

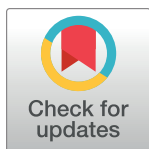
RESEARCH ARTICLE

Closing target trimming and CTTdocker programs for discovering hidden superfamily loci in genomes

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Data Availability Statement: The full CTT and CTTdocker packages are available in two repositories hosted by GitHub at <https://github.com/hua-lab/ctt> and <https://github.com/hua-lab/cttdocker>, respectively.

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Abstract

The contemporary capacity of genome sequence analysis significantly lags behind the rapidly evolving sequencing technologies. Retrieving biological meaningful information from an ever-increasing amount of genome data would be significantly beneficial for functional genomic studies. For example, the duplication, organization, evolution, and function of superfamily genes are arguably important in many aspects of life. However, the incompleteness of annotations in many sequenced genomes often results in biased conclusions in comparative genomic studies of superfamilies. Here, we present a Perl software, called Closing Target Trimming (CTT), for automatically identifying most, if not all, members of a gene family in any sequenced genomes on CentOS 7 platform. To benefit a broader application on other operating systems, we also created a Docker application package, CTTdocker. Our test data on the *F-box* gene superfamily showed 78.2 and 79% gene finding accuracies in two well annotated plant genomes, *Arabidopsis thaliana* and rice, respectively. To further demonstrate the effectiveness of this program, we ran it through 18 plant genomes and five non-plant genomes to compare the expansion of the *F-box* and the *BTB* superfamilies. The program discovered that on average 12.7 and 9.3% of the total *F-box* and *BTB* members, respectively, are new loci in plant genomes, while it only found a small number of new members in vertebrate genomes. Therefore, different evolutionary and regulatory mechanisms of Cullin-RING ubiquitin ligases may be present in plants and animals. We also annotated and compared the *Pkinase* family members across a wide range of organisms, including 10 fungi, 10 metazoa, 10 vertebrates, and 10 additional plants, which were randomly selected from the Ensembl database. Our CTT annotation recovered on average 14% more loci, including pseudogenes, of the *Pkinase* superfamily in these 40 genomes, demonstrating its robust replicability and scalability in annotating superfamily members in any genomes.

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Introduction

Genome growth and innovation is largely attributed to the expansion of gene superfamilies [1–3]. Through both whole-genome duplication (WGD) and small-scale duplication (SSD) events, members of a gene family can increase dramatically [4–6]. Newly synthesized members in a family have been commonly recognized to experience either pseudogenization or fixation in a genome through four processes: 1) conservation if gene dosage is beneficial [1], 2) neofunctionalization if a novel function is acquired in one copy [1], 3) subfunctionalization if daughter copies split the function of the ancestral copy [7], or 4) specialization if all daughter copies perform new functions differing from their ancient copy [8]. Previous studies have suggested that dosage-balance constraints guarded duplicated copies against loss by determining the stoichiometry of duplicated products [9, 10]. Interestingly, families involving different cellular functions have various birth over death ratios. Transcription factors tend to have a much higher fixation rate than others, such as G-protein-coupled chemosensory receptors for finding food, odorants and pheromones and the immunoglobulins involving in the primary immune defense in vertebrates, and the nucleotide-binding site-leucine-rich repeat (NBS-LRR) receptors whose functions are largely found in defending pathogens in plants [11–16]. The significant contribution in genome evolution and the striking variance of evolutionary processes of members within and between families place the studies of gene duplication an important role in decoding the function of genomes. To reach this, precisely finding the total members of a gene family in a genome would be the first effort.

Since the era of draft sequencing of human genome, finding genes in massive genomic DNA sequences has drawn significant attention in understanding the structure and function of genomes [17]. Early in this century, many computational methods were developed in order to predict genes in a piece of DNA or in an entire genome precisely. Overall, these methods can be divided into two categories: sequence similarity-based extrinsic methods and machine-learning curated intrinsic approaches. The former employs known query sequences to align a target sequence to discover the structure of an unknown locus, while the latter utilizes dynamic programming to identify genes by locating the gene elements (e.g., specific signals in the 5'- or 3'-ends of genes) [18]. Due to the extreme variability of gene elements, the intrinsic (*ab initio*) methods often yield more false predictions than the extrinsic evidence-based predictions [18]. For example, prior *ab initio* methods predicted *Caenorhabditis elegans* and *Homo sapiens* genes with an accuracy rate of 50% and 20%, respectively [19, 20]. Certainly, the accuracy of both types of gene annotation methods has been significantly increased since both the scale and the information of known genes are now dramatically expanded and improved. Recently developed next generation mRNA sequencing-based methods have increased the accuracy of *de novo* genome annotations to an average of 57.6% on *C. elegans* genes [21, 22]. However, the human ENCODE genome annotation assessment project, EGASP, suggested that the mRNA and protein sequences-based annotation programs still yielded the most accurate prediction of gene models [20].

With the booming of next generation sequencing technologies [23], large-scale genome sequencing has become routine in many individual laboratories, while genome annotation and analysis remains challenging. Considering the importance of gene superfamily studies and the contemporary large accumulation of accomplished genome projects, we developed a Perl package, named Closing Target Trimming (CTT), for superfamily annotation. This package incorporates the well-established sequence similarity-based GeneWise annotation tool [24] to automatically identify and re-annotate most, if not all, members of a gene superfamily in a genome, thus benefiting comparative genomic studies.

The algorithm of CTT was first developed during the course of studying the phylogenetics of the *F-box* gene superfamily in plants [5]. When we retrieved the *F-box* gene members in prior annotations from 18 sequenced plant genomes, including both seedless and seed plants, we noticed that there were many novel *F-box* putative loci left unannotated in genomes. Those loci are either lacking clear gene signals that allow them to be discovered *ab initio* or too close with one another to be uncovered by GeneWise annotation, since the latter only finds the best homologue of a known query protein sequence [5, 24]. Recently, we have also employed this algorithm to study the duplication mechanism of the ubiquitin and ubiquitin-like protein (in whole called ubiquitin) family in 50 sequenced plant genomes [6]. CTT has been demonstrated as an effective tool to retrieve most, if not all, members of a gene superfamily in genomes we have analyzed. To make this program readily available for the biological science community, we organized and improved the scripts used in the previous two studies and compiled it into one integrated package that allows researchers with little programming experience to use.

Materials and methods

Package development

The CTT package is written in Perl (Version 5) and has been tested on the CentOS 7 Linux operating system. The CTTdocker is a Docker application package of CTT that has been tested on CentOS 7, Ubuntu 18.04.2 LTS, and macOS Mojave (version 10.14). The results described in this paper are based on the performance of the CTT package running on the CentOS 7 Linux operating system.

Similarity-based gene annotation

Based on our previous studies on CTT-based re-annotation of *F-box* and *ubiquitin* genes in plant genomes [5, 6], we designed to retrieve a 10,000 nucleotide (nt) genomic sequence sandwiching the start coordinate of a target domain for identifying a novel family locus. Since a non-plant gene may be longer than a plant gene on size, we also examined the effectiveness of CTT prediction by expanding the size of a putative locus to 100,000 nts. A putative novel locus is positioned by tBLASTn search using the query of a collection of seed sequences, which represent the domain signature of a target family, against the genome sequence. Only if a new locus identified by CTT iteration search (see algorithm below) encompasses the tBLASTn-defined locus would the genomic sequence be further analyzed. To eliminate a potential false prediction due to the inaccuracy of one reference protein sequence, we applied at least the top two best homologous protein sequences, based on bit scores of BLASTx search [25], to back search the genomic DNA sequence for predicting the transcript model of a putative novel locus using GeneWise [24]. If the locus is consistently predicted as a protein-coding gene by at least two reference sequences, the coding sequence identified with the highest GeneWise score was taken as the best prediction of this putative new locus. The resulting peptide sequence is further examined for the presence of a target family domain using HMMER search (<http://hmmer.org>) against the Pfam-A database (Version 32.0, <https://pfam.xfam.org>, E-value <1).

CTT iteration search algorithm

The genomic DNA sequence with 10,000 nts is likely to contain more than one locus. This is particularly common in many superfamily genes that were tandemly duplicated. This prevents the identification of a putative new locus by GeneWise because GeneWise only aligns a reference protein sequence to the best homologous region [24]. To expose this putative new locus defined by tBLASTn search, unrelated adjacent DNA sequences are iteratively trimmed and

removed by CTT. First, the 10,000 nt genomic DNA sequence is used to identify a locus that best matches to a homologous protein sequence by GeneWise (GeneWise score ≥ 50). The DNA sequence of a predicted locus is trimmed and removed if it does not encompass the tBLASTn-defined region. The remaining DNA sequence is then iterated for a sequential BLASTx-GeneWise-trimming search until the tBLASTn-defined region is identified to be within the GeneWise predicted locus. Otherwise, the tBLASTn-defined region is not considered to be a new family locus.

Data resources

To test the effectiveness of this CTT package, a genome sequence file, a generic feature format 3 (GFF3) file, and a protein sequence file from in total 63 sequenced genomes (S1 Table) were retrieved from Phytozome (Version 12, <https://phytozome.jgi.doe.gov>) or Ensembl (Release 94, <https://useast.ensembl.org>). The seed files for three test superfamilies, the F-box (PF00646), the BTB (PF00651) and the Pkinase (PF00069) protein families, and the Pfam-A database were downloaded from Pfam (Version 32.0, <https://pfam.xfam.org>).

Results

Structure and implementation of CTT

The CTT package is built on the CentOS 7 Linux operating system. The source code is permitted to use and distribute under the GNU General Public License Version 3.0. The package requires the Pfam-A database and six program dependencies, including BioPerl (<https://bioperl.org>), BLAST [25], HMMER (<http://hmmer.org>), GeneWise [24], PfamScan (<https://pfam.xfam.org>) and CD-HIT [26]. The users are strongly recommended to follow the steps described in a *README.md* file under the main directory */ctt* to install the database and the dependencies on CentOS 7. We found that compiling BioPerl could be really problematic for researchers with little programming experience. The instructions provided in “Install BioPerl” in the *README.md* file can significantly reduce this hassle. After BioPerl and BioPerl-Run are installed, the “make all” function in *makefile* under the directory */ctt/dependencies* should help to install the Pfam-A database and the remaining program dependencies smoothly.

Before running the program, the users need to collect the information of target genomes and seed sequences for a family of interest as input files into the directories */ctt/species_databases* and */ctt/seeds*, respectively. Since we are interested in both known and unknown members of a family, a genome sequence file, a generic feature format 3 (GFF3) file, and a protein sequence file from a previous genome annotation are required to be deposited in the directory of */ctt/species_databases*. The family members from prior annotations are used as reference peptide (Ref_Pep) sequences to predict the gene structure of an unknown locus using GeneWise. Therefore, we recommend adding the same set of three files from well-annotated genomes to increase the prediction accuracy. The package is capable of working on multiple genomes at one time. Once the number of genomes is decided and all the required information is collected, a tab file, named “*organismal_genome_gff3_proteome_files.tab*”, needs to be prepared with each row containing the names of a genome sequence file, a GFF3 file, and a protein sequence file of one genome, each of which is separated with a tab space. The seed file of a family can be found and downloaded from the family webpage on Pfam (<https://pfam.xfam.org>). For example, a FASTA-formatted seed file of the *F-box* family can be found at <https://pfam.xfam.org/family/F-BOX#tabview=tab3>. A detailed method is also available on protocols.io ([27]; S1 Fig). This file should be placed in the directory */ctt/seeds*. All the input sequence files are in a FASTA format, which allows a BioPerl module, Bio::DB::Fasta, to parse the sequences. The species genome and protein sequence files should also be converted to BLASTable databases in the same directory

using the *makeblastdb* function as instructed in the *README.md* file or as described in the manual of BLAST [25]. A step-by-step protocol can be found on protocols.io [27].

The operation code of this program is formulated as one Perl command in the terminal under the directory of the package */ctt*. The users simply need to enter a code formatted as “*perl ctt.pl -seed family_seed_file.txt -f Pfam_family_id -superfamily simplified_family_id_you_named*” to start the program. The *ctt.pl* should automatically read the input files from */ctt/species_databases* and */ctt/seeds* to finish the annotation. There are in total seven steps, each of which is operated by an annotation module saved in the directory */ctt/annotation_modules*. In accordance, there are 7 outputs that the program produces and saves stepwise in a final */ctt/ctt_output* directory (Table 1).

In Step 1, *prior_annotation_search.pm* applies the seed sequences as query to BLASTp [25] a previously-annotated protein database. Pfam domains present in each hit protein sequence are obtained by *PfamScan.pl* search against the Pfam-A database that is automatically downloaded, processed, and saved under the directory */ctt/databases/pfam*. The Pfam domain search in *PfamScan.pl* is essentially done by HMMER3 (<http://hmmer.org>). The latter applies profile hidden Markov models (profile HMMs) to determine the presence of a sequence homologous to a Pfam domain, whose information is represented by a multiple sequence alignment and a hidden Markov model (HMM) in the Pfam-A database (<https://pfam.xfam.org>). In the current version of CTT, we apply 1e-5 and 1 as an E-value cutoff for the BLASTp and HMMER searches, respectively. Both values are artificially determined based on our previous studies that may assist to include most known members in both the *F-box* and the *ubiquitin* families [5, 6].

The module of Step 2, named *reference_pep_best_dm_search.pm*, is designed to organize the full-length protein sequences of family members predicted in Step 1 and retrieve the best family domain sequence in each protein sequence as a new seed sequence. Both the full-length sequences and the combined new and Pfam family seed sequences are subject to CD-HIT [26] to remove redundant ones. The retaining full-length and seed sequences are used as reference and seed sequences for later GeneWise annotation and tBLASTn search, respectively.

Step 3 applies the non-redundant seed sequences as queries to search a genome database using tBLASTn with an e-value cutoff $\leq 1e-5$. Since the tBLASTn search generates a large number of hits, we considered two hits the same if the middle coordinate of one resides in the region of the other. In addition, we use AWK (comes with CentOS) to compare the start and the end coordinates of one tBLASTn hit with those of the previously-annotated genes, whose information is retrieved from the GFF3 file. Since tBLASTn finds a region aligned with a family domain, the hit should reside in an annotated gene if it is part of the gene. Those hits are not considered as new loci and are removed from further analyses. This step is accomplished using a module named *finding_putative_new_loci.pm*.

Table 1. Annotation modules and outputs of CTT.

Step	Module	Output
1	<i>prior_annotation_search</i>	Protein sequences of a target family
2	<i>reference_pep_best_dm_search</i>	Non-redundant reference peptide sequences and combined new and Pfam seed sequences
3	<i>finding_putative_new_loci</i>	Genomic DNA sequences of putative new loci each having 10,000 nts
4	<i>closing_target_trimming</i>	Trimmed genomic DNA sequences only containing the target locus
5	<i>annotate_the_best_model</i>	GeneWise annotation results: the genomic DNA, transcript, and peptide sequences for each protein coding gene are predicted. Pseudogenes are also identified.
6	<i>correct_gdna_coordinates</i>	Fine tune the genomic coordinates of a protein coding gene
7	<i>pfamscan</i>	Finalize the new gene members

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Step 4 runs the CTT algorithm which is coded in a module called *closing_target_trimming.pm*. In order to gain a genomic region long enough to cover a putative new locus, we added a sequence of 5,000 nts on both sides of the start point of a retaining tBLASTn hit in Step 3. Based on our previous study on *F-box* genes, the longest *F-box* locus in plants is less than 10,000 nts [5]. A DNA sequence of 10,000 nts should be sufficient to contain a new locus in plants. For other organisms, the users can modify *finding_putative_new_loci.pm* in Step 3 to retrieve a longer region if needed. This 10,000 nt DNA sequence containing an unknown locus is used to BLASTx the Ref_Pep database obtained in Step 2. The best hit is then used as a protein template in GeneWise to predict the structure of a new gene. However, the best homology search algorithm may not let GeneWise find the locus containing the target hit if there are multiple homologous sequences in this DNA fragment. To expose the target locus, this module applies up to six iterations of GeneWise annotation and trimming to shorten the genomic sequence until a hit-containing locus is discovered (Fig 1). The iteration stops if the GeneWise score is lower than 50 or no BLASTx hit is obtained.

To reduce the bias caused by a problematic reference sequence, Step 5 calls the module, *annotate_the_best_model.pm*, to utilize the protein sequences of the three best BLASTx hits as GeneWise templates to annotate the gene structure of an unknown target locus uncovered in Step 4. Only if one of the three gene models (peptide-coding gene, pseudogene without GeneWise transcript, or pseudogene containing early stop codons) is consistently predicted by at least two reference sequences would we consider it a good annotation. The transcript predicted with the highest GeneWise score is taken as the final prediction for a peptide-coding gene.

Step 6 retrieves the correct genomic coordinates of a putative new member and Step 7 confirms the presence of a family domain in the new annotated protein sequence (Table 1).

Sensitivity and specificity test

We tested both sensitivity and specificity of CTT on gene finding using the *F-box* gene family (Pfam ID: PF00646) as an example in two well annotated plant genomes, *Arabidopsis thaliana* and rice (*Oryza sativa*). First, we used *prior_annotation_search.pm* to identify the full set of *F-*

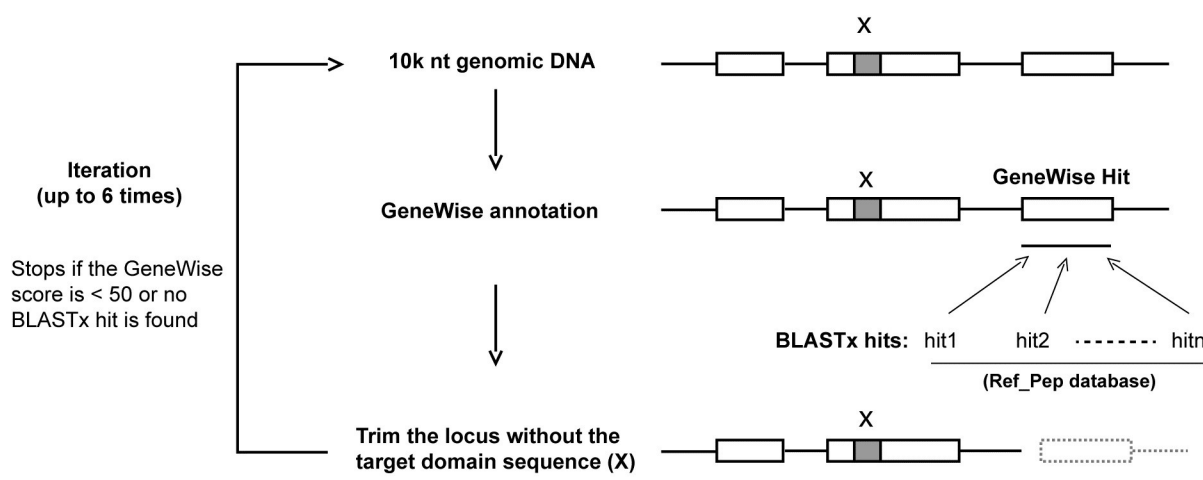


Fig 1. A schematic illustration of the CTT algorithm. The "x" indicates a genomic region that encodes a target family domain. The coordinates of "x" is defined by a tBLASTn search using the query of seed sequences of the target family. "Hit1, 2, ... n" indicate the top hits from a BLASTx search using a 10,000 nt genomic DNA sequence containing "x" as query against protein sequences of previously-annotated family members. The process is iterated for up to six times to shorten the genomic sequence until a new locus containing "x" is discovered. The iteration stops if the GeneWise score is lower than 50 or no BLASTx hit is obtained.

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Table 2. The sensitivity and specificity of CTT in finding plant *F-box* gene members.

Species*	Sensitivity		Specificity			(Sn+Sp)/2 (%)
	Pseudo-genes	Protein Coding	Pident (%)	Integrity (%)	Mean (%)	
<i>Ath</i>	15	59	99.5 ± 1.5	95.1 ± 12.1	97.3 ± 6.2	78.2
<i>Osa</i>	20	65	99.2 ± 2.2	86.9 ± 20	93.0 ± 10	79

* The full names of the species along with their abbreviations in Tables 2–6 and 8 are as listed in S1 Table.

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box gene members in the previously predicted proteomes of Arabidopsis and rice. To assess the performance, we randomly selected 100 *F-box* members in each species and removed their annotations in the corresponding proteome. We then ran through CTT to examine 1) how many of these 100 *F-box* genes were rediscovered in each genome, and 2) how good their annotations are compared to the previous protein sequences. To increase both the sensitivity and specificity of CTT, we collected the annotation datasets from 18 plant genomes (S1 Table) as we did previously [5].

We defined that a test *F-box* locus was identified if the midpoint coordinate of the CTT-annotated locus is within the test locus. We found that 75 and 85 test *F-box* loci were rediscovered by CTT in Arabidopsis and rice genomes, respectively. We also found by CTT that 15 and 20 test *F-box* loci from Arabidopsis and rice, respectively, were predicted to have no transcript available or express a protein with premature stop codons, indicative of pseudogenes (Table 2). This result further confirmed our previous discoveries showing that a significant number of *F-box* loci are pseudogenized [5, 10].

To test the specificity of CTT, we compared both sequence identity and integrity of the predicted protein sequences with previous annotations using BLASTp analysis. Between the two genomes compared, CTT seems to perform slightly better in *Arabidopsis* to predict the full length of a test *F-box* locus (Table 2, *p-value* = 0.005, Student's *t*-test). This is likely due to a close relationship between a reference genome, *A. lyrata*, and *A. thaliana*. However, for all the protein sequences predicted, CTT yielded >99% sequence identities with the original predictions, which are consistent with the outstanding performance of GeneWise annotation if a good reference sequence is available [24]. After averaging both specificity and sensitivity, CTT obtained 78.2 and 79% accuracy in finding a new *F-box* locus in Arabidopsis and rice, respectively (Table 2).

Size comparison of the *F-box* and the *BTB* superfamilies in plants

We next applied CTT to search the members of two gene superfamilies in 18 selected plant genomes. In addition to the *F-box* gene family, we selected the *bric-a-brac/tramtrack/broad complex* (*BTB*) family (Pfam ID: PF00651) for comparison. The criteria of selecting these two families are 1) that both encode a substrate receptor in a Cullin-RING E3 ubiquitin ligase complex important for regulating a vast number of metabolic and signaling pathways in all eukaryotic organisms [28], and 2) that both families are largely expanded in plant genomes [5, 29].

Consistent with our phylogenetic studies on the *F-box* gene superfamily, we obtained a similar number of *F-box* genes in all 18 plant genomes ($\rho = 0.96$, *p-value* = 6.7e-06, Spearman's correlation test) (Fig 2A and S1–S4 Files). The slight difference in the number of *F-box* genes found in the two studies may have resulted from 1) the upgrade of genome and *F-box* seed sequences, and 2) the improvement of CTT in this package. For example, in this package, we incorporated GFF3 files to precisely locate known loci in a prior annotation (Step 3, *finding_putative_new_loci.pm*, Table 1). However, our previous study used BLAT search [30] to locate an annotated gene, which may result in inaccurate genomic coordinates [5].

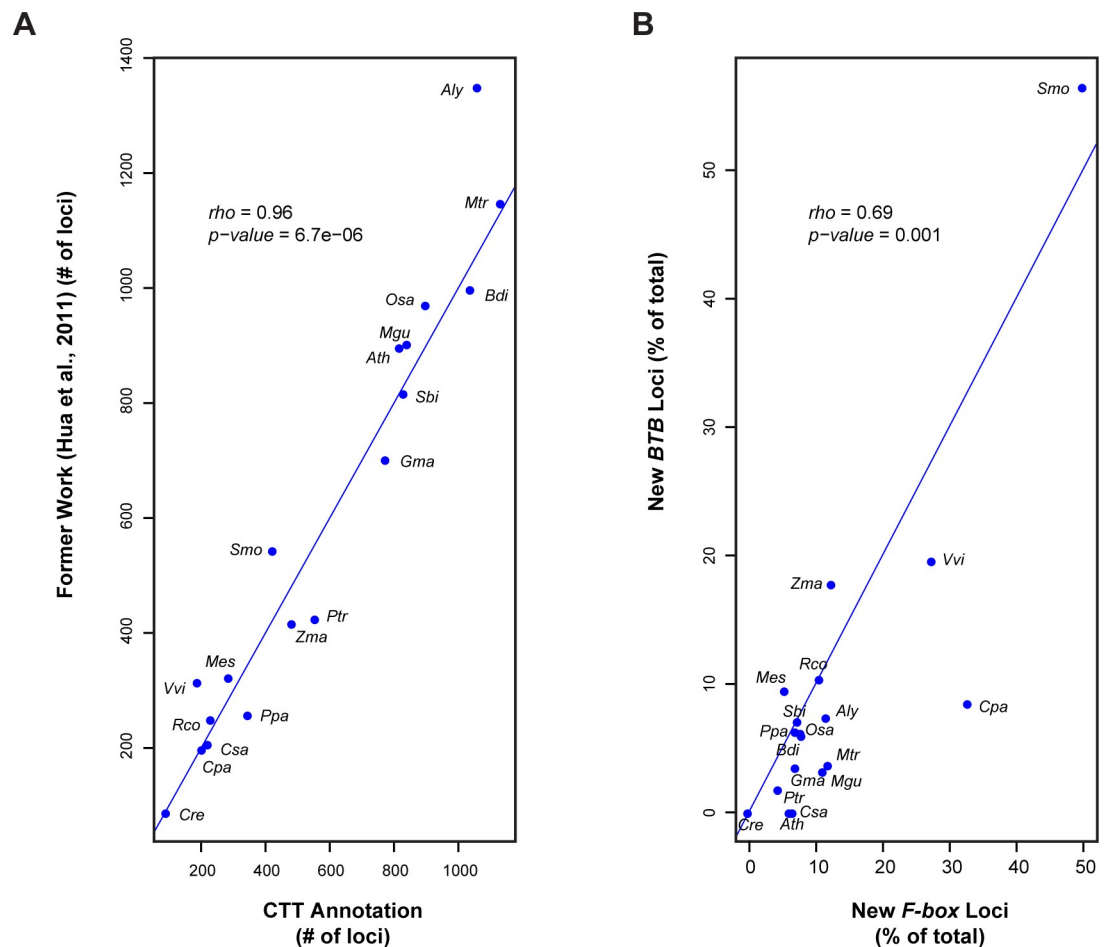


Fig 2. Performance test on the CTT program. (A) Annotation comparison of the *F-box* gene superfamily in 18 plant genomes between a previous work [5] and the output of CTT automatic annotation in this work. (B) Number correlation of new *F-box* and new *BTB* loci discovered in 18 plant genomes. The blue line indicates equal x axis and y axis values. The full names of the species along with their abbreviations are as listed in S1 Table.

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After CTT annotation, we discovered that, on average, 12.7 and 9.3% of the total members in the *F-box* and *BTB* families, respectively, are new loci (Tables 3 and 4 and S5–S8 Files). Although a slightly higher proportion of *F-box* genes were discovered than that of *BTB* genes ($p\text{-value} < 0.05$, Student's t -test), the percentiles of new members in these two families are significantly correlated ($\rho = 0.69$, $p\text{-value} = 0.001$, Spearman's correlation test) (Fig 2B). Therefore, there are various annotation qualities in different sequenced genomes, further highlighting the effectiveness of this package in helping identify most, if not all, superfamily members in a genome for comparative genomic studies.

CTT annotation of the *F-box* and the *BTB* superfamilies in non-plant genomes

Since the CTT algorithm was first developed to study the *F-box* gene superfamily in plants [5], we questioned whether this newly designed CTT package was also able to annotate new family members in non-plant genomes. We selected the genomes of human and its two-close relatives, the chimpanzee and mouse, as well as nematode and budding yeast as a test dataset (S1

Table 3. CTT annotation of *F-box* genes in 18 plant genomes.

Species	Prior Annotation	CTT Annotation		Total	New Finding (% of total)
		Pseudogenes	Protein Coding Genes		
<i>Aly</i>	940	63	57	1060	11.3
<i>Ath</i>	771	33	12	816	5.5
<i>Bdi</i>	960	41	33	1034	7.2
<i>Cpa</i>	139	53	10	202	31.2
<i>Cre</i>	95	0	0	95	0.0
<i>Csa</i>	210	3	7	220	4.5
<i>Gma</i>	723	39	14	776	6.8
<i>Mes</i>	274	5	4	283	3.2
<i>Mgu</i>	750	49	35	834	10.1
<i>Mtr</i>	1000	81	35	1116	10.4
<i>Osa</i>	831	34	34	899	7.6
<i>Ppa</i>	325	6	18	349	6.9
<i>Ptr</i>	534	18	6	558	4.3
<i>Rco</i>	209	12	8	229	8.7
<i>Sbi</i>	772	35	25	832	7.2
<i>Smo</i>	213	80	133	426	50.0
<i>Vvi</i>	140	14	37	191	26.7
<i>Zma</i>	426	36	23	485	12.2

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Table). For a strict comparison, we applied CTT to annotate the *F-box* and the *BTB* families in these five genomes.

Unlike the large and unstable expansion of the *F-box* superfamily in plants (Table 3; [5]), we found that its size in three closely related vertebrates is fairly small and stable (Table 5 and

Table 4. CTT annotation of *BTB* genes in 18 plant genomes.

Species	Prior Annotation	CTT Annotation		Total	New Finding (% of total)
		Pseudogenes	Protein Coding Genes		
<i>Aly</i>	75	2	3	80	6.3
<i>Ath</i>	86	0	0	86	0.0
<i>Bdi</i>	197	11	1	209	5.7
<i>Cpa</i>	43	2	2	47	8.5
<i>Cre</i>	70	0	0	70	0.0
<i>Csa</i>	55	0	0	55	0.0
<i>Gma</i>	194	4	3	201	3.5
<i>Mes</i>	67	3	2	72	6.9
<i>Mgu</i>	60	0	2	62	3.2
<i>Mtr</i>	105	2	2	109	3.7
<i>Osa</i>	173	7	4	184	6.0
<i>Ppa</i>	89	1	5	95	6.3
<i>Ptr</i>	163	2	1	166	1.8
<i>Rco</i>	43	2	2	47	8.5
<i>Sbi</i>	171	9	3	183	6.6
<i>Smo</i>	30	2	36	68	55.9
<i>Vvi</i>	45	7	3	55	18.2
<i>Zma</i>	125	20	6	151	17.2

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S9–S12 Files), suggesting that plants and vertebrates may have adapted a different mechanism in regulating the evolution and the function of the F-box-mediated protein ubiquitylation system. Consistent with the broad post-translational regulatory function of the ubiquitin-26S proteasome system (UPS), CTT annotation discovered that the three vertebrates have a larger BTB family than many flowering plant species (Table 6 and S13–S16 Files). In addition, the expansion of this family in these genomes is like the *F-box* superfamily showing little size variation. More interestingly, only a small proportion of members from both families were newly discovered by CTT in these five non-plant genomes analyzed (Tables 5 and 6).

We initially thought that the low number of new loci detected by CTT in these five non-plant genomes might be due to a larger genomic size of vertebrate genes than that of plant genes. We revised the *finding_putative_new_loci.pm* module in Step 3 to retrieve a genomic DNA sequence of 100,000 nts instead of 10,000 nts for downstream CTT annotation. This modification did not make any improvement. Indeed, we were able to use the same size of genomic sequences to identify a large proportion of new *Pkinase* members in 40 randomly selected genomes (see below). Therefore, we think a genomic DNA sequence of 10,000 nts, albeit arbitrarily, would be a good size for searching the presence of a superfamily gene. In general, this size can also be rationalized based on the longest locus of known family members in a genome and easily adjusted by changing *\$flank_head* and *\$flank_tail* values in the module of Step 3, *finding_putative_new_loci.pm*.

Replicability and scalability test

Our CTT program was initially designed to study the duplication mechanism of *F-box* genes in plants. In this paper, we demonstrated its capability of finding both *F-box* genes and *BTB* genes in five non-plant genomes as well. To further test its replicability and scalability, we examined its performance in finding a UPS-independent large family, *Pkinase*, in a large set of sequenced genomes, ranging from 10 fungi, 10 metazoa, 10 vertebrates, and 10 additional plants, which were randomly selected from the Ensembl genome collection (<https://www.ensembl.org>). The *Pkinase* family proteins involve protein phosphorylation, a process for adding a phosphate group onto a substrate. Within this family, the catalytic domain is called *Pkinase*, which contains an average of 267 amino acids, 2.5 and 5.5 fold longer than the *BTB* (108 amino acids) and the *F-box* domain (48 amino acids), respectively. To benchmark the performance of CTT, we also carefully recorded and compared its execution time in each of the 7 modules among these four groups of genomes (Table 7). As expected, the total execution time varied significantly amongst the four groups, ranging from 2.33 hr to 45.28 hr, which in part depended on the sizes of the proteomes and genomes being annotated. Interestingly, CTT finished the annotation in the fungi and metazoa with a speed similar to that in the plants (0.04 ms/nt) and vertebrates (0.01 ms/nt), respectively, probably reflecting their similar genome organizations.

Table 5. CTT annotation of *F-box* genes in 5 non-plant genomes.

Species	Prior Annotation	CTT Annotation		Total	New Finding (% of total)
		Pseudogenes	Protein Coding Genes		
<i>Cae</i>	388	0	1	389	0.3
<i>Hom</i>	70	0	0	70	0.0
<i>Mus</i>	82	0	0	82	0.0
<i>Pan</i>	69	4	0	73	5.5
<i>Sac</i>	14	0	0	14	0.0

<https://doi.org/10.1371/journal.pone.0209468.t005>

Table 6. CTT annotation of *BTB* genes in 5 non-plant genomes.

Species	Prior Annotation	CTT Annotation		Total	New Finding (% of total)
		Pseudogenes	Protein Coding Genes		
<i>Cae</i>	126	0	0	126	0.0
<i>Hom</i>	151	0	0	151	0.0
<i>Mus</i>	183	1	0	184	1.1
<i>Pan</i>	155	6	4	165	6.1
<i>Sac</i>	1	0	0	1	0.0

<https://doi.org/10.1371/journal.pone.0209468.t006>

Our annotation results in these four large groups of diverse organisms again showed its merit in finding new superfamily members. We discovered that an average of 4.4%, 15.2%, 27.6%, and 9.9% more *Pkinase* members in the fungi, metazoa, vertebrates, and plants, respectively (Table 8). To validate they were truly new loci, we took *Amborella* as an example to visualize its 34 new peptide loci in JBrowse that is available on Phytozome (<https://phytozome.jgi.doe.gov>) (S17–S19 Files and Fig 3). Interestingly, no previous loci were duplicated in any one of these new members. Encouragingly, exons in eight of them indeed contain an expression sequence tag (EST), suggesting they are transcribed. Moreover, 16 of them overlap with a BLASTx hit when searched against the proteomes of Arabidopsis, rice and Chlamydomonas, providing additionally evolutionary evidence. Our CTT annotation also discovered that 33.6%, 29.0%, 32.8%, and 64.5% of the new members in fungi, metazoa, vertebrates, and plants, respectively, contain in-frame shift and early stop codons, which are characteristics of pseudogenes. Collectively, our CTT annotation of the *Pkinase* family in these 40 genomes further highlighted the importance of comprehensive annotations in understanding the evolutionary process of superfamilies.

Discussion

The invention of next-generation sequencing technologies revolutionized genomic studies by dramatically reducing the cost and increasing the speed in various genome sequencing

Table 7. Performance benchmark test of CTT in annotating *Pkinase* genes in 40 genomes.

	Fungi	Metazoa	Vertebrates	Plants
Step 1 (hr)*	0.99	1.65	2.92	8.13
Step 2 (hr)	0.00	0.00	0.00	0.00
Step 3 (hr)	0.17	2.13	17.68	6.75
Step 4 (hr)	0.33	2.50	7.92	7.53
Step 5 (hr)	0.82	5.45	15.97	13.67
Step 6 (hr)	0.00	0.05	0.33	0.13
Step 7 (hr)	0.02	0.22	0.47	0.25
Total (hr)	2.33	12.00	45.28	36.47
Size of Proteome (# of proteins)	8.0E+04	2.3E+05	2.9E+05	3.4E+05
Size of Genomes (# of nt)	2.2E+08	5.2E+09	3.2E+10	3.5E+09
Annotating Speed (ms/nt)	0.04	0.01	0.01	0.04

*Performance was evaluated based on execution time of each step for annotating *Pkinase* genes in 10 genomes that were randomly selected from each organismal group. Abbreviations: ms, milliseconds; hr, hour; nt, nucleotides. The program was performed in a Dell PowerEdge R710 server with X5670 2 x 12-core CPUs (2.93 GHz) and 64GB ram.

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Table 8. CTT annotation results of *Pkinase* genes in 40 genomes.

Group	Species*	Prior Annotation	CTT Annotation		Total	New Finding (% of total)
			Pseudogenes	Protein Coding Genes		
Fungi	<i>Asp</i>	134	3	8	145	7.6
	<i>Bip</i>	116	1	3	120	3.3
	<i>Cer</i>	96	1	2	99	3
	<i>Cry</i>	91	1	2	94	3.2
	<i>Cyb</i>	91	3	1	95	4.2
	<i>Ent</i>	40	1	3	45	11.1
	<i>Esc</i>	90	1	2	93	3.2
	<i>Sug</i>	86	0	6	92	6.5
	<i>Tal</i>	186	2	2	190	2.1
	<i>Yar</i>	85	0	0	85	0
Metazoa	<i>Cae</i>	458	27	75	560	18.2
	<i>Cra</i>	239	7	44	290	17.6
	<i>Dro</i>	572	1	0	573	0.2
	<i>Hel</i>	337	1	7	345	2.3
	<i>Lep</i>	178	6	21	205	13.2
	<i>Nem</i>	244	8	31	283	13.8
	<i>Pri</i>	380	22	70	472	19.5
	<i>Sar</i>	167	1	27	195	14.4
	<i>Ste</i>	225	15	193	433	48
	<i>Tri</i>	187	6	4	197	5.1
Vertebrates	<i>Cal</i>	814	64	37	915	11
	<i>Cho</i>	243	216	299	758	67.9
	<i>Cyp</i>	680	29	123	832	18.3
	<i>Fic</i>	329	13	98	440	25.2
	<i>Hap</i>	764	13	75	852	10.3
	<i>Ict</i>	558	144	99	801	30.3
	<i>Mes</i>	507	40	90	637	20.4
	<i>Mus</i>	930	40	60	1030	9.7
	<i>Pel</i>	387	10	112	509	24
	<i>Tup</i>	287	165	254	706	59.3
Plants	<i>Amb</i>	460	107	38	607	24.2
	<i>Chl</i>	538	0	0	538	0
	<i>Gal</i>	77	0	0	77	0
	<i>Man</i>	1097	42	39	1178	6.9
	<i>Mus</i>	803	37	20	860	6.6
	<i>Ory</i>	1118	52	8	1178	5.1
	<i>Pru</i>	1105	38	12	1155	4.3
	<i>Sel</i>	784	144	168	1132	30.7
	<i>The</i>	927	10	5	942	1.6
	<i>Vig</i>	693	84	82	859	19.3

*Genomes were randomly selected from each organismal group available at the Ensembl database.

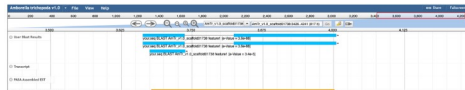
<https://doi.org/10.1371/journal.pone.0209468.t008>

projects. The recently-emerging third generation (or long-read) sequencing technologies would further this revolution by sequencing genomes in a much more affordable, accurate, and effective manner [23]. The contemporary genome sequencing technologies have already

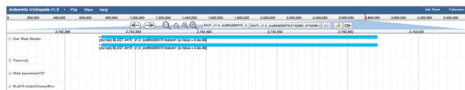
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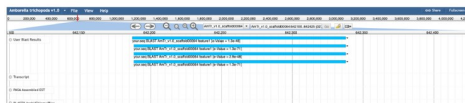
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AmbPK_N0007



AmbPK_N0010



AmbPK_N0013



AmbPK_N0016



AmbPK_N0019



AmbPK_N0022



AmbPK_N0025



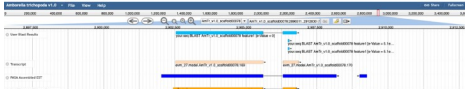
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AmbPK_N0031



AmbPK_N0034



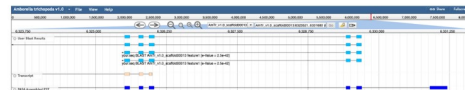
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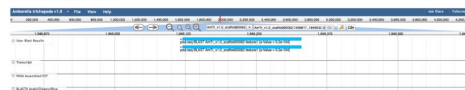
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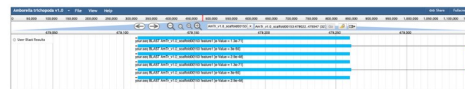
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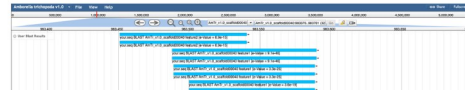
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AmbPK_N0017



AmbPK_N0020



AmbPK_N0023



AmbPK_N0026



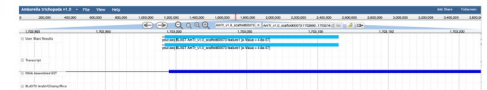
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AmbPK_N0032



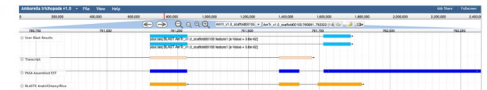
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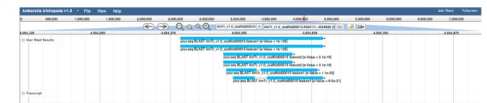
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AmbPK_N0009



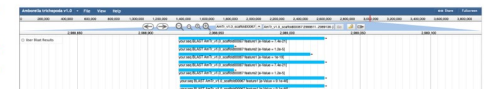
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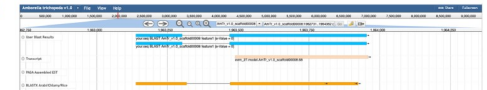
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AmbPK_N0018



AmbPK_N0021



AmbPK_N0024



AmbPK_N0027



AmbPK_N0030



AmbPK_N0033



CTT Annotation
Prior Annotation

PASA Assemble EST
BLASTx Ath/Osa/Cre

Fig 3. JBrowse images showing 34 truly new *Pkinase* loci identified by CTT in the *Amborella* genome. The transcript of each CTT-annotated *Pkinase* locus was used as query to BLASTn the *Amborella* genome. The best hit was visualized in JBrowse available on phytozome. Species abbreviation: *Ath*, *Arabidopsis thaliana*; *Osa*, *Oryza sativa*; *Cre*, *Chlamydomonas reinhardtii*.

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made it possible to sequence the genomes of every organism on the earth, such as the Earth BioGenome Project (EBP) initiated this year [31]. However, a sequencing machine only produces a lengthy genome book composed of 4 or 5 letters, i.e., A, T, C, G, or N. To understand the organization, evolution, functions, and interactions of numerous genomes, post genome sequencing data analysis is much more challenging than ever before. For example, many biological laboratories are not yet able to take full advantage of the power of whole-genome sequencing because of the lack of bioinformatic tools and/or skills.

While many state-of-the-art bioinformatic tools have been developed to annotate genomes, our studies have shown that superfamily members were often missing in various accomplished genome projects [5, 6] (Tables 3 and 4). Studying gene duplications of a family across genomes can shed light on the evolutionary and functional mechanisms of intracellular regulatory pathways [4, 6, 10]. Yet, a biased conclusion could be made if a comprehensive understanding of the members of a gene family in genomes is not achieved. For example, it was concluded that herbaceous annual plants have a larger *F-box* gene family than woody perennial plants from a study of few plant genomes [32]. However, our later CTT-based comprehensive re-annotation of the *F-box* genes in a broad range of plant genomes did not fully support this conclusion [5]. Our previous data and the data in this work clearly show that the size of the *F-box* superfamily is not significantly correlated with the growth behavior of a plant species, indicative of a genomic drift evolutionary mechanism ([5]; Table 3). The *F-box* family in herbaceous plants, papaya (*Carica papaya*), cucumber (*Cucumis sativus*), and Castor bean (*Ricinus communis*), is smaller than that in the woody plants *Populus trichocarpa* and *Medicago truncatula* (Table 3).

The effectiveness of CTT in annotating the *F-box* and the *BTB* superfamilies in both plants and non-plant organisms suggests a broad application of this software. Our further analysis of its performance in annotating the *Pkinase* family in 40 diverse genomes demonstrated its robust replicability and scalability (Tables 7 and 8 and Fig 3). The availability of this CTT package will benefit both evolutionary and functional genomic studies of superfamily genes in eukaryotic organisms.

Package availability

The full CTT and CTTdocker packages are available in two repositories hosted by GitHub at <https://github.com/hua-lab/ctt> and <https://github.com/hua-lab/cttdocker>, respectively.

Supporting information

S1 Table. List of 18 plant genomes and 5 non-plant genomes used in this work.
(XLSX)

S1 Fig. Stepwise description for retrieving a FASTA-formatted seed sequence file of a family.
(TIF)

S1 File. Protein sequences of the *F-box* genes identified by CTT in prior annotations of 18 plant species.
(FA)

- S2 File. Protein sequences of novel *F-box* genes reannotated by CTT in 18 plant species.**
(FA)
- S3 File. Transcript sequences of novel *F-box* genes reannotated by CTT in 18 plant species.**
(FA)
- S4 File. Genomic DNA sequences of novel *F-box* genes reannotated by CTT in 18 plant species.**
(FA)
- S5 File. Protein sequences of the *BTB* genes identified by CTT in prior annotations of 18 plant species.**
(FA)
- S6 File. Protein sequences of novel *BTB* genes reannotated by CTT in 18 plant species.**
(FA)
- S7 File. Transcript sequences of novel *BTB* genes reannotated by CTT in 18 plant species.**
(FA)
- S8 File. Genomic DNA sequences of novel *BTB* genes reannotated by CTT in 18 plant species.**
(FA)
- S9 File. Protein sequences of the *F-box* genes identified by CTT in prior annotations of five non-plant species.**
(FA)
- S10 File. Protein sequences of novel *F-box* genes reannotated by CTT in five non-plant species.**
(FA)
- S11 File. Transcript sequences of novel *F-box* genes reannotated by CTT in five non-plant species.**
(FA)
- S12 File. Genomic DNA sequences of novel *F-box* genes reannotated by CTT in five non-plant species.**
(FA)
- S13 File. Protein sequences of the *BTB* genes identified by CTT in prior annotations of five non-plant species.**
(FA)
- S14 File. Protein sequences of novel *BTB* genes reannotated by CTT in five non-plant species.**
(FA)
- S15 File. Transcript sequences of novel *BTB* genes reannotated by CTT in five non-plant species.**
(FA)
- S16 File. Genomic DNA sequences of novel *BTB* genes reannotated by CTT in five non-plant species.**
(FA)

S17 File. Protein sequences of novel *Pkinase* genes reannotated by CTT in *Amborella*.
(FA)

S18 File. Transcript sequences of novel *Pkinase* genes reannotated by CTT in *Amborella*.
(FA)

S19 File. Genomic DNA sequences of novel *Pkinase* genes reannotated by CTT in *Amborella*.
(FA)

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Investigation: Zhihua Hua.

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Software: Zhihua Hua.

Supervision: Zhihua Hua.

Validation: Zhihua Hua.

Visualization: Zhihua Hua.

Writing – original draft: Zhihua Hua.

Writing – review & editing: Zhihua Hua.

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