A tail of two pandas— Whole Genome K-mer Signature Analysis of the Red Panda (*Ailurus fulgens*) and the Giant Panda (*Ailuropoda melanoleuca*)

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Abstract

Background: The red panda (*Ailurus fulgens*) is a riddle of morphology, making it hard to tell whether it is an ursid, a procyonid or a member of its own family. Previous genetic studies have given contradictory results as to its phylogenetic placement.

Results: Therefore, a recently developed whole genome-based algorithm, the Whole Genome K-mer Signature algorithm was used to analyze the genomes of 28 species of Carnivora, including *A. fulgens* and several felid, ursid, mustelid, one mephitid species. This algorithm has the advantage of holistically using all the information in the genomes of these species. Being a genomics-based algorithm, it also reduces stochastic error to a minimum.

The results show that *A. fulgens* is a member of the mustelid clade ($p = 9 \cdot 10^{-97}$). *A. fulgens* also separates from the mephitid *Spilogale gracilis*. The giant panda, *Ailuropoda melanoleuca* also clusters away from *A. fulgens*, together with other ursids ($p = 1.2 \cdot 10^{-62}$). This could be due to the geographic isolation of *A. fulgens* from other mustelid species.

Conclusions: The main conclusion that we can draw from this study is that on a whole genome level *A. fulgens* belongs to the mustelid clade, and not an ursid or a mephitid. This despite the fact that previously some researchers classified *A. fulgens* and *A. melanoleuca* as relatives. Since the genotype determines the phenotype, molecular-based classification takes precedence over morphological classifications. This affirms the results of some previous studies, which studied smaller portions of the genome. This study is more substantial because it takes the whole genome into account.

Keywords: red panda, giant panda, whole genome k-mer signature, Pearson correlation, mustelid, ursid

Background

The red panda (*Ailurus fulgens*) is an enigmatic animal and is hard to classify based on its morphology. It lives in parts of India, Nepal and China, and has a distinct red-white coloration, and a striped, bushy tail. It goes by several nicknames, such as the ‘bear-cat’, the ‘cat-bear’, the ‘lesser panda’ or the ‘fire-fox’. Some researchers think *A. fulgens* is a relative of the giant panda (*Ailuropoda melanoleuca*) based on several physical characteristics. These include an almost exclusive diet of bamboo (both species eat meat on occasion), and have an enlarged radial sesamoid bone, which they use to process bamboo [1,2].

Because of these similarities, the giant panda even received its name from the red panda. According to other opinions, *A. fulgens* has been classified as a member of the family Procyonidae (raccoons). Yet others put the red panda into its own family (Ailuridae). *A. fulgens* also has some unique characteristics: a large zygomatic arch, a powerful jaw, and complex cheek teeth, following a P2-3 pattern [1].

Previous genetic studies

According to new genetic evidence, there are two species of red panda, the Himalayan red panda (*A. fulgens*), and the Chinese red panda (*A. styani*) [3]. Due to reduced numbers, the red panda is an endangered species. Previous studies based on different combinations of nuclear and mitochondrial genes have given contradictory results as to the taxonomic relationship of *A. fulgens* with other carnivores. This may be because only several mitochondrial and/or nuclear genes were analyzed, and not the entire whole genome sequence (WGS).

The red panda's classification as a procyonid or procyonid-relative is based on immunological, DNA-DNA hybridization, and isozyme evidence [4]. A phylogenetic tree based on Bayesian analysis of cytochrome-b put *A. fulgens* next to Canidae [5].

For example, Peng et al. classify *A. fulgens* either as a mustelid, placing them next to the American marten (*Martes americana*), or as a mephitid, next to the striped skunk (*Mephitis mephitis*). This was based on the analysis of 12 concatenated proteins, based on neighbor-joining (NJ) and maximum likelihood (ML) phylogenetic methods, respectively [6]. In a study of three mtDNA genes (12S rRNA, 16S rRNA and cytochrome b) and intron 1 of the nuclear transthyretin gene, Flynn et al. also found that *A. fulgens* is neither an ursid, nor a procyonid, nor a mephitid, but a mustelid [1]. Another study including three mitochondrial and three nuclear genes by Fulton and Strobeck, based on 16 arctoid species, with *Canis lupus* as an outlier, placed *A. fulgens* in close relationship to *M. mephitis* [7].

Yu and Zhang studied introns 4 and 7 from the nuclear gene β -fibrinogen (FGB) as well as the mitochondrial gene NADH dehydrogenase subunit 2 (ND2) in 17 species from the order Carnivora. In their results, these researchers found that *A. fulgens* is most closely related to procyonids based on analysis of intron 4 of the FGB gene. But when intron 7 was analyzed, it clustered towards ursids. Classification based on the ND2 gene *A. fulgens* clustered with mustelids, but these results had poor bootstrapping support. When the two introns were combined with analysis of the genes IRBP and TTR, *A. fulgens* was closest to mustelids [8].

Sato et al. analyzed a 5.5 Kbp segment of DNA coding for five genes, AOPB, BRCA1, RAG1, RBP3, and VWF, and found that *A. fulgens* clusters together with procyonids and mustelids, and not with mephitids (skunks and stink badgers) [9]. An earlier, similar result was attained when studying a 3.2 Kbp segment containing the genes APOB, RAG1 and IRBP [10]. Genomically, *A. fulgens* shares several apomorphic chromosome fusions with mustelids, namely F2+C1p and A1p+C1q [11]. However, *A. fulgens* differs in several other chromosomal rearrangements indicating that it diverged early from other mustelids.

Interestingly, several genes have been found in both species which show convergent development. For example, changes in the amino acid composition of the DYNC2H1 and PCNT proteins lead to polydactyly in humans and mice, but to the pseudthumb in the giant and red pandas. Three other convergent genes (PRSS1, PRSS36, and CPB1) are responsible for more efficient uptake of nutrients from bamboo, which makes up a large part of their diet as well. Four other genes, ADH1C, CYP3A5, CYP4F2, and GIF also enable the more effective utilization in the giant and red pandas of vitamins A and B12 as well as arachidonic acid, which are absent or very low in bamboo [2].

Intron analysis is useful, since these sequences are not under mutational constraint. An analysis of 22 Kbp of nuclear intron sequences from 16 carnivore species groups *A. fulgens* with Musteloidea *sensu stricto* (Mustelidae+Procyonidae) to the exclusion of mephitids [12]. These results, however, contradict results coming from mtDNA analyses [13].

Principle of analysis

Since morphology-based classification of *A. fulgens* is ambiguous, it would be helpful to analyze the precise taxonomic status of this species based on a whole genome-based algorithm. To this end, the Whole Genome K-mer Signature (WGKS) algorithm [14] is used to analyze the genomes of five bear species, eleven cat species and ten species from the family Mustelidae (weasels, otters, martens, and badgers), *Spilogale gracilis*, a mephitid species, as well as *A. fulgens*, making 28 species in total.

The advantages of using a genomics-based algorithm to analyze the WGS of these organisms is that it takes all the information present in the WGS, as opposed to just a handful of genes. Whole genome-based algorithms also have the advantage that they greatly reduce stochastic error, due to the vast number of characters (DNA bases) that they analyze [15]. Using this algorithm can provide definitive results as to the phylogenetic classification of *A. fulgens*.

Results and Discussion

Pre-clustering analysis

The list of species and the PCC matrix can be seen in Additional File 1 online. The Hopkins statistic is 0.9, which means that the data set is of very good quality for clustering. The silhouette plot (Supplementary figures 1 and 2) gave a maximum average silhouette width of 0.82 for three clusters. This value was 0.8 for four clusters. The average silhouette width was studied for two to seven clusters. The only difference was the placement of the mephitid, *S. gracilis* into its own group (cluster 4 in Supplemental figure 2).

Whole genome analysis

In Figure 1 we can see three visible clusters, felids, ursids and mustelids, with *S. gracilis* in between the mustelids and the ursids. Based on the results in Table 1, *A. fulgens* clearly clusters together with the mustelids, although on average, it has a lower mean PCC value compared to all the other species, 0.89 ± 0.03 , whereas mustelids have a mean PCC value of 0.95 ± 0.04 .

This difference is not too significant. If we compare *Felis nigripes* (the black-footed cat) with other cats, it has a mean PCC value of 0.89 ± 0.02 , whereas felids having an even greater mean PCC of 0.97 ± 0.03 . Yet we know that cats are a monophyletic group. Table 2 shows the minimum, mean, maximum PCC for all three putative clades, as well as the p-value, which is statistically significant for all three groups.

Based on this evidence, *A. fulgens* belongs to mustelids as a monophyletic group. Since it has such a low mean PCC is because it may have diverged early from other mustelids, possibly due to its isolated mountainous habitat in parts of Myanmar, Burma and China. This can also be seen well in Figure 2A, which shows the UPGMA-based phylogenetic tree for the mustelids.

Also important is that the skunk species *S. gracilis* does not cluster with mustelids. When compared with mustelids, *S. gracilis* has a mean PCC value of 0.78 ± 0.02 . *A. fulgens* has a PCC value of 0.79 with this species as opposed to a mean PCC value of 0.89 with mustelids, reported previously. This also indicates that mustelids and mephitids form separate clades.

The giant panda, *Ailuropoda melanoleuca* is a clearly a member of a clade which includes the ursids, as shown in Figure 2B. It has a mean PCC value of 0.97 ± 0.003 with the other ursids. Other genetic evidence classifies the giant panda as a member of Ursidae. This includes mtDNA, chromosome banding patterns, and serological and immunological evidence [20, 21].

Conclusion

In conclusion, we have seen that *A. fulgens* belongs to the mustelids. This species also clusters away from *S. gracilis*, indicating that mustelids and mephitids are separate clades. This is based on whole genome data as opposed to the contradictory results in previous studies involving just a handful of genes, one even in two different exons of the same gene. This demonstrates the utility of the WGKS algorithm, which takes a holistic approach of analyzing the WGS. *A. melanoleuca*, on the other hand, belongs to the ursids, as shown consistently in the WGS results.

Materials and Methods

Data and programs used

The Python script `motif_analysis_k-1.py` at github.com/csmaty/motif_analysis was used to generate WGKS profiles. Version 3.6.0. of R was used. The heatmap was generated using the R command 'heatmap', using the ward.D clustering algorithm for the WGKS analysis. Clusters were generated using the 'cutree' command and were depicted in phylogenetic trees using the UPGMA method [16]. To determine the optimal number of clusters, the 'cluster' and 'factoextra' libraries and the `fviz_nbclust` command were used, setting the method parameter to 'wss'. The 'fviz_silhouette' plot was used to construct the Silhouette plot. Additional Excel files and figures as well as the mitochondrial genome fasta file can be found online at github.com/csmaty/ailurus.

Description of algorithm

The WGKS algorithm that was used in the analysis is an alignment-free k-mer sequence comparison method [17]. These methods involve the statistical comparison of k-mers between species. A k-mer is a segment of DNA k bp long, which can correspond to the core segment of a transcription factor binding site, a repeat element or other regulatory element. These elements take part in protein binding and gene regulation and are conserved across different species. The advantages of using a k-mer based alignment-free algorithms over alignment-based ones is that they process input much faster and are unbiased by guide trees imposed upon the data [18, 19].

For a lengthy description of the algorithm, the reader is referred to Cserhati et al., 2019 [14]. However, a short description is provided here for better understanding. The WGKS algorithm is divided into three steps.

First, all possible k-mers in the genome of a given species are enumerated to give the observed occurrence O . Then, based on the background base pair distribution (A/C/G/T%), the expected occurrence E can also be calculated. Thus, the score value S can be calculated in the following way:

$$(1) S_{k-mer} = \frac{O-E}{O+E}$$

Score values can be interpreted in three ways:

- (2) $O \gg E : S_{k-mer} \rightarrow 1$ (overrepresented k-mer)
- (3) $O \ll E : S_{k-mer} \rightarrow -1$ (underrepresented k-mer)
- (4) $O = E : S_{k-mer} \approx 0$ (randomly occurring k-mer)

Even if the genome is partially or completely duplicated, then the score value will not change. This is because both the Observed and Expected values will increase by the proportion that the duplicated genome is compared to the pre-duplication genome.

The next step involves comparing the k-mer signature between two species. The k-mer signature is simply a list of all k-mers ordered in lexicographical order from AA...A to TT...T, together with their score values. For a given value k , there are 4_k possible k-mers. Thus, the k-mer signature also corresponds to a vector of 4_k numbers. Since octamers were analyzed, this corresponds to 65,536 possible octamers. Two of these vectors can be compared to one another for two different species using the Pearson Correlation Coefficient (PCC). PCC values closer to 1 represent a pair of closely related species, within the same clade. Lower PCC values denote two unrelated species. This step is performed between all possible pairs of species to derive a square PCC matrix. P-values for clusters were calculated by comparing the PCC values for all species pairs within the cluster with all PCC values for all species pairs where one species came from the cluster, and the other species was outside the cluster.

The last step involves visualizing the PCC in a heatmap and using clustering algorithms to detect monophyletic groups. Clustering can be done for example using the k-means clustering algorithm, or the Partitioning Among Medoids (PAM) algorithm.

Abbreviations

Mephitid: a member of the family Mephitidae, or skunks.

mtDNA: mitochondrial DNA.

Mustelidae: a member of the family Mustelidae, or a group of animals including weasels, otters, ferrets, minks, martens and wolverines.

PCC: Pearson Correlation Coefficient.

Procyonid: a member of the family Procyonidae, or raccoons.

UPGMA: unweighted pair group method with arithmetic mean, an agglomerative hierarchical clustering method.

Ursid: a member of the family Ursidae, or bears.

WGS: whole genome sequence.

WGKS: whole genome k-mer signature.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The Python script `motif_analysis_k-1.py` at github.com/csmaty/motif_analysis was used to generate WGKS profiles. Additional Excel files and figures can be found online at github.com/csmaty/ailurus.

Competing interests

The author declares that he has no competing interests.

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Author contributions

M.C. designed the whole study, ran all calculations and scripts, and wrote the article.

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Tables

Table 1. Classification of the 28 species used in the WGKS analysis.

Species	cluster
<i>Ailurus fulgens</i>	1
<i>Enhydra lutris</i>	1
<i>Gulo gulo</i>	1
<i>Lontra canadensis</i>	1
<i>Lutra lutra</i>	1
<i>Mellivora capensis</i>	1
<i>Mustela ermine</i>	1
<i>Mustela putorius furo</i>	1
<i>Neovison vison</i>	1
<i>Pteronura brasiliensis</i>	1

<i>Taxidea taxus</i>	1
<i>Ailuropoda melanoleuca</i>	2
<i>Ursus americanus</i>	2
<i>Ursus arctos</i>	2
<i>Ursus maritimus</i>	2
<i>Ursus thibetanus</i>	2
<i>Acinonyx jubatus</i>	3
<i>Felis catus</i>	3
<i>Felis nigripes</i>	3
<i>Lynx canadensis</i>	3
<i>Lynx pardinus</i>	3
<i>Panthera leo</i>	3
<i>Panthera onca</i>	3
<i>Panthera pardus</i>	3
<i>Panthera tigris</i>	3
<i>Prionailurus bengalensis</i>	3
<i>Puma concolor</i>	3
<i>Spilogala gracilis</i>	4

Table 2. Statistical measures for each of the three clusters in the WGKS analysis.

baramin	name	species	min	mean	max	stdev	p-value
1	mustelids	11	0.841	0.954	0.999	0.04	8.97E-97
2	ursids	5	0.966	0.983	0.997	0.012	1.23E-62
3	felids	11	0.879	0.965	0.998	0.032	6.17E-95

Figure legends

Figure 1. Heatmap depicting baraminic relationships for 28 species based on results from the WGKS algorithm. Brighter colors represent species pairs which are in the same baramin, with a PCC value closer to 1. Darker colors represent species pairs which are in different baramins, with a PCC less than 1.

Figure 2. A. UPGMA-based phylogenetic tree of mustelids based on PCC values. B. UPGMA-based phylogenetic tree of ursids based on PCC values.

Supplementary Figure 1. Silhouette plot for three clusters. The average silhouette width is 0.82.

Supplementary Figure 2. Silhouette plot for four clusters. The average silhouette width is 0.8.

Additional Files

Additional File 1: Results of whole genome analysis of 28 species. The files includes a list of species, and the genome sequence files downloaded from NCBI, the PCC matrix which is a result of the WGKS algorithm, as well as the species clusters and the cluster statistics.

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