Report

Current Biology

Phylogenomics of the world's otters

Highlights

- Phylogenomic relationships among the world's otters were resolved
- Genera *Lutra*, *Aonyx*, *Amblonyx*, and *Lutrogale* should be synonymized under *Lutra*
- The tropical African endemic *Aonyx congicus* was supported as a distinct species
- Demographic history varied but common patterns seem related to paleoclimatic cycles

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In brief

de Ferran et al. employ 24 complete genomes of all living otter species to investigate the evolutionary history of this group of semiaquatic mammals. The results shed light on their phylogenomic relationships, taxonomy, demographic history, and current levels of genomic diversity, with implications for the conservation of these threatened species.

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Phylogenomics of the world's otters

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SUMMARY

Comparative whole-genome analyses hold great power to illuminate commonalities and differences in the evolution of related species that share similar ecologies. The mustelid subfamily Lutrinae includes 13 currently recognized extant species of otters,[1–5] a semiaquatic group whose evolutionary history is incompletely understood. We assembled a dataset comprising 24 genomes from all living otter species, 14 of which were newly sequenced. We used this dataset to infer phylogenetic relationships and divergence times, to characterize patterns of genome-wide genealogical discordance, and to investigate demographic history and current genomic diversity. We found that genera *Lutra*, *Aonyx*, *Amblonyx*, and *Lutrogale* form a coherent clade that should be synonymized under *Lutra*, simplifying the taxonomic structure of the subfamily. The poorly known tropical African *Aonyx congicus* and the more widespread *Aonyx capensis* were found to be reciprocally monophyletic (having diverged 440,000 years ago), supporting the validity of the former as a distinct species. We observed variable changes in effective population sizes over time among otters within and among continents, although several species showed similar trends of expansions and declines during the last 100,000 years. This has led to different levels of genomic diversity assessed by overall heterozygosity, genome-wide SNV density, and run of homozygosity burden. Interestingly, there were cases in which diversity metrics were consistent with the current threat status (mostly based on census size), highlighting the potential of genomic data for conservation assessment. Overall, our results shed light on otter evolutionary history and provide a framework for further in-depth comparative genomic studies targeting this group.

RESULTS AND DISCUSSION

To address outstanding questions regarding the evolutionary history of otters, we aimed to (1) conclusively resolve the phylogenetic relationships and divergence times among the extant species; (2) assess the genomic support for the validity of poorly known species, such as the Congo clawless otter (*Aonyx congicus*) from equatorial rainforests of sub-Saharan Africa; (3) reconstruct the demographic history of each species; and (4) assess and compare genome-wide levels of genetic diversity among the species. We built a dataset comprising 24 whole-genome sequences (14 of which are novel) representing all extant otter species (Figure 1; Table S1). This dataset was generated using modern and museum samples (required in the case of

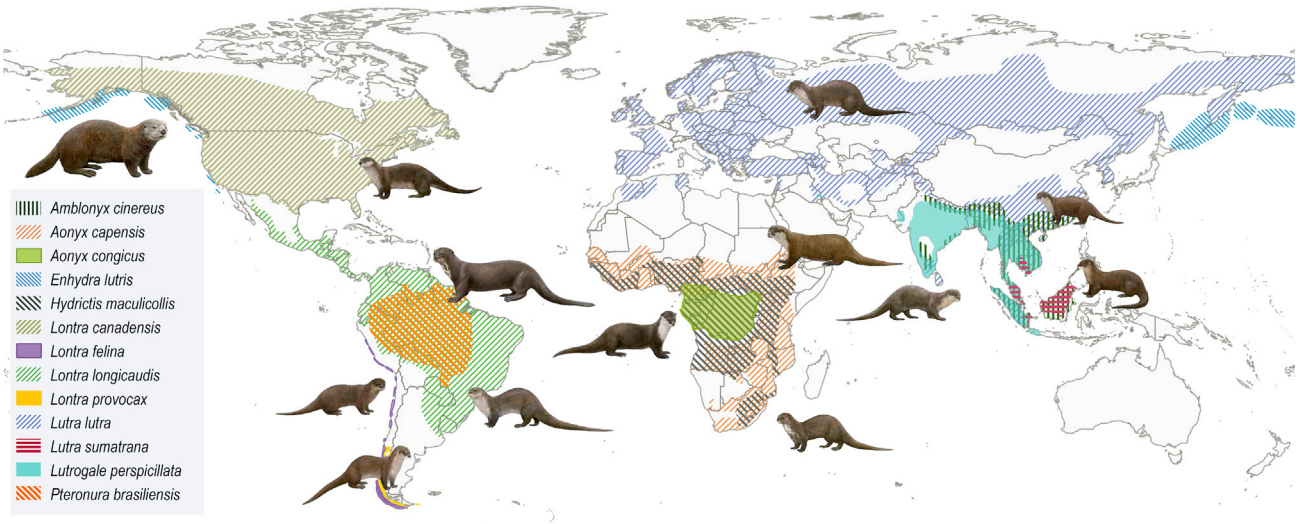

Figure 1. Distribution of the world's otters
Geographic distribution of all 13 extant otter species (adapted from IUCN Red List data[6]).

two rare species), which were sequenced using Illumina technology to an average depth of ~28× and 0.015–1.4×, respectively (Table S1).

Phylogenomic analyses of four datasets comprising different schemes of non-overlapping genomic fragments (GFs) (10 kb and 100 kb, contiguous or skipping 100 kb) applied to taxon set 2 (one individual per species, 11 species with modern samples allowing higher-quality genomes; Table S1) reconstructed three main topologies in the same order of frequency (Table S2). Additional analyses with multiple nuclear supermatrices as well as mitogenomes for all 13 species (including museum samples) recovered the most prevalent topology (Figure S1), which was also reconstructed as the genome-wide species tree using ASTRAL-III (Figure 2). This highly supported phylogeny divided otters into three primary lineages: (1) eight species found in Africa and Eurasia, including the sea otter (*Enhydra lutris*), which is distributed across the North Pacific Rim; (2) a lineage comprising the four species of genus *Lontra* distributed in the Americas; and (3) the South American giant otter (*Pteronura brasiliensis*), which diverged from the other lineages 10.5 million years ago (mya). These results are consistent with previous studies based on single-locus or multilocus datasets.[1–3] All currently recognized genera containing at least two species were recovered as monophyletic.

The three main topologies differed only in the relative position of the sea otter (*Enhydra lutris*) and spotted-necked otter (*Hydrictis maculicollis*) as basal species in the Africa/Eurasia clade (Table S2), a node that has been unresolved in previous studies.[1–5] Topological discordance among different markers is a well-known phenomenon that can be caused by incomplete lineage sorting (ILS), gene duplication, or introgression.[7,8] Considering the relatively short (~530,000-year) interval between the branching of *Enhydra* and *Hydrictis*, we investigated the two most likely causes: ILS and introgression. Our analyses with Dsuite[9] did not detect any evidence of introgression affecting this or any other node in the phylogeny (i.e., D statistics were low, Z scores were <3 and non-significant, f_4 ratios were <0.004, and all f-branch estimates were zero), indicating that ILS is most likely the main cause of the genealogical discordance affecting the relative positions of *Enhydra* and *Hydrictis*, as well as other localized instances of topological variation in the otter tree.

In light of the extensive historical hybridization seen in other carnivoran groups, such as felids, ursids, and canids (e.g., Li et al.,[10] Kumar et al.,[11] and Chavez et al.[12]), the lack of genomic signatures of historical interspecies admixture in otters is noteworthy. Two non-exclusive factors may drive this pattern: (1) predominance of allopatric speciation, followed by extended periods of allopatry that minimized opportunities for hybridization; and (2) rapid evolution of reproductive isolation, possibly related to habitat use and/or behavior. In support of the former, it may be noted that most extant otters have non-overlapping ranges and have likely diverged in allopatry, including multiple independent intercontinental dispersal events of ancestral lineages.[2] Notably, species that are extensively sympatric have deep divergence times (e.g., *P. brasiliensis* versus *Lontra longicaudis* [10.5 mya divergence]; *H. maculicollis* versus *Aonyx capensis* [6 mya divergence]; Figures 1 and 2) and/or very distinct ecological features, such as habitat preferences.[13–15] For example, the Asian small-clawed otter (*Amblonyx cinereus*) and the smooth-coated otter (*Lutrogale perspicillata*) diverged recently (1.42 mya) and their ranges overlap in Southeast Asia. However, the former usually occupies smaller water bodies and feeds mostly on crabs, while the latter is piscivorous and uses larger water bodies.[16,17] Although in this study we did not test for introgression between sister species, it is noteworthy that these two otters have been reported to hybridize,[18–20] but this seems to be a localized phenomenon, possibly driven by occasional human-induced disruption of behavioral/ecological reproductive barriers. Another interesting example comprises the marine otter (*Lontra felina*) and the southern river otter (*Lontra provocax*), whose distributions overlap in southern Chile. They

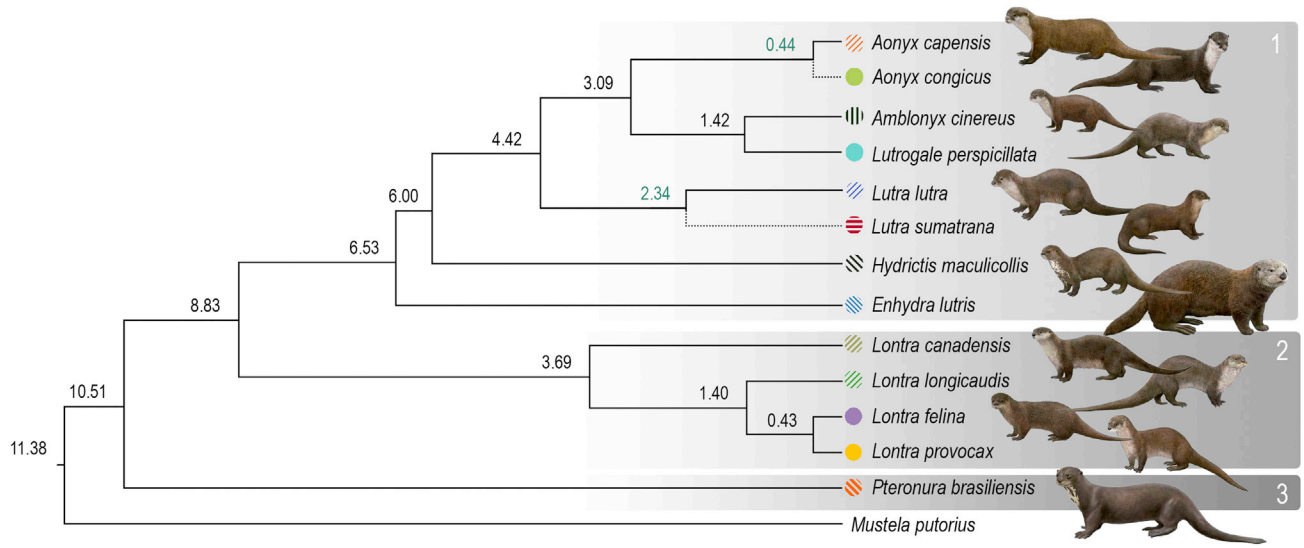

Figure 2. Phylogeny of the world's otters

For the 11 species represented by modern samples (solid terminal branches), a genome-wide species tree was inferred with ASTRAL-III from maximum likelihood (ML) phylogenies of non-overlapping genomic fragments (GFs). All four combinations of GF size and spacing (see text and Table S2 for details) yielded the same most common tree. In the four ASTRAL-III trees, all nodes were supported by 100% bootstrap values. Numbers above branches are estimated divergence times for the adjacent nodes, calculated as the mean value across all GFs. Species with dashed terminal branches were represented by museum specimens and had lower depth of coverage. Hence, their positions were reconstructed with ML from supermatrices of concatenated nuclear fragments (Figure S1) and a concordant mitogenome-based analysis; their divergence times (indicated in green) were based on the mitogenome dataset (see text and Figure S2 for details). Colors and patterns of circles at the end of terminal branches correspond to those shown in Figure 1 for each species.

diverged only 0.43 mya, possibly via parapatric speciation,[14] and currently occupy different habitats,[21] with no reported cases of potential hybridization. These pairs of recently diverged otters should provide interesting systems to investigate the evolution of reproductive isolation in this group.

Divergence time estimates obtained from our datasets (Figures 2 and S2) corroborated previous estimates for most nodes[2,22] and allowed the assessment of additional nodes that had not been sampled in earlier studies. Interestingly, when the museum samples from the Congo clawless otter (*Aonyx congicus*) and hairy-nosed otter (*Lutra sumatrana*) were included in the nuclear dataset, the dates for several nodes were strongly overestimated, possibly due to substitutional biases introduced during read mapping caused by their low depth of coverage. This issue was not observed in the mitogenome dataset, in which their depth of coverage was much higher (~20×) (Table S1). Therefore, although the nuclear data reliably placed these two species within the Lutrinae phylogeny (Figure S1), they did not allow accurate estimation of divergence times. Therefore, for the nodes connecting them to their respective sister species, we consider the mitogenome-based divergence times to be more accurate, since they strongly agree with the genome-wide estimates for all other nodes (Figure S2).

The fact that the 13 species of otters are currently divided into eight genera, of which five are monospecific, has led to considerable taxonomic complexity in this group. Previous studies[3,4] have suggested different taxonomic rearrangements for the Africa/Eurasia lineage. However, they either did not collect data from all species and/or were not able to resolve some of the relationships among them. In light of our very well-supported phylogeny, we suggest synonymizing *Aonyx*, *Amblonyx*, and *Lutrogale* under *Lutra* Brisson, 1762.[23] Interestingly, the species constituting these genera (along with *Hydrictis* and *Lontra*) were originally classified within *Lutra* before they were subsequently divided into separate genera by taxonomists in the 19th and 20th centuries.[24] Such a scheme would simplify the taxonomic structure of Lutrinae, thus conveying the monophyletic nature of this Africa/Eurasia lineage and its similar depth of divergence (4.42 mya) and degree of morpho-ecological disparity relative to the genus *Lontra* in the Americas (3.69 mya divergence) (Figures 2 and S2). If this change is implemented, the correct specific epithets for these species would be *capensis*, *congica*, *cinerea*, and *perspicillata*, respectively.

The well-resolved phylogeny and divergence time estimates among otter species allowed comparative assessments of their biogeographic history. Within the genus *Lontra*, the split time between the North American river otter (*Lontra canadensis*) and the three Neotropical species (*L. longicaudis*, *L. felina*, and *L. provocax*), 3.7 mya (95% HPD = 2.7–4.6 mya), coincides with the formation of the Panama Isthmus ~3 mya,[25] supporting the view that the three latter species diversified during and after the Great American Biotic Interchange.[2,26] Consistent with previous analyses,[2] two endemic African otter genera, *Hydrictis* and *Aonyx*, independently colonized that continent. The spotted-necked otter (*H. maculicollis*) and African clawless otter (*A. capensis*) overlap across much of their respective distributions, but the latter is replaced by the Congo clawless otter (*A. congicus*) in the Congo River basin. The latter has been classified as a subspecies of *A. capensis* in the past,[24,27] but the two otters are distinguishable by their fur coloration, shape of the

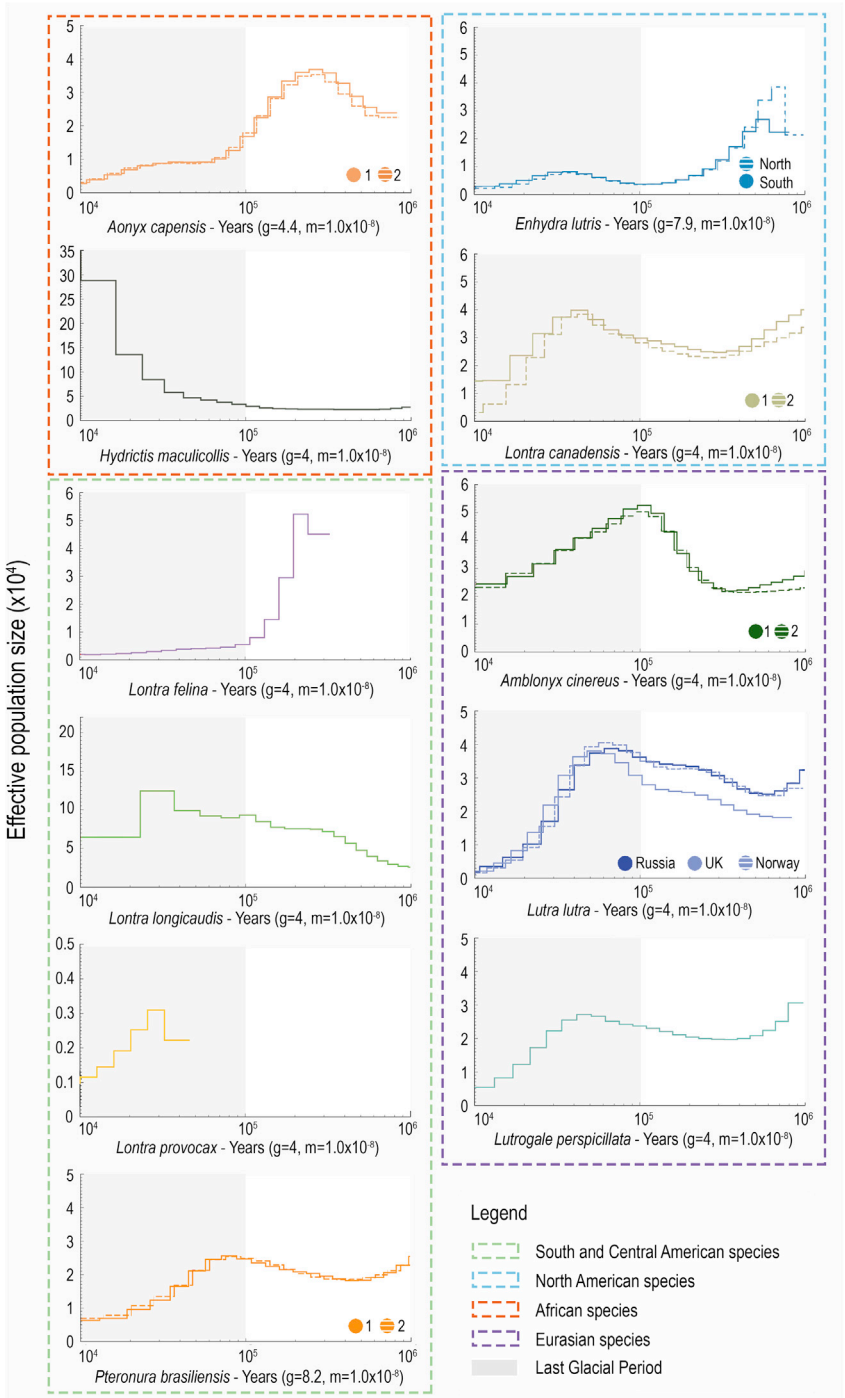

Figure 3. Demographic history of otter species

Demographic history of 11 otter species inferred from whole-genome sequences of 18 individuals with the PSMC approach.

Time (in years) is shown along the x axis and effective population size ($N_e \times 10^4$) is shown along the y axis. Note the different scales of N_e for the different otter species. The generation times and mutation rate assumed here were based on the data provided in Table S3. Bootstrap results are presented in Figure S3. The mapping references were *Amblonyx cinereus* 3 for *Amblonyx* and *Lutrogale* genomes; *Enhydra lutris* North for *Enhydra* individuals; *Lontra canadensis* 3 for *Lontra* genomes; *Pteronura brasiliensis* 3 for *Pteronura* individuals; and *Lutra lutra* UK for the remaining species.

rhinarium (nose pad), and cranial and dental measurements, suggesting distinction at species level.[28,29] However, genetic comparisons between them were limited to a short mitochondrial DNA segment from one individual each, highlighting the need for additional data to bear on this question.[28] Our genomic data resolved the two *Aonyx* species as reciprocally monophyletic lineages that diverged 0.44 mya (95% HPD = 0.25–0.70 mya), similar to the 0.43 mya (95% HPD = 0.26–0.70 mya) split age between *L. felina* and *L. provocax* (Figures 2 and S2). This result supports recognition of *A. congicus* as a valid species, with important implications for the prioritization of conservation strategies on its behalf.

Next, we investigated genome-wide historical trends in effective population size (N_e) among 11 otter species with genome coverages >19× (taxon set 3; Table S1). Interestingly, we observed a similar pattern (initial decline, rebound, and secondary decline beginning 40–100 thousand years ago [kya]) in six species with widely different geographic distributions and habitat associations: *E. lutris*, *L. canadensis*, *A. cinereus*, *P. brasiliensis*, *L. lutra*, and *L. perspicillata* (Figures 1 and 3). In most cases, the secondary decline was sharper than the initial one, and in all six species its timing coincides with the cooling trend of the Last Glacial Period (115–11.7 kya). We hypothesize that these trajectories, observed in both tropical and boreal species, may have been driven by Pleistocene glacial-interglacial cycles with alternating periods of globally cooler/arid versus warmer/humid climates,[30–32] which in turn affected the extent, quality, and productivity of freshwater and coastal marine environments inhabited by otters. A slightly different trend (initial increase followed by sharp decline beginning ca. 200 kya) was observed for *A. capensis*, which we speculate could have been driven by the previous glacial cycle and/or influences of biotic interactions (see below). The most extreme declines were observed for the two southernmost South American species (*L. provocax* and *L. felina*), likely driven by habitat changes (e.g., extensive ice sheet coverage) during the Last Glacial Period that may have severely affected their population numbers, particularly given that *L. provocax* has the smallest range among all otters[21] and *L. felina* has a linear distribution restricted to rocky shores along the western coastline of South

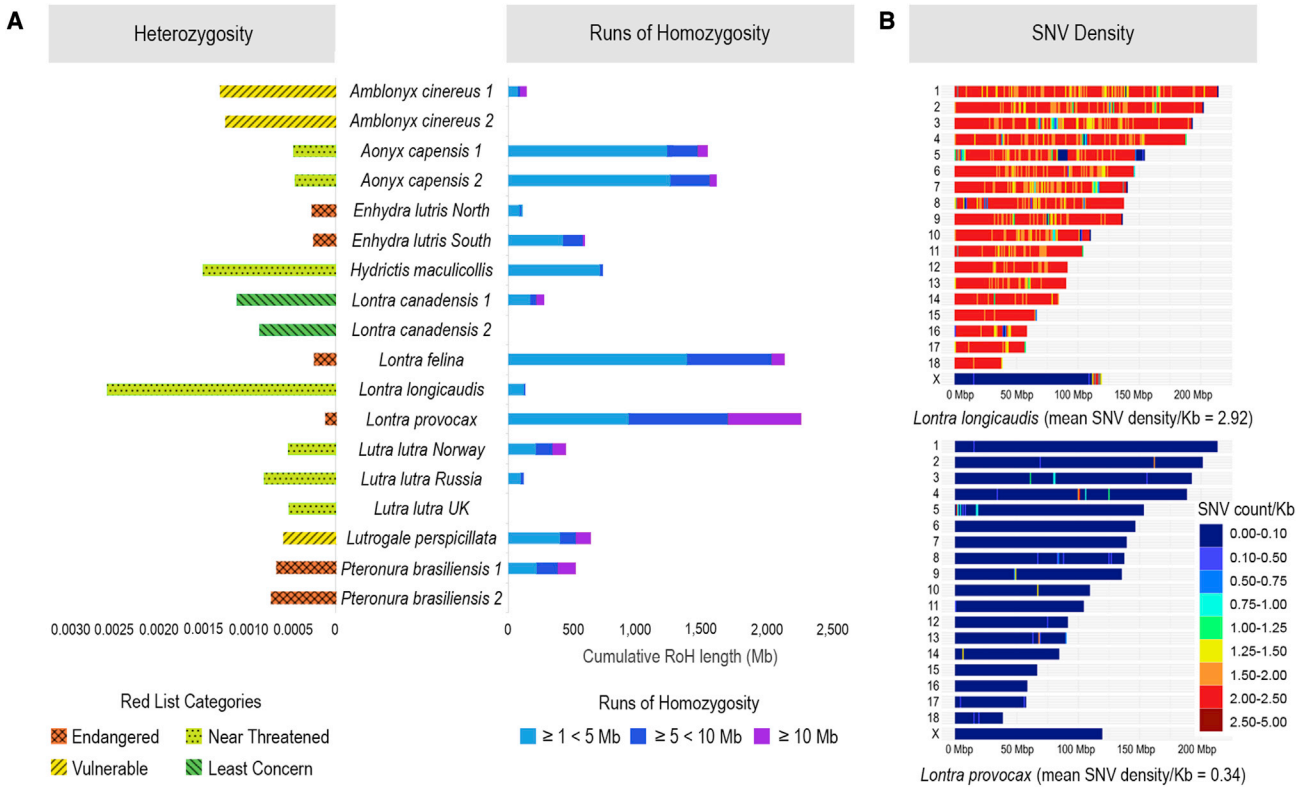

Figure 4. Genomic diversity of otter species

(A) Genome-wide heterozygosity (left) and runs of homozygosity (right) of 18 individuals representing 11 otter species. Bars showing heterozygosity values are colored according to the species' IUCN Red List category.

(B) Two representative plots showing the distribution of SNV density (per 1 Kb window) in the Neotropical otter (top) and southern river otter (bottom); the mean autosomal SNV density of each individual is indicated in parentheses; SNV density plots for all the assessed individuals are shown in Figure S4.

America.[33] We note that otters inhabiting higher latitudes in both the northern and southern hemispheres show low recent N_e values (e.g., 2,000 individuals for *L. lutra*; 2,500 for *E. lutris*; 2,000 for *L. felina*), with that of *L. provocax* (1,000 individuals) being the lowest among all analyzed species.

In contrast to the other otters, two tropical/subtropical species, the Neotropical otter (*L. longicaudis*) and the spotted-necked otter (*H. maculicollis*), showed distinctive trends of increasing N_e toward the present, which in the former was followed by a more recent decline (Figure 3). It is interesting to observe the contrasting demographic histories of sympatric species such as *L. longicaudis* and *P. brasiliensis* in South America, and *H. maculicollis* and *A. capensis* in Africa, whose distinct ecological features may have led to different population dynamics in response to the same paleoclimatic cycles. For example, *L. longicaudis* is more of a habitat generalist than *P. brasiliensis*,[34] while *H. maculicollis* tends to use larger and more permanent water bodies compared to *A. capensis*,[13] factors that may have facilitated population expansions in the former species during cooler/drier periods, while possibly driving population declines in the latter species.

For six of the species for which we had genomes of two or more individuals, N_e trajectories were essentially identical. The sole exception was a lower N_e trajectory of the Eurasian otter (*L. lutra*) from the UK prior to 50 kya compared to two individuals sampled in Norway and Russia (Figure 3). This intriguing difference remained visible regardless of the reference genome or the filtering parameters employed in the analysis (Figure S3B). This suggests that the UK sample may represent a distinct demographic lineage, an inference that is corroborated by the phylogenetic analyses of mitochondrial and nuclear data (Figures S2D and S2F), which place it as the most divergent *L. lutra* lineage, having split from the Norway/Russia pair at least 0.37 mya. If affirmed by the analysis of additional individuals from across the *L. lutra* range, and after considering potential anthropogenic biases (e.g., a possible effect of captive-bred releases in the UK during the 1980s and 1990s[35–37]), this observation could suggest that these populations were isolated in different refugia during Pleistocene glacial periods, consistent with patterns reported for multiple species of European plants and animals (e.g., Hewitt[38,39]). Interestingly, the three N_e trajectories become similar and begin to simultaneously decline precipitously ~40–70 kya, coinciding with the advance of the Last Glacial Period, well before the island of Britain became separated from mainland Europe ca. 6 kya.[40]

We then assessed current levels of genome-wide diversity (Figure 4) and observed that it was generally lower in species with low N_e estimates closer to the present (Figure 3), demonstrating congruence among these assessments. In particular, the two species with the lowest N_e trajectories (*L. felina* and

L. provocax) exhibited the lowest heterozygosity and the largest burden of runs of homozygosity (RoH). Overall, the estimated RoH burden was quite variable, even among individuals from the same species, in some cases likely reflecting idiosyncratic histories of inbreeding (including possible *ex situ* effects in the *A. capensis* samples). Examination of the genome-wide distribution of heterozygous single-nucleotide variants (SNVs) among otter species showed patterns largely consistent with mean heterozygosity, as expected, but provided a detailed view of the extent of SNV density along chromosomal scaffolds (Figures 4 and S4). For example, the genome of *L. longicaudis* is characterized by many large blocks of high SNV density (mean = 2.92/Kbp), while that of *L. provocax* shows small and sparse blocks of high SNV density against a background density that is almost 9-fold lower (mean = 0.34/Kbp). Taken together, our analyses of genetic diversity suggest dramatically different adaptive potentials among otter species.

From a conservation perspective, we observed relatively low genome-wide heterozygosity for most species, and cases of taxa allocated in a high-threat IUCN category (which is based on estimated census size and projected population trends, and considered an imperfect predictor of extinction risk[41]) that exhibited particularly low variation (Figure 4). Specifically, the three species with the lowest heterozygosity (*L. provocax*, *L. felina*, and *E. lutris*) are in the most threatened IUCN category (Endangered). On the other hand, the fourth "Endangered" otter (*P. brasiliensis*) presented higher levels of diversity, likely due to its reasonably large population sizes in its Amazon stronghold, in spite of severe threats across its range (including extirpation in many areas). In contrast, *L. lutra* has similar levels of diversity and is in a lower-risk IUCN category (Near Threatened), also being considered less threatened than the more variable *A. cinereus* (Vulnerable). This may be due to the broad distribution of *L. lutra*, which tends to decrease its species-wide risk category despite severe threats faced by regional populations. In contrast, *A. cinereus*, which also suffers from various threats (e.g., habitat loss and degradation, animal trafficking, and reduction of prey base by over-exploitation[42,43]), has a smaller range (Figure 1), which may have influenced this assessment.

Such comparisons illustrate situations in which genome-wide diversity could be used to predict extinction risk (e.g., for *L. provocax*, *L. felina*, and *E. lutris*) and cases in which other factors (e.g., severe current threats not yet impacting genetic diversity) may dominate. Although the IUCN Red List does not directly consider genetic diversity as a criterion to assess threat status, the relevance of incorporating genomic data in conservation assessments and management actions is a current focus of discussions in this field (e.g., Scott et al.[44,45]). For example, an analysis of 130 mammal species showed that overall heterozygosity was lower in species at higher-risk IUCN Red List threat categories.[46] Improved assessments of this relationship will be enabled as whole-genome data are generated for more taxa varying in their biological properties, as well as multiple individuals and populations within each species, as has been recently shown with an in-depth analysis of sea otter population genomics.[47]

Overall, our results allowed a comprehensive assessment of the evolutionary history of the world's otters, including insights into their phylogeny, biogeography, demographic history, and genomic diversity. This has allowed the proposition of a new genus-level taxonomy for this group, supported the recognition of an endemic otter from equatorial Africa, and indicated how different species were affected demographically by past climatic shifts, influencing current levels of genomic diversity. We suggest that comparative assessments of entire clades comprising multiple related species, providing a historical backdrop against which present-day diversity can be assessed, are a useful avenue to pursue as this field progresses to fully incorporate genome-wide approaches in evolutionary and conservation biology.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2022.06.036.

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AUTHOR CONTRIBUTIONS

Conceptualization, E.E., K.-P.K., and V.d.F.; data curation, V.d.F., H.V.F., and F.d.J.T.; formal analysis, V.d.F., H.V.F., F.d.J.T., and S.K.; funding acquisition,

E.E., K.-P.K., and M.T.P.G.; investigation, V.d.F., O.S., M.-H.S.S., and G.Z.L.; methodology, V.d.F., H.V.F., O.S., M.-H.S.S., K.-P.K., and E.E.; project administration, E.E. and K.-P.K.; resources, C.S.T., G.V., J.A.V., F.B., N.S., T.B., O.A.R., and M.T.P.G.; supervision, E.E., K.-P.K., and M.T.P.G.; writing – original draft, V.d.F., E.E., and K.-P.K.; writing – review & editing, H.V.F., F.d.J.T., O.S., M.-H.S.S., C.S.T., G.Z.L., G.V., J.A.V., F.B., S.K., N.S., T.B., O.A.R., and M.T.P.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Amblonyx cinereus	This study	London Zoo
Aonyx capensis	This study	San Diego Zoo Wildlife Alliance
Aonyx capensis	This study	San Diego Zoo Wildlife Alliance
Aonyx congicus	This study	MNHN-ZM-MO-1947-31
Aonyx congicus	This study	MNHN-ZM-2005-612
Hydrictis maculicollis	This study	Brookfield Zoo
Lontra canadensis	This study	Brookfield Zoo
Lontra felina	This study	Algarrobo, Chile
Lontra longicaudis	This study	Amazonas, Brazil
Lontra provocax	This study	Parque Nacional Nahuel Huapi, Argentina
Lutra lutra	This study	Narvik, Norway
Lutra lutra	This study	Tyumen Oblast, Russia
Lutra sumatrana	This study	KU CN4494
Lutrogale perspicillata	This study	Pak6 (Pisa Collection)
Critical commercial assays		
QIAmp DNeasy Blood & Tissue kit	QIAGEN	Cat# 69504
TruSeq DNA PCR-Free	Illumina	Cat# 20015962
TruSeq Nano DNA kits	Illumina	Cat# 20015964
Deposited data		
Amblonyx cinereus	This study	NCBI Bioproject: PRJNA841998
Amblonyx cinereus	DNAZoo	NCBI SRA: SRR12437584
Amblonyx cinereus	DNAZoo	NCBI SRA: SRR12437579
Aonyx capensis	This study	NCBI Bioproject: PRJNA841998
Aonyx capensis	This study	NCBI Bioproject: PRJNA841998
Aonyx congicus	This study	NCBI Bioproject: PRJNA841998
Aonyx congicus	This study	NCBI Bioproject: PRJNA841998
Enhydra lutris	[48]	NCBI Bioproject: PRJNA388419
Enhydra lutris	[49]	NCBI Bioproject: PRJNA472597
Hydrictis maculicollis	This study	NCBI Bioproject: PRJNA841998
Lontra canadensis	This study	NCBI Bioproject: PRJNA841998
Lontra canadensis	Canseq150 program	NCBI SRA: SRR10409165
Lontra canadensis	DNAZoo	NCBI SRA: SRR12437593
Lontra felina	This study	NCBI Bioproject: PRJNA841998
Lontra longicaudis	This study	NCBI Bioproject: PRJNA841998
Lontra provocax	This study	NCBI Bioproject: PRJNA841998
Lutra lutra	This study	NCBI Bioproject: PRJNA841998
Lutra lutra	[50]	NCBI Bioproject: PRJEB35339
Lutra lutra	This study	NCBI Bioproject: PRJNA841998
Lutra sumatrana	This study	NCBI Bioproject: PRJNA841998
Lutrogale perspicillata	This study	NCBI Bioproject: PRJNA841998
Pteronura brasiliensis	Broad Institute	NCBI Bioproject: PRJNA399365
Pteronura brasiliensis	DNAZoo	NCBI SRA: SRR12437585
Pteronura brasiliensis	DNAZoo	NCBI SRA: SRR12437583
Mustela putorius furo	Broad Institute	NCBI Bioproject: PRJNA59869

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
FastQC	[51]	https://github.com/s-andrews/FastQC
PALEOMIX 1.2.13.2	[52]	https://github.com/MikkelSchubert/paleomix
AdapterRemoval 2.0.0	[53]	https://github.com/MikkelSchubert/adapterremoval
Picard	N/A	http://broadinstitute.github.io/picard/
BWA	[54,55]	http://bio-bwa.sourceforge.net/
mapDamage	[56]	https://ginolhac.github.io/mapDamage/
ANGSD 0.921	[57]	https://github.com/ANGSD/angsd
RepeatMasker 4.0.9	[58]	https://www.repeatmasker.org
SAMtools	[59]	http://www.htslib.org
trimAl 1.4	[60]	http://trimal.cgenomics.org
RAxML 8.2	[61]	https://cme.h-its.org/exelixis/web/software/raxml/
Dsuite	[9]	https://github.com/millanek/Dsuite
ASTRAL-III	[62]	https://github.com/smirarab/ASTRAL
PAML 4.5	[63]	http://abacus.gene.ucl.ac.uk/software/
PSMC	[64]	https://github.com/lh3/psmc
bcftools 1.10	N/A	https://samtools.github.io/bcftools/bcftools.html
vcftools v0.1.16	[65]	https://vcftools.github.io/
ggplot2	[66]	https://ggplot2.tidyverse.org

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Eduardo Eizirik (eduardo.eizirik@pucrs.br).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Newly generated raw fastq files have been deposited at NCBI and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The current study uses 24 whole genomes sequences, representing all 13 currently recognized extant otter species. Detailed information is provided in Table S1.

METHOD DETAILS

Genome sequencing

We sequenced 14 whole genomes representing 11 of the 13 extant otter species (including two individuals each of *Lutra lutra*, *Aonyx capensis*, and *Aonyx congicus*). For *Pteronura brasiliensis*, *Enhydra lutris,* and *Lutra lutra*, we used previously reported genomes (Table S1). For the demographic history and genome-wide diversity analyses, we included additional genomes that became available while we were conducting this study. This led to an overall dataset comprising 24 genomes (three of which were only used as reference genomes) representing all extant otter species, to which we added a domestic ferret (*Mustela putorius furo*) genome to be used as an outgroup (see Table S1).

For nine species, genomes were sequenced using blood or tissue from modern samples, while for the remaining two species (*Lutra sumatrana* and two individuals of *Aonyx congicus*), we used museum samples (Table S1). For modern samples, DNA was extracted using a phenol/chloroform protocol[67] or commercial kits (QIAmp DNeasy Blood & Tissue kit [QIAGEN]), genomic libraries with 350-base pair (bp) inserts were prepared with TrueSeq DNA PCR-Free or TrueSeq Nano DNA kits (Illumina), and sequenced with 150 bp paired-end reads on an Illumina HiSeq X Ten instrument. DNA extractions of museum samples followed Salleh et al.[68] and library preparations were carried out according to the BEST method described by Carøe et al.[69] Sequencing was performed

using 50 bp paired-end reads for *Aonyx congicus* 1 and 80 bp single-end reads for *Lutra sumatrana* and *Aonyx congicus* 2 on an Illumina HiSeq 4000 instrument.

Mapping

We used FastQC to assess sequence quality,[51] and the PALEOMIX pipeline version 1.2.13.2[51] to filter reads and map them against reference genomes (see below). This pipeline included steps for trimming reads, removing adapters with AdapterRemoval version 2[52] and filtering PCR duplicates with Picard MarkDuplicates (http://broadinstitute.github.io/picard/). For modern samples, we discarded reads shorter than 100 bp and with quality scores lower than 30. The remaining reads were mapped against reference genomes using the BWA 0.7.1-Mem algorithm.[54] For the museum samples, we used BWA-backtrack[55] and the following parameters: for both *Aonyx congicus* samples, we discarded reads shorter than 15 bp, and for *Lutra sumatrana*, reads shorter than 18 bp, not setting any minimum quality threshold. The three museum samples were checked with mapDamage 2.0[55] and no significant evidence of DNA damage was observed.

For all phylogenomic analyses, we mapped the reads against the domestic ferret reference genome (*Mustela putorius furo*[70]), since we considered it important to use a reference that was equidistant from all ingroup species. For the demographic history and genome-wide diversity analyses, we tested the impact of employing different reference genomes (data not shown) and decided to use the closest reference available for each species, which were: *Amblonyx cinereus* 3 for *Amblonyx* and *Lutrogale* genomes; *Enhydra lutris* North for *Enhydra* individuals; *Lontra canadensis* 3 for *Lontra* genomes; *Pteronura brasiliensis* 3 for *Pteronura* individuals; and *Lutra lutra* UK for the remaining species (see Table S1).

To reconstruct the mitochondrial genomes, we mapped the reads against the *Mustela putorius furo* mitogenome using the PALEOMIX pipeline version 1.2.13.2,[52] with the same parameters described above for the modern samples. To assess the robustness of the reconstructed mitochondrial sequences, we also mapped the reads against different otter mitogenomes and performed the same phylogenetic and dating analyses (see below), yielding very consistent results (data not shown). We therefore only present the results based on the external, equidistant *M. p. furo* reference, consistent with the nuclear phylogenomic analyses described below.

Consensus and masking

We generated consensus sequences of all genomes using ANGSD version 0.921[57] with the parameters doFasta= 2, doCounts= 1, and explode= 1. For the modern samples, we also used the quality parameters setMinDepth= 10 and minMapQ= 20. For the three museum samples, we tested different minimum depth (1, 2, 4, or 10), mapping quality 10, and base quality 20 settings, and for the mitogenomes we used setMinDepth= 5 and minMapQ= 20. For modern samples, low complexity DNA sequences and repeats were masked using the RepeatMasker version 4.0.9[58] carnivore database. Museum samples were not masked.

QUANTIFICATION AND STATISTICAL ANALYSIS

Phylogenomic analysis

Nuclear genome

We selected the outgroup (*Mustela putorius furo*) as the reference to use in our main set of phylogenomic analyses, since it is equidistant from all otter sequences, thus avoiding any potential bias induced by employing an ingroup reference.

Because the three museum samples had much lower coverage than the modern ones, we used two different taxon sets for the phylogenomic analyses (see Table S1):

Taxon set 1 – This included 21 individuals and all 13 otter species plus *Mustela putorius furo* as an outgroup. We used Samtools mpileup[59] with the following parameters: -d 0 -q 10 -Q 20 -s. Using an in-house shell script, the Samtools file was filtered for the following combinations of minimum depth (D), minimum mapping quality (MapQ) and minimum base quality (BQ) parameters for the two *Aonyx congicus* genomes: (a) D=1, MapQ=10, BQ=20; (b) D=2, MapQ=10, BQ=20; (c) D=4, MapQ=10, BQ=20; and (d) D=10, MapQ=10, BQ=20. We considered only the sequenced regions that were shared by both *A. congicus* genomes and extracted those same regions from all the other individuals. We then concatenated the alignments from these regions using an in-house python script, creating a supermatrix for each combination of depth/quality parameters listed above (a-d). Subsequently, we filtered sites according to the amount of missing data, using trimAl version 1.4[60] with the following parameter combinations: (i) allowing any amount of missing data (no filter); (ii) keeping only sites with 40% or less missing data, (iii) keeping only sites with 20% or less missing data and (iv) keeping only sites with no missing data. For each data set (derived from the 16 combinations of 4 depth/quality and 4 filtering schemes - see Figure S1), we estimated a maximum-likelihood (ML) tree with RAxML HPC-PTHREADS 8.2[61] using a GTR+GAMMA model of nucleotide substitution, with 100 bootstrap replicates.

Taxon set 2 – This included 1 individual per species, considering only modern samples (total of 12 genomes, including 11 otter species and the outgroup). Using an in-house python script, we created four datasets comprising non-overlapping genomic fragments (GFs) consisting of contiguous 10 kb and 100 kb windows, as well as of non-contiguous 10 kb and 100 kb windows (in both cases skipping 100 kb between sampled GFs). We filtered sites using trimAl version 1.4,[60] allowing for a maximum of 33% missing data, i.e. allowing a maximum of 4 species to have missing data at a given site. After this step, windows smaller than 50% of the original size were excluded. The remaining total size of each dataset was: for 10 kb GFs - 1,189,814,856 bp; for 10 kb GFs skipping 100 kb - 109,014,723 bp; for 100 kb GFs - 1,237,896,213 bp; for 100 kb GFs skipping 100 kb - 620,526,295 bp.

For each GF in each dataset, we estimated a maximum-likelihood (ML) tree with RAxML HPC-PTHREADS 8.2, using a GTR+GAMMA substitution model, with 100 bootstrap replicates. We surveyed the frequency of different topologies retrieved with the different datasets, and used the GF trees to estimate a species tree applying a multi-species coalescent approach. For that, we used the ML tree obtained for each GF, along with its bootstrap results, as the input for an ASTRAL-III[62] analysis. To investigate potential cases of interspecies introgression leading to genealogical discordance, we employed the Dsuite package[9] with default settings.

Mitochondrial genome

For this analysis, we included the same individuals from Taxon set 1 and 2 described above. The alignment was generated using an in-house python script. We estimated a maximum-likelihood tree for the mitogenome alignment excluding the control region (15,442 bp), using RAxML HPC-PTHREADS 8.2 with a GTR+GAMMA model of nucleotide substitution and 100 bootstrap replicates to assess nodal support.

Divergence time

We estimated the divergence time for the mitochondrial and nuclear genomes with a Bayesian Markov Chain Monte Carlo algorithm using MCMCtree.[63] For the nuclear genome, we used Taxon sets 1 and 2. For Taxon set 1, we ran MCMCtree for the concatenated data with the following filtering parameters: minimum D = 4, MapQ = 10, BQ = 20, including only sites with 40% or less missing data. For Taxon set 2, we ran MCMCtree for each of the contiguous 100 kb GFs, assuming the tree topology resulting from its own RAxML analysis (described above), and including only GFs for which one of the three main topologies was retrieved. The mitogenome was analyzed as a single dataset. For both datasets (nuclear and mitochondrial), we used the same parameters: ndata = 1; seqtype = 0: nucleotides; usedata = 1: seq like; clock = 2: independent rates; RootAge = '>.057<.118'; model = 4: HKY85; alpha = 0.02; ncatG = 4; cleandata = 0: no; BDparas = 1 1 0.1; kappa_gamma = 6 2; alpha_gamma = 1 1; rgene_gamma = 1 6.89; sigma2_gamma = 1 10; finetune = .1_ .1 .1 .1 .1 .1 .1; print =1; burnin = 200000; sampfreq = 5; nsample = 50,000. We used three calibration points: (1) root age between 5.7 and 11.8 MYA;[71] (2) 3.6 MYA as the minimum age for *Lutra*;[72] and (3) 3.8 MYA as the minimum age for *Lontra*.[73]

Demographic history

We inferred the demographic history for 18 individuals of the 11 otter species for which we had modern samples (Taxon set 3 – Table S1) by applying the Pairwise Sequentially Markovian Coalescent (PSMC) approach.[64] After assessing the impact of using different reference genomes and removing the sex chromosomes, we performed final analyses only considering autosome data and using the closest available reference, as described in the mapping section. We used the default settings recommended in the PSMC manual (https://github.com/lh3/psmc) and performed 100 bootstrap replicates to assess variance in the demographic trajectories (Figure S3).

The resulting graphs were plotted using the psmc_plot.pl script, assuming an absolute mutation rate per nucleotide of 1.0×10^{-8} per generation.[74] To assume realistic, species-specific generation times, we used available data from field and/or captive breeding studies or estimated them based on available information on each species' life history (Table S3). When no data were available for a given species, we used information from a closely related species with a similar life history and/or weight/size (Table S3).

For *Lutra lutra*, to test if the difference found among individuals was an artifact, we mapped each sample against three reference genomes (*Amblonyx cinereus* 3, *Enhydra lutris* North and *Lutra lutra* UK) and tested three different maximum depth parameters (40, 50 and 100) (see Figure S3).

Single nucleotide variant calling

Variant calling was performed using bcftools v1.10 with the following parameters for mpileup: -a AD, INFO/AD, ADF, INFO/ADF, ADR, INFO/ADR, DP, SP, -d 250 -q 30 -Q 30 –adjust-MQ 50; -m -v -f GQ,GP for bcftools call; and -e 'QUAL<20.0 || FORMAT/SP>60.0 || FORMAT/DP<5.0 || FORMAT/GQ<20.0' for bcftools filter (following Totikov et al.[75]).

Genome-wide diversity

To estimate genome-wide diversity parameters for Taxon set 3, which comprises 18 modern samples (see Table S1), we used the genomes mapped against the closest reference available for each species, as described in the mapping section. Heterozygosity was calculated using ANGSD[57] with the parameters minmapq 30, C 50, gl 1, -dosaf 1, -fold 1 and using the output (saf.idx file) to run the realSFS program in ANGSD. We then calculated the heterozygosity by dividing the number of heterozygous genotypes by the total number of sites.

We identified the presence and extent of runs of homozygosity (RoH) with bcftools roh using the -M 1e-3, –AF-dflt 0.1, –skip-indels, and -G30 parameters. We then grouped the RoH in three categories according to their size: 1) $\geq$ 1 to < 5 Mb, 2) $\geq$ 5 to < 10 Mb, and 3) $\geq$ 10 Mb.

We also estimated the density and distribution of variable sites across each chromosome, excluding the Y chromosome and following the chromosome order used by Totikov et al.[75] SNV density plots were constructed using the "snpden" function in VCFtools,[65] calculating the number of heterozygous SNVs in 1 Mb non-overlapping windows. Variant density plots were created using the ggplot2 package in R.[71]