Exon Capture - Principle and Applications to Phylogenomics and Population Genomics of Fishes

By Chenhong Li¹,²,*

¹Shanghai Universities Key Laboratory of Marine Animal Taxonomy and Evolution, Shanghai Ocean University, Shanghai 201306, China
²Shanghai Collaborative Innovation for Aquatic Animal Genetics and Breeding, Shanghai Ocean University, Shanghai 201306, China

ABSTRACT Phyllogenetic reconstruction based on one locus or a few loci can be misleading due to gene-tree/species-tree discordance. Species delimitation and intraspecific studies also often suffered from low resolution because of insufficient statistic power when few loci were used. Exon capture method is one of the most efficient way to collect genome-scale data, which can significantly augment studies that aimed to investigate patterns and histories of organisms at both intraspecific and high level. Here, I showed the advancement of shifting from single-gene method to genomic approach and the benefit of applying exon capture method comparing to alternative genomic techniques. Then, I explained the principle of exon capture method as well as providing detailed recommendations for applying this method. Finally, I demonstrated exon capture method using two applications and discussed future perspectives of this technology.

Key words: Target enrichment, exon capture, species delimitation, population structure, population dynamics, adaptation, molecular systematics, phylogenetics

INTRODUCTION

Because there are more molecular data available than morphological characters, and homology of molecular data usually is easier to be identified than the morphological characters, molecular data have become the predominant evidence to reconstruct tree of life. Molecular data also have been wildly used as the indispensable source for studying population structure and dynamics. Traditional molecular markers include mitochondrial genes or a few nuclear loci. Gene genealogy estimated from a single gene does not necessarily consistent with evolutionary history of species. Processes, such as gene introgression and incomplete lineage sorting can mislead species-level phylogenetic reconstruction based on single gene. For example, in our study of the Chinese perch (Siniperca spp.) using Cytochrome c oxidase I (COI) gene, we found that different species were mixed together on the tree (Liu et al., 2017).

One individual of *S. obscura* was more closely related to *S. kneri* than to other individuals of *S. obscura,* and individuals of the *S. kneri* and *S. chuatsi* were all mixed together (Fig. 1), but the problems were resolved when more independent nuclear loci were used (Liu et al., 2017).

High-level phylogenies based on single locus also can be misleading. In my dissertation work (Li, 2007), I showed that maximum likelihood phylogenies based on single-copy nuclear genes were not consistent with each other, that is, no two trees had the same topology (Fig. 2). Paralogy (Maddison, 1997), incomplete lineage sorting (Funk and Omland, 2003; Maddison and Knowles, 2006), horizontal gene transfer (Kurland et al., 2003) and stochastic errors all can led to the inconsistent results. Using genome-scale data can help to sort out nonphylogenetic noise and recover the true phylogenetic signals. With a large number of characters, the stochastic errors associated with the estimations decreases (Delsuc et al., 2005), and many independent nuclear genes can reduce some systematic errors (Collins et al., 2005; Maddison and Knowles, 2006).

Species delimitation methods, such as DNA barcoding
also are based on single locus, most commonly COI gene. We have showed that single locus sometimes cannot distinguish recently diverged species or sister species if there was mild gene flow between them (Liu et al., 2017). The genetic distance between species was not greater than the genetic distance between individuals within species when one locus or a few loci were used. When more independent loci were added, the species can be correctly distinguished and a clear gap between the within-species distance and the between-species distance revealed (Fig. 3).

As showed above, using a single gene or a few loci to study evolutionary history and population genetics of fishes often lead to erroneous results at both species and higher level. Using genome-scale data can improve estimation of population parameters (Luikart et al., 2003) and resolve inconsistency in phylogenetic reconstruction due to gene-tree/species-tree discordance (Li, 2007).

**EXON CAPTURE AND OTHER GENOMIC APPROACHES FOR POPULATION GENETICS AND PHYLOGENOMICS**

There many ways to acquire genome-scale data for population genetics and phylogenomic studies. The straight-
forward way is to sequence the whole genome, such as reconstructing phylogeny of birds (Jarvis et al., 2014) and ruminants (Chen et al., 2019) using whole genome data. Nonetheless, it is still costly to sequence whole genome for population studies or phylogenetics and it is not necessary most of the time.

Reduced-representation sequencing is a technique used to extract parts of the genome, often orthologous in different samples and then make those into sequencing libraries for the next generation sequencing. It has been wildly used for phylogenetics and population studies in recent years (Emerson et al., 2010; Faircloth et al., 2012; Lemmon et al., 2012; Li et al., 2013). Two strategies are commonly found in reduced-representation sequencing. First is related to restriction site, such as Restriction-site Associated DNA (RAD) Sequencing (Baird et al., 2008) and double digest RADseq (ddRAD) markers (Peterson et al., 2012). These methods could be used to generate a large amount of data from anonymous loci that are particularly useful in studying population genomics or evolution history under species level (Davey and Blaxter, 2010), but homology of the anonymous loci becomes uncertain, when divergent species were compared. Furthermore, for each species, a new set of loci need to be developed, which makes comparison between studies difficult. The other strategy of reduced-representation sequencing is gene capture, also known as target enrichment, which often result in less missing data than the restriction site-related methods do (Collins and Hrbeck, 2015). Target loci can be applied across highly divergent taxonomic groups (Faircloth et al., 2012; Lemmon et al., 2012; Li et al., 2013). Gene-capture methods can be used to enrich highly anonymous conserved regions and utilize variable flanking regions for data analyses, such as the method of Ultraconserved Element (UCE) captures (Faircloth et al., 2012) and Anchored Hybrid Enrichment (AHE) (Lemmon et al., 2012). Nevertheless, gene capture also can be used to target exons directly (Bi et al., 2012; Li et al., 2012; Hedtke et al., 2013).

Exons have been more commonly used for phylogenetics than anonymous noncoding regions, and evolution propriety of protein-coding sequences has been well studied. Furthermore, the flanking region of exons make these markers can also be used for population genetic studies. The papers listed in Table 1 were retrieved from PubMed (https://pubmed.ncbi.nlm.nih.gov/, accessed on Oct 15, 2021) using the keywords: gene + capture + fish for publications from 2011 to 2021. There are 29 papers published on fish population genetics and phylogenomics using exon capture methods and two papers on studying functional genes. These studies covered both osteichthyans and chondrichthyans. Eighteen papers were focused on high-level phylogenetics, and 11 papers on population genetics or phylogeographics. Application of exon capture methods on population-level studies is yet fully exploited.

**PRINCIPLE OF EXON CAPTURE METHOD AND RECOMMENDATIONS**

Exon capture is based on hybridizing RNA/DNA baits (probes) to DNA libraries of targeted species and enriching sequences similar to the baits for subsequent high-throughput sequencing. Exon capture method involves three major aspects: target selection and baits design, library preparation, and gene capture and amplification.

Exon capture markers have been developed for osteichthyans as well as chondrichthyans (Li et al., 2013; Ilves and López-Fernández, 2014; Nielsen et al., 2017; Jiang et al., 2019; Hughes et al., 2021). Ilves and López-Fernández (2014) developed 923 exon markers for cichlids based on genome sequence of *Oreochromis niloticus*. They compared single-copy exons found in *O. niloticus* with genomic data of four additional African cichlid species and chose the single-copy exons across all five cichlid species as their targets. Those markers worked well and were applied in
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study of Neotropical cichlids (Ilves et al., 2018). However, their exon markers were selected to target only cichlids, not intended to be used for other fish species. Moreover, they chose target exons that were relatively long (750–2000 bp), so limited the number of potential exon markers, because most exons of vertebrates are around 200 bp (Li et al., 2013). Similarly, taxon-specific exon markers were used for studying archived tiger shark (Galeocerdo cuvier). Nielsen et al. (2017) designed baits on 44,794 transcriptome sequences of the lesser spotted catshark (Scyliorhinus canicula) and used those to test gene capture on archived tiger shark samples.

Taxon-specific exon markers not only limit the potential applicable taxonomic range of these markers, but also make the results not comparable or integrable from different fish lineages. Exon-capture targets for all vertebrates were explored (Li et al., 2013). Genomes of six vertebrate: human (Homo sapiens), chicken (Gallus gallus), western clawed toad (Xenopus tropicalis), green anole (Anolis carolinensis), zebrafish (Danio rerio), and elephant shark (Callorhinus milii) were compared to identify putatively orthologous genes within each respective genome. A total of 1,449 exon loci were selected and tested in different vertebrate lineages with various success rate (Li et al., 2013). Nonetheless, few studies applied those “vertebrate markers” on fish studies, probably due to the small number of applicable loci and that those are not fish-specific markers. Subsequently, Song et al. (2017) developed 17,817 single-copy nuclear coding sequence (CDS) markers for percomorph fishes and applied those in the siniperids with great success. Hughes et al. (2018) selected 1,721 exon markers > 200 bp from the 17,817 markers and retrieved their sequences from hundreds of transcriptomic and genomic datasets in silico. Recently, exon capture markers for all ray-finned fishes were optimized and tested (Jiang et al., 2019; Hughes et al., 2021). Exon capture markers specific for all chondrichthians are yet to be developed.

Exon markers can be identified by comparing genomes of model species (Fig. 4). Many considerations were taken into baits design, such as uniqueness and conservativeness of markers, length and complexity of markers, and genetic distance between baits and target sequences (Bi et al., 2012; Faircloth et al., 2012; Lemmon et al., 2012; Li et al., 2013; Hugall et al., 2016; Campana, 2018). I have four recommendations for selecting exon capture markers: (1) single-copy genes are preferred, because they are less susceptible to paralogy problem; (2) relax the length requirement for exon markers, because most exons of vertebrates are short; (3) final set of in silico identified markers should be tested empirically. Once the target exons have been selected, their sequence information can be send to company for refined bait designing and synthesizing, such as ArborBiosciences (Ann Arbor, MI, USA); (4) use the sequences of a close relative to your species of interest for bait design if available.

The second aspect of exon capture is library preparation, which is the process of fragmenting DNA of target species and adding adapters to both end of the fragments. There are many ways for library preparation. The most commonly used protocol involves ultrasonic shearing DNA, blunt end, ligation and fill-in steps (Meyer and Kircher 2010; Huang et al., 2021). Other protocols, such as tagmentation library prep (https://www.illumina.com/techniques/sequencing/ngs-library-prep/tagmentation.html) involves less hands-on time, but those commercial kits are very expensive. Using the common protocol and recipe for homemade reagents can significantly lower the cost of exon capture (Huang et al., 2021). Usually 30–300 ng starting materials are enough for library prep, and extra DNA would not significantly increase the product. If there are only trace amount of DNA available, pre-amplification genomic DNA using MALBAC method can be applied (Zong et al., 2012). Size of inserted DNA usually is around 500 bp. If fragments larger than 1000 bp were detectable, size selection should be carried out to remove those long fragments to improve sequencing results. Eighteen cycles of the last amplification step is recommended, because PCR duplication is less of concern than inadequate material before gene capture steps.

The gene capture steps should follow manual of commercial kits, such as myBaits (https://arborbioscience.com/wp-content/uploads/2021/03/myBaits_v5.01_Manual.pdf). Since the number of targeting loci usually is much lower than
regular whole-exome capture, one tenth of the bait recommended volume could be used. Because fish exon capture usually is applied across species, a touch-down temperature scheme should be used and the washing temperature must be lowered accordingly (Li et al., 2013). During the hybridization and washing step, we should keep in mind that the purpose of this procedure is to separate the target DNA fragments from non-specific ones, so precise temperature control should be managed and discarding unwanted should be complete.

APPLICATIONS APPLYING EXON CAPTURE METHOD AND FUTURE PERSPECTIVE

With the development of exon-capture markers and lowering cost of library preparation and sequencing, more and more phylogenomic and population genomic studies involved exon capture approaches (Maisano Delser et al., 2016; Corrigan et al., 2017; Liu et al., 2017; Song et al., 2017; Kuang et al., 2018; Li et al., 2018; Straube et al., 2018; White et al., 2018; Betancur et al., 2019; Cheng et al., 2019; Rincon-Sandoval et al., 2019; Yin et al., 2019; Sarker et al., 2020; Ai et al., 2021; Arcila et al., 2021; Atta et al., 2021; Kolmann et al., 2021; Rou-Varón et al., 2021; Sarker et al., 2021). Here I illustrate what we can do with exon capture methods using two examples from my lab.

The Odontobutidae is a group of sleeper fishes with six genera and 15–22 species. The composition of the Odontobutidae and the interrelationship of the odontobutids were unresolved. We collected sequence data of 4,434 single-copy exons from 41 specimens of odontobutids and reconstructed a robust phylogeny of the Odontobutidae (Li et al., 2018). After removing PCR duplicates, in average, 57% were unique reads. After discarding loci that cannot be aligned well, 4,397 were left for further analyses. Phylogenetic tree had 100% bootstrap support for all nodes, and species tree generated the same result (Fig. 5). One hundred most clock-like loci were used to calibrate a time tree. The most recent common ancestor of odontobutids was estimated at 30.8 Ma (20.7–41.9 Ma, 95% HPDs). Additionally, DEC analysis implemented in RASP showed that ancestor of the odontobutids was distributed in southern China and Indo-China Peninsula (Li et al., 2018).

In another study, we tested hypotheses about freshwater invasion of Coilia nasus and identified loci adapted to non-migratory freshwater habitat (Cheng et al., 2019). We captured 4,434 exon for the Coilia species complex. Read assembly and data filtering produced 1,813 loci for each sample on average, and 2,869 clean target were kept for subsequent analyses. We found that both C. nasus and C. brachygnathus were valid species using Bayes factor species delimitation (BFD*). Two independent freshwater invasion events with subsequent gene flow between adjacent populations were supported by fastsimcoal2 analyses, with the first event occurring around 4.07 Ma and the second happened around 3.2 Ka (Fig. 6). F-DIST analyses singled out 120 outliers by comparing migratory C. nasus and C. brachygnathus, and 21 outlier between migratory and land-locked C. nasus. Nine of those loci were shared between the two comparisons, suggesting that those might play a conserved and important role in adaptation of C. nasus to freshwater habitat (Cheng et al., 2019). Furthermore, population structure and migration between populations were estimated using the exon-capture data.

The two examples illustrated that exon-capture data can be used to trace evolutionary history at both species level and higher level. It can also be used to identify adaptive loci given good comparative setting. Given the decreasing cost for sequencing, one might argue that sequencing the whole genome is easier than exon capture method. Nonetheless, the cost is still higher for whole genome sequencing than exon capture, particular for many fish species that have large genome size. Furthermore, for archive samples or samples with contamination from environment and low proportion of endogenous DNA, exon capture is the best way to enrich the target loci. Finally, gene capture can be used to enrich both mitochondrial and nuclear DNA fragments in environmental DNA research (Jensen et al., 2021).
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엑손 포획 - 원리와 어류의 계통유전체학 및 집단유전체학으로의 응용

Chenhong Li\textsuperscript{1,2}

\textsuperscript{1}상하이해양대학교 해양동물 분류 및 진화 핵심연구소, \textsuperscript{2}상하이해양대학교 수생동물 유전 및 육종 협력 혁신센터

요 약 : 한 유전자 또는 소수의 유전자에 기반한 계통발생학적 재구성은 분자 계통수/종 계통수의 불일치로 인해 오해를 불러일으킬 수 있다. 종의 구분과 종내 연구에서도 적은 유전자를 사용할 때 통계적 부족으로 해상도가 낮은 경우가 많다 발생한다. 엑손 포획법은 게놈 규모의 데이터를 수집하는 가장 효율적인 방법 중 하나로, 종내 및 상위 수준에서 생물의 패턴과 역사를 구명하는 연구에 크게 이바지할 수 있다. 이 논문에서는 단일 유전자 방법에서 게놈 접근으로의 전환의 절차와 개선을 구명하는 연구에 크게 이바지할 수 있다. 또한 엑손 포획법의 원리를 설명하고 이 방법의 작용을 위한 개선한 계산을 기술하였다. 최종적으로, 두 가지 작용을 활용한 엑손 포획법을 설명하고 이 기술에 대한 미래 전망을 논의하였다.

찾아보기 낱말 : 표적농축, 엑슨 포획, 종 경계, 집단 구조, 개체군 동태, 적응, 분자계통분류학, 계통발생학