Zootoca vivipara as a model for testing evolutionary transition from oviparity to viviparity

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Introduction

The lizard *Zootoca vivipara* is one on the few example in Nature which shows, within the same species, populations with different reproductive modalities. Oviparous populations live in the southern part of its distributional range (the newly discovered *Z.v.carniolica* in Eastern-Italian Alps and *Z.v.louislantzi* in the Pyrenees), while viviparous subspecies (e.g. *Z.v.vivipara* and *Z.v.sachalinensis*) are widely distributed from British Isles and central France to Scandinavia and north-eastern Asia [1](Fig.4). This species is, therefore, particularly well suited for studying the evolutionary shift in reproductive mode.

Results and Discussion

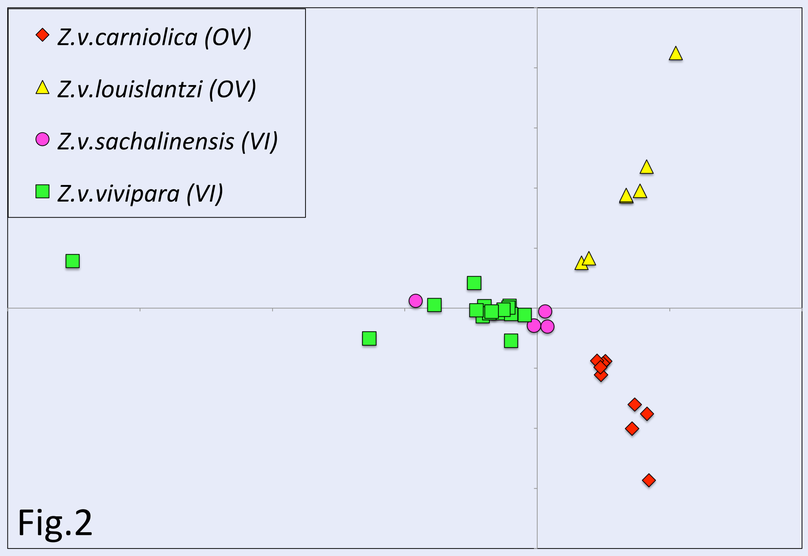

Fig.2 MultiDimensional Scaling based on genetic distances between individuals according to 19k SNPs

Bioinformatic analysis performed using the pipeline software Stacks produced about 100.000 local alignments and about 260.000 Single Nucleotide Polymorphisms (SNPs). In order to minimize the total number of missing data, we selected 19.013 SNPs. These markers were used to describe the overall genetic variation between subspecies. The result of MDS (Fig.2) seems to confirm (according to [1]) the existence of two parapatric oviparous clades (*Z.v.carniolica* and *Z.v.louislantzi*) and one viviparous clade (composed by *Z.v.vivipara* and *Z.v.sachalinensis*).

Additionally, a Minor Allele Frequency Spectrum has been calculated dividing the whole dataset according to the phenotype (Viviparous/ Oviparous). This analysis allows to underline polymorphisms that show low frequency in one phenotype and high frequency in the other, and vice versa. About 2.000 SNPs were selected according to MAFS. Furthermore, these polymorphisms were analyzed with TASSEL software [3] using GLM method to test for significant association between genotypes and phenotypes. After multiple-comparison correction, 289 SNPs showed a significant association with phenotype.

Genomic sequences (200-500 bp long, achieved with Illumina Paired-end protocol) physically linked to these markers were BLASTed against the whole NCBI Nucleotide Collection, with particular interest in looking for sequence similarities in *Anolis Carolinensis* genome (the only reptile genome available at the moment). Results are summarized in Figure 5 and Table 1.

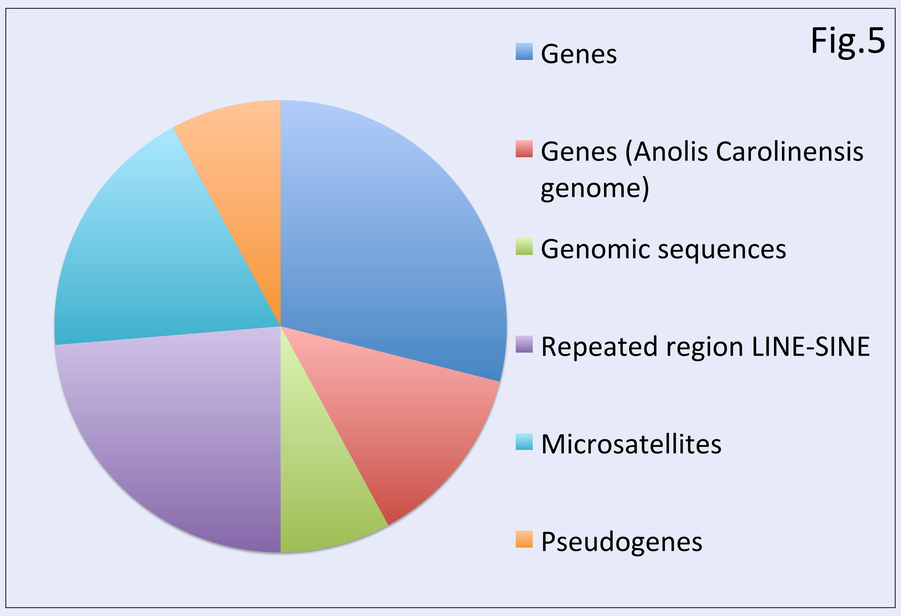

Fig.5 Pie-chart that summarizes sequence categories obtained from BLASTing about 300 genomic sequences (250 of them were unknown)

Genes (Predicted protein)	Genome
Toll-like receptor (immune system)	*Anolis Carolinensis*
Mitoferrin-1-like transporter	*Anolis Carolinensis*
Protein phosphatases	*Anolis Carolinensis*
Fibrinogen beta chain (coagulation)	*Anolis Carolinensis*
Aspartate beta-hydroxylase domain	*Gallus gallus*
Estrogen-related receptor gamma	*Homo sapiens*

Table 1. Some examples of genes with high values of sequence similarity with sequences selected in *Zootoca vivipara* according to TASSEL and MAFS

Materials and Methods

A Next Generation Sequencing technique has been used to analyse 40 samples of *Zootoca vivipara* to cover the overall genetic variation of the species. RADtag sequencing (Fig.1) uses Illumina HiSeq technology to simultaneously discover and analyse thousands of SNPs at genome level [5]. Bioinformatic analyses have been conducted using the pipeline software Stacks v0.9995 [6].

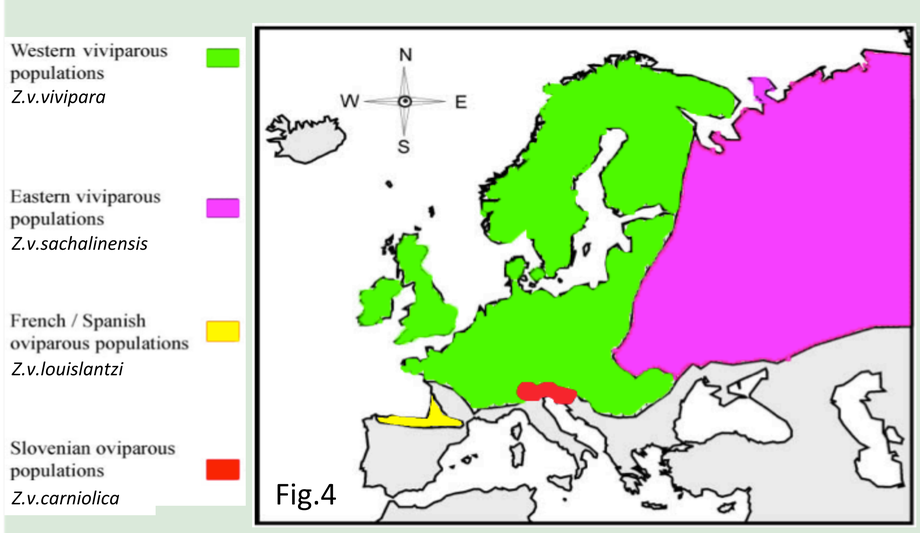

Fig.4 *Zootoca vivipara* European distribution and subspecific pattern (modified from [2])

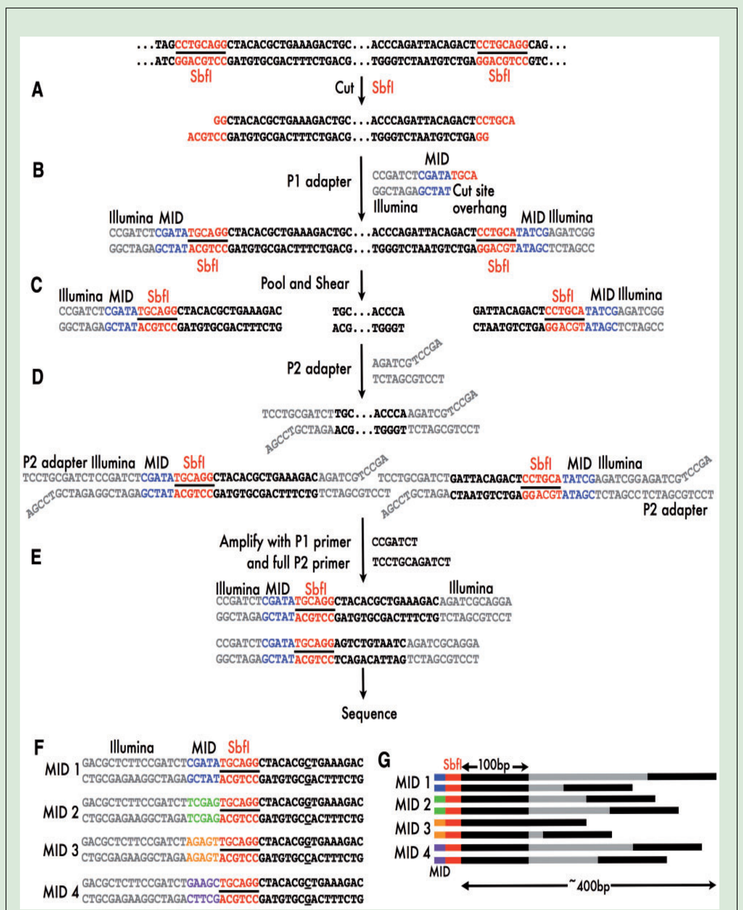

Figure I: The process of RADSeq. (**A**) Genomic DNA is sheared with a restriction enzyme of choice (SbfI in this example). (**B**) PI adapter is ligated to SbfI-cut fragments. The PI adapter is adapted from the Illumina sequencing adapter (full sequence not shown here), with a molecular identifier (MID; CGATA in this example) and a cut site overhang at the end (TGCA in this example). (**C**) Samples from multiple individuals are pooled together and all fragments are randomly sheared. Only a subset of the resulting fragments contains restriction sites and PI adapters. (**D**) P2 adapter is ligated to all fragments. The P2 adapter has a divergent end. (**E**) PCR amplification with PI and P2 primers. The P2 adapter will be completed only in the fragments ligated with PI adapter, and so only these fragments will be fully amplified. (**F**) Pooled samples with different MIDs are separated bioinformatically and SNPs called (C/G SNP underlined). (**G**) As fragments are sheared randomly, paired end sequences from each sequenced fragment will cover a 300–400 bp region downstream of the restriction site.

Conclusions

Looking for genes and markers showing signals of selection is becoming relatively straightforward with the advent of NGS; RADtag, together with Paired-end sequencing, is a useful method to assemble millions of genomic reads into contigs which can be compared to known sequences in existing databases. However, not all genes can be identified, since non-model species may contain unknown genes or the closest reference genome may not be so close.

In *Zootoca vivipara*, the matching proportion of contigs physically linked to SNPs which show signal of selection is around 14%. This value decrease to 6% when the entire dataset of contigs is analyzed.

However, about 20 conserved genes have been identified as possibly related to the two different reproductive modalities.

In order to investigate this topic, previous studies have been mainly focused on differential gene expression between mammals and viviparous squamate reptiles [7].

Evolution of viviparity poses a major immunological hurdle for mother and foetus. For instance, cytokines (Interleukin-1α and IL-1β are responsible of materno-foetal tolerance) seem to play a similar role in mammals and viviparous squamates. Tool-like receptor, identified in this study, together with the Interleukin-1 receptor forms a receptor superfamily, an important molecule in immune system. Moreover, genes like Hβ58 and HoxA10 may be involved in the placenta development of both mammals and reptiles. Hormone receptors have, for sure, an essential role in evolution of viviparity, in fact thay may regulate follicolar development and oocyte maturation [7]. One example of this category has been identified in this study (Tab.1).

So far, only gene-by-gene or protein-by-protein approaches have been taken; this study is the first attempt to analyze the oviparity/ viviparity transition at genomic level, with the consciousness that this shift is a very complex physiological process, probably mediated by thousands of genes.

Acknowledgments

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