Footsteps in the snow -

Pilot study for future monitoring of individual lynx (*Lynx lynx*) from eDNA in snow tracks

AquaBiota Report 2019:10

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NATUR VÅRDS VERKET

NORDENS ARK

WildlifePark Ranua – Lapland

AquaBiota

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SUMMARY

Traditionally, information on lynx (*Lynx lynx*) population dynamics either require the close physical encounter to the animal or destructive/unspecific methods. These methods are intrusive for the target animal. Tracking of footsteps and using automatic cameras are traditional tools used for Lynx inventories. However, if many individuals walk in each other's' tracks or if cameras do not detect all individuals the populations may be underestimated. In areas of Sweden with temporary or intermittent snow cover traditional surveys of tracks are restricted in time and it is sometimes difficult to separate family groups.

A new promising method to assess the lynx population is to collect the environmental DNA (eDNA) that is present in a lynx's footprints (tracks) in the snow.

AquaBiota has engaged in a pilot project for narrowing the challenging gap between identifying lynx as a species; to distinguishing lynx individuals from each other. The main challenge is the transition from mtDNA to nDNA, which this far has been unsuccessful.

In this study, snow samples of lynx track were collected in zoos in Scandinavia as well as in the wild in Sweden. In order to develop the field and laboratory methods, the aims were to (1) Collect samples from zoo individuals (Ranua Zoo, Finland) and in the wild. (2) Identify lynx and their food from snow and (3) Verify the occurrence of enough high-quality nDNA in taken samples by using microsatellites and concurrent retrieval of correct size bands on gels as preliminary markers for positive results.

One of the taken samples from zoos was analysed using metabarcoding and it proved to contain both Lynx and several food items which reflected the feed given in the zoo. Nuclear DNA in lynx was for the first time successfully amplified from snow steps initially from a zoo and later from samples in the wild. The method is nondestructive and non-invasive. After the next stage of sampling and molecular fine tuning, the method will be a great tool in identifying lynx on a large scale in order to retrieve population data as to be used for protection and proper management of the species. Furthermore, when the field methods are developed enough for providing consistent results, the next step will be to train hunters and snow trackers to sample snow of lynx individuals on a large geographic scale.

SAMMANFATTNING

Information om storleken på lodjursfamiljer och hur många individer det finns i olika län ar viktiga för underlag i beslut om skydd och jakt av arten. Information om individer används även för att ta reda på om ett eller flera lodjur har skadat tamboskap. För att samla data på individnivå för stora rovdjur som lo (*Lynx lynx*) krävs antingen metoder som innebär närkontakt med djuret eller destruktiva eller ospecifika metoder. Vissa metoder kan ha påverkan på eller till och med vara direkt skadliga för måldjuren. Spårning i snö samt användning av automatiska viltkameror är två traditionella icke

skadliga metoder som används vid lodjursinventeringar. Vanliga svagheter med dessa är att om flera individer går i varandras spår eller om kamerorna inte upptäcker alla individer kan populationerna underskattas. I områden i Sverige med tillfälligt eller oregelbundet snötäcket är traditionella spårundersökningar begränsade i tiden och det är ibland svårt att separera familjegrupper.

En ny lovande metod för att bedöma vilt på populationsnivå är att samla in miljö-DNA (eDNA) som finns i snöspår (tassavtryck) av exempelvis lo. AquaBiota har deltagit i ett pilotprojekt för att minska gapet mellan att identifiera lo på artnivå till att skilja loindivider från varandra. Den största utmaningen i detta är att det kräver en övergång från att analysera mtDNA (mitokondriellt DNA) till nDNA (nukleärt DNA). I denna pilotstudie samlades snöprover av lospår in i en nordisk djurpark samt i naturen i Sverige.

Syftet med undersökningen var att utveckla fält- och laboratoriemetoder med följande mål: (1) att samla in prover från djurparksdjur (på Ranua Zoo, Finland) samt i naturen, (2) att identifiera lo och deras födodjur från snö samt, (3) att kontrollera förekomsten av tillräckligt högkvalitativt nDNA i tagna prover med hjälp av genetiska analyser på individnivå (i detta fall mikrosatelliter). Ett av de tagna proverna från Ranua zoo analyserades med hjälp av analyser på artnivå och detta visade sig innehålla både lo och samt flera arter som getts som foder i djurparken. Analyserna på individnivå som utfördes både på djurparksprover samt prover insamlade i naturen gav utslag, vilket är en preliminär indikation för positiva resultat för att identifiera lodjursindivider med hjälp av prover från snöspår. Fem av fem testade markörer för mikrosatelliter för lodjur fungerade. För att säkert skilja individer från varandra behövs normalt ca 20 fungerande mikrosatelliter.

Till vår kännedom är detta första gången någonsin en lyckad amplifiering av kärn-DNA utförts från snöspår från lodjur, till en början från djurparken och därefter från prov från naturen. Metoden är icke-förstörande och icke-invasiv. Efter ytterligare en etapp av provtagning och molekylär finjustering, kommer metoden att vara ett effektivt verktyg för att kunna identifiera lo i stor skala i syfte att samla in populationsdata som kan användas i förvaltning av arten. När insamlings- och analysmetoderna utvecklats tillräckligt kommer det vara möjligt att att utbilda jägare och spårare i snöpårsprovtagning av lodjursindivider på en mer omfattande geografisk skala.

1. BACKGROUND

Traditionally, information on lynx (*Lynx lynx*) population dynamics either require close physical encounter to the animal or destructive/unspecific methods. In order to gain genetic materials from lynx, urine, feces or dead or sedated lynx have been needed to access hair, blood and skin samples (McKelvey et al, 2006; Diefenbach et al 2015)). These methods are either intrusive for the target animal or materials collected with large effort or over a long period of time.

In order to manage the Lynx populations and to identify specific individuals that are prone to attack domestic livestock it is of high importance to understand individual movement patterns as well as the population size and health of the lynx. Hunting permits and estimated populations are traditionally based on camera detections and trackers. These methods are not precise and may miss individuals or correct estimates of den sizes. Furthermore, the use of automated camera surveillance in Sweden is regulated.

One promising method is to sample the environmental DNA (eDNA) that is present in a lynx's footprints (tracks) in the snow. This far, the use of eDNA from snow has only been used to identify the presence/absence at the species level only (Franklin et al. 2019), but has, to date, been unsuccessful at identifying individuals. The Franklin (et al. 2019) article proved to be useful in separating different felines on species level in a region where several species co-exist. Kinoshita (et al. 2019) managed to identify four mammal species from snow-tracks in Japan.

Within every eukaryotic cell there is only a single set of double stranded nuclear DNA (nDNA) present; whereas the copy number of mitochondrial and ribosomal DNA (mtDNA) in a cell is much higher, the magnitude of 1000-2000 copies per cell. Species-specific analyses (that is at a resolution level where for example lynx DNA can be distinguished from fox or wolf DNA) using eDNA have focused on the mitochondrial region, whereas individual genotyping is a greater challenge due to the much lower number of copies in the nuclear regions. Therefore, there is a need to obtain excellent quality eDNA to generate individual genotypes that can be applied to management and conservation issues involving lynx population dynamics.

AquaBiota has engaged in a pilot project for narrowing the logistical gap between identifying lynx as a species to distinguishing lynx individuals from each other. The largest challenge is the transition from mtDNA to nDNA.

In this study snow samples of lynx track were collected in zoos in Scandinavia as well as in the wild in Sweden. In order to develop the field and laboratory methods, the aims were to (1) Collect samples in zoos (Ranua Zoo, Finland) as well as in the wild. (2) Identify lynx and their food from snow and (3) Verify the occurrence of enough high-quality nDNA in taken samples by using microsatellites and concurrent retrieval of correct size bands on gels as preliminary markers for positive results.

The main aim of this first study was to successfully extract nDNA and to show that nuclear marker can be used for eDNA by showing right band sizes on gels after PCR. Sequencing data from the project will be presented in the final report.

2. METHODOLOGY

2.1. Sample collection

The zoo samples were collected April 19th, 2018 in Ranua Zoo, Finland (snow tracks) and in Nordens Ark, Sweden (saliva), map in Figure 1. Twenty-five snow samples from the wild were collected from 16 locations in Western Sweden. Plastic containers and snow spades were cleaned using 10% hypochlorite (in order to remove all possible traces of DNA), rinsed and washed with 70% molecular grade EtOH.

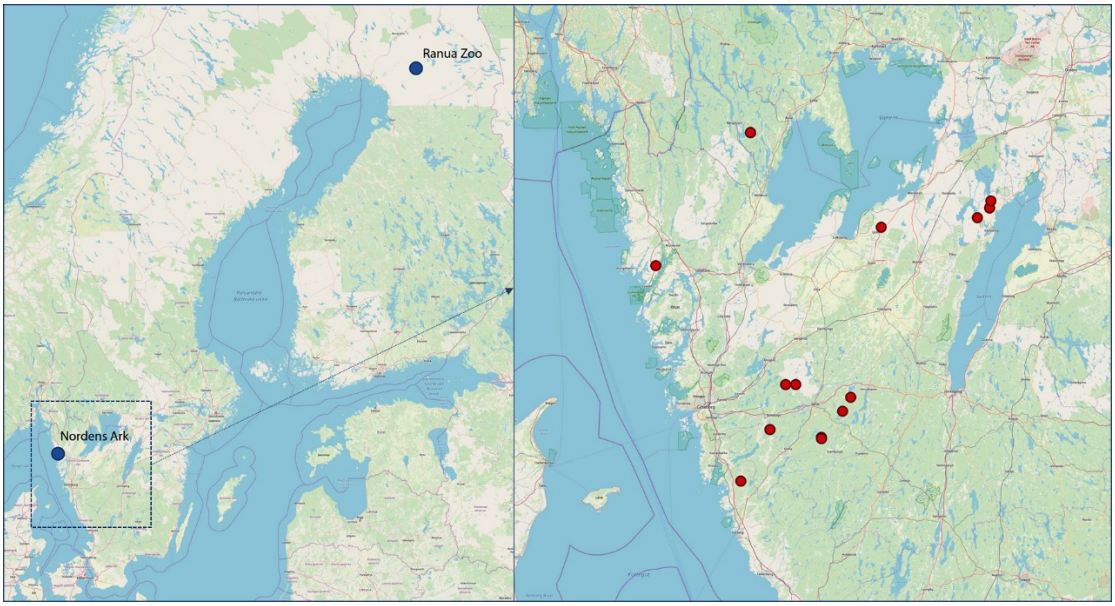

Figure 1. Sampling locations in zoos (left) and in the wild (right).

Detailed information on sampling conditions and lynx information is presented in Table 1 . In Nordens Ark, saliva was collected from a sedated lynx for use as a positive control. In Rauna, snow was collected from one cage and 20 lynx steps were collected for each sample. In the wild, samples were collected from as many footsteps as possible (typically 10-20) for each sample making sure that only steps from one individual were collected per sample. Additionally, snow samples without footsteps and water were used as negative controls to detect possible contamination.

The snow was melted in room temperature and filtered immediately while melting. Capsuled Sterivex 0.22 µm filters and NatureMetrics capsuled double water filters 0.7 µm were used. The samles were immediately fixated with DNA preservative and sent to the eDNA laboratories at MoRe Research and NatureMetrics for further processing.

Table 1. Collected lynx snow tracks samples. Air temperature, location, number of steps collected per sample, coordinates and volume melted snow water filtered (V H_2O) were recorded.

Environ-ment	Location	T °C air	Sample name	Date collected	# tracks	V H_2O (ml)	Lat	Lon
Zoo	Nordens Ark	N/A	Lo Saliv*	N/A	N/A	N/A	58.442900	11.435604
Zoo	Ranua Zoo	9	LX 16 B	2018-04-19	20	280	65.943976	26.466729
Wild	Härserud	0	LO 01	2018-12-19	9	150	58.986389	12.363574
Wild	Härserud	0	LO 02	2018-12-19	10	255	58.986389	12.363574
Wild	Härserud	0	LO 03	2018-12-19	10	432	58.987340	12.363331
Wild	Härserud	0	LO 04	2018-12-19	10	585	58.987340	12.363331
Wild	Brinta	2	LO 05	2018-12-19	15	158	57.813086	12.675898
Wild	Brinta	2	LO 06	2018-12-19	12	225	57.813086	12.675898
Wild	Hedared	2	LO 08	2018-12-19	12	135	57.813445	12.765751
Wild	Hedared	2	LO 09	2018-12-19	7	125	57.813445	12.765751
Wild	Stättared	-6	LL 015	2019-01-24	17	130	57.353150	12.277220
Wild	Stättared	-6	LL 016	2019-01-24	22	120	57.353150	12.277220
Wild	Svenljunga	-4	LL 017	2019-01-24	20	155	57.562752	12.993976
Wild	Svenljunga	-4	LL 018	2019-01-24	20	160	57.556288	12.994331
Wild	Svenljunga	-4	LL 019	2019-01-24	15	120	57.556288	12.994331
Wild	Svenljunga	-4	LL 020	2019-01-24	18	140	57.556280	12.994348
Wild	Brastad	N/A	LL 023	2019-01-27	N/A	140	58.372000	11.521000
Wild	Karlsborg	-4	LL 025	2019-01-31	12	210	58.639473	14.489366
Wild	Götene	-2	LL 026	2019-01-31	12	220	58.550764	13.525636
Wild	Dalsjöfors	-4	LL 033	2019-01-31	16	150	57.686722	13.182195
Wild	Ulricehamn	-4	LL 035	2019-01-31	16	150	57.753083	13.254508
Wild	Karlsborg	-4	LL 039	2019-01-31	12	180	58.641224	14.489341
Wild	Karlsborg	-3	LL 046	2019-01-31	12	280	58.594834	14.380837
Wild	Bollebygd	-1	LL 048	2019-01-31	N/A	180	57.599480	12.536191
Wild	Bollebygd	-1	LL 049	2019-01-31	N/A	95	57.599480	12.536191
Wild	Bollebygd	N/A	LL 050	2019-01-31	N/A	170	57.599480	12.536191
Wild	Karlsborg	-3	LL 054	2019-01-31	12	280	58.672757	14.500881

* Positive control

Figure 2. Lynx tracks in the forest near Härserud, Sweden.

Figure 3. Sampling. First and second rows shows sampling in the wild with field staff including trackers. Third row shows lynx footsteps, the left image including likely ferret tracks. Fourth row: lynx in Ranua zoo and filtering of snow sample.

2.2. Genetic analysis

The outline of the workflow is shown in the flowchart in figure 3.

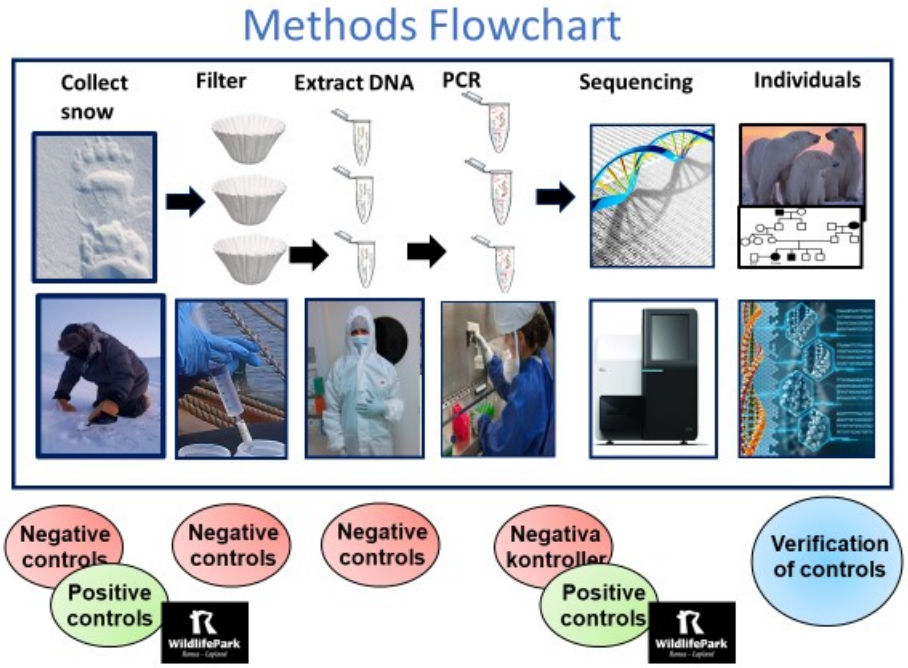

Figure 3. *Flowchart showing the different steps from sample collections to analysis. The positive samples originated from snow at Rauna zoo.*

2.3.1 DNA extractions

The filters were immediately fixated after filtering and extracted following Spens et al (2017). eDNA concentration (ng/µL) was measured using Qubit Fluorometric Quantitation System (Fisher Scientific).

2.3.2 Metabarcoding

The metabarcoding and the microsatellite analyses took place at NatureMetrics eDNA laboratories in London which are designed for the purpose. The metabarcoding determined the species and additionally reveal recently ingested food items. The method was used for the zoo individual in Rauna Zoo.

2.3.3 Microsatellites

Five microsatellites markers (out of 20 sufficient loci) were selected and tested on five samples including a feces sample in order to determine whether nuclear markers for the

first time can be retrieved from DNA traces left in snow. The samples were tested for microsatellites following the molecular protocols in Polanc et al. (2012). The PCR products were run out on an agarose gel in order to determine right sizes of bands on the gels.

3. RESULTS & DISCUSSION

3.1. DNA extractions

The DNA samples from the saliva and snow in the parks showed high concentrations (2-50 ng/uL) whereas the samples from the wild contained low amounts of DNA (0,8-7 ng/ul). All the negative samples were negative and DNA concentrations were below detection rates, which means that both field and laboratory samples were clean. There was no difference in DNA performance between the different filters.

Table 2. *DNA extraction results. # MS loci amplified shows the number of microsatellite loci that amplified out of 5.*

Location	Environment	Sample type	Sample ID	# MS loci amplified
Nordens Ark	Zoo	Saliva	Lo Saliv	5 / 5
Ranua	Zoo	Snow	LX 16 B	5 / 5
Härserud	Wild	Snow	LO 01	5 / 5
Härserud	Wild	Snow	LO 02	5 / 5
Brastad	Wild	Snow	LL 023	5 / 5
Dalsjöfors	Wild	Snow	LL 033	5 / 5
Karlsborg	Wild	Snow	LL 046	5 / 5

3.2. Metabarcoding results

Metabarcoding was performed on the snow sample from Ranua Zoo. The species detected in the sample are shown in figure 4.

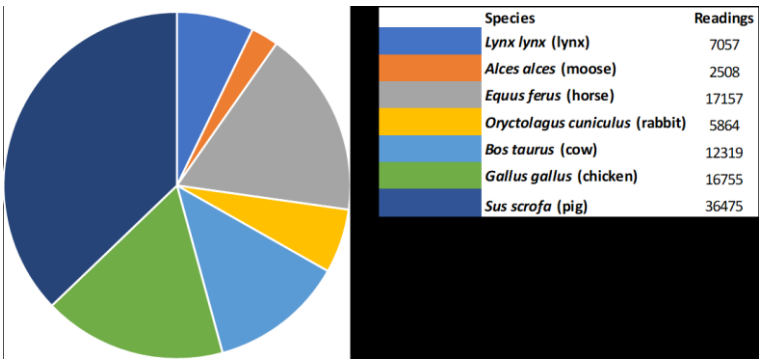

Figure 4. Species detection within the sample from Ranua Zoo.

The sample from Ranua Zoo was, not unexpectedly contaminated with food items which represented a large proportion of the DNA. The types of food item were verified with the zoo which was reflected in the results. The negative control sample did not contain any

vertebrate DNA. The results from the metabarcoding showed that the snow sample from the zoo was dominated with food items which had been given to the animal in the zoo.

3.3. Preliminary microsatellite results

Nuclear DNA from lynx was for the first time successfully amplified from the snow tracks in all the tested samples. Microsatellites are species specific and controls from other species did not amplify. The main take home message from the results was the success to access nuclear DNA and therefore opens up the possibility to further fine tune the method in order to choose markers for individual determination.

The method is nondestructive and shows a great potential to identify lynx individuals on a large scale in order to retrieve population data as to be used for protection and proper management of the species.

All the PCRs for the lynx samples samples for 5 microsatellite loci gave the right sized bands on the gels, which is indicated in table 2. The bands on the gels (Figure 5) give a very strong indication that the nuclear DNA amplified only for targeted lynx DNA and that the samples can be used for detection of individuals. Sequencing data from two of the samples showed the right sequences.

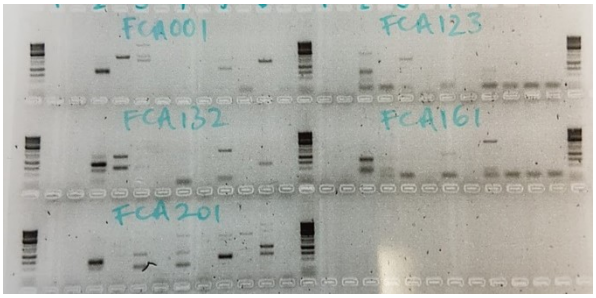

Figure 5. Gel image showing the amplification of the five microsatellite loci showing accurate band sizes.

In order to distinguish individual lynx from each other by high certainty a sufficient number of different loci are needed (Polanc et al. 2012). The main results from this study show that small quantities of snow DNA are sufficient to target the nuclear region which can be analyzed with microsatellites or SNPs (small nucleotide polymorphisms; Nussberger et al. 2014). The laboratory methods will need further fine tuning, which is standard procedure for optimizing molecular workflows.

In addition to the collaborative sampling between trackers and AquaBiota staff with extensive experience in (aquatic) eDNA sampling, pilot tests were also made where trackers collected samples according to a written protocol. Unfortunately, several of these samples were sent unlabeled or with confusing labels, without possibility for backtracking of taken locations. Some samples had also likely been taken using other equipment as other snow containers than the ones supplied were used, likely leading to high risk of contamination. These experiences indicate that very clear instructions need to be provided and training is likely required if trackers and hunters are to collect samples

on large scale. The sampling of snow tracks is more challenging and much more prone to contamination than sampling of feces samples.

The next steps include to demonstrate that individuals can be identified from snow tracks taken in the wild by optimizing the analyses for more microsatellites (enough to distinguish individuals) or use SNPs. Furthermore, it would also be interesting to try metabarcoding on samples from the wild, possibly identifying food choice including potential traces of domestic animals in problem areas. Many issues regarding field sampling also remain unknown, e.g.: what is the minimum amount of tracks needed for analysis, how old can the tracks be, does it matter if tracks have undergone thawing/freezing cycles, what is the best and useable in practice sampling equipment?

4. CONCLUSIONS

The method shows great promise as two major obstacles are overcome; 1) retrieving sufficient amounts of DNA and; 2) for the first time, managing to access the nuclear DNA from snow (both from captive and wild animals) which is important for population studies.

The laboratory methods need further fine tuning and when these are developed enough, more focus can be placed on the field sampling methods, including training of trackers and hunters in eDNA sampling.

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Micaela Hellström was responsible for the logistics and design of the project.

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APPENDIX 1

Metabarcoding

Purified DNAs were amplified with 12 replicate PCRs for a ~230 bp hypervariable region of the 12S rRNA gene using the Kelly vertebrate primer set (Kelly et al 2014) which is designed to detect vertebrates. All PCRs were performed in the presence of both a negative control and a positive control sample.

PCR replicates were pooled and purified, and sequencing adapters were added. Success was determined by gel electrophoresis.
Comment: All samples were successfully indexed; electrophoresis bands were strong and of the expected size. No repeat reactions were necessary.

Amplicons were purified and checked by gel electrophoresis, these were then quantified by using a Qubit (Qubit Fluorometric Quantitation (Fisher Scientific) high sensitivity kit according to the manufacturer's protocol.
Comment: All amplicons were successfully purified and were of high yield (Worksheet "Quality Control").

All purified index PCRs were pooled into a final library with equal concentrations. The final library was sequenced using an Illumina MiSeq V2 kit at 10 pM with a 10% PhiX spike in.

Bioinformatics for metabarcoding

Each species has a unique genetic barcode or sequence in hypervariable gene regions which gives the species a molecular identity. The resolution of these identities are improving with the rapid advancement of molecular techniques in combination with growing reference databases. The different sequences were initially matched against a large and publicly available database based on GenBank and maintained by National Center for Biotechnology Information, NCBI (https://www.ncbi.nlm.nih.gov/). Sequences for more than 370 000 known species are available (Benson et al. 2017) displaying 0,6 billion sequences and 2,6 trillion base pairs according to NCBI's homepage. The different sequences are matched against this database and reveal the identity of fish, amphibians or mammals.

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