Lomako Forest in the Democratic Republic of Congo. One community was previously studied (for example, Dupain *et al.* 2002) but has only been intermittently monitored since the Second Congo War that ended in 2003. The primary aim of this initial survey was to identify individuals for consistent long-term monitoring, future behavioral studies, and to obtain preliminary assessments on the demography of the Iyema bonobos. We developed two research objectives. The first was to identify unique

individuals, determine their sex, and evaluate the genetic diversity in our sample. Our second objective was to conduct repeated sampling of individuals to estimate the size of the bonobo population in our study area using genetic capture-recapture. Previous behavioral research produced a size estimate for the main study community at this field site; however, not all of the community members were identified. Dupain *et al.* (2002) were able to visually identify 12 distinct individuals and speculated the community was composed of approximately 50 individuals. Genetic analyses can provide a second estimate, especially when differences in capture probabilities are considered. Additionally, we collected GPS data in association with fecal samples and constructed an association matrix to assess whether our collected samples represent individuals from a single community or multiple communities.

Methods

Study area and sample collection

The Lomako Forest is located between the Lomako and Yekokora rivers in the Équateur province of the Democratic Republic of Congo. The forest is approximately 3,800 km², and consists primarily of polyspecific evergreen rainforest, in addition to swamp forest and seasonally flooded forests (White 1992). There are two bonobo study sites in the Lomako Forest: Iyema and N'dele, which are separated by 15 km.

Fifty-three fecal samples were collected near the Iyema site (00°55'N, 21°06'E) in eight days in June and July 2014. Samples were collected within a 15 km² area (Fig. 2). Fecal samples were collected as part of our ongoing effort to habituate one community of bonobos at the field site. Bonobos were located in the morning before leaving their night nests. Most fecal samples were collected under these nests, and additional

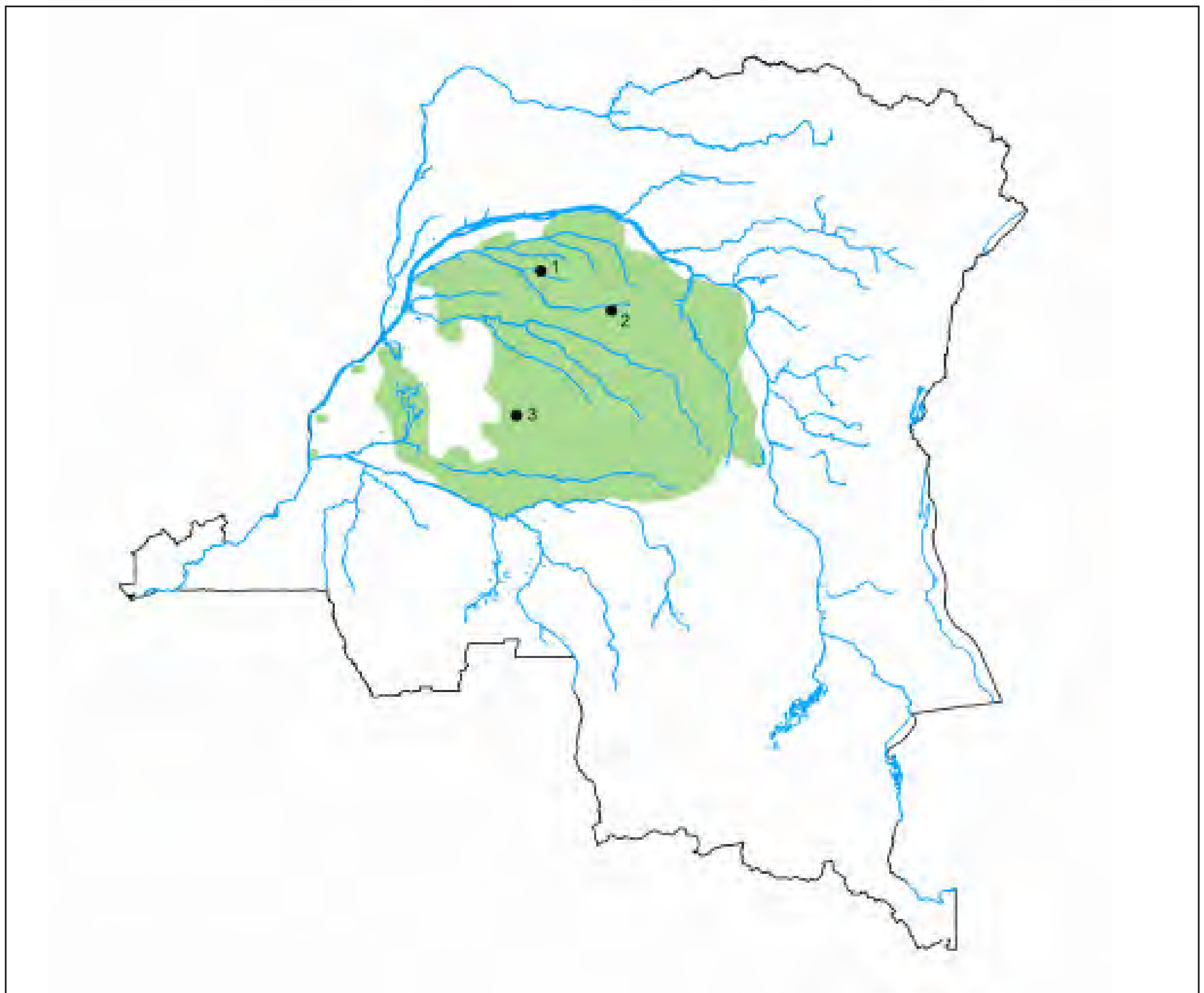

Figure 1. Distribution of *Pan paniscus* (green) and the locations of the long-term bonobo research areas: 1 - Lomako, 2 - Wamba, 3 - LuiKotale.

samples were collected opportunistically during party follows. The GPS location of each sample was recorded using a Garmin GPS unit. To minimize contamination, a mask and gloves were worn when collecting samples. Samples were placed in a 50 mL Falcon tube with 20 mL of RNAlater (Thermo Fisher Scientific, Waltham, MA, USA). All samples were stored at ambient temperature until they could be shipped to the Ting Laboratory (Molecular Anthropology Group, University of Oregon). Samples were then immediately frozen at -20°C until genomic DNA extraction.

DNA extraction and quantification

Genomic DNA was extracted using a QIAamp DNA Mini Stool Kit (Qiagen, Valencia, CA, USA). We made several modifications to the manufacturer's extraction protocol following Archie *et al.* (2003). DNA was eluted in 75 µL of buffer AE following Wikberg *et al.* (2012). Endogenous DNA extracted from noninvasive samples is often degraded, present at low concentrations, and susceptible to allelic dropout during amplification via PCR (Taberlet *et al.* 1996; Morin *et al.* 2001). We thus quantified the DNA in each sample using a quantitative PCR (qPCR) assay following Morin *et al.* (2001). Samples were amplified in 20 µL reactions containing 1X TaqMan Mastermix (Applied Biosystems, Foster City, CA, USA), 200 nM TAMRA™ probe (Applied Biosystems), 300 nM F primer, 300 nM R primer, 8 µg/mL BSA, 5.6 µL H_2O, and 2 µL of DNA. Reactions were carried out using a StepOnePlus qPCR thermocycler (Applied Biosystems). We genotyped samples that contained >0.005 ng/µL of DNA following Morin *et al.* (2001).

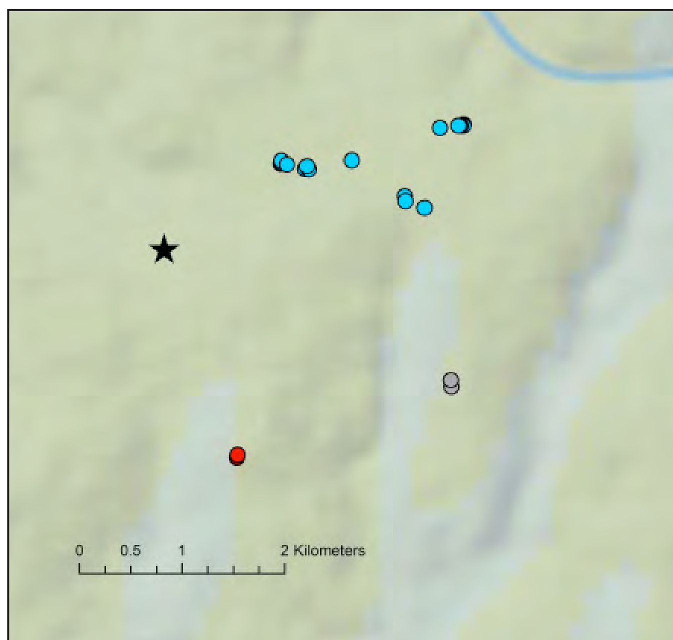

Figure 2. Map of fecal sample collection locations. Each circle represents a successfully genotyped fecal sample. Colors indicate the association network for each sample and correspond to Figure 3. The base camp is represented by a star.

Genotyping

Fifteen dinucleotide and tetranucleotide Short Tandem Repeat (STR), or microsatellite, loci were screened for amplification success and polymorphism (see Table 1, Bradley *et al.* 2000; Arandjelovic *et al.* 2009; Schubert *et al.* 2011; Wikberg *et al.* 2012; Ruiz-López *et al.* 2016). Two markers did not amplify (D1s207, D4s2408) and two (D10s611, FESPS) were found to be monomorphic in our sample. Polymorphic markers were organized into five multiplex sets consisting of two or three markers: set 1 (D5s1457, D14s306), set 2 (D3s1299, D1s548), set 3 (C19a, D6s474 D10s676), set 4 (D8s260, D11s2002), and set 5 (D3s1766, D6s311). The forward primer for each marker was fluorescently labeled with either 6-FAM, HEX or NED. Samples were amplified in 12.5 µL reactions containing 1X QIAGEN Multiplex PCR Master Mix (Qiagen, Valencia, California), 0.2 µM of each primer, 1 µg/mL BSA, 1.75 µL H_2O, and 2 µL of DNA. Initial incubation was carried out at 95°C for 15 min. Amplification was performed using 35 cycles with denaturation at 94°C for 30 sec, annealing at 58°C for 90 sec, and elongation at 72°C for 60 sec. Final extension was carried out at 60°C for 30 min. Successful amplification of each sample was verified using gel electrophoresis with a 1% agarose gel. PCR products were run with a size standard (GeneScan™ 500 Rox™, Applied Biosystems) and separated by capillary electrophoresis using a 3730 DNA sequencer (Applied Biosystems). Allele sizes were determined using the software GENEMAPPER 5.0 (Applied Biosystems) and verified by eye. To control for allelic dropout and ensure accuracy, each sample was independently amplified and genotyped at least three times at every locus. Following Morin *et al.* (2001), samples containing greater than 0.1 ng/µL of endogenous DNA were replicated three times, samples containing between 0.1 and 0.05 ng/µL of endogenous DNA were replicated four times, and samples with 0.05 and 0.005 ng/µL of endogenous DNA were replicated seven times.

Samples were determined to be heterozygous at a locus if two alleles were observed in at least two replicates, while samples were determined to be homozygous if only one allele was observed in at least three replicates. Consensus genotypes were generated using GIMLET (Valiére 2002). Unresolved consensus genotypes were determined manually using alleles with the highest frequency. GIMLET was also used to determine the allelic dropout rate and false allele rate (Supplement 1).

GENALEX 6 (Peakall and Smouse 2012) was used to compare final consensus genotypes. Samples that matched at all but one or two loci were rechecked for allelic dropout and genotyping errors. If human genotyping errors occurred, genotypes were rectified and reanalyzed for matches. This conservative approach was used to avoid identifying individuals based on erroneous genotypes.

Finally, we tested the power of our set of markers to identify unique individuals using GENALEX. We calculated the power to differentiate between random individuals P(ID) and the power to differentiate between siblings P(ID)sib (Waits *et al.* 2001). As bonobos are social primates that live in groups

Table 1. Genetic diversity measures per locus.

Marker	Motif	*Na*	*Ne*	*Ho*	*He*	*UHe*	F_{IS}	HWE
C19a	4	5	2.03	0.368	0.507	0.521	0.2981	Non-significant
D1s548	4	5	2.57	0.737	0.611	0.627	-0.1803	Non-significant
D3s1229	2	6	4.66	0.789	0.785	0.807	0.0217	Non-significant
D3s1766	4	4	2.37	0.526	0.578	0.593	0.1155	Non-significant
D5s1457	4	6	3.47	0.684	0.712	0.731	0.0659	Non-significant
D6s311	2	9	6.17	0.789	0.838	0.861	0.0847	Non-significant
D6s474	4	4	2.33	0.895	0.571	0.586	-0.5494	Non-significant
D8s260	2	6	3.76	0.789	0.734	0.754	-0.0485	Non-significant
D10s676	4	6	4.12	0.895	0.758	0.778	-0.1547	Non-significant
D11s2002	4	8	5.51	0.684	0.819	0.841	0.1903	Non-significant
D14s306	4	4	2.71	0.368	0.632	0.649	0.4388	Non-significant
Mean		5.7	3.61	0.684	0.686	0.704	0.025	

Motif, repeat motif; Na, number of alleles; Ne, number of effective alleles; Ho, Observed heterozygosity; He, expected heterozygosity; UHe, unbiased expected heterozygosity; F_{IS}, Inbreeding coefficient; HWE, results for Hardy-Weinberg equilibrium test with "Non-significant" meaning the locus did not deviate from HWE.

with related individuals, we used the more conservative measure, P(ID)sib, using a cutoff of 0.001. This approach ensures the accurate identification of unique individuals from fecal samples.

We used GENEPOP 4.2 (Raymond and Rousset 1995) to test for deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) across all markers. Fisher's exact test was used to test for a deviation from HWE across all loci. We used the program's Markov chain algorithm to determine departures from HWE and LD for each marker using 100 batches and 1000 iterations. Significance levels were adjusted using a Bonferroni correction. MICROCHECKER (Van Oosterhout *et al.* 2004) was used to test for evidence of null alleles. We used GENALEX (Peakall and Smouse 2012) to determine various measures of genetic diversity: allelic richness, number of effective alleles, observed heterozygosity, and unbiased expected heterozygosity. We also calculated the inbreeding coefficient (F_{IS}) following Weir and Cockerham (1984) for each locus using GENEPOP (Raymond and Rousset 1995).

Sex identification

Following the identification of distinct individuals within our sample, the sex of each individual was assessed using an amelogenin assay (Bradley *et al.* 2001) modified for visualization via gel electrophoresis. Extracted DNA was amplified in 15 µL reactions containing 1X GoTaq® (Promega, Madison, WI, USA), 200 µM F primer, 200 µM R primer, 12 µg/mL BSA, 2.7 µL H_2O, and 3 µL of DNA. We electrophoresed DNA using a 4% agarose gel at 100 volts for 2.5 hrs. Validation of the methodology was conducted on human samples of known sex (see Supplement 2), and each bonobo sample was amplified and visualized on a gel twice to ensure correct sex identification.

Community size estimation

We used two approaches to estimate the number of individuals at Iyema: the software Capture (Otis *et al.* 1978) and the R Package Capwire (Miller *et al.* 2005). We used a comparative approach to contrast a traditional capture-recapture method (Capture) with a program developed specifically for genetic capture-recapture (Capwire). Traditional methods, such as Capture, estimate population size based on the occurrence of captures per session. We used the m(h) model from Chao (1989), which accounts for heterogeneity in individual capture probabilities. We also used both models from the R Package Capwire to estimate the number of individuals: 1) the Equal Capture Model (ECM), that assumes that all individuals in a population have an equal probability of being sampled (Miller *et al.* 2005); and 2) the Two-Innate Rates Model (TIRM) which assumes that two classes of individuals exist—individuals that are easy to capture and those that are difficult to capture (Miller *et al.* 2005). We also used a likelihood ratio test to determine which model best fit the data. Finally, we generated 95% confidence intervals for both ECM and TIRM models using maximum population estimates of 50, 200, 500, and 1000 individuals. We report the confidence intervals for a maximum population estimate of 200 because the intervals varied little for maximum population sizes greater than this value.

Association analysis

We created an association matrix to estimate how many bonobo communities were represented in our sample. Dyads were scored as "0" if fecal samples were not found in association and scored as "1" if fecal samples were collected from individuals in the same party (nests within a 30-m radius) on the same day (McCarthy *et al.* 2015). We used the software NetDraw 2.155 (Borgatti 2002) to visualize associations.

Results

Thirty-eight samples yielded a sufficient quantity of DNA for genotyping, and 33 samples were genotyped at all 11 loci. Our sample success rate was thus 62% (33/53). Probability of identity analyses revealed that at least nine loci are needed to identify unique individuals. P(ID) was 1.8×10^{-8} and P(ID)sib

Table 2. Sample capture and recaptures.

Times Captured (N)	Individuals (N)
1	12
2	4
3	1
4	1
6	1

was <0.001 at 9 loci. Of the 33 samples collected, we identified 19 distinct individuals (13 females and six males).

Microsatellite diversity

Allelic richness ranged from four to nine different alleles for each locus (Table 1). Mean allelic richness across all markers was 5.7. Our sample deviated significantly from Hardy-Weinberg equilibrium across all loci (χ^2 = 47.27, df = 22, P <0.01). However, subsequent tests for heterozygote deficiency and heterozygote excess yielded no significant results (p-value = 0.08 and p-value = 0.92, respectively), and individually, all 11 loci conformed to Hardy-Weinberg equilibrium (Table 1). No loci showed evidence of linkage disequilibrium. The presence of null alleles was detected in one marker: D14s306 (Oosterhaut = 0.1934). Mean expected heterozygosity was 0.69 and expected unbiased heterozygosity was 0.70 (Table 1). F_{IS} ranged from -0.18 to 0.44, averaging 0.025 across all markers.

Population size estimation

Out of 19 individuals, 12 were captured once, four individuals were captured twice, one individual was captured three times, one individual four times, and one individual six times (Table 2). Using the m(h) model in Capture, the program estimated 36 individuals were present in this area (95% CI: 24−81). The equal capture model (ECM) indicated approximately 26 individuals were present (95% CI: 19−36), whereas the two innate rates model (TIRM) indicated approximately 38 individuals present (95% CI: 26−66). Finally, we tested both Capwire models and found TIRM to better fit our data (LR = 8.72, bootstraps = 500, P <0.05). While these models generated both point estimates and confidence intervals, we focus on the latter for the remainder of the paper.

Patterns of association

Associations between dyads are displayed in Figure 3. Thirteen individuals were found to form the largest association (blue nodes). Six individuals formed two smaller, separate networks; one containing four individuals (red nodes) and the other containing two individuals (gray nodes). Individuals in these smaller networks were never found in association with individuals in the larger network.

Discussion

These results highlight our ability to obtain genetic information using non-invasive sampling from this population of

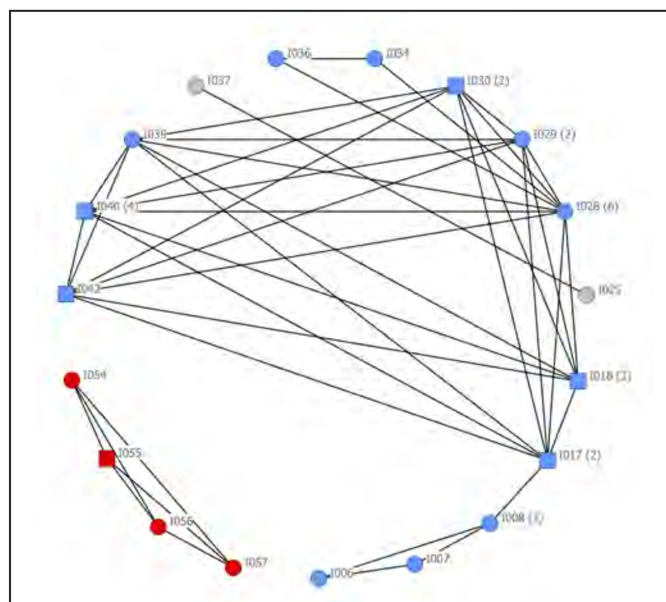

Figure 3. Association Network. Each node is an individual; circular nodes are female and square nodes are male. Numbers in parentheses indicate the number of times an individual was captured for individuals that were sampled more than once. Lines connecting two nodes indicate the pair of individuals was found in association at least once. Individuals with blue nodes are members of the main study community and were collectively sampled on six days. Individuals with gray nodes were sampled on a different day as were individuals with red nodes. These individuals (gray and red nodes) may represent individuals who are not members of the main study community.

bonobos. Our success rate (62%) for sample viability was comparable to other studies of African apes (Arandjelovic *et al.* 2010; Basabose *et al.* 2015; McCarthy *et al.* 2015; Moore and Vigilant 2014). Additionally, our microsatellite panel yielded a sufficient number of loci to confidently identify individuals. Taken together, this study represents the beginning of long-term bonobo monitoring at the Iyema site, with the identification of unique individuals and the first genetic capture-recapture population size estimates.

While some markers exhibited relatively high rates of allelic dropout and false alleles (Supplement 1), only one marker (D14s306) showed evidence of null alleles. Null alleles describe mutations in the regions that flank target nucleotide sequences that can prevent primers from properly annealing (Chapuis and Estoup 2007). While null alleles typically do not impact basic population analyses, they can affect accurate assessment of relatedness and paternity analyses (Dakin and Avise 2004). As we hope to use microsatellite data to determine infant paternity in the future, we will exclude this marker in future analyses.

The genetic diversity represented by our sample is similar to wild-born captive bonobos and other wild bonobo populations. Reinartz *et al.* (2000) examined the autosomal diversity in 14 wild-born captive bonobos (founders). Across 28 polymorphic microsatellite loci, mean allelic richness was 5.2 and mean expected heterozygosity was 0.58. Schubert *et al.* (2011) analyzed genetic diversity at 19 autosomal markers among five groups of wild bonobos around LuiKotale and found a mean allelic richness of 7.3 and mean expected

heterozygosity of 0.75. Our study of 19 wild bonobos resulted in similar findings to both studies (mean allelic richness: 5.7; mean expected heterozygosity: 0.69). These results highlight the genetic diversity at Iyema and are very encouraging from a conservation standpoint, although more work needs to be done to assess threats to bonobos in this area since taxa with long generation times can be affected and threatened by human disturbance prior to visible declines in genetic diversity (e.g., see Ruiz-López *et al.* 2016).

All three mean estimates of population size fell near the previous estimate based on behavioral observations (approximately 50 individuals). Indeed, the population size estimate and confidence intervals from the m(h) model in Capture and the TIRM estimate in Capwire are very similar (24–81 and 26–66, respectively). These estimates are also consistent with known maximum bonobo community sizes: Lomako (Bakumba): 36; Lomako (Eyengo): 21; LuiKotale: 35; Wamba (E1): ~28; Wamba (E2): ~45 (Kano 1992, Surbeck *et al.* 2011, White and Wood 2007). While these samples may represent a single community, our analyses cannot exclude the possibility that more than one community was sampled. Of the two Capwire models, TIRM fit the data better than the ECM; a result that is congruent with previous research (Arandjelovic *et al.* 2010). TIRM is a better model for estimating population or community size in bonobos than ECM because it accounts for heterogeneity in capture probabilities largely due to the fission-fusion social structure of bonobos. While consistent estimates were generated, our small sample size resulted in large confidence intervals further supporting that more accurate census measures require repeated sampling of nearly all individuals present in a community (Basabose *et al.* 2015). As the main bonobo community is not fully habituated, it is highly unlikely that all individuals were sampled. Additionally, heterogeneity of capture probabilities can greatly influence size estimates and confidence intervals. Basabose *et al.* (2015) noted that the two-innate rates model accounted for this heterogeneity, as did the m(h) model in Capture. One particular demographic group that exhibited a higher probability of avoiding genetic capture was infants, who nest with their mothers for several years (Fruth and Hohmann 1994), may not defecate outside of the nest, and whose feces are smaller and more difficult to find. Collectively, these factors can result in infrequent infant fecal sampling, and thus result in inaccurate and underestimated population sizes.

One challenge of using genetic capture-recapture approaches to estimate community size rather than population size is the possibility of sampling more than one community. Prior to habituation, distinguishing between different communities of bonobos and chimpanzees can be difficult. Both bonobos and chimpanzees fission-fusion and form parties that may last several days (Goodall 1986; Kano 1992). Particular parties or individuals may be more difficult to sample. Basabose *et al.* (2015) used a nest sharing analysis to overcome the challenge of distinguishing communities. While chimpanzees are generally considered to maintain strictly defined territories, bonobo ranges appear to frequently overlap (Kano 1992; Waller 2011). This makes it difficult to discern whether or not unhabituated animals belong to a particular community. Our analysis of association revealed the possibility that more than one community was sampled. The samples that constitute the two smaller networks (gray and red) were all collected on two separate days that did not involve any sampling of the main network (blue). Additionally, these six individuals were sampled much farther away from the remaining samples. We are unsure, therefore, whether or not these individuals are members of the same community from which the other 13 individuals were sampled. The association network illustrates three separate networks; however, this may reflect fission-fusion dynamics, especially considering the small sampling period. These results highlight the difficulty of determining the number of communities sampled when collecting bonobo fecal samples over a short time period.

While our genetic survey is preliminary, this study is an important first step for resuming longitudinal bonobo research in the Lomako Forest. We identified 19 individuals and estimated the population size in the study area to be between 26 and 66 individuals. Continued non-invasive sampling will enable us to identify and monitor specific individuals in addition to assessing the number of bonobo communities present at Iyema. Future research will also use this study as a starting point for the use of relatedness data to better understand bonobo social organization and community membership, the effects of kinship on social behavior, and bonobo reproductive strategies.

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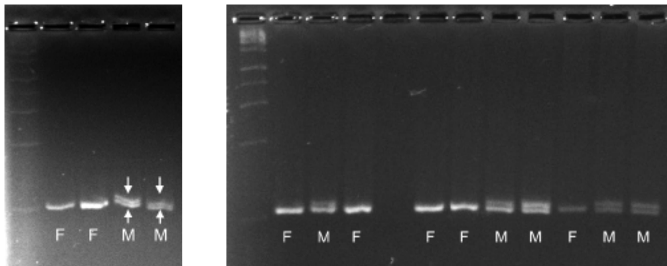
Supplement 1. Dropout and False Allele Rate per Locus.

Marker	Allelic Dropout Rate	False Allele Rate
C19a	0.217	0.054
D1s548	0.097	0.303
D3s1229	0.077	0.253
D3s1766	0.155	0.085
D5s1457	0.080	0.293
D6s311	0.139	0.133
D6s474	0.125	0.310
D8s260	0.068	0.293
D10s676	0.077	0.221
D11s2002	0.191	0.365
D14s306	0.136	0.082

Supplement 2. Amelogenin Assay Visualization Validation



Amplified products from the amelogenin assay (Bradley *et al.* 2001). The gel on the left displays four human control samples and the gel on the right displays multiple bonobo samples. The presence of one band indicates female, while two bands indicates male. The sex of each sample is identified below the product.