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AB12PHYLO: an integrated pipeline for Maximum Likelihood phylogenetic inference from ABI trace data

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Abstract

Multi-gene phylogenies constructed from multiplexed and Sanger sequencing data are regularly used in mycology and other disciplines as a cost-effective way of species identification and as a first means to investigate genetic diversity samples.

Today, a number of tools exist for each of the steps in this analysis, including quality control and trimming, the generation of a multiple sequence alignment (MSA), extraction of informative sites, and the construction of the final phylogenetic tree. A BLAST search in a reference database is often performed to identify sequences of type specimens to compare the samples with in the phylogeny. Made over the past decades, these tools are all independent from and often not perfectly adapted to one another.

We present AB12PHYLO, an integrated pipeline that can perform all necessary steps from reading in raw Sanger sequencing data through visualizing and editing phylogenies. In addition, AB12PHYLO can calculate basic summary statistics for each gene in the phylogeny.

AB12PHYLO is designed as a wrapper of several open access and commonly used tools for each of the intermediate stages, and intended to simplify the phylogenetic pipeline while still allowing a high degree of access. It comes as a command-line version for the highest reproducibility and an intuitive graphical user interface (GUI) for easy adoption by IT-agnostic end-users. The use of AB12PHYLO significantly reduces the hands-on working time for these analyses.

Main text

Multi-gene phylogenies obtained through Sanger sequencing are a cost-effective and fast way method to aid species identification of fungal samples collected in the field, get the first insight into their genetic diversity, and as a means to select subsets of samples for more expensive and labor-intensive whole genome sequencing approaches. Such analyses often

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use barcode sequences, specific genic or intergenic fragments of well-defined genes, that have been widely used over the past decades. Examples are regions of the Internally Transcribed Spacer (ITS) (White et al. 1990), Elongation Factor 1 alpha (EF1) (Carbone and Kohn 1999) or RNA polymerase II subunit (RBP2) (Liu et al. 1999). These genes have been sequenced for a large number of type specimens, and sequence comparison of the samples in question with stored type specimens either through direct local alignments or database searches such as NCBI-BLAST (Johnson et al. 2008), can help confirm species identity. Often, sequence data of a single barcode gene is not sufficient to specifically determine fungal identity on species level, whereas a combination of three or more barcodes can reliably determine which species the sample belongs to (see e.g. Woudenberg et al. 2015). In another example, construction of multi-gene phylogenies formed the basis for phylogenetic reclassification: the fungal plant pathogen genus *Ulocladium* appears morphologically different from the genus *Alternaria*, but multi-gene phylogenies did not result in monophyletic clades, suggesting to rename the *Ulocladium* spp, which now fall under the broader *Alternaria* genus (Woudenberg et al. 2013). The method is also in use to get better insights into pathogens in the field: Two recent studies used multi-gene phylogenetic analyses to confirm the nature and relationship of the pathogens *A. alternata* and *A. solani* in potato fields in Wisconsin or similarly in Brazil (Adhikari et al. 2020; Ding et al. 2020). Other recent studies used the method to identify and compare *Colletotrichum* spp. on tea (Orrock et al. 2019), strawberry (Chen et al. 2019), and a variety of hosts (He et al. 2019) or to re-assess the taxonomic classification of *Mycosphaerella* spp on persimmon.(Hassan and Chang 2018).

The analyses presented above often involve manual inspection of the sequence quality, followed by manual data trimming. Further steps often require additional hands-on work as well, if only to prepare the output of one tool as input for the next. Such manual processing slows down analysis and hampers reproducibility in general, as many parameters are often not properly recorded. In order to speed up data analyses of this kind and increase their reproducibility, we constructed a fully customizable pipeline which we call AB12PHYLO.

AB12PHYLO is developed as a Python 3 package around widely-used open source tools. It takes raw ab1 (ABI) files. Additionally, the user can provide a template specifying the corresponding sample names, which can be formatted in a 96-well plate format, to represent the way the samples are often loaded for sequencing. When no sample template is specified, AB12PHYLO uses regular expressions that the user can modify to search for and extract the file and gene names.

Its command-line version is assembled from the following eight main stages: i) File input: After the command line is supplemented with default configurations, the tables mapping plate coordinates to sample IDs are read to memory. Ab1 trace files are read using Biopython Bio.SeqIO (Cock et al. 2009), matched to their original sample ID and gene, and passed to quality control. Reference sequences are saved to the respective per-gene dataset. ii) Quality control was modeled after SeqTrace (Stucky 2012): Read ends are trimmed until a user-defined proportion of characters in the chromatogram have a phred quality score at or above another user-defined threshold, with 8 / 10 and 30 the pre-set default values. End trimming can discard reads. Consecutive stretches of characters with a score below the phred threshold will be replaced by an equal-length stretch of unknown N characters if they are longer than the last user-definable limit in trace processing; pre-set at 5. Reverse reads are replaced by their reverse complement. iii) Edited sequences are passed to a multiple sequence alignment tool in per-gene datasets. AB12PHYLO is able to interface with local installations of MAFFT (Katoh et al. 2002), Clustal Omega (Sievers et al.

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2011) or MUSCLE (Edgar 2004); or an EMBL-EBI online service for any of them (Notredame et al. 2000) at https://www.ebi.ac.uk/Tools/msa. iv) The alignments are trimmed with Gblocks (Castresana 2000). Requirements for a conserved site can be set at four different levels, from 90% identiy to the most relaxed permissible parameters, and a fifth option skipping trimming entirely. The per-gene MSAs are concatenated into a supermatrix alignment. v) A BLAST similarity search of data from the first gene in the analysis is carried out to identify source species. If this search is to be run locally, AB12PHYLO employs BLAST+ (Camacho et al. 2009), which will download, update or check a user-defined database before searching it. Per default, AB12PHYLO will query the NCBI nucleotide database for sequences not found in the local database with Biopython Bio.Blast (Cock et al. 2009), and BLAST can also be run entirely via the public NCBI BLAST API, but this approach is not suitable for large datasets. Two more directly related options are available: Skip BLAST altogether, or parse one or several XML files from a previous analysis or a web BLAST. vi) The maximum likelihood (ML) tree is inferred from the concatenated alignment with RAxML-NG (Kozlov et al. 2019). While the evolutionary model is pre-set to GTR+ Γ and the numbers of ML tree searches to 10 with random or parsimony starting trees each, these parameters can be user-defined. Also, the number of parallel threads can be limited. vii) With RAxML-NG, bootstrap replicates are generated from the best ML phylogeny found in the previous step. FBP and TBE support values for the best ML tree are computed from the bootstrap trees constructed in parallel threads. viii) Output: The generated phylogeny is plotted with Toytree (Eaton 2020) and shown alongside other results in an HTML results page. A CGI script allows interactive searching of taxa and selecting populations while computing diversity statistics and the Tajima's D neutrality test. A more detailed model of the command-line AB12PHYLO program flow is shown in Figure 1.

The GUI version of AB12PHYLO implements the same process while giving users direct control over each step: visualizations of sequence trimming and MSAs allow immediate identification of out-of-register samples, and carefully balanced MSA trimming to prevent both signal loss and trimming artifacts. Furthermore, the graphical AB12PHYLO enables comfortable export of the computation-heavy ML tree inference to a more powerful computer, faster calculation of diversity statistics, and more as well as easier tree modifications.

As a proof of concept, we obtained the data from the above-mentioned study by Ding et al. (2020). We ran AB12PHYLO with default settings, providing both the raw ab1 files and the sequence data of the type specimens as used by Ding et al (2020). Two samples did not pass the default quality controls. With the remaining 74 samples, we resolved a phylogenetic tree similar to the one in the original work, in which the same genotype groups can be annotated (Figure S1). The MSA used for the phylogeny was 1822 bp long and included 74 samples. Our analysis was run on 12 threads on a system with 64 GB RAM. The total run time for this analysis, including parallelized bootstrapping and BLAST was less than 10 minutes.

Thus, we conclude that AB12PHYLO can produce high-quality multi-gene phylogenies rapidly. The use of AB12PHYLO significantly reduces hands-on working time for these analyses, and overall runtime by parallelization of computation-heavy maximum likelihood tree inference.

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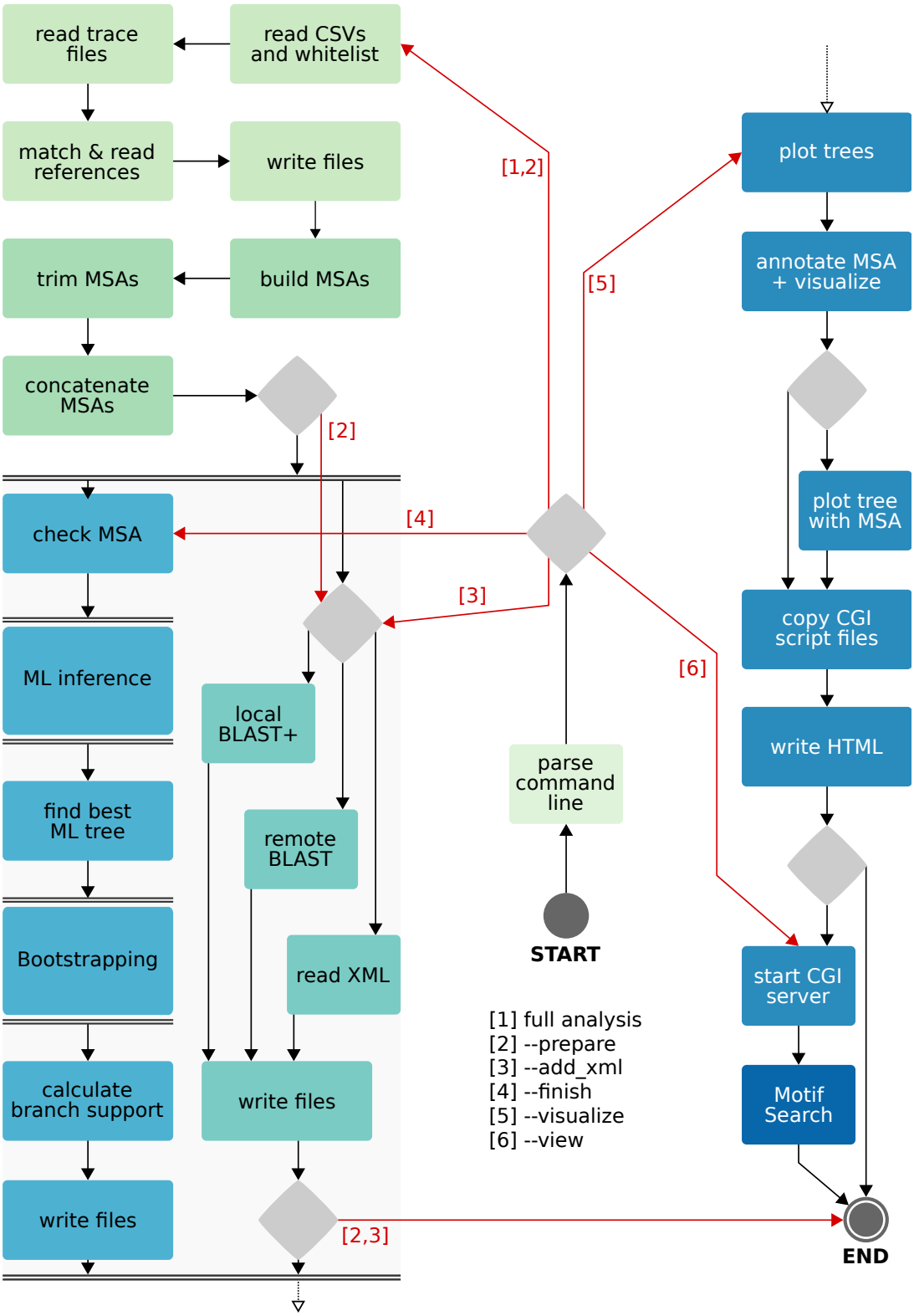

Figure 1

Flowchart of command-line AB12PHYLO. Red arrows represent run modes listed below START, and diamonds mark decisions. The dashed arrow at the bottom signifies that the pipeline continues at the dashed arrow at the top right. Pairs of double horizontal lines indicate the activity between the runs in parallel threads, and the color of the activity rectangles points to the source Python 3 module.

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Data Availability

AB12PHYLO is published under the GPLv3 license. It runs on standard desktop computers either under Linux, MacOS or Windows operating systems and can be installed via the pip or conda package-managment systems, the latter also allowing easy installation of an environment with all external tools. Installation instructions and source code are available at https://github.com/lkndl/ab12phylo

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Supplements

Figure S1

A multi-gene phylogeny constructed with graphical AB12PHYLO from the Alt a 1, EF1, ITS1F and GAPDH loci in the Ding et al. 2020 dataset. The maximum likelihood tree was inferred from 80 ML tree searches, 40 starting at random and 40 at maximum-parsimony trees. The General Time-Reversible (GTR) model of DNA substitution was used in conjunction with four gamma-distributed (+ Γ4) rate classes. Branch support was calculated from 1000 bootstrap iterations and is shown here as Transfer Bootstrap Expectation (TBE, Lemoine et al 2019). Regular samples are labeled with their sample ID and the annotated species of their best BLAST hit, with label color brightening with decreasing percent identity along a black-body spectrum. Reference sequences are labeled in blue. Internal node size and color reflect bootstrap support from 1000 replications, with TBE support > 70% in blue. This is the default style for AB12PHYLO trees.

The ML tree was rooted at the reference *Alternaria molesta* strain CBS 548.81 and accurately agrees with Fig. 3 from Ding et al. 2020. Previously identified genotypes I – V could be resolved and are labeled accordingly.

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References

Adhikari, T. B., Ingram, T., Halterman, D., and Louws, F. J. 2020. Gene Genealogies Reveal High Nucleotide Diversity and Admixture Haplotypes Within Three Alternaria Species Associated with Tomato and Potato. Phytopathology®. 110:1449–1464

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., and Madden, T. L. 2009. BLAST+: architecture and applications. BMC Bioinformatics. 10:421

Carbone, I., and Kohn, L. M. 1999. A Method for Designing Primer Sets for Speciation Studies in Filamentous Ascomycetes. Mycologia. 91:553–556

Castresana, J. 2000. Selection of Conserved Blocks from Multiple Alignments for Their Use in Phylogenetic Analysis. Mol. Biol. Evol. 17:540–552

Chen, X. Y., Dai, D. J., Zhao, S. F., Shen, Y., Wang, H. D., and Zhang, C. Q. 2019. Genetic Diversity of Colletotrichum spp. Causing Strawberry Anthracnose in Zhejiang, China. Plant Dis. 104:1351–1357

Cock, P. J., Antao, T., Chang, J. T., Chapman, B. A., Cox, C. J., Dalke, A., Friedberg, I., Hamelryck, T., Kauff, F., Wilczynski, B., and de Hoon, M. J. 2009. Biopython: freely available Python tools for computational molecular biology and bioinformatics. Bioinformatics. 25:1422–1423

Ding, S., Meinholz, K., and Gevens, A. J. 2020. Spatiotemporal Distribution of Potato-Associated Alternaria Species in Wisconsin. Plant Dis. 105:149–155

Eaton, D. A. R. 2020. Toytree: A minimalist tree visualization and manipulation library for Python. Methods Ecol. Evol. 11:187–191

Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32:1792–1797

Hassan, O., and Chang, T. 2018. Phylogenetic and Morphological Reassessment of Mycosphaerella nawae, the Causal Agent of Circular Leaf Spot in Persimmon. Plant Dis. 103:200–213

He, L., Li, X., Gao, Y., Li, B., Mu, W., and Liu, F. 2019. Characterization and Fungicide Sensitivity of Colletotrichum spp. from Different Hosts in Shandong, China. Plant Dis. 103:34–43

Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S., and Madden, T. L. 2008. NCBI BLAST: a better web interface. Nucleic Acids Res. 36:W5-9

Katoh, K., Misawa, K., Kuma, K., and Miyata, T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 30:3059–3066

Kozlov, A. M., Darriba, D., Flouri, T., Morel, B., and Stamatakis, A. 2019. RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. Bioinformatics. 35:4453–4455

Liu, Y. J., Whelen, S., and Hall, B. D. 1999. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerse II subunit. Mol. Biol. Evol. 16:1799–1808

Notredame, C., Higgins, D. G., and Heringa, J. 2000. T-coffee: a novel method for fast and accurate multiple sequence alignment11Edited by J. Thornton. J. Mol. Biol. 302:205–217

Orrock, J. M., Rathinasabapathi, B., and Spakes Richter, B. 2019. Anthracnose in U.S. Tea: Pathogen Characterization and Susceptibility Among Six Tea Accessions. Plant Dis. 104:1055–1059

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J. D., and Higgins, D. G. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. 7:539

Stucky, B. J. 2012. SeqTrace: A Graphical Tool for Rapidly Processing DNA Sequencing Chromatograms. J. Biomol. Tech. JBT. 23:90–93

White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. 38 - AMPLIFICATION AND DIRECT SEQUENCING OF FUNGAL RIBOSOMAL RNA GENES FOR PHYLOGENETICS. Pages 315–322 in: PCR Protocols, M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White, eds. Academic Press, San Diego.

Woudenberg, J. H. C., Groenewald, J. Z., Binder, M., and Crous, P. W. 2013. Alternaria redefined. Stud. Mycol. 75:171–212

Woudenberg, J. H. C., Seidl, M. F., Groenewald, J. Z., de Vries, M., Stielow, J. B., Thomma, B. P. H. J., and Crous, P. W. 2015. Alternaria section Alternaria: Species, formae speciales or pathotypes? Stud. Mycol. 82:1–21