Automatic annotation of the bHLH gene family in plants

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Abstract

Background: The bHLH transcription factor family is named after the basic helix-loop-helix (bHLH) domain that is a characteristic element of their members. Understanding the function and characteristics of this family is important for the examination of a wide range of functions. As the availability of genome sequences and transcriptome assemblies has increased significantly, the need for automated solutions that provide reliable functional annotations is emphasised.

Results: A phylogenetic approach was adapted for the automatic identification and functional annotation of the bHLH transcription factor family. The bHLH_annotator for the automated functional annotation of bHLHs was implemented in Python3. Sequences of bHLHs described in literature were collected to represent the full diversity of bHLH sequences.

Previously described orthologs form the basis for the functional annotation assignment to candidates which are also screened for bHLH-specific motifs. The pipeline was successfully deployed on the two *Arabidopsis thaliana* accessions Col-0 and Nd-1, the monocot species *Dioscorea dumetorum*, and a transcriptome assembly of *Croton tiglium*.

Depending on the applied search parameters for the initial candidates in the pipeline, species-specific candidates or members of the bHLH family which experienced domain loss can be identified.

Conclusions: The bHLH_annotator allows a detailed and systematic investigation of the bHLH family in land plant species and classifies candidates based on bHLH-specific characteristics, which distinguishes the pipeline from other established functional annotation tools. This provides the basis for the functional annotation of the bHLH family in land plants and the systematic examination of a wide range of functions regulated by this transcription factor family.

Key words: bHLH, *Arabidopsis thaliana*, *Croton tiglium*, *Dioscorea dumetorum*, orthology, phylogeny, annotation

Introduction

A basic helix-loop-helix (bHLH) domain is the characteristic and name-giving feature of the bHLH transcription factor family. This gene family is found in three major eukaryotic lineages (animals, plants, fungi). In plants, the bHLH family is one of the largest groups of transcription factors only second to MYBs [1]. To modulate gene expression, the bHLH transcription factors bind as dimers to specific DNA sequences. This function is conserved through two functionally distinct regions in the basic helix-loop-helix domain: The basic region is located at the N-terminus of the domain and consists of mainly basic residues [2]. It functions as a DNA binding domain [3], recognizing a hexanucleotide motif in the major groove [4–6]. At the C-terminus of the domain, the helix-loop-helix region is located. It consists of two amphipathic helices separated by a loop [2]. Through the interaction of hydrophobic residues, it mediates protein-protein interactions used for dimerization [4–6].

The most recognized target sequence is the E-box CANNTG [7–11]. While recognizing the E-box, each monomer of the bHLH dimer binds one half of the motif in the major groove [5, 6]. Both the formation of homo- and heterodimers was reported for bHLH transcription factors [4, 5, 12]. In addition, the bHLHs are also able to interact with other classes of transcription factors [1]. A famous example is the MYB-bHLH-WD40 (MBW) complex [13], but the formation of other multimeric complexes is possible too [14, 15].

The functionality of the different elements of a bHLH is determined by certain amino acid residues, which form a conserved motif (Figure 1).

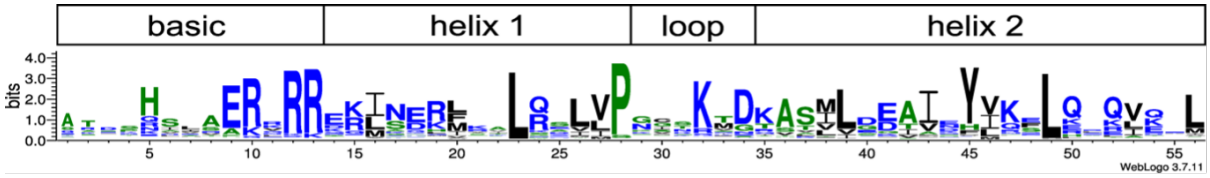


Figure 1: Conservation of amino acid residues of plant bHLH domain. Hydrophobic amino acids are coloured black. Hydrophilic amino acids are coloured blue and neutral amino acids green. The motif is based on conserved residues of bHLHs in Arabidopsis thaliana, Oryza sativa, Physcomitrella patens and Selaginella moellendorffii [9, 10]. Constructed with Weblogo [16].

In plants, the basic region of a bHLH is represented by the first 10 to 19 amino acids [1, 8–11, 17]. Conserved positions in this region determine the DNA binding behaviour [4–6, 18]. Crucial are the positions 5, 9 and 13 in the conserved motif (Figure 1), which mainly define the hexanucleotide target motif [4–6, 18]. The positions 10 and 12 are occupied by arginine residues contacting the DNA backbone [4, 18].

In the helix regions, the hydrophobic amino acids form the core at dimer formation, thus stabilising the interaction [4]. Consistent with this function, analyses of the animal bHLH residues have shown less sequence variability for buried helix positions than for exposed [19]. The loop region shows high variation regarding length and sequence [1, 8–11, 17]. In eukaryotes, the minimum found loop length is five amino acids long [4, 19]. Conserved positions in the loop have been shown to stabilise the shape of the loop [4, 5]. Also, for animal bHLHs, a lysine residue in the loop was reported to participate in DNA binding by interacting with the DNA backbone [4] or being mandatory for DNA binding [20].

Based on their DNA binding properties, members of the bHLH family can be categorised into groups. The first distinction is between DNA binding bHLHs and non-binding HLHs. The latter group is also called ‘atypical bHLHs’. For the prediction of DNA binding ability, the number of basic residues in the basic region is inspected [8]. Most studies use a minimum of 5 basic residues as cutoff to consider candidates as binding bHLHs [10, 11, 21]. The DNA binding bHLHs are further differentiated based on their hexanucleotide target motif. The amino acid composition at specific positions in the basic region determines thes motif that is recognized by a bHLH [8]. The most abundant motif in plants and animals is the E-box CANNTG [7–11]. E-box binders are distinguished from non-E-box binders based on the presence of glutamic acid and arginine at the positions 9 and 12 [8, 10, 11, 21–23]. The glutamic acid contacts the first two bases (CA) of the E-box [4–6, 18] and has been shown to be important for DNA binding [24]. This interaction is stabilised by the arginine residue, which directs the side chain of the glutamic acid while contacting the DNA backbone [5]. In plants, as well as animals, the most common E-box motif is the G-box CACGTG [7–11]. Therefore, the group of E-box binders is categorised into G-box binders and non-G-box binders. G-box binders are identified by histidine, glutamic acid and arginine at the positions 5, 9, and 13 [8, 10, 11, 21–23]. The histidine residue binds to the last base (G) of the motif [4, 6, 18]. The arginine residue at position 13 distinguishes the G-box from the E-box motif CAGCTG [4, 6]. In terms of DNA binding specificity, the interaction of bHLH and DNA is not limited to the target motif or basic region. Flanking bases outside the target motif have been shown to discriminate binding for certain animal bHLHs [24] and to be recognized by amino acids in the basic region [5, 6, 18]. Also, residues outside the basic region participate in DNA binding. Contacts with the

phosphate groups of the DNA backbone have been observed for residues in the loop and helix 2 region [4, 6], like the lysine residue in the loop mentioned before.

The atypical bHLHs are defined by their lack of basic residues in the basic region and therefore predicted not to be able to bind DNA. Instead, HLHs have shown to contain proline residues in the basic region [8]. Because the HLH region and therefore the ability to dimerize is still intact, they are proposed to be negative regulators of DNA binding by forming heterodimers with DNA binding bHLHs [8, 10]. While this behaviour had been found in both plant and animal bHLHs [14, 25], the groups of these two species showed no similarity regarding the sequence [8, 10].

Based on their phylogenetic relationship, the bHLH transcription factor family can be grouped into subfamilies. Depending on the analysed species and criteria, the number of subfamilies varies. Multispecies analyses have predicted 26 [9] or 32 subfamilies [10]. Because the deep notes of the phylogenetic trees in the analyses have low statistical support, no strong conclusion about the relationship between the subfamilies should be drawn. The subfamilies themself are highly supported, allowing examination of subfamily specific characteristics and evolutionary relationship [1, 7–10, 26, 27]. The plant subfamilies are proposed to be monophyletic, as they do not cluster together with other eukaryotic bHLHs [8, 9]. Furthermore, they are conserved among different plant species and most families have been shown to be present in early land plants before the evolution of mosses [9]. Subfamily characteristics are the number and positions of introns [1, 8, 10, 27], the predicted protein length, and the position of the domain [1]. Conserved motifs outside the bHLH domain can be associated with individual subfamilies [1, 8–10, 27]. Some motifs can be observed in different subfamilies, but the relative spatial location is subfamily associated [9]. Most subfamilies belong to the same group with respect to their DNA binding properties. Some distant subfamilies share DNA binding properties which suggests that some features might have developed independently in separate lineages [8, 10]. The functional diversification within the subfamilies is variable. Some subfamily members are involved in similar biological processes or have redundant functions, while other subfamilies contain highly functionally specialised members [1, 10].

In plants, the bHLH family has been expanded through gene duplication [1, 8] and domain shuffling [8–10]. Evidence for this theory are the presence of the same conserved motifs in different subfamilies [9, 10], as well as the sequence diversity outside of the bHLH domain [8]. Domain shuffling also has been suggested for animal bHLHs [26, 28]. Further named arguments regarding animal bHLHs are the loss of the basic domain in some subgroups and the spatial variation of the domain [28], which also apply to plant bHLHs [1, 8, 10].

136 The bHLH transcription factor family controls a wide range of biological processes, which
137 warrants in-depth investigations of functions and characteristics. The availability of plant
138 sequence data has increased significantly with development of new sequencing technologies
139 like next-generation sequencing (NGS) [29] and more recently long read sequencing
140 technologies [30, 31]. While structural annotation can be achieved automatically with the
141 integration of external hints, the functional annotation of predicted genes remains a challenge.
142 This reinforces the need for automated annotation of sequences.
143
144 Various approaches for the automated annotation of sequences are deployed. Established
145 tools assign a functional description via sequence similarity to annotated protein sequences
146 or the detection of hidden Markov Model (HMM) profiles. HMM profiles for this annotation
147 method can be retrieved from Pfam [32, 33] which is a comprehensive protein domain profile
148 database. InterProScan5 is a tool that assigns Pfam domains and other annotation ontology
149 terms to given sequences [34]. To provide standardised vocabularies, Gene Ontology (GO)
150 terms [35, 36], the KEGG Orthology (KO) entries from the KEGG database [37] or the plant
151 specific MapMan BIN ontology [38, 39] can be utilised. The automated functional annotation
152 pipeline Blast2GO assigns GO terms to a set of given sequences based on sequence similarity
153 to previously characterised reference sequences [40]. For KO entries, the KEGG Automatic
154 Annotation Server (KAAS) [41] can be deployed, which is conceptually similar to Blast2GO.
155 Mercator4 [39, 42] is a plant specific functional annotation pipeline, assigning MapMan BIN
156 annotations to novel sequences [43]. The input sequences are classified by scanning for BIN
157 specific HMM motifs. Input sequences that cannot be assigned to a MapMan BIN are
158 annotated by performing a BLASTP search against the Swiss-Prot database [39, 44].
159
160 Other functional annotation initiatives rely on the concept that orthologs are likely to have the
161 same function [45], thus specifically identifying orthologs. One approach is the identification
162 via clustering, which is performed by OrthoMCL [46, 47]. Another approach is the investigation
163 of the phylogenetic relationship through identification of homologs, the alignment of all
164 sequences, and the construction of a phylogenetic tree. Based on the inferred relationships in
165 the tree, functional predictions and further analysis can be performed [48]. A similar approach
166 was developed for the identification of gene families based on massive collections of
167 transcriptome assemblies [49]. OrthoFinder implements this approach for the identification of
168 orthologs between species [50]. KIPEs can identify enzymes in a pathway based on orthology
169 to previously characterised sequences and downstream inspection of functionally important
170 amino acid residues [51, 52]. SHOOT can identify the ortholog of a given sequence in a
171 collection of sequence data sets that represent a range of species [53]. The MYB_annotator
172 represents a pipeline for the automated functional annotation of the MYB transcription factor

173 family by phylogenetic identification of orthologs and a detailed characterisation of identified
174 candidates based on specific sequence features [54].
175 Here, the objective was to develop a pipeline for the automated identification and functional
176 annotation of bHLHs in plants based on orthology to previously characterised sequences.
177 Initial candidates are recognized through similarity to a collection of bait sequences or by a
178 bHLH-specific HMM motif. The pipeline harnesses a phylogenetic approach to define
179 orthologs as final candidates. Special characteristics of the bHLH family like the subfamily-
180 specific motifs or DNA binding properties are analysed. Screens of data sets with numerous
181 isoforms like *de novo* transcriptome assemblies are enabled by a parallelization option.
182

183 Materials and methods

184 Development of a workflow for automatic bHLH annotation

185 An automatic basic helix-loop-helix (bHLH) annotation pipeline was implemented in Python3
186 [55]. The bHLH_annotator builds on a strategy derived from the MYB_annotator [54] and
187 includes the identification of bHLH candidates based on sequence similarity revealed through
188 BLASTp v2.12.0+ [56], the construction of a global alignment of candidate sequences with
189 MAFFT v7 [57] or Muscle5 [58], the construction of phylogenetic trees with FastTree2 v2.1.10
190 [59] or RAxML-NG v0.9.0 [60], the identification of orthologs and paralog clusters with
191 DendroPy 4.5.2 [61], the domain check and screening for subfamily specific motifs with
192 HMMER 3.3.2 [62] and the prediction of DNA binding group based on residues in the basic
193 region (Figure 4).

194 Input data sets

195 If run with default settings, the reference sequences used to annotate ortholog candidates are
196 the *Arabidopsis thaliana* bHLHs. These sequences were annotated by the latest public data
197 release on The Arabidopsis Information Resource (TAIR) website [63, 64]. Also, the subfamily
198 name of each reference sequence was retrieved from a multi species study of the bHLH family
199 [9]. From the same study [9], sequences of subfamily specific motifs were collected, aligned
200 with Muscle5 [58], and a HMM motif specific for a subfamily was built with the HMMER 3.3.2
201 [62] function “hmmbuild” if possible. For the prediction of DNA binding groups, AT1G09530
202 was chosen as reference. Residue positions of the candidate bHLH domains are determined
203 based on this polypeptide sequence.

204 ## Bait sequence collection

205 Previously described bHLH sequences from various plant species were retrieved to form the
206 bait sequence collection. Sequences were taken from the supplementary data of the
207 respective publication or extracted from the plants predicted polypeptide sequences specified
208 in the respective publication (Table 1). Duplicate sequences were removed from the bait
209 collection. bHLHs with a high grade of sequence divergence were identified in the well studied
210 species *Arabidopsis thaliana* [1, 8, 10] and *Oryza sativa* [10, 27] (see Additional file 1 for
211 details). The BLASTp-based Python script "collect_best_BLAST_hits.py" in version v0.29 [65]
212 was used to find the best BLAST hits in predicted polypeptide sequences of *Brassica napus*
213 for the diverged sequences in *A. thaliana* and *Zea mays* in *O. sativa.* The obtained sequences
214 were added to the bait sequence collection to represent the diverged sequences with a higher
215 phylogenetic diversity.
216
217 *Table 1: Plant polypeptide sequences used to collect bHLH sequences. The species, version*
218 *and database source are given.*

Species	**Version**	**Source**
Aquilegia coeruela	v3.1	Phytozome [66, 67]
Arabidopsis thaliana	TAIR10	Phytozome [63]
Brassica napus	v5	Brassica napus Genome Browser [68]
Brassica rapa	v1.2	Brassicaceae Database [69]
Camellia sinesis	-	Tea Plant Information Archive [70]
Citrus grandis	v1.0	Phytozome [66, 71]
Cucumis melo	v3.5.1	Cucurbit Genomics Database [72]
Eucalyptus grandis	v2.0	Phytozome [66, 73]
Gossypium hirsutum	"TM-1" genome NAU-NBI_v1.1	Cottongen [74, 75]
Musa acuminata	DH-Pahang v4	Banana Genome Hub [76]
Nicotiana tabacum	v4.5	SolGenomicsNetwork [77]
Oryza sativa	7.0	Rice Genome Annotation Project [78]
Phaseolus vulgaris	v2.1	Phytozome [66]

Physcomitrella patens	JGI v1.1	Phytozome [66]
Populus trichocarpa	v1.1	Phytozome [66]
Prunus persica	v2.1	Phytozome [66, 79]
Vitis vinifera	v2.1	Phytozome [66, 80]
Zea mays	B73 RefGen_v3	MaizeGDB [81]

Additional bHLH-like outgroup sequences were identified based on sequence similarity to bHLH sequences and a phylogenetic placement outside of the monophyletic bHLH clade (see Additional file 2 for details). The collection of bait and outgroup sequences was reduced to represent the full sequence diversity with a minimal number of sequences. This optimisation of the bait sequence collection substantially reduces the computational costs and run time of the analysis (see Additional file 2 for details). With the HMMER 3.3.2 [62] option “hmmbuild” a HMM motif of the optimised bait collection was created.

The bHLH bait collection, outgroup collection and reduced collections represent the bait collection v0. For the bait collection v1, the bHLH candidates (type 1 and 2) identified in the benchmarking of the *A. thaliana* Col-0 accession and not described in the literature [1, 8, 10] were added. Also, the remaining bHLH sequences of *B. napus* and *Z. mays* were added, as these species are only represented by the diverged sequences in v0. The final bait collection v1.1 was obtained by removing bait sequences with an interchanged phylogenetic placement regarding the bHLH baits and outgroup.

Parameter optimisation

Optimal BLAST parameters would allow a comprehensive identification of all bHLHs while minimising the number of false-positive candidates that need to be filtered out in the following steps. To avoid overfitting of parameters, the optimisation was performed on *A. thaliana* as representative of eudicot plants as well as *O. sativa* as representative of monocot plants (see Additional file 1 for details).

Proof of concept and benchmarking

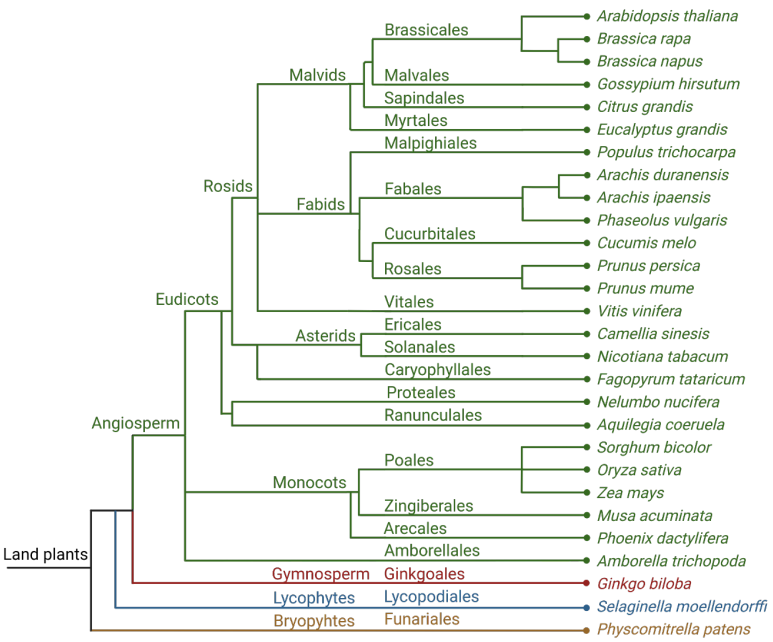
As proof of concept, the pipeline in v1.01 with the bait collection v0 was deployed on the Araport11 polypeptide sequence collection of the *A. thaliana* accession Col-0 [64]. *A. thaliana* bait sequences were temporarily removed from the bait sequence set before deployment. Afterwards, the pipeline was deployed on polypeptide sequences of the *A. thaliana* accession Nd-1 [82], *Dioscorea dumetorum* [83] and *Croton tiglium* [84] with the complete bait collection.

The identified candidates in the *A. thaliana* Nd-1 accession were assigned to the Col-0 candidates by using them as baits for the BLASTp-based Python script "collect_best_BLAST_hits.py" v0.29 [65] to compare the results of both accessions.

Results

bHLH and outgroup bait collection

The initial bait collection consists of 4,545 bHLH sequences of 28 plant species [85]. The optimisation process resulted in an optimised bait collection of 318 sequences representing 27 species. For each sequence the species name, the reference labelling it as bHLH, the accession identifier, and the source of the sequence was documented. As representatives of bryophytes, lycophytes, gymnosperm, and several angiosperm species are included, both the bHLH bait collection and the optimised bait collection cover a wide phylogenetic range of land plant species (Figure 2). The collection of outgroup sequences contains 141 identified non-bHLH sequences of 16 species. The optimised outgroup collection contains 89 non-bHLHs and still represents the same 16 species.



	bHLH	optimised bHLH	outgroup	optimised outgroup
Arabidopsis thaliana	175	17	2	1
Brassica rapa	230	16	10	8
Brassica napus	510	-	-	-
Gossypium hirsutum	430	14	15	2
Citrus grandis	128	8	8	5
Eucalyptus grandis	153	3	9	7
Populus trichocarpa	183	4	7	2
Arachis duranensis	132	7	-	-
Arachis ipaensis	127	9	-	-
Phaseolus vulgaris	155	1	9	8
Cucumis melo	118	4	9	8
Prunus persica	95	3	8	5
Prunus mume	95	5	-	-
Vitis vinifera	94	4	9	7
Camellia sinesis	134	20	12	7
Nicotiana tabacum	100	2	10	5
Fagopyrum tataricum	163	11	-	-
Nelumbo nucifera	115	8	-	-
Aquilegia coeruela	118	8	7	5
Sorghum bicolor	174	19	-	-
Oryza sativa	175	17	8	6
Zea mays	211	35	-	-
Musa acuminata	244	12	11	7
Phoenix dactylifera	126	11	-	-
Amborella trichopoda	84	6	-	-
Ginkgo biloba	85	25	-	-
Selaginella moellendorffi	94	21	-	-
Physcomitrella patens	97	28	2	1
	4,545	318	136	84

Figure 2: Phylogenetic relationship of all plant species represented in the bait sequence collection v1.1. Number of sequences in bait collection, optimised bait collection, outgroup collection and optimised outgroup collection.

Orders of plant species were retrieved from WFO [86]. Phylogenetic relationship of orders and families revised by [87, 88].

The phylogenetic relationship shows the outgroup sequences forming a group separated from the monophyletic bHLH sequences (Figure 3, Additional file 3). The phylogenetic separation of the outgroup sequences is also shown in a phylogenetic tree inferred with IQ-TREE 2 [89] (Additional file 4). A few bHLH sequences of the optimised bait collection are not matched by the HMM motif of the optimised bait collection.

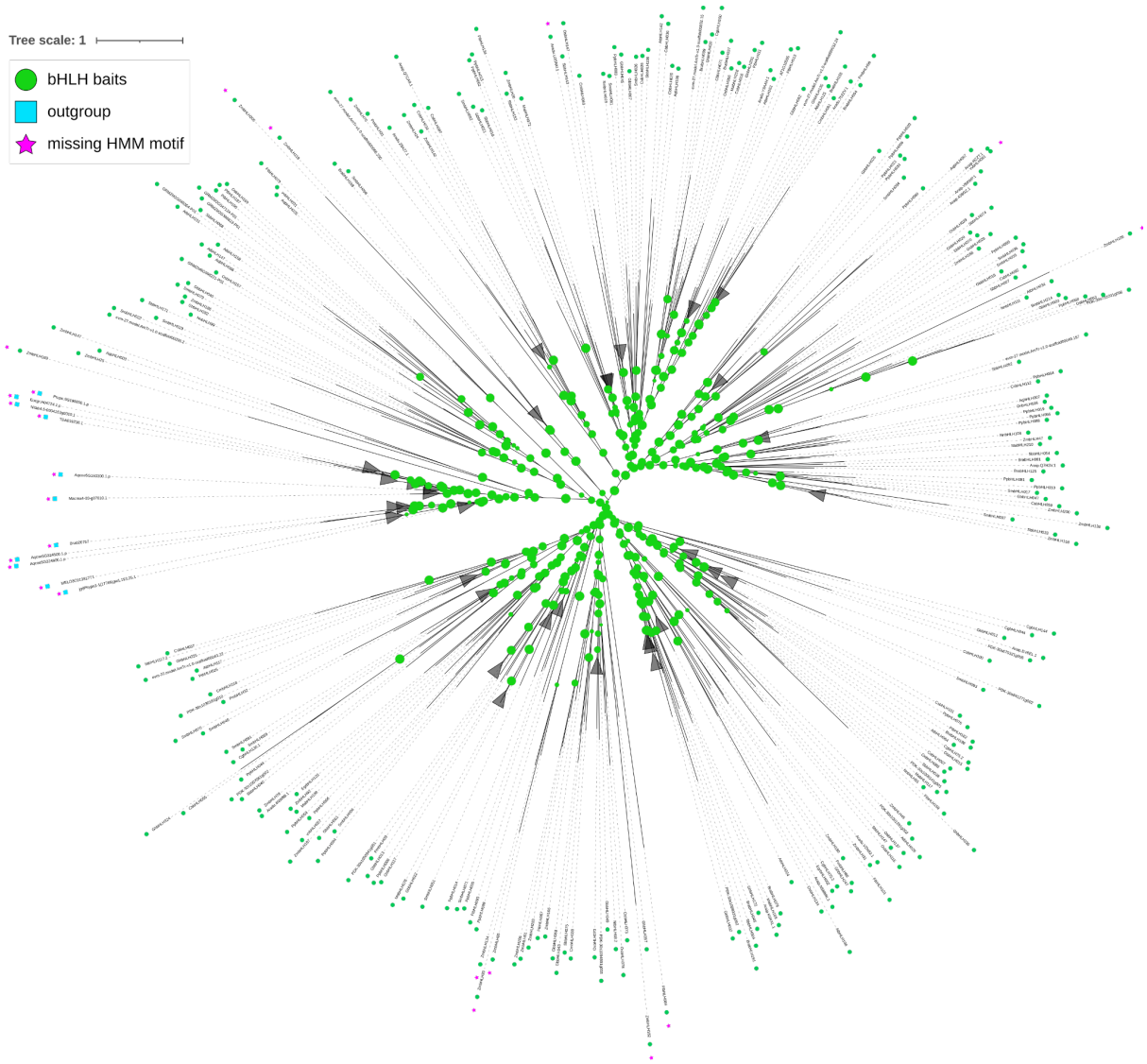


Figure 3: Maximum likelihood tree showing the phylogenetic relationship between optimised bHLH bait collection and optimised outgroup collection v1.1. Bootstrap values are represented by the size of green circles. Clades with an average branch length distance below one are collapsed. Figure created with iTOL [90].

bHLH identification and annotation workflow

275 The pipeline comprises several mandatory and optional steps (Figure 4). In step 1, initial
276 candidates are identified via BLAST [91, 92] or HMMER [62]. In step 2, the candidates are
277 classified as ingroup (bHLH) or outgroup (non-bHLH) members. A phylogenetic tree
278 containing the clean candidate and bait sequences is constructed (step 3). Based on the tree,
279 orthologous baits and orthologous bHLH reference sequences are assigned to each candidate
280 (step 4). In the following steps, an analysis of the bHLH domain is performed based on a HMM
281 motif and the DNA binding group is predicted (step 5). Also based on HMM motifs, subfamily
282 specific motifs are identified in the candidate sequences (step 6). Next, phylogenetic trees are
283 constructed containing clean candidate sequences and *A. thaliana* bHLHs (step 7),
284 respectively. Step 8 provides the option to collapse large groups of similar sequences by
285 defining one representative per clade. This option is intended for the analysis of *de novo*
286 transcriptome assemblies, which are usually rich in transcript isoforms. In step 9, another
287 phylogenetic tree of the representative candidates retained in step 8 and *A. thaliana* bHLHs is
288 created. The bHLH_annotator pipeline is publicly available through a GitHub repository:
289 https://github.com/bpucker/bHLH_annotator.

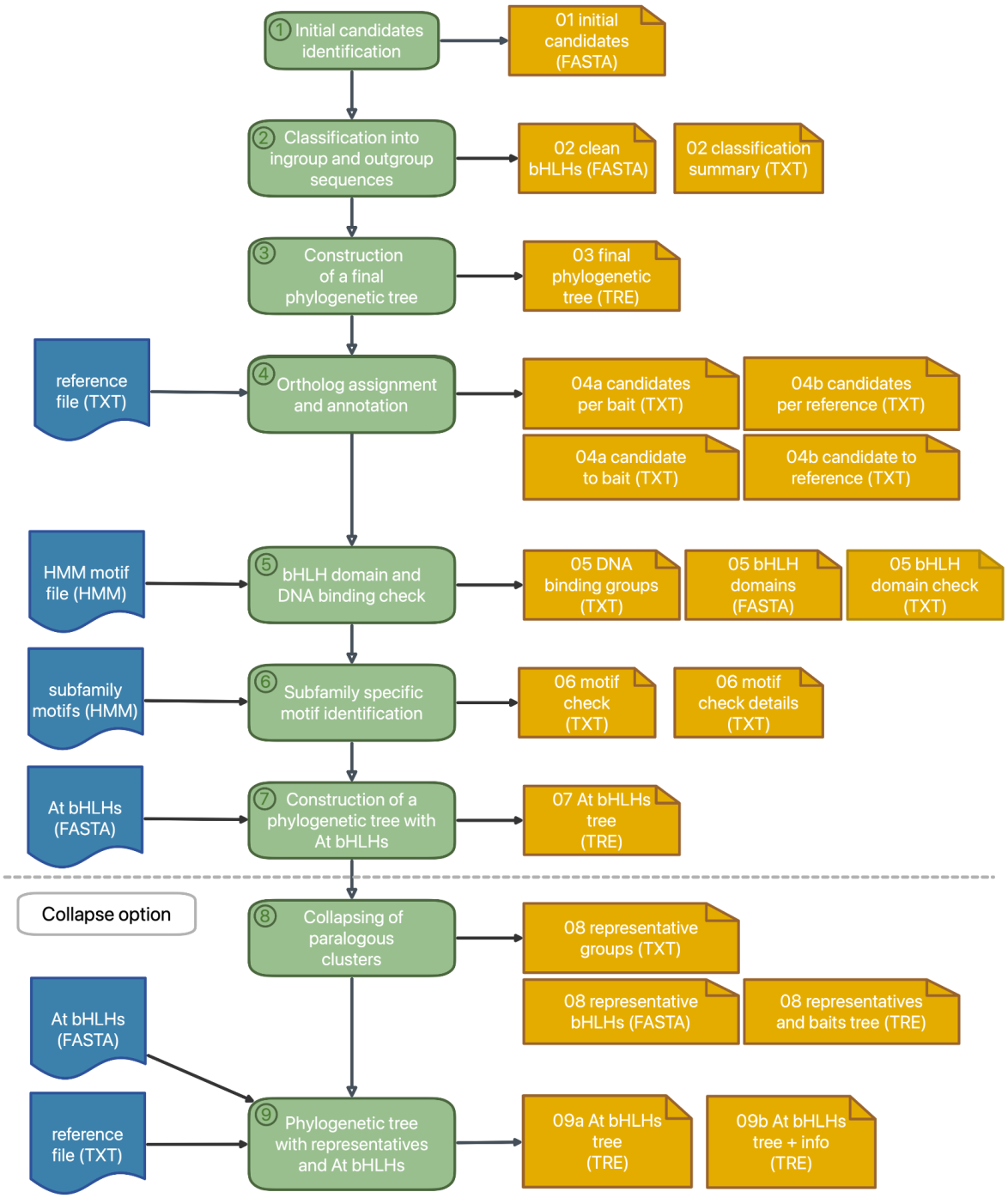


Figure 4: Schematic illustration of the bHLH_annotator pipeline. The pipeline steps and output files are numbered. Input files are coloured blue. The pipeline steps are coloured green. All output files with format are numbered and coloured yellow. Step 8 and step 9 are only performed if the collapse option is chosen.

Step 0: Input data and integrity check

Prior to execution of the bHLH_annotator, several inputs are checked. Users need to supply an input FASTA file containing coding or peptide sequences (subject file) and to specify an output folder. Additional input files like the HMM motif for the HMMER search can be defined in a config file or as arguments.

The defined files are checked for forbidden characters in the sequence identifiers and for consistency of identifiers across files. The input FASTA file is cleaned to remove forbidden characters. A mapping file is created to document the connection between cleaned identifiers and the original identifiers of the user-supplied subject file.

Step 1: Identification of initial candidates

Initial candidates can be identified via BLAST [91, 92] or HMMER [62]. For HMMER, a HMM motif must be defined (the HMM motif of the bait collection is defined as default in the config file). As default option, BLAST is used to perform a search with the bait sequence collection against the user-supplied subject file. The hits are filtered based on bit score, alignment length and percentage of identical matches (described as similarity) to retain the initial bHLH candidates.

Step 2: Classification into ingroup and outgroup sequences

A phylogenetic tree of the initial candidates and bait collection is constructed. As an alignment tool, Muscle5 [58] or MAFFT v7 [57] can be selected with Muscle5 [58] being the default option. Each alignment is trimmed by removing positions with less than 10% occupancy in a given alignment position. Tree construction can be done via default FastTree2 [59] with option "-wag" or RAxML-NG [60]. In case of a high number of candidates, parallel tree construction with a fixed number of candidates per tree is possible. All candidates are screened for the presence of the defined HMM motif. The classification of candidates is based on the phylogenetic distance to neighbouring ingroup and outgroup leaves representing bait sequences (Figure 5).

	Candidate 1	Candidate 2	Candidate 3
ingroup baits	3	2	1
outgroup baits	0	1	2
score	1.0	0.67	0.33

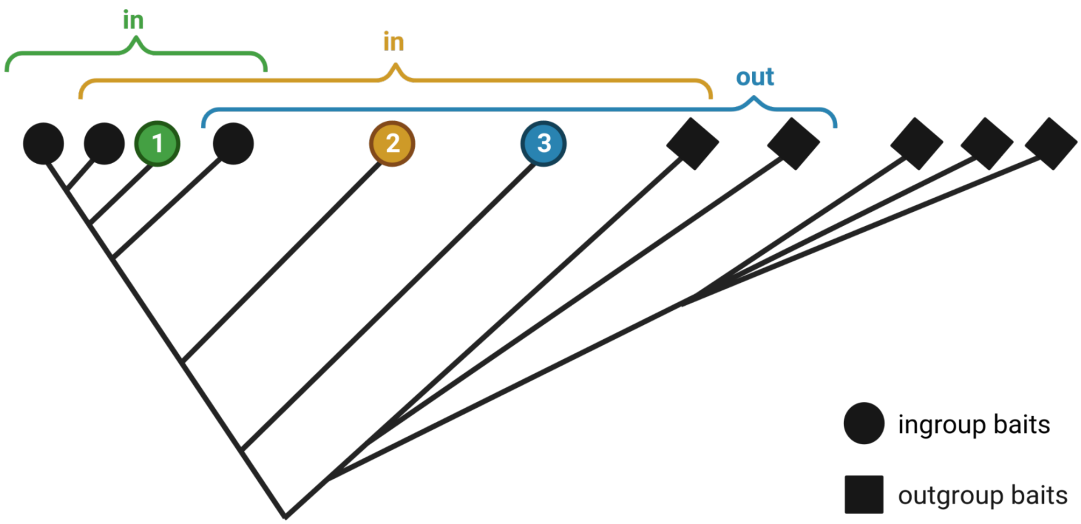


Figure 5: Schematic illustration of the classification into ingroup and outgroup candidates. The brackets denote the neighbouring leaves considered for the classification of each candidate. The number of considered neighbouring leaves for classification is 3. Candidates with a score higher than 0.5 are considered as ingroup bHLHs (in); candidates with a score below 0.5 are considered as outgroup candidates (out).

With DendroPy 4.5.2 [61], patristic distance and edge count of each candidate leaf to all other leaves of the trees are computed. The classification is performed on each candidate leave separately. Neighbouring leaves are sorted based on ascending edge count and a defined number of these leaves is selected for classification. Singular leaves with a patristic distance longer than the mean nearest taxon distance (calculated with DendroPy v4.5.2 [61] multiplied by a defined factor are removed from the selection to exclude outliers. The ingroup and outgroup baits among the selected leaves are counted and the relative contribution of ingroup sequences is calculated as a score. Based on a defined minimum score and a minimum number of ingroup and outgroup baits in the selection, the candidate leaf is classified as ingroup or outgroup. Also, candidates missing the bHLH motif can be excluded from the ingroup if the user specified the presence of this motif as mandatory.

With the candidates classified as ingroup, a second classification with a newly constructed tree is started to filter candidates that were previously falsely accepted. The second phylogenetic tree is expected to be of higher quality due to the reduced number of non-bHLHs in the multiple sequence alignment. The ingroup candidates of the second classification are accepted as final bHLH candidates.

341 ## Step 3: Construction of a final tree

342 A phylogenetic tree of the final candidate and bait sequences is constructed as described
343 above. Because the contribution of non-bHLH sequences is the smallest among all analyses,
344 the resulting tree is assumed to represent the phylogenetic relationship of the candidates with
345 the highest possible reliability.

346 ## Step 4: Assignment of orthologs and annotation

347 An ortholog bait sequence is assigned to each final bHLH candidate. DendroPy v4.5.2 [61] is
348 used to calculate the edge and patristic distances from each candidate to all baits in the final
349 tree (step 3). The bait sequence with the minimum edge distance to each candidate is defined
350 as ortholog. As a result, all ortholog candidates per bait sequence are collected. Also, for all
351 candidates, the assigned bait sequences are listed together with edge distance and patristic
352 distance.
353 In the following step, the final bHLH candidates are annotated based on orthologous
354 relationships to the reference sequences. The reference file defines the functional annotation
355 description, an alternative sequence name, and the name of the phylogenetic subfamily for all
356 reference sequences. Based on the assigned orthologs, the candidates are annotated with
357 this information from the reference file. While annotated *A. thaliana* bHLHs are provided as
358 standard references, users could also run the analysis with their own references. The
359 reference sequences must be included in the complete bait collection, or the *A. thaliana*
360 bHLHs file (step 7).

361 ## Step 5: bHLH domain check and prediction of binding group

362 All final candidates are checked for the presence of the bHLH domain using HMMER [62] and
363 the defined HMM motif (the HMM motif of the bait collection is defined as default in the config
364 file). The results are summarised and identified bHLH domains are exported into a FASTA file.
365 For prediction of DNA binding groups, the crucial domain positions are analysed for each
366 candidate. Based on determined positions in the reference bHLH AT1G09530, the crucial
367 locations are identified in the trimmed alignment that served as basis for the final phylogenetic
368 tree (step 3). For each candidate, several information are listed: (1) the number of basic
369 residues in the basic region, (2) the amino acids at the positions of 5, 9 and 13 (based on
370 AT1G09530), (3) DNA binding ability, (4) E-box binding ability, and (5) G-box binding ability.

371 ## Step 6: Identification of motifs

372 All final candidates are screened for the presence of subfamily specific motifs using HMMER
373 [62]. For each candidate, the recognized motifs are listed, and the motif sequences are

374 extracted. The subfamily specific motifs are defined in the motifs file and the collection can be
375 extended by users.

376 Step 7: Construction of a tree with *A. thaliana* bHLHs

377 A phylogenetic tree of the final candidates and *A. thaliana* bHLHs is constructed as described
378 above. While most users might consider the well-studied *A. thaliana* bHLHs helpful, it is also
379 possible to provide the bHLHs sequences of a different species or bHLH sequences of multiple
380 species through this option.

381 Step 8: Collapsing paralogous clusters

382 *De novo* transcriptome assemblies are characterised by a high level of sequence redundancy
383 due to large numbers of transcript isoforms per gene. Large groups of similar sequences
384 (expected paralogs or isoforms) are collapsed by defining one representative sequence per
385 clade. For each leaf of the final tree (step 3), the mean nearest taxon distance and patristic
386 distance to all other leaves of the trees is calculated using DendroPy v4.5.2 [61]. The
387 neighbouring leaves are sorted by ascending edge distance and are analysed in this order. All
388 candidate leaves with a patristic distance less than the mean nearest taxon distance multiplied
389 by a defined cutoff factor are identified as members of the paralog group. The analysis is
390 stopped at the first bait sequence. All members of the paralog group are excluded from further
391 group member identifications to prevent overlapping clusters. Each paralog is collapsed by
392 defining the member with the longest sequence as representative. A phylogenetic tree of the
393 representatives and bait sequences is constructed under the same conditions as described
394 above.

395 Step 9: Construction of phylogenetic tree with representatives and *A.* 396 *thaliana* bHLHs

397 A phylogenetic tree of the representative candidates and the *A. thaliana* or other landmark
398 bHLH sequences is constructed as described above (step 7). Also, another phylogenetic tree
399 is constructed containing the subfamily and alternative sequence names of all reference bHLH
400 sequences in their labels. Based on the phylogenetic relationship to the references,
401 subfamilies are assigned to the representative candidate sequences.

Proof of concept and benchmarking

A. thaliana accession Col-0

As a technical validation, the Col-0 sequences were screened for bHLHs. The pipeline identified 571 initial candidates in the *A. thaliana* accession Col-0 [64]. After the first phylogenetic classification in the pipeline, this number was reduced to 249 candidates. The second classification identified 241 possible bHLH candidates (Figure 6), as eight candidates were placed differently in the phylogenetic tree of the more stringent second classification round.

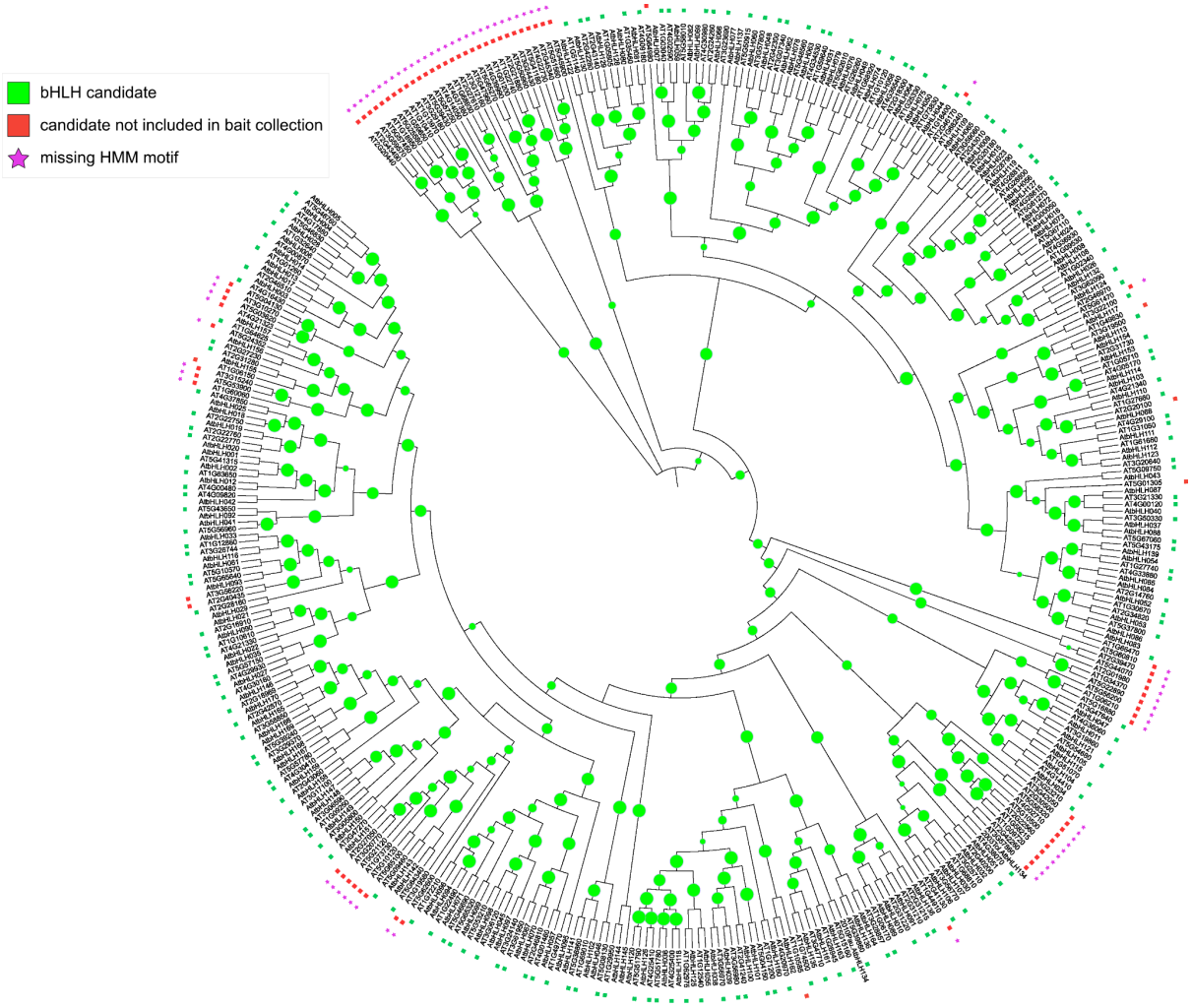


Figure 6: Cladogram derived from a maximum likelihood tree showing the phylogenetic relationship between identified candidates in A. thaliana accession Col-0 and A. thaliana bHLHs of the bait collection. Bootstrap values are represented by green circle size. Figure created with iTOL [90].

These possible bHLH candidates included 165 of the 167 *A. thaliana* bHLHs that were described in the literature [1, 8, 10]. The two candidates not identified are AT1G25310 and AT5G50010, which were not detected by BLAST due to a sequence similarity below 40% and/or bit score below 60. The 76 candidates identified in addition to previous reports in the

418 literature can be divided into four types: **(1)** 5 bHLH candidates that are not mentioned in
419 publications [1, 8, 10], but are annotated as bHLHs in TAIR; **(2)** 3 sequences not annotated
420 as bHLH but harbouring the bHLH domain and placed within bHLH clades; **(3)** 13 candidates
421 missing the bHLH motif and not being annotated as bHLH, but placed within bHLH clades; (**4)**
422 55 outliers on singular long branches or in groups on long branches with not more than one
423 bait sequence. With the filter option to exclude candidates missing the bHLH motif, the false
424 positives were reduced to members of the first type (AT1G10585, AT1G06150, AT5G01305,
425 AT2G20100, AT1G49830) i.e. bHLHs not mentioned in the publication, but annotated as bHLH
426 in TAIR and the second type (AT2G40435, AT3G56220 and AT5G64980) i.e. not annotated
427 as bHLH but harbouring the characteristic domain.
428 When deploying the pipeline with the option to search for initial candidates via HMMER [62]
429 instead of BLAST [91, 92], 198 initial candidates were identified. The classification reduced
430 this number to 186 possible bHLH candidates. These candidates included all 167 *A. thaliana*
431 bHLHs of the bait collection and 19 additional candidates. The additional candidates included
432 all members of type one and two identified in the BLAST search.

433 *A. thaliana* accession Nd-1

434 To test the pipeline on a well defined annotation without perfectly matching sequences,
435 another *A. thaliana* accession was analysed. The pipeline identified 584 initial candidates in
436 the *A. thaliana* accession Nd-1 [82]. After the first classification, this number was reduced to
437 239 candidates. The second classification identified 235 bHLH candidates (Figure 7).

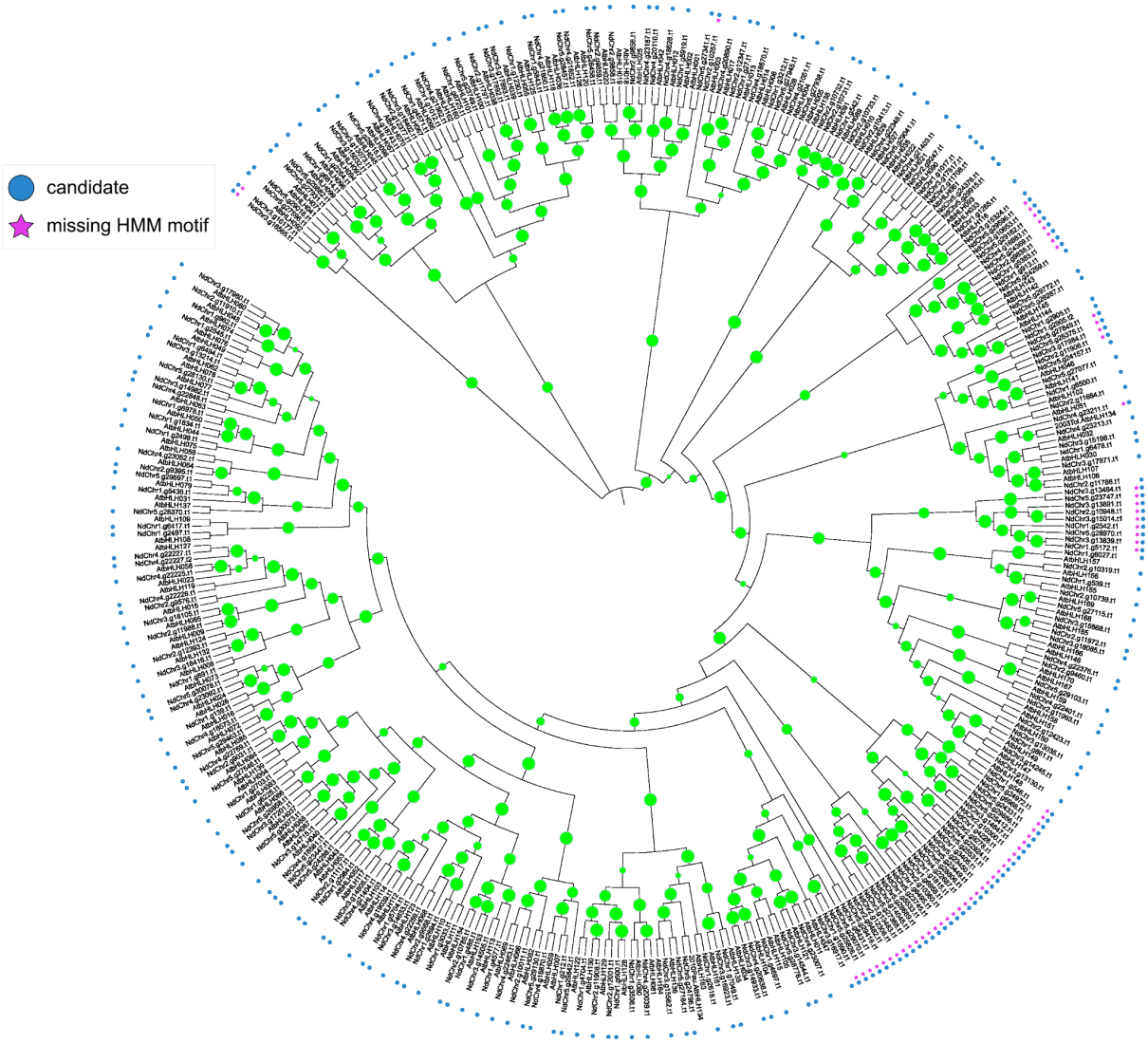


Figure 7: Cladogram derived from a maximum likelihood tree showing the phylogenetic relationship between identified candidates in the A. thaliana accession Nd-1 and A. thaliana bHLHs of the bait collection. Bootstrap values are represented by green circle size. Figure created with iTOL (Letunic & Bork, 2021)

Of these candidates, 213 were directly assigned to candidates identified in the Col-0 accession. Two other candidates (NdChr1.g2497.t1, NdChr5.g28287.t1) were assigned to the *A. thaliana* bHLHs of the bait collection not identified in Col-0 (AT1G25310 and AT5G50010). The 20 remaining candidates were not assigned to Col-0 orthologs. 18 of these candidates fitted the description of candidates with unclear phylogenetic placement (type 4 candidates in *A. thaliana* Col-0), as they were outliers on very long branches or in groups located on long branches with not more than one bait sequence. Of the identified Col-0 candidates, 30 candidates did not have an assigned Nd-1 candidate. Of these candidates, 27 were outliers with unclear phylogenetic placement (type 4), while one bHLH candidate described in the literature [1, 8, 10] (AT4G28790) and two candidates grouping into bHLH clades (type 3) were without assignment.

Monocot species *Dioscorea dumetorum*

To test the performance on a crop with huge phylogenetic distance to the model organism *A. thaliana*, the monocotyledonous species *Dioscorea dumetorum* was analysed. The pipeline identified 747 initial candidates in *D. dumetorum* [83]. After the first classification, this number was reduced to 254 candidates. The second classification identified 235 possible bHLH candidates (Figure 8). Of these candidates, 200 contained the bHLH motif of the optimised bait collection.

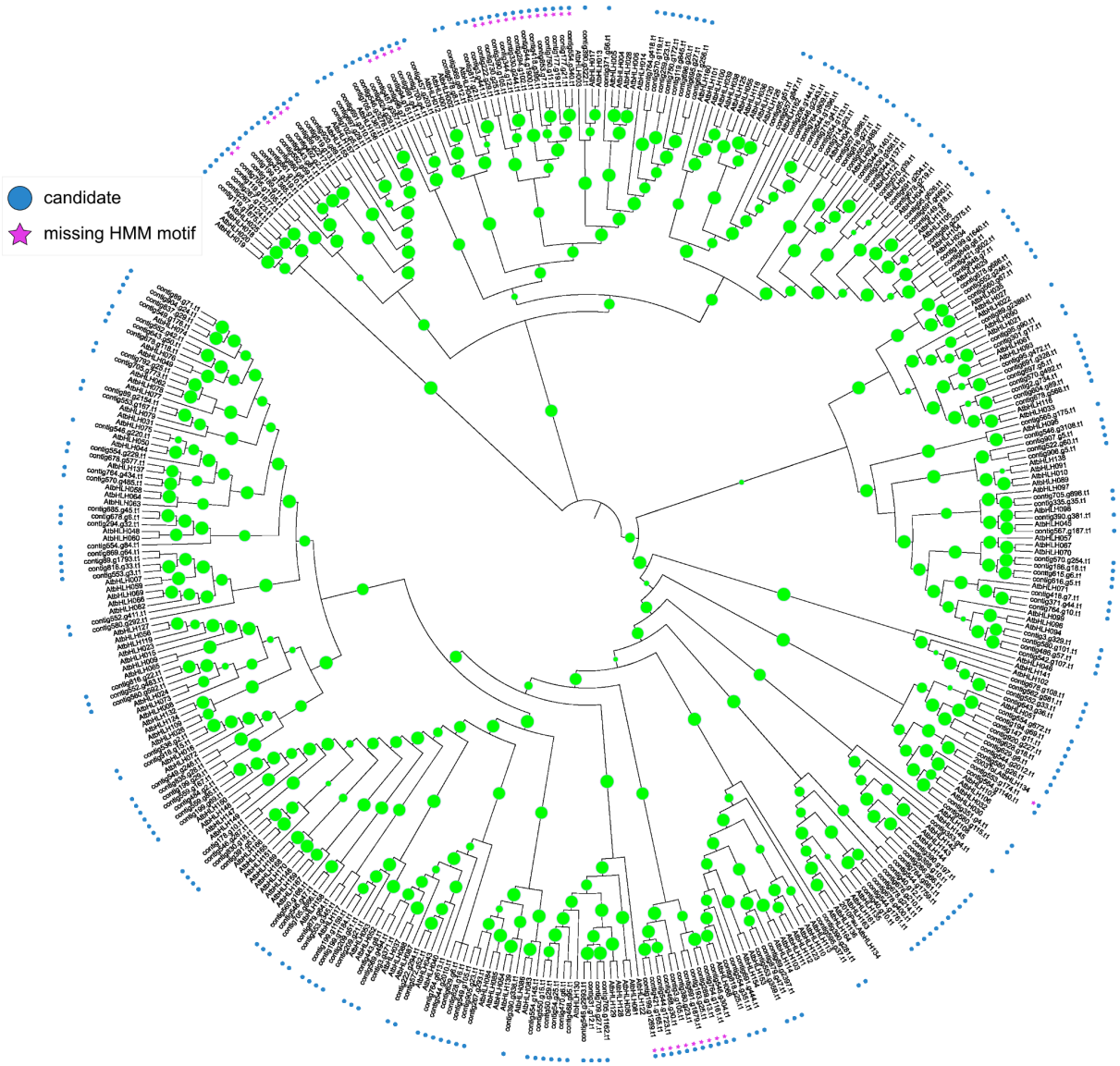


Figure 8: Cladogram derived from a maximum likelihood tree showing the phylogenetic relationship between identified candidates in D. dumetorum and A. thaliana bHLHs of the bait collection. Bootstrap values are represented by green circle size. Figure created with iTOL [90].

Transcriptome assembly of *Croton tiglium*

A transcriptome assembly was screened for bHLHs to validate the performance of the pipeline on a highly redundant sequence data set. The pipeline identified 1458 initial candidates in the

transcriptome assembly of *C. tiglium* [84]. In a first attempt without the parallel option, the pipeline was aborted in the first classification as the alignment with Muscle5 [58] was too memory consuming when aligning the 1893 sequences used for the classification. With the parallel option, the first classification was distributed onto five phylogenetic trees, which reduced the number of candidate sequences per analysis. As a result, 648 candidates were identified. The second classification was distributed onto three phylogenetic trees and identifies 552 possible bHLH candidates of whom 308 harboured the bHLH motif of the bait collection. By utilising the collapsing option of the pipeline, 178 representative candidates for the isoforms of the transcriptome assembly were determined, of whom 120 contain the bHLH motif (Figure 9).

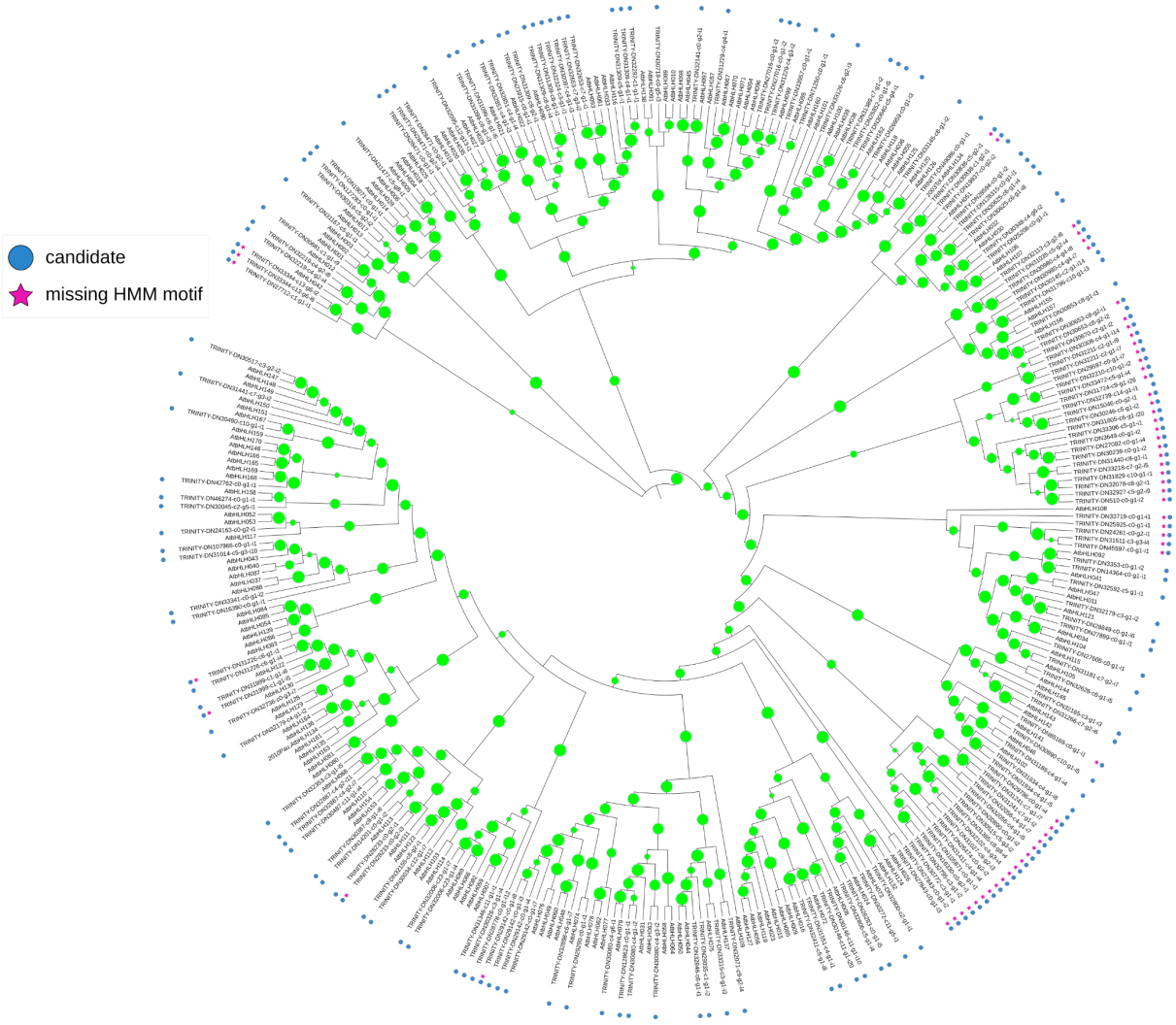


Figure 9: Cladogram derived from a maximum likelihood tree showing the phylogenetic relationship between identified representative candidates in C. tiglium and A. thaliana bHLHs of the bait collection. Bootstrap values are represented by green circle size. Figure created with iTOL [90].

481 # Discussion

482 Our bHLH_annotator pipeline enables the automatic identification and annotation of the bHLH
483 gene family in a plant species. The bHLH bait collection covers a wide phylogenetic range of
484 land plant species, including early branching species like *Physcomitrella patens.* The diversity
485 of bHLH lineages with a minimal number of sequences is represented by the optimised bHLH
486 bait collection. This optimization reduced the computation costs and run time of the analysis
487 substantially. As observed in other studies [9], bHLH sequences from various species cluster
488 together in groups of orthologous sequences that are often considered to share a common
489 function. In agreement with the assumption that the bHLH family is monophyletic [8, 9], the
490 outgroup sequences form a distinct clade separated from the bHLH sequences.
491
492 A major challenge has been the construction of an accurate multiple sequence alignment due
493 to low sequence conservation in certain regions of bHLHs. Alignments produced by Muscle5
494 have been shown to lead to phylogenetic trees with the most consistent separation of the
495 outgroup sequences. This is in agreement with the Muscle5 publication that claims a higher
496 accuracy than other established alignment tools [58]. However, Muscle5 has been shown to
497 be time- and resource consuming which makes it less well suited for large datasets [58]. Thus,
498 the number of sequences aligned must be minimised by utilising a minimal but diversity
499 representing collection of bait sequences for the alignments. The number of initially identified
500 candidates is another factor that contributes to the computational costs. The bHLH_annotator
501 comes with a parallel option, which assigns the initial candidates to separate bins to perform
502 several parallel classifications. The successful deployment of the pipeline on the *Croton tiglium*
503 sequences with the parallel option demonstrates the ability to functionally annotate the bHLH
504 transcription factor family in transcriptome assemblies with large numbers of transcript
505 isoforms.
506
507 Deploying the pipeline on the *A. thaliana* accession Col-0 resulted in the identification of 165
508 out of 167 bHLHs. This means that some sequences are missed via BLAST [91, 92]. Because
509 the bHLH family in *A. thaliana* is well studied [1, 8, 10], it is very likely that also bHLHs with a
510 high degree of specialisation and corresponding sequence divergence are described in
511 literature. These sequences can be challenging to identify via similarity to bait sequences due
512 to their high degree of sequence divergence. The addition of diverged *Brassica napus* and
513 *Zea mays* sequences to the bait collection promised a more extensive identification in closely
514 related monocot and eudicot species. However, HMMER [62] is a sensitive approach to
515 identify even diverged candidate sequences in all species and outperformed BLAST in this
516 respect. Regardless, a comprehensive identification of the bHLH family is demonstrated in the

517 Col-0 accession of *A. thaliana,* which is also supported by the identification of eight additional
518 bHLHs not described in the literature (type 1+2, see results for details). The observation that
519 all but one candidate of the bHLH candidates described in the literature [1, 8, 10] and identified
520 in Col-0 were also identified in Nd-1 demonstrates that the identification is not prevented by a
521 small number of sequence variations.
522 Through BLAST [91, 92], 68 additional candidates were identified in the Col-0 accession that
523 miss the bHLH domain and are not described in literature. While 13 of these candidates were
524 placed into clades of bHLHs (type 3, see results for details), 55 candidates represented
525 outliers on long branches or were placed into groups of long branches with not more than one
526 bHLH (type 4, see results for details). Some of these were recognized because they were
527 placed between the outgroup sequences and bHLHs in the phylogenetic tree and were not
528 filtered in the classification. There are several explanations for the identification of the
529 remaining candidates: One possible explanation is that members of the bHLH family lost their
530 characteristic domain and perhaps function. A possible example is AT5G50960, which was
531 functionally annotated by TAIR as a nucleotide binding protein that homodimerizes. An
532 argument against the domain loss theory for this protein is the localisation in the cytosol
533 predicted on TAIR [63, 64]. Another explanation is the evolution of the bHLH family via domain
534 shuffling [8–10]. If the affected bHLH gene is the result of domain shuffling, orthologs of the
535 gene the bHLH domain encoding exons were inserted into can be recognized. It is also
536 possible that some of the candidates represented pseudogenes, which have also been
537 observed in other studies [10, 27], or artefacts. Furthermore, some candidates can be
538 misplaced in the phylogenetic tree because no member with a close phylogenetic relationship
539 was contained in the bait sequence collection. In total, 30 of the Col-0 bHLH candidates,
540 mainly representing outliers on long branches, were assigned to any identified bHLH
541 candidate in Nd-1. The same applies to 20 candidates in Nd-1. Keeping this observation in
542 mind, the interpretation of candidates on long branches must be performed with high caution
543 and justifies an in-depth investigation of their phylogenetic relationship. The candidates
544 missing the HMM motif and with a phylogenetic placement inside the bHLHs (type 3, see
545 results for details) can be interpreted with a higher certainty. These might represent good
546 candidates for the investigation of domain loss events.
547 However, if the identification of bHLH candidates with domain loss is not an objective of the
548 study, candidates missing the HMM motif can be excluded by applying the filter domain option
549 or utilising the HMMER [62] search option. As the HMMER [62] search identified all *A. thaliana*
550 bHLHs described in the literature [1, 8, 10] and the additional identified bHLHs (type 1+2, see
551 results for details) in the Col-0 accession, it demonstrated a sensitive identification of
552 sequences harbouring the bHLH domain. This included even diverged sequences and
553 reduced the number of additional candidates.

554
555 The bHLH_annotator pipeline was developed to functionally annotate the bHLH transcription
556 factor family. This is also possible with other available tools intended for the functional
557 annotation of sequences: The Pfam database [32, 33] provides the HMM motif PF00010,
558 which represents the bHLH domain [93]. InterProScan5 [93] can be applied to identify
559 members of the bHLHs. Also, motifs like IPR045239 are provided by InterPro, which is
560 described as a transcription factor bHLH95-like bHLH domain, identifying a specific group of
561 plant bHLHs [93]. Although no detailed functional annotation is provided by Pfam, it can be
562 useful for initial identification of bHLHs comparable to the initial results of the bHLH_annotator
563 pipeline. A functional annotation can be assigned to sequences by the Mercator4 pipeline [39,
564 42]. Sequences assigned to bin 15.5.32 are annotated as bHLHs with no further functional
565 description. An example for a bin providing a functional description is 26.1.2.1.3, which
566 describes bHLHs involved in the regulation of blue light perception, like cryptochrome
567 interacting bHLHs (CIBs) [39, 42]. Also, loss of function can be detected by identifying bins
568 with no assigned sequences [39]. But as the pipeline only provides functional annotation and
569 does not put the sequences into phylogenetic context, no further information can be obtained.
570 A phylogenetic approach is recommended for the annotation of entire gene families [48, 94].
571 Through the investigation of the phylogenetic relationship, gene duplications can be
572 recognized and functional annotations can be placed into context [48]. Moreover, bHLH family
573 members that experienced loss of function can be identified through a phylogenetic approach.
574 But there are also some pitfalls that must be kept in mind: Functional diversification might
575 cause orthologs to have (slightly) different functions in different species. Furthermore,
576 paralogs can appear as orthologs, if their respective ortholog is lost [45]. OrthoFinder is a tool
577 that enables the phylogenetic identification of orthologs if clear one-to-one relationships exist
578 between genes in different species [50]. But as no direct functional annotation is provided, this
579 step has to be done manually for example by inferring the Gene Ontology (GO) terms of
580 identified orthologs [35, 36]. The bHLH_annotator pipeline was developed for the functional
581 annotations of the bHLH transcription factor family in plants. During conceptualization and
582 implementation, bHLH-specific characteristics have been considered. Additional
583 functionalities are provided by the recognition of subfamily specific motifs and the prediction
584 of DNA binding properties. This allows an automatic and detailed investigation of the bHLH
585 transcription factor family in a wide range of plant species.
586 For future improvement of the pipeline, a more diverse outgroup could possibly lead to a
587 refinement of the candidate classification. Adding reference sequences of other species than
588 *A. thaliana* to the reference collection used for functional annotation would be helpful for the
589 annotation of species that are distantly related to *A. thaliana*. Therefore, users are enabled to
590 add their own collection of landmark bHLH sequences to the set of reference sequences.

591 # Conclusion

592 With the bHLH_annotator, a pipeline for the automatic identification and functional annotation
593 of the basic helix-loop-helix (bHLH) transcription factor family in plants is available. Along with
594 the pipeline, a comprehensive collection of bHLH sequences is provided, which represents
595 the full diversity of the bHLH gene family. An optimised collection of the bHLH sequences
596 containing only one representative sequence of each bHLH lineage saves time and resources
597 during analysis. The pipeline performs functional annotation through the phylogenetic
598 identification of orthologs. Depending on the utilised search option, former bHLHs which
599 experienced domain loss can be identified via BLAST. An identification limited to bHLHs
600 harbouring the bHLH domain is provided by HMMER. Phylogenetic trees constructed with the
601 optimised bait sequence collection and *A. thaliana* bHLHs allow a detailed investigation of the
602 annotated bHLHs. Characteristics like the subfamily specific motifs and prediction of DNA
603 binding properties are analysed. Furthermore, the bait collections, reference sequences
604 utilised for the annotation, and *A. thaliana* bHLH sequences can be customised for the
605 intended purpose. This provides a powerful set of options for the investigation of the bHLH
606 transcription factor family in land plants, even for transcriptome assemblies. For analysing the
607 evolution of the bHLH family, identifying domain loss events or understanding the development
608 of biological function, a wide range of functions regulated by this transcription factor family can
609 be examined.

Availability and requirements

Project name: bHLH_annotator
Project home page: https://github.com/bpucker/bHLH_annotor
Operating system(s): Linux
Programming language: Python3
Other requirements: dendropy, BLAST, HMMER, MAFFT, MUSCLE, RAxML or FastTree2
License: GNU General Public License v3.0
Any restrictions to use by non-academics: none

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The pipeline and the required input data sets are freely available via github: https://github.com/bpucker/bHLH_annotator. Additionally, the released version was archived via zenodo(10.5281/zenodo.7903959). The analysed transcriptomic and genomic resources are publicly available: *Arabidopsis thaliana* Col-0 (https://www.arabidopsis.org/), *A. thaliana* Nd-1 (https://doi.org/10.5447/IPK/2019/4), *Dioscorea dumetorum* (https://doi.org/10.4119/unibi/2941469), and *Croton tiglium* (PRJNA416498).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CT and BP planned the study. CT wrote the software and performed the bioinformatic analysis. CT and BP interpreted the results, and wrote the manuscript. All authors have read the final version of the manuscript and approved its submission.

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