Diachronic monitoring of snow leopards at Sarychat-Ertash State Reserve (Kyrgyzstan) through scat genotyping: a pilot study

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Abstract

Snow leopards (*Panthera uncia*) are a keystone species of Central Asia's high mountain ecosystem. The species is listed as vulnerable and is elusive, preventing accurate population assessments that could inform conservation actions. Non-invasive genetic monitoring conducted by citizen scientists offers avenues to provide key data on this species that would otherwise be inaccessible. From 2011 to 2015, OSI-Panthera citizen science expeditions tracked signs of presence of snow leopards along transects in the main valleys and crests of the Sarychat-Ertash State Reserve (Kyrgyzstan). Scat samples were genotyped at seven microsatellite loci, which allowed estimating a minimum of 11 individuals present in the reserve from 2011 to 2015. The genetic recapture of 7 of these individuals enabled diachronic monitoring, providing indications of individuals' movements throughout the reserve. We found putative family relationships between several individuals. Our results demonstrate the potential of this citizen science program to get a precise description of a snow leopard population through time.

Keywords: snow leopard, noninvasive genotyping, population dynamics, microsatellite, relatedness, diachronic monitoring, citizen science, Central Asia

Introduction

The most important threats facing snow leopard (*Panthera uncia*) populations include habitat loss, loss of prey-resources, human-wildlife conflicts, and poaching [1-4]. Because of these, snow leopards were listed as Endangered by the IUCN from 1986 to 2016. Thanks to recent conservation efforts, the species was downlisted to Vulnerable (C1) in 2017. Rough estimations of the total number of individuals indicate between 2,700 and 3,400 snow leopards (https://www.iucnredlist.org/species/22732/50664030). However, many range countries are missing up-to-date information regarding snow leopard population sizes and demographic trends because they are highly elusive carnivores who live in remote mountainous locations, making ecological, behavioural and population studies challenging [4,5]. Research that seek to provide current and accurate demographic trends is the main priority highlighted in the Snow Leopard Survival Strategy [2].

In Kyrgyzstan, snow leopard numbers have decreased at an alarming rate over the last few decades, with 600 – 700 individuals estimated in the late 1980s, against 150 – 200 in 2000 [6]. Latest estimates are around 350 – 400 individuals (National Academy of Sciences of Kyrgyzstan, unpublished data) [4]. So far, protection efforts in this country have mainly focused on preventing poaching, one of the most important threats to wildlife since the break-up of the Soviet-Union [6]. The main protected area, the Sarychat-Ertash State Reserve, established in 1995, is located in the Tien-Shan mountain range of Kyrgyzstan (**Fig. 1**). Beside poaching, major threats on biodiversity in the Sarychat-Ertash State Reserve (SESR) include climate change, mining, overgrazing, and overhunting [7]. The SESR highlands are also surrounded by hunting concessions, which increases pressure on snow leopards and their prey. The SESR is divided in fourteen districts, each monitored by a ranger. Several studies based on genetic analyses and camera trapping estimated the snow leopard population size in the SESR to be around 20 individuals in 2011 [4,8,9]. However, an accurate picture of population trends on the long term is lacking.

Non-invasive genetic analyses are suitable tools to provide estimations of demographic parameters necessary to frame conservation plans [10-13]. The main objectives of the present study were to assess the suitability of non-invasive genetic techniques to study the snow leopard population living in the SESR, and to obtain information on family relationships and movement patterns. Despite the practical constraints associated with this kind of studies for such an elusive species, we were able to identify several individuals, follow individuals' movements over several years, and determine the level of relatedness among them.

Materials and methods

Study Area

Before 1995, the SESR was a grazing area for USSR shepherds who lived there year-long with flocks containing thousands of animals. The SESR boundary encompasses 1,340 km², with a 720 km² core zone and a 620 km² buffer zone [7] (**Fig. 1**). The relief is characterized by large flat valleys about 1 km wide surrounded by high mountains of altitudes ranging from

2,000 to 5,500 m [7]. The climate is continental, with low average temperatures even during the summer months (-21.5 °C in January; +4.5 °C in June). Vegetation types include arid grasslands and alpine meadows, with a majority of bushy and blanket cover type plants that are able to sustain the harsh and windy climate [7]. Beside the snow leopard (*Panthera uncia*), several large and meso-carnivore species are found in the reserve: wolf (*Canis lupus*), red fox (*Vulpes vulpes*), Tian Shan brown bear (*Ursus arctos isabellinus*), Manul (*Felis manul*), Eurasian lynx (*Lynx lynx isabellinus*) and several mustelids. Large and medium herbivores which are snow leopards' favorite prey species, such as the Siberian ibex (*Capra sibirica*), the argali (*Ovis ammon*), the grey marmot (*Marmota baibacina*), as well as smaller rodents, are also found. Several species of birds are present, a few of which that represent prey species for snow leopards, such as snowcock (*Tetraogallus himalayensis*) and chukar partridge (*Alectoris chukar*) [4,7].

Monitoring of snow leopard presence

In this study, snow leopards of SESR were monitored during citizen science expeditions led by the OSI-Panthera research program (osi-panthera.org). These 2-week expeditions were executed by local rangers and guides, scientific educators, and volunteers. Monitoring effort increased over time, with 2 expeditions in 2011 (July and August), 3 in 2012 and 2013 (June, July, and August), and 4 in 2014 and 2015 (June, early July, July-August and late August).

Snow leopard presence was recorded based on specific signs (presence of scats, hairs, scratch marks, tracks, urine sprays on rocks, and carcasses of prey species), and based on pictures from camera traps set at known locations. Incidental species were also recorded to obtain information on prey presence and biodiversity level.

The protocol consisted of prospecting for snow leopards signs along transects (**Fig. 1**). As snow leopards are more likely found in steep and rocky environments and travel along topographic edges [4], transects were designed along waterbodies, ridgelines and cliffs, as well as in narrow valleys and canyons [9]. Most transects were set within a sampling area of about 500 km² within the SESR core zone, around the main valley in which the Ertash River flows, and at the entry of secondary valleys (**Fig. 1**).

Glaciers, which are not considered high quality habitat for snow leopards [4] were only prospected once due to both low accessibility and time constraints. As snow leopards are territorial and travel several kilometers each day [4], our large sampling area enabled us to estimate the number of individuals in the whole reserve and to assess their movements. The list of prospected ridgelines and information on the presence of putative snow leopards signs and collected feces can be found in **Table S1**.

Collection of scat and hair samples

Putative snow leopard scats were identified based on size, shape, vegetation content, as well as proximity to tracks, scratch marks and carcasses. Scats were collected with latex gloves, and then stored into silica beads containers at room temperature until DNA extraction. In the OSI participation conditions (http://www.vacances-scientifiques.com/Conditions-de-Participation.html), it is stated that data gathered by participants during these expeditions will be used for scientific purposes.

A total of 137 putative snow leopard scat samples were collected and subsequently analyzed (14 in 2011, 24 in 2012, 45 in 2013, 41 in 2014, and 13 in 2015). A feces collection authorization was signed by the head of Sarychat Ertash State Reserve.

Hair samples from 2 captive snow leopards (1 male and 1 female) with known pedigrees were collected by the staff of Jungle Cat's World (Ontario, Canada), and were used as positive controls for species identification and sexing. Additional scat and hair samples from other captive snow leopards were collected non-invasively on the floor by the staff of the Toronto Zoo (Ontario, Canada) with the permission of the Animal Care director of the Toronto Zoo and were also used as positive controls during genetic analyses.

DNA extraction

DNA from both hair and scat samples was extracted with a Qiagen DNeasy tissue extraction kit. Depending on the sample, 10 hairs with visible roots or the outer mucosal layer of dried scat (obtained by swabbing scats with water hydrated cotton-tipped applicators) were used for extraction. These were suspended in 180 µl of lysis buffer (2 M urea, 0.1 M NaCl, 0.25% N-laurylsarcosine, 5 mM CDTA and 0.05 M Tris HCl pH 8), an amount that is more efficient for extraction of low template DNA. For scat, protein digestion was performed by adding 20 µl of proteinase K (600 U/mL) to each sample, and by incubating the samples in a 65 °C water bath for 1.5 hours, followed by a second spike of proteinase K, and overnight incubation at 37 °C. For hair, protein digestion was conducted by adding 25 µl of proteinase K (600 U/mL) and 10 µl of 1M DDT to each sample, and by incubating the samples in a 65 °C water bath for 1.5 hours. This was followed by a second spike of proteinase K and DTT, and overnight incubation at 37 °C. Subsequent extraction steps were carried out as per the manufacturer's instructions, following the protocol with final DNA elution to 50 µl TE.

Species identification

Snow leopard identification through scat was carried out using two snow leopard specific primers, CYTB-SCT-PUN-F' (5'-TGGCTGAATTATCCGATACC) and CYTB-SCT-PUN-R' (5'- AGCCATGACTGCGAGCAATA) which amplify a 150 bp fragment of the mitochondrial cytochrome b gene [14].

To assess the sensitivity of these primers at low DNA yields, dilutions were carried out on the Toronto control samples as well as on field samples with concentration eluted down to 0.5 ng/µl, 0.05 ng/µl, 0.005 ng/µl, and 0.0005 ng/µl. A 20 µl polymerase chain reaction (PCR) was then prepared containing 2 µl of PCR Buffer (10X), 2 µl of 2 mM dNTPs, 1.33 µl of 3 µg/µl BSA, 0.8 µl of 50 mM MgCl2, 0.4 µl of each 10 µM primer, 0.2 µl of 5 U/µl Taq enzyme, 10.87 µl of autoclaved sterile ultra-pure water (ddH$_2$O), and 2 µl of DNA extract.

To optimize our amplification conditions, a first PCR reaction was carried out on the eluted control samples with a temperature gradient from 50 °C to 60 °C at the annealing step. Thermocycling conditions were set as follows: 94 °C for 5 minutes, followed by 35 cycles of each 94 °C for 30 seconds, 50 °C to 60 °C for 60 seconds (annealing step with temperature gradient) and 72 °C for 60 seconds with a final extension at 60 °C for 45 minutes. Along with the known snow leopard samples, a "no DNA template" negative control was included. PCR

products were visualized under ultraviolet light on a 1.5% agarose gel stained with ethidium bromide. As all eluted control samples amplified well, the optimal annealing temperature for the amplification of field samples was selected based on the brightness of the control samples that were at the lowest DNA concentrations. DNA extracted from field samples was amplified using the same PCR and thermocycling conditions, but with an annealing temperature of 55 °C. For each sample, the dilution for which amplification was successful was selected for subsequent analyses.

Forward and reverse sequences were obtained by using BigDye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, Inc.). Sequencing was performed on an automated DNA sequencer (ABI 3730; Applied Biosystems, Inc.).

Gender identification

Sex identification was performed using felid-specific primers, targeting a zinc-finger region of the x and y-chromosomes. (Zn-finger F: AAGTTTACACAACCACCTGG and R: CACAGAATTTACACTTGTGCA). A fluorescent dye-label (HEX) was applied to the forward primer as the product size of Zfx at 166 base-pairs and 163 for Zfy were to close together to be distinguished by agarose gel [15].The 10ul Zn-finger PCR reaction was prepared containing of 2 µL of 5X reaction buffer (Promega), 0.8 µL of 2.0 mM MgCl2, 1µL of 0.2 mM of each dNTP, 0.1 µL of each primer at 10 µM, 0.67 µL 0.2 ug/ul BSA, 0.1µl of 5U/µl Taq polymerase(Promega) and 2µL of DNA. The PCR profile was 94 ℃ / 5 min, [94 ℃ / 1 min, 59 ℃ / 1 min, 72 ℃ / 1 min] x 34 cycles. PCR products were run in the ABI 3730 system. For each test, a "no DNA template" negative control was included. Positive control samples were included using high quality tissue from known cougar males and females, as well as scat and hair samples from known males and females snow leopards.

Individual genotyping

Seven microsatellite loci (PUN100, PUN124, PUN132, PUN225, PUN229, PUN327 and PUN935 [16]) were genotyped when DNA samples were identified as snow leopard DNA. A 10 µl PCR was prepared containing 2.0 µl of PCR Buffer (5X), 1.0 µl of 2 mM dNTPs, 1.0 µl of 3 µg/µl BSA, 0.6 µl of 25 mM MgCl2, 0.3 µl of each 10µM primer, 0.1 µl of 5U/µl Taq enzyme, 2.7 µl of autoclaved ddH$_2$O, and 2 µl of DNA extract.

For each sample, both PCR amplification and genotyping were duplicated with thermocycling conditions set at 95 °C for 10 minutes, followed by either 49 or 34 cycles at 95 °C for 15 seconds, 55 °C for 30 seconds, and 72 °C for 60 seconds. To verify amplification, PCR products were visualized under ultraviolet light on a 1.5% agarose gel stained with ethidium bromide. The amplified products were then fractionated and sized using GENEMAPPER v 4.0 on the ABI 3730 (Applied Biosystems, Inc.). We included hair and scat samples from one know individual with each ABI 3730 injection to ensure consistency in allele size and included positive and negative controls with each analysis. In order to prevent genotyping error, serial dilutions were conducted. This technique allows avoiding losing or miss-assigning alleles when signals from stock solutions are too strong. Under these circumstances, samples are unscorable because of wide peaks. When stock solutions that provided these wide peaks are diluted, peaks become clear, and the samples scorable. Serial dilutions also provide

an opportunity to control for artifacts or errors in morphology from the ABI at different signal strengths. In this study, serial dilutions of samples that were overloaded on the ABI when their stock solution was used were set at 1/2, 1/5, 1/10, and 1/20 of the stock solution.

Distribution of pairwise genetic distances according to kinship

To monitor individuals, we need to be able to discriminate them based on their genotypes. Hence, first, we estimated the probability P_{uni} that two unrelated individuals have the same genotype using the following formula:

$$P_{id} = \sum_{i=1}^{n} (f_i^2)^2 + \sum_{i=1}^{n-1} \sum_{j>i}^{n} (2f_i f_j)^2$$

$$P_{uni} = \prod_{k=1}^{L} P_{id_k}$$

where P_{id} is the probability that two unrelated individuals have the same genotype at a given locus, n is the number of alleles at a given locus, f_i is the frequency of allele i and L is the number of loci.

Whereas it is simple to compute the probability that two unrelated individuals have the same genotype, it is much more difficult to compute the expected distribution of the genetic distance, that is the number of different alleles between two individuals according to their kinship (*i.e.* unrelated, parent-descendant, full siblings, half siblings) and there are necessarily some inside a given population. An R script has been written to compute these distributions using simulations (script_GenerateFamiliesIndividualRealFreqs-SupMaterials-Panthera.R in GitHub "https://github.com/jmorode/OSI-Panthera_genetics_scripts"). Pairs of genotypes were generated for different kinships. For two unrelated individuals, at each locus, the genotype of each individual was generated by randomly sampling two alleles, taking into account allele frequencies in the population. For a parent and its descendant, the genotype of the parent was generated as described above, and the genotype of the descendant was generated by sampling at random one allele from this parent and the other allele taking into account allele frequencies in the population. For full siblings, two unrelated parents were first generated, then each descendant was generated by randomly sampling one allele from each parent. For half siblings, one mother and two unrelated fathers were first generated, then one descendant was generated by randomly sampling one allele from the mother and one allele from one father, and the other descendant was generated by randomly sampling one allele from the same mother and one allele from the other father. For each kind of relationship, one million simulations were performed to estimate the empirical distribution of pairwise distances based on kinship. All data analyses were carried out in the R statistical environment (version 3.4.3)[17].

Estimation of genealogical relationships

The software ML Relate [18] was used to find related snow leopards. The accuracy of inferred family relationships was evaluated in parallel using simulations of the genotypes of related

individuals (script_GenerateIndividualRealFreqs-SupMaterials-Panthera.R in GitHub "https://github.com/jmorode/OSI-Panthera_genetics_scripts"). Two thousand families - composed by a mother, two unrelated fathers, two full siblings and two pairs of half siblings – were generated using the method described above, allowing testing the four relationships inferred by ML Relate: parent-offspring, full siblings, half siblings and unrelated. The genotypes of the members of these 2,000 families were written in an input file for ML Relate to evaluate the accuracy of the inferred relationships. For each pair of individuals in each family, the known relationship was compared with the one inferred by ML Relate. In each family, 6 unrelated individuals, 1 pair of full siblings, 2 pairs of half siblings and 6 pairs of parent-offspring were expected. The percentage of known relationships found by the program over the 2,000 families was taken as an estimation of the accuracy of this program to identify the relationships in the population of snow leopards with the microsatellite loci analyzed.

Results

Scat genotypes

DNA was extracted for 137 scats. A snow leopard specific fragment of the *cytb* gene could be amplified for 107 samples. Among them, 51 samples were successfully genotyped at 7 microsatellite loci. The other samples had missing data, with 13 samples with one missing locus, 5 samples with two missing loci, and 38 samples with between 3 and 6 missing loci. Samples genotyped with no more than one missing locus are listed in **Table 1** (the complete set of genotypes can be found in the file **supp_mat_data1_Genotypes.xls** in supplementary material). Thirty-two unique genotypes were identified, potentially corresponding to the same number of individual snow leopards. Eight of them were sampled several times (**Table 1** and **Fig. 2**).

Distributions of pairwise genetic distances according to genealogical relationships

Allele frequencies and expected heterozygosity were estimated using all the snow leopard samples (**Table 2**). Pairwise distances between the different genotypes were also computed (**Table 3** and **Fig. 3**). Most of the distance distribution is included between five and ten differences between genotypes. However, some pairs of genotypes differ by only one or two alleles (10 pairs and 18 pairs, respectively).

Based on allele frequencies, it could be computed that the probability (P_{uni}) that two unrelated individuals would have the same genotype was very low (2.73 x 10^{-5}). Moreover, simulations showed that the expected distance between individuals that come from a population with the observed allele frequencies would be (taking only into account number of differences with a probability of occurrence above 5%): 4 to 9 differences for unrelated individuals, 2 to 6 differences for parent offspring pairs, 1 to 6 differences for full siblings, and 3 to 8 differences for half siblings (**Fig. 4**).

Minimum number of snow leopards

Although collected samples were carefully genotyped, with such material some genotyping errors are possible. When only considering samples genotyped at all loci, or all loci minus one, and assuming no genotyping errors, 31 snow leopards were identified. This number dropped to 11 when we considered only samples for which a complete genotype was available, and assumed that up to two differences could be the result of genotyping errors (**Fig. 5**). The number of genotypes identified grew steadily from 2011 to 2015, with no evidence of saturation (**Fig. 5**).

Relationships between snow leopards

Relationships between snow leopards were estimated using ML Relate. With complete genotypes (no missing data), eighteen parent-offspring, thirty-three full siblings and eighteen half siblings relationships were found. When the genotypes with missing data at one locus were added, thirty-six parent-offspring, fifty-one full siblings and twenty-eight half siblings relationships were found. In both cases, each individual was related to between two and twelve other individuals (**Fig. S1** in supplementary material). Relationship matrices can be found in the file **supp_mat_data2_MLRelateOutput.xls** in supplementary material.

The assessment of ML Relate using simulated families showed that the program had an overall 62% accuracy. Parent-offspring relationships were correctly found in 71% of cases, unrelated individuals in 69% of cases, full siblings in 48% of cases, and half siblings in only 20% of cases. When the true relationship was not found (38% of the time), 32% were indicated as unrelated, whereas 13% were parent-offspring, 6% full siblings, and 13% half siblings; 31% were indicated as parent-offspring, whereas 3% were unrelated, 22% full siblings, and 6% half siblings; 10% were indicated as full siblings whereas 2% were unrelated, 6% parent-offspring, and 2% half-siblings; 27% were indicated as half-siblings whereas 11% were unrelated, 11% parent-offspring, and 5% full siblings.

Diachronic monitoring of individuals

Monitoring of individuals inside the reserve was performed using scat samples for which six to seven loci were genotyped as the probability that two individuals share the same genotype at at least loci is very low. Assuming no genotyping errors, seven individuals were sampled more than once (**Fig. 2**). A total of 14 scats belonging to the individual SL1 were collected between 2011 and 2014 in Bir Baital (P), Uch Baital (N), Chomoi (G), Saryetchki (Q) and Orto-Bordu (E). This snow leopard was present all around the main valley, and was able to cross the Ertash River, as its feces were found on both river banks. A total of 4 scats belonging to the individual SL2 were collected in 2014 in the south-eastern part of SESR on the southern side of the Ertash River, in the Solomo (U) and Sirdibai (T) areas. A total of 3 scats belonging to the individual SL3 were collected in 2014 in Jili Boulak (V). A total of 10 scats belonging to the individual SL4 were collected in 2013 and 2014 in the core part of the main valley on the eastern side of the Ertash River (Kirk-choro (H), Gueuleu (L) and Sary etchki (Q)). A total of 3 scats belonging to the individual SL5 were collected in 2011, 2014 and 2015 in Solomo (U). A total of 8 scats belonging to the individual SL6 were collected in 2012 and 2013 in Bordu (C), Chomoi (G), Kirk-choro (H) and

Gueuleu (L). Finally, a total of 2 scats belonging to the individual SL7 were collected in 2011 and 2014 in Solomo (U). The maps corresponding to each individual can be found in the **supplementary materials (maps SL1 to SL7)**.

DiscussionThanks to reiterated OSI-Panthera expeditions, snow leopard individuals were identified by genotyping scat samples collected over multiple years. Through this non-invasive capture-recapture method, it was possible to follow individuals' movements. This study shows that citizen science expeditions are a powerful way to gather field data over long periods of time and across large areas when logistical and financial demands cannot be met via academic research projects [19].

Scats sampled were often degraded when collected because of harsh weather and high altitude. This hindered the optimization of the genotyping process, as DNA was sometimes not of good enough quality for individual identification. Nonetheless, with seven microsatellite loci, a minimum of 11 snow leopards were identified, seven of which were followed over time and space within the SESR.

New genotypes were steadily identified each year with no evidence of saturation. This could be due to three different factors: *i*) not enough scats were collected to be able to identify most snow leopards in the sampled area, *ii*) the birth of cubs during the sampling period, who could be some of the new individuals sampled and/or iii) individuals are regularly coming from outside the sampling area into SESR. A more extensive survey is necessary to disentangle the importance of these factors.

The most conservative estimation of the number of snow leopard identified in this study (at least 11 individuals, is similar to 15 individuals identified via camera trapping in 2014 [4], and 18 individuals identified with genetic markers across a 1,341 km² area in 2009 [8].

When including complete genotypes and genotypes with only one missing locus, 16 males and 11 females were identified (**Table 1**). Although this sex ratio (1.45) is not significantly different from a one male to one female ratio (chi-squared test, p = 0.33), it will be important to assess its evolution over a longer period of time in SESR, as snow leopard sex ratio can be highly dynamic and it could affect the renewal potential of the population [20].

Using the software STRUCTURE v2.3.4 [21], as expected at the geographic scale of the SESR, no evidence of genotype clustering was found (data not shown). This confirms the results from a recent range-wide snow leopard phylogeographic study, which found three clusters corresponding to the geographic subspecies *Panthera uncia irbis* (Mongolia), *Panthera uncia uncia* (India, Pakistan, Tajikistan, and Kyrgyzstan), and *Panthera uncia uncioides* (Qinghai, Tibet, Bhutan, and Nepal). Within each subspecies, genetic differentiation was very low, in particular among four individuals from Kyrgyzstan belonging to the subspecies *Panthera uncia uncia* [22].

Genealogical relationships

Many relationships between individuals were found by ML Relate (62 relationships when complete genotypes were used, and 115 when genotypes containing up to one locus with

missing data were used). All individual snow leopards were related to at least two other individuals. Some individuals were shown to have ten relationships, although some of them were in fact inconsistent (for example, individual 2_2015 was identified by ML Relate as being both full sibling with 13_2015 and 35_2013, which were indicated as being parent-offspring).

Simulated pairs of individuals with known relatedness showed that the overall accuracy of ML Relate was low (62%), indicating that the dataset used in this study was not large enough to reliably identify the relationships between snow leopards. This is particularly the case for estimated full siblings and half sibling relationships, which showed an accuracy level of only 48% and 20%, respectively. In contrast, parent-offspring and unrelated individuals were identified more accurately (71% and 69%, respectively). A better knowledge of the relationships between individuals could be valuable information to estimate the level of inbreeding, the number of descendants per males and females, but more data are necessary to achieve this goal.

Despite the lack of accuracy when trying to identify genealogical relationships between individuals, the expected distribution of pairwise distances according to relationships suggests that if only one or two differences are found between two genotypes, it is unlikely that these individuals are unrelated. One or two allelic differences between two individuals would most likely represent either a close relationship, or the genotype of only one individual with genotyping errors. Ten pairs of genotypes showed only one allelic difference, and eighteen showed two allelic differences. Among them, two genotypes with one difference and five with two differences involve both a male and a female, meaning these pairs of genotypes cannot be the results of genotyping the same individual with errors. In addition, all these pairs of genotypes were identified as full siblings by ML Relate. However, among the pairs of genotypes with one or two allelic differences, there is an excess of pairs with the same sex (8/10 and 14/18 respectively, whereas we expect that half the full siblings pairs are of the same sex, that may be evidence that some closely related genotypes are the result of genotyping errors. Thus, at least seven out of the twenty-nine closely related pair of genotypes are probably not the results of genotyping errors, but more likely represent related snow leopards which may be full siblings as inferred by ML Relate.

Diachronic monitoring of individuals

Some individuals were recaptured several times, across multiple years. Two individuals, SL1 (male) and SL6 (male), were found throughout the main valley of the SESR core, over a broad territory. They could be two dominant males of the valley. Male and female territories overlapped in the study area, as it has been already observed in other studies [4]. SL7 was sampled twice in 2011, at two very close locations, and was never sampled again; expeditions may have not cross its road again, his main territory could have moved outside the reserve, or he could have died.

In conclusion, our analyses allowed a better understanding of the snow leopard population dynamics in the SESR from 2011 to 2015. In the near future, we could deepen our analyses and refine our parameter estimations by continuing scat sampling efforts and by using other

approaches such as camera trapping. It will allow a deeper understanding of the SESR snow leopard population dynamics, and thus, further inputs for the conservation of this species in Kyrgyzstan.

Author contributions:

J.R., A.P., S.R., A.L.C., A.O., B.C. designed the study. J.R, A.P., S.R., A.L.C., A.O., B.C. and A.V. collected the samples. A.P., B.W., M.H. and N.T.X. performed PCR amplifications, sequencing and scoring of the microsatellite markers. J.R., J.F. and D.C. analyzed the data. J.R., A.P. and D.C. wrote the manuscript. All authors revised the manuscript and approved the final version.

Competing Interests:

The authors declare no competing interests.

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Tables

Table 1 Scat genotypes (no missing data or only at one locus)

Individual	Sexe	Locus													Samples number
		PUN100		PUN124		PUN132		PUN225		PUN229		PUN327		PUN935		
33_2014	Female	87	87	90	90	120	122	179	179	106	110	78	86	115	115	1
1_2012	Female	87	89	90	90	120	122	177	177	110	110	86	86	115	115	1
SL6 / 4_2012	Female	87	89	90	90	120	122	177	179	110	110	86	86	115	115	8
26_2013	-	87	89	90	90	120	122	179	179	110	110	86	86	115	115	1
2_2015	Male	87	89	90	96	118	122	177	181	106	110	78	86	119	119	1
13_2015	Male	87	89	90	96	120	120	181	181	106	106	78	86	119	119	1
35_2013	Male	87	89	90	96	120	122	177	181	106	110	78	86	119	119	1
42_2013	Female	87	89	90	96	120	122	177	181	110	110	78	86	119	119	1
34_2013	Male	87	89	90	96	120	122	181	181	106	110	78	86	119	119	1
9_2015	Male	89	89	88	96	120	122	177	177	**	**	78	88	115	119	1
17_2012	Male	89	89	90	90	120	122	179	179	**	**	86	86	115	115	1
6_2015	Female	89	89	90	96	120	122	175	181	110	110	78	86	119	119	1
2_2013	Male	89	89	90	96	120	122	177	177	104	108	78	88	115	119	1
SL1 / 12_2012	Male	89	89	90	96	120	122	177	177	106	106	78	88	115	119	14
23_2012	Male	89	89	90	96	120	122	177	181	106	106	78	88	115	119	1
SL2 / 2_2014	Male	89	89	90	96	120	122	177	181	106	110	78	86	119	119	4
14_2011	-	89	89	90	96	120	122	**	**	106	106	78	88	115	119	1
SL3 / 6_2014	Male	89	89	100	102	112	120	177	179	106	110	78	86	115	119	3
3_2015	Male	89	91	90	96	120	122	177	181	106	110	78	86	119	119	1
SL5 / 13_2011	Female	89	91	90	100	112	118	175	175	110	110	78	86	115	119	3
5_2015	Female	89	91	90	100	112	122	175	179	106	108	78	86	119	119	1
SL4 / 17_2013	Male	89	93	90	90	120	122	175	179	106	110	86	86	119	119	10
23_2013	Male	89	93	90	96	120	122	175	179	106	106	86	86	119	119	1
31_2014	Female	89	93	90	96	122	122	175	179	**	**	86	86	119	119	1
11_2012	Female	89	93	90	100	112	122	175	177	**	**	78	86	119	119	1
20_2014	Male	89	93	**	**	120	122	175	179	106	110	86	86	119	119	1
8_2015	-	91	95	90	90	120	122	177	177	108	108	86	86	119	119	1
7_2011	-	93	93	90	90	120	120	181	181	**	**	86	86	115	119	1
SL7 / 6_2011	Male	93	93	90	90	120	122	175	181	110	110	78	86	115	119	2
10_2011	Female	93	93	90	90	120	122	181	181	110	110	78	86	115	119	1
22_2013	-	93	93	90	90	122	122	173	175	106	106	86	86	**	**	1

Table 2 Allele frequencies and expected heterozygosity

	Locus												
	PUN100		PUN124		PUN132		PUN225		PUN229		PUN327		PUN935	
Number of alleles	5		7		7		8		4		4		3	
	87	0.161	88	0.015	110	0.015	171	0.015	104	0.019	78	0.333	115	0.339
	89	0.548	90	0.647	112	0.076	173	0.015	106	0.404	86	0.591	119	0.629
	91	0.081	96	0.221	114	0.015	175	0.206	108	0.077	88	0.076	127	0.032
Allele size and frequency	93	0.194	100	0.074	116	0.015	177	0.324	110	0.500				
	95	0.016	102	0.015	118	0.045	179	0.206						
			106	0.015	120	0.409	181	0.235						
			108	0.015	122	0.424								
He	0.63		0.53		0.64		0.75		0.58		0.53		0.49	

Table 3 Pairwise distances between genotypes

|	33_2014	1_2012	13_2013	26_2013	2_2015	13_2015	35_2013	42_2013	34_2013	9_2015	17_2012	6_2015	2_2013	9_2014	23_2012	4_2014	14_2011	6_2014	3_2015	13_2011	5_2015	12_2014	23_2013	31_2014	11_2012	20_2014	8_2015	7_2011	6_2011	10_2011	22_2013
33_2014	0																														
1_2012	5	0																													
13_2013	4	1	0																												
26_2013	3	2	1	0																											
2_2015	7	7	7	8	0																										
13_2015	8	9	9	9	4	0																									
35_2013	6	6	6	7	1	3	0																								
42_2013	7	5	5	6	2	4	1	0																							
34_2013	6	7	7	7	2	2	1	2	0																						
9_2015	10	8	9	10	8	9	7	7	8	0																					
17_2012	5	5	4	3	10	10	9	9	9	7	0																				
6_2015	8	7	7	7	4	5	3	2	3	7	8	0																			
2_2013	9	7	8	9	7	8	6	6	7	3	8	6	0																		
9_2014	8	7	8	9	6	6	5	6	6	3	8	6	2	0																	
23_2012	8	8	8	9	5	5	4	5	5	4	8	5	3	1	0																
4_2014	7	7	7	8	2	4	1	2	2	6	8	2	5	4	3	0															
14_2011	8	9	9	9	7	6	6	7	6	5	8	6	4	2	2	5	0														
6_2014	7	8	7	8	7	8	6	7	7	7	8	7	7	6	6	5	7	0													
3_2015	7	7	7	8	2	4	1	2	2	7	9	3	6	5	4	1	6	6	0												
13_2011	9	8	8	8	7	9	8	7	8	10	10	6	9	9	9	8	9	6	7	0											
5_2015	8	10	9	9	6	7	6	7	6	10	9	6	8	8	8	6	8	6	5	5	0										
12_2014	6	6	5	5	6	7	5	6	5	10	6	5	9	8	8	5	8	7	5	8	5	0									
23_2013	8	8	7	7	6	5	5	6	5	9	7	5	8	6	6	5	6	8	5	9	5	2	0								
31_2014	10	9	8	8	7	8	7	7	7	8	6	6	9	9	9	7	9	10	7	9	6	4	3	0							
11_2012	10	9	9	10	6	8	6	6	7	7	8	6	8	8	8	6	9	7	6	6	4	6	6	4	0						
20_2014	8	8	7	7	7	8	6	7	6	10	8	6	10	9	9	6	9	7	6	9	6	2	3	5	7	0					
8_2015	9	6	7	8	8	9	7	7	8	9	8	8	7	8	9	7	10	10	6	10	7	6	7	8	8	8	0				
7_2011	9	8	8	8	10	7	9	9	8	9	6	9	10	10	9	9	10	10	9	10	11	7	8	7	8	9	8	0			
6_2011	6	6	6	6	7	8	6	5	6	9	8	4	8	8	7	6	8	8	6	6	8	5	7	8	7	7	8	5	0		
10_2011	6	6	6	6	7	7	6	5	5	9	8	5	8	8	7	6	8	8	6	7	9	6	8	9	8	8	8	4	1	0	
22_2013	9	9	9	9	10	10	10	11	10	13	9	10	12	10	10	10	10	12	10	11	9	6	6	7	9	8	9	8	7	8	0

Figures
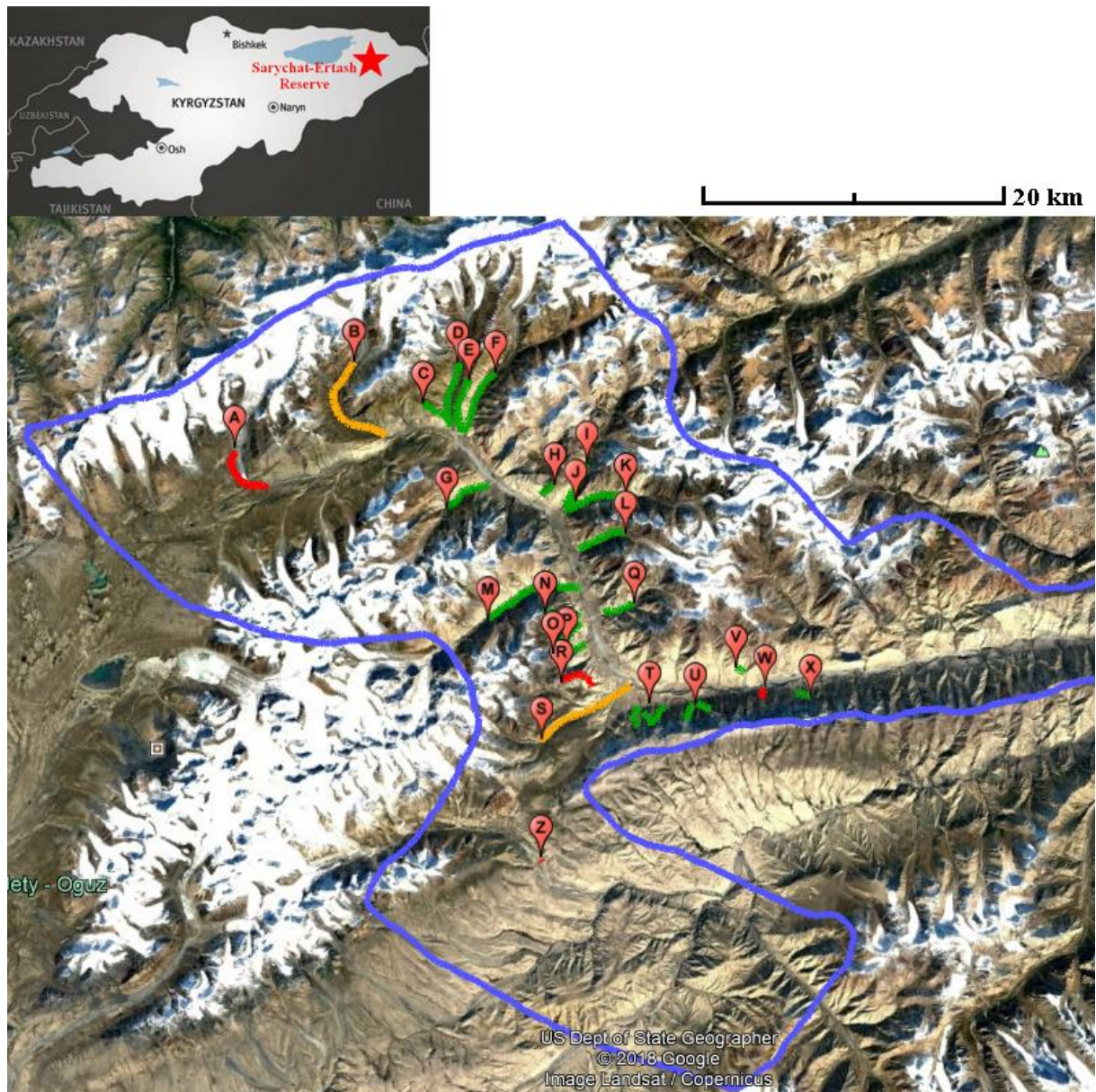
Figure 1. Sampling area. A) Sarychat-Ertash State Reserve location in Kyrgyzstan; B) snow leopard signs and sampled scats along transects within SESR from 2011 to 2015. Blue line: SESR boundary; red line: transects where no snow leopard signs were found; orange line: transects where snow leopard signs was found; green line: transects where snow leopard scats were sampled. Capital letters refer to transect indexes which are compiled in table S1 in supplementary materials. The framed area is shown in Fig.2.
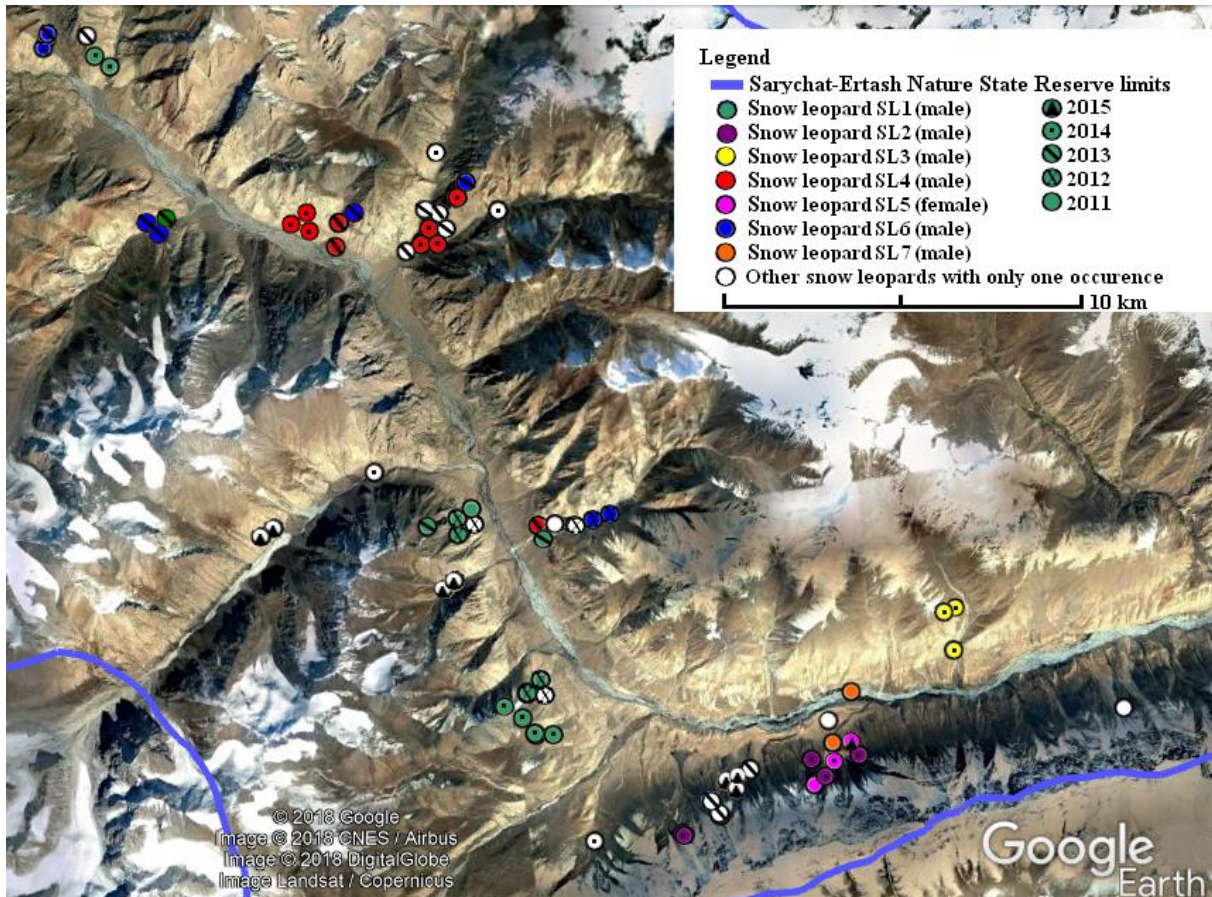
Figure 2. Location of the genotyped snow leopard scats in the Sarychat-Ertash State Reserve from 2011 to 2015.
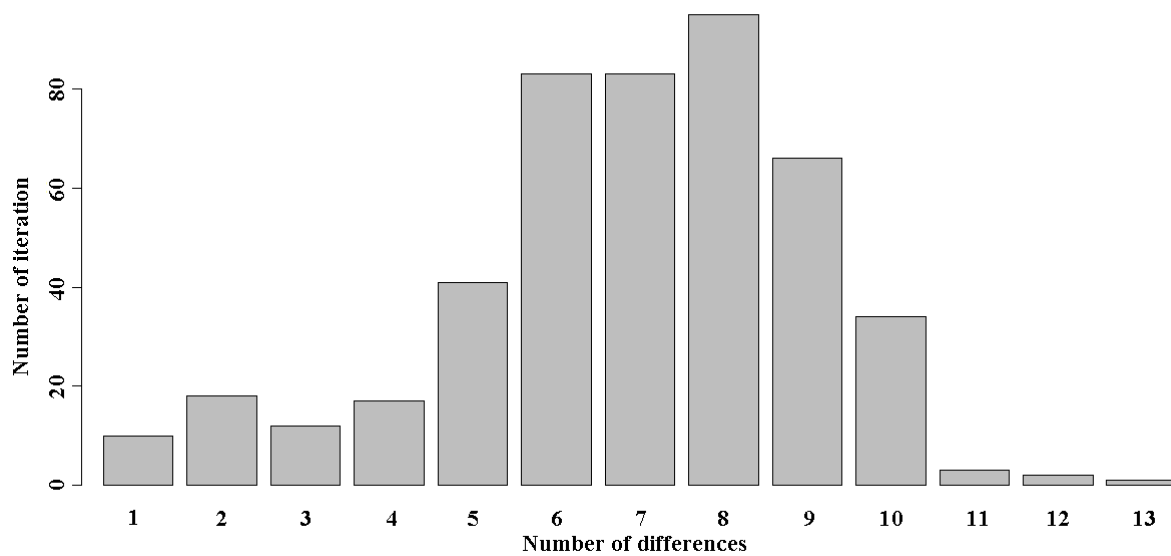

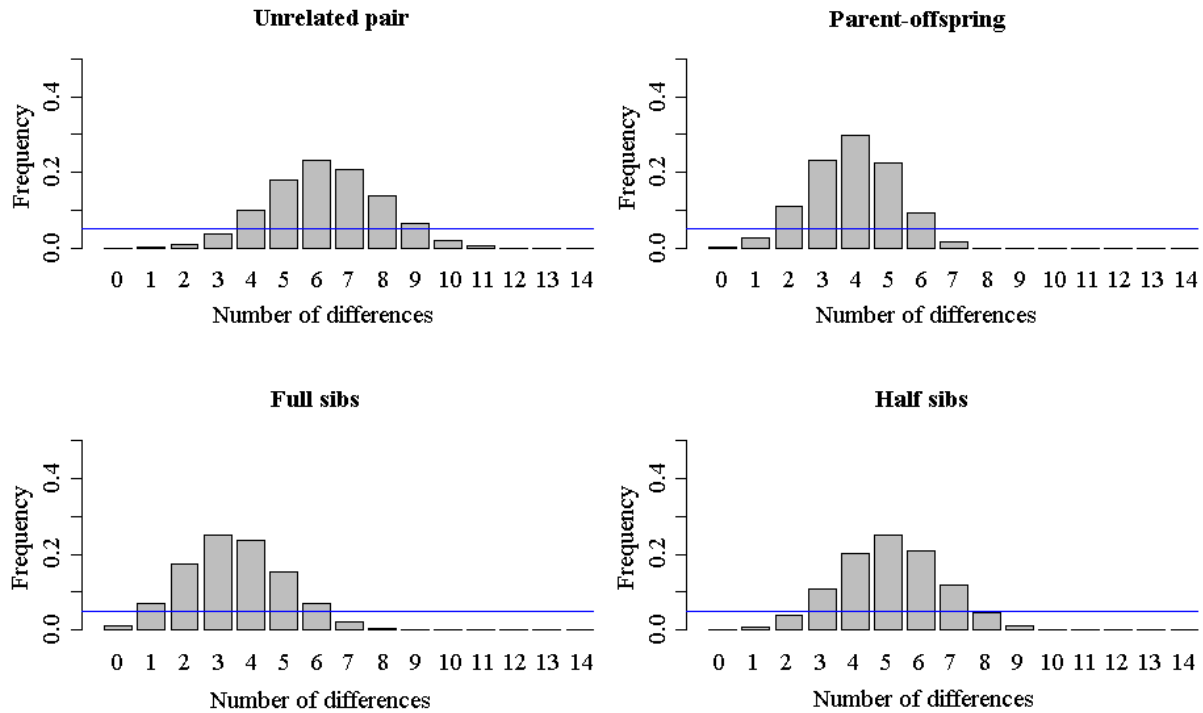**Figure 3.** Pairwise distances between genotypes.

Figure 4. Expected distribution of pairwise genetic distance according to genealogical relationship.
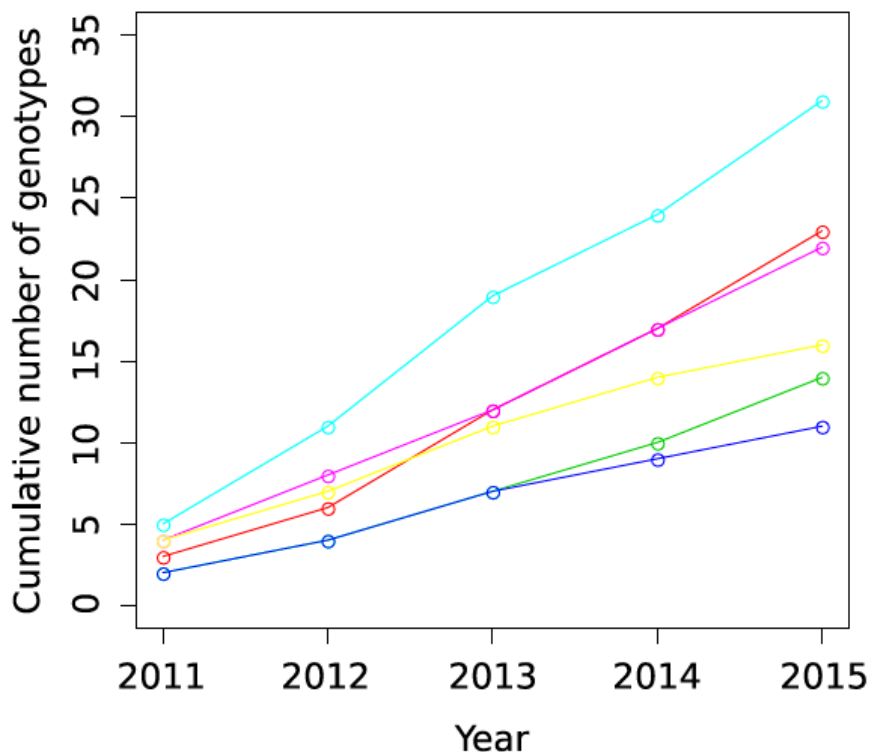

Figure 5. Cumulative number of snow leopard different genotypes identified from 2011 to 2015. Red: complete genotypes only; green: complete genotypes with the possibility of one genotyping error; blue: complete genotypes with the possibility of two genotyping errors; light blue: complete genotypes and genotypes with one locus not genotyped; purple: complete

genotypes and genotypes with one locus not genotyped and with the possibility of one genotyping error; yellow: complete genotypes and genotypes with one locus not genotyped and with the possibility of two genotyping errors.