Empirical Assessment of RAD Sequencing for Interspecific Phylogeny

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Abstract

Next-generation sequencing opened up new possibilities in phylogenetics; however, choosing an appropriate method of sample preparation remains challenging. Here, we demonstrate that restriction-site-associated DNA sequencing (RAD-seq) generates useful data for phylogenomics. Analysis of our RAD library using current bioinformatic and phylogenetic tools produced 400× more sites than our Sanger approach (2,262,825 nt/species), fully resolving relationships between 18 species of ground beetles (divergences up to 17 My). This suggests that RAD-seq is promising to infer phylogeny of eukaryotic species, though potential biases need to be evaluated and new methodologies developed to take full advantage of such data.

Key words: **phylogenomics, next-generation sequencing, restriction-site associated DNA sequencing, empirical data, Insecta, Carabidae.**

Brief Communication

Next-generation sequencing opened up new possibilities for phylogenetics, allowing rapid and cost-effective generation of millions of reads from different loci on nonmodel species; however, choosing an appropriate method of library construction remains challenging. Consequently, Sanger sequencing of a few genes is still widely used to infer species phylogenies (McCormack et al. 2013). Sequencing complete eukaryotic genomes to infer species phylogenies remains expensive, time consuming, and unrealistic. Sequencing mitochondrial genomes is more affordable, but trees can be misleading due to introgression and heteroplasmy. Thus, methods based on reduced representations of the nuclear genome appear most adequate to generate large amounts of data across many individuals at reasonable costs.

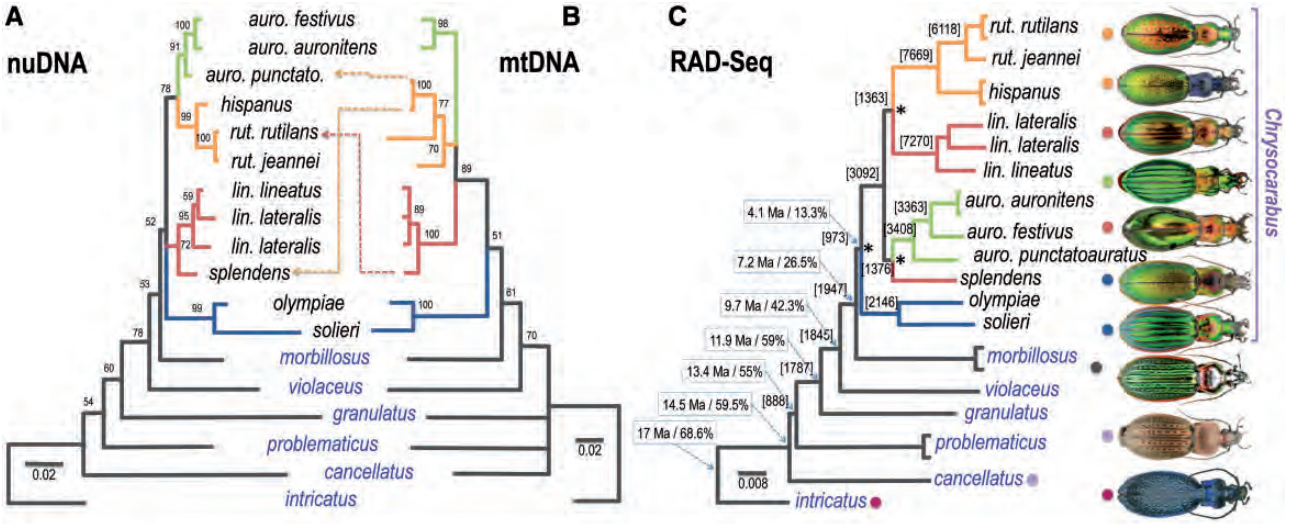
Restriction-site-associated DNA sequencing (RAD-seq, Baird et al. 2008; supplementary fig. S1, Supplementary Material online) has been used to infer the recent evolutionary history (<3 My) of few organisms (e.g., Jones et al. 2013; Nadeau et al. 2013). However, with increasing genetic distances, mutations in the restriction sites may reduce the number of orthologous loci, making RAD-seq inappropriate to infer deeper relationships (McCormack et al. 2012). Conversely, recent in silico studies suggested that a sufficient number of markers could be obtained from distant species (up to 60 My old, Rubin et al. 2012). Here, we empirically tested this prediction by comparing the power of Sanger and RAD sequencing approaches to resolve relationships between 18 nonmodel species of ground beetles (*Carabus*), whose divergences ranged from 1.2 to 17 My (supplementary table S1, Supplementary Material online).

Details regarding data generation and analysis are described in the supplementary materials, Supplementary Material online.

Sanger—After a lengthy process of screening loci for variability, primer design and PCR optimization, maximum likelihood (ML) analyses of sequences from three mitochondrial and six nuclear markers led to poorly resolved and conflicting topologies, highlighting possible mitochondrial introgression between species (fig. 1*A* and *B*).

RAD-seq—After 4 days of library preparation following the protocol by Etter et al. (2011) (*Pst*I enzyme), 2 weeks of sequencing on one lane of a HiSeq 2000 flowcell and a week of data processing on a standard computer (using Stacks; Catchen,et al. 2011), we obtained 400× the volume of Sanger data and 270× more informative sites. Our data set resulted from a stringent loci selection to ensure for homology and minimize the amount of missing data. More than half of the individuals should have sequences for a loci to be included in our analysis, and the number of mismatches allowed when merging loci from all individuals varied from 4 to 10 (parameter *n*, cstacks)

Whatever the value of *n*, ML analysis of the RAD-seq data sets (up to 25,425 loci, i.e., one every 12,000 nt, supplementary table S2, Supplementary Material online) produced the same fully resolved topology (fig. 1C and supplementary fig. S2, Supplementary Material online),



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	Sanger		RAD ($n_{cstacks}$ = 10)	
Matrix :	*Carabus* (18 ind./13 spp.)	*Chrysocarabus* (12 ind./7 spp.)	*Carabus* (18 ind./13 spp.)	*Chrysocarabus* (12 ind./7 spp.)
Number of loci :	9		25 425	
Alignment length :	5 584 bp		2 262 825 bp	
Data set size :	100 512 nt	67 008 nt	40 730 850 nt	27 153 900 nt
Variable sites :	1030 (18.4%)	535 (9.6%)	367 813 (16.3%)	216 300 (9.6%)
Informative sites :	495 (8.9%)	264 (4.7%)	132 186 (5.8%)	91 420 (4.0%)
Missing data :				
without old DNA sample	21.8%	17.9%	26.9%	13.3%
of old DNA sample (*C. olympiae*)	65.5%		75.4%	
Sample preparation and sequencing :	1 week / ca. 1 200 USD (without marker screening)		3 weeks / ca. 3 600 USD	
Data Analysis (CBGP cluster) :	2-3 days		1-2 weeks	

Fig. 1. ML phylogenies (RAxML 7.2.8-ALPHA, Stamatakis 2006; 10,000 bootstrap replicates) (*A*) from the six nuclear (3,659 bp) and (*B*) three mitochondrial (1,925 bp) markers sequenced using Sanger technology. Nodes with bootstrap supports (BP) < 50% are collapsed (see also supplementary fig. S4, Supplementary Material online). Dashed arrows show possible cases of mitochondrial introgression. (*C*) ML phylogeny (RAxML, 10,000 bootstrap replicates, $n_{cstacks} = 10$) inferred from the RAD data sets (the same topology was recovered whatever the value of *n*, see supplementary fig. S2, Supplementary Material online). BP = 100 for all nodes but three (stars) for which BP = 99. Synapomorphies supporting nodes as calculated with MacClade 4.08 (Maddison DR and Maddison WP 2005) are between brackets. Node mean ages, taken from Deuve et al. (2012), and missing data are given as follows: ages/percentage of missing data. (*D*) Comparison between Sanger and RAD-seq data sets. Percentages of missing data were obtained using Geneious 6.1.6. (Drummond et al. 2010). Percentages of variable and informative sites were calculated using MEGA 5.2.2 (Tamura et al. 2011). See details for all data sets in supplementary table S2, Supplementary Material online.

different from the nuclear and mitochondrial Sanger trees. These two last topologies were rejected by statistical tests ($P < 0.001$). Although significant loss of RAD markers occurred for the oldest DNA sample (78.3%–75.4%, *Carabus olympiae*, extraction performed in 1998), enough signal remained for its placement. Loss of RAD markers also occurred with increasing genetic distances, though enough information was retained to accurately resolve the relationships within *Carabus* (Deuve et al. 2012). When *n* was set to 10, more polymorphic loci were included and missing data reached 68.6% for a divergence of 17 My (fig. 1C and supplementary fig. S2, Supplementary Material online). Preliminary tests are thus recommended to optimize the amount of allowed mismatches within a RAD locus to avoid loci overkill while ensuring loci homology across individuals. Nevertheless, percentages of missing data were comparable between Sanger and RAD-seq data sets (fig. 1D, supplementary table S2, Supplementary Material online). Furthermore, missing data did not bias reconstruction, as topologies obtained from the complete matrices (loci shared by all 18 species) were similar to those inferred from the incomplete matrices (supplementary fig. S3, Supplementary Material online).

Overall, this study illustrates the power of RAD-seq to infer shallow relationships, and we believe that this approach may be generalized to many groups (~90% of the insect genomes range between 100 and 800 Mb, supplementary fig. S5, Supplementary Material online). Here, we used only one partition and relied on current evolutionary models to analyze RAD-seq data. Testing alternative partitioning schemes or developing more appropriate models to deal with the specificities of such data (e.g., modeling gain/loss of restriction sites) might be promising to improve inferences.

Finally, RAD-seq definitely opens new avenues for phylogeneticists but possibly also a Pandora's box of analytical issues. These issues will need to be explored to avoid being

misled by systematic (Lemmon EM and Lemmon AR 2013) or other biases (Arnold et al. 2013), which may differ depending on the level of genetic divergence among samples.

Supplementary Material

Supplementary figures S1–S5 and tables S1 and S2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org.)

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