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TECHNICAL ADVANCE

PREPACT 2.0: Predicting C-to-U and U-to-C RNA Editing in Organelle Genome Sequences with Multiple References and Curated RNA Editing Annotation

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Abstract: RNA editing is vast in some genetic systems, with up to thousands of targeted C-to-U and U-to-C substitutions in mitochondria and chloroplasts of certain plants. Efficient prognoses of RNA editing in organelle genomes will help to reveal overlooked cases of editing. We present PREPACT 2.0 (http://www.prepact.de) with numerous enhancements of our previously developed Plant RNA Editing Prediction & Analysis Computer Tool. Reference organelle transcriptomes for editing prediction have been extended and reorganized to include 19 curated mitochondrial and 13 chloroplast genomes, now allowing to distinguish RNA editing sites from "pre-edited" sites. Queries may be run against multiple references and a new "commons" function identifies and highlights orthologous candidate editing sites congruently predicted by multiple references. Enhancements to the BLASTX mode in PREPACT 2.0 allow querying of complete novel organelle genomes within a few minutes, identifying protein genes and candidate RNA editing sites simultaneously without prior user analyses.

Keywords: pyrimidine substitutions, RNA editing prediction, plants, protists, mitochondrial DNA, chloroplast DNA, BLASTX

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Introduction

The term RNA editing has originally been coined for the surprising discovery that translatable reading frames are created by targeted uridine insertions in mitochondrial pre-mRNAs in the trypanosomes.[1] After this exciting original finding numerous other processes of RNA editing, adding twists to the known pathways of gene expression, have been discovered in many evolutionary disparate groups of life.[2] Like in trypanosomes, RNA editing is particularly abundant in the mitochondrial genetic systems of diverse eukaryotes. A prominent example along those lines is the slime mold *Physarum polycephalum,* which features hundreds of cytidine insertions as the dominant type of RNA editing.[3] Similar in abundance is RNA editing in certain land plants, for which the lycophytes, which represent the most ancient surviving branch of vascular plant evolution, reveal particular astonishing examples. More than 2,000 events of mitochondrial C-to-U RNA editing have been identified in the club moss *Selaginella moellendorffii.*[4] The sister lycophyte *Isoetes engelmannii* shows only slightly fewer editing events, but in this case they occur in both directions of pyrimidine exchange.[5] Such "reverse" U-to-C editing events are also particularly abundant in ferns and hornworts. A transcriptome analysis of the hornwort *Anthoceros formosae* chloroplast, for example, has revealed nearly 1,000 C-to-U and U-to-C conversions.[6]

Pyrimidine-exchange editing in organelles of photosynthetic organisms has previously been regarded as a gain in early land plant evolution, given that no events were reported for ancestral green algae. However, two events of C-to-U RNA editing have recently been discovered in the mitochondrial transcriptome of the protist *Naegleria gruberi*[7] and it is very likely that these RNA editing events are executed by the same nuclear factors as in plants, the so-called "DYW-type" pentatricopeptide repeat (PPR) proteins.[8] This discovery opens up the possibility that many more instances of RNA editing may remain undiscovered in organelle genome sequences.

The plant-type of organelle RNA editing can be reasonably predicted given that the pyrimidine transitions mostly serve to re-establish evolutionary conserved codons in genes of the core standard set of chloroplast and mitochondrial gene complements. Particular obvious examples are the occasional creation of methionine start (AUG) or stop (UAR, UGA) codons by C-to-U editing from threonine (ACG) and from glutamine (CAR) or arginine (CGA) codons, respectively. Alternative examples include the removal of in-frame stop codons by reverse U-to-C editing. Aside from start and stop codon conversions, however, pyrimidine-exchange RNA editing can result in 22 other possible switches in meaning between codons for alanine and valine, arginine and cysteine, histidine and tyrosine, threonine and isoleucine or methionine and in any direction among the leucine, phenylalanine, proline and serine YYN codons. Predicting, comparing and annotating these RNA editing events is cumbersome, notably when they appear in large numbers.

The prognosis of mRNA editing events based on comparison with orthologous genes from non-editing taxa or from confirmed cDNA data of taxa utilizing RNA editing becomes more reliable with an increasing number of diverse reference sequences. Here we present a significant extension in functionality of our WWW server PREPACT, the "Plant RNA Editing Prediction & Analysis Computer Tool".[9] The PREPACT reference database has been entirely reshaped and extended and now relies on coding sequences directly translated from revised GenBank entries after taking RNA editing events into account, which have been manually curated. The latter step is necessary given that no standard annotation for RNA editing sites has hitherto been introduced for primary database accessions. We introduce a novel feature "RNA_editing" for annotation and internal representation of editing sites in the PREPACT reference database, which may be adapted in the future for primary database entries. The PREPACT 2.0 update now allows users to freely select from multiple organellar reference genomes for prediction of editing in their query sequences. In its BLASTX mode, PREPACT 2.0 even allows for comfortable simultaneous prediction of coding regions and RNA editing sites for entire organelle genome sequence queries within a few minutes.

Material and Methods

PREPACT core functionality

Like the initial version,[9] the core of PREPACT 2.0 is written in the PHP hypertext preprocessor scripting language (http://php.net) using a MySQL (http://mysql.com) database backend and running on an Apache web server (http://httpd.apache.org). The graphical interface uses JavaScript elements (http://jquery.com, http://jqueryui.com, http://github.com/carhartl/jquery-cookie, http://github.com/flaviusmatis/flexibleArea.js, http://laktek.com/2008/10/27/really-simple-color-picker-in-jquery) for comfortable usage. Raster graphics output is generated by PHP scripts using ImageMagick (http://imagemagick.org). The PREPACT core functionality has been partly re-implemented, but still relies on the comparison of edited "reference" sequences aligned to unedited "test" sequence queries. Essentially, PREPACT suggests pyrimidine transitions (C-to-U, optionally U-to-C) in codons to result in improved amino acid matches in the reference sequences. Alignments for analysis can be provided directly by the user in FASTA format or can be generated by the BLASTX program,[10,11] provided by the NCBI (http://blast.ncbi.nlm.nih.gov). The latter is enhanced with new functionality in PREPACT2 allowing simultaneous identification of protein coding genes and RNA editing sites without prior user analyses in non-annotated sequences.

Revision of the reference structure and database generation for BLASTX mode

The protein reference sequence database was entirely revised for PREPACT 2.0 and now relies on translated coding sequences of full organelle genomes taking known editing events into account. Original GenBank accessions (Table 1) were retrieved from NCBI, split into their various elements such as header, feature list, qualifiers and sequence origin and saved into the internal MySQL database after format checking. The fully-functional hierarchical tree of feature and qualifier objects within sequence objects is retained. A complete set of associated methods for position calculation, information retrieval and manipulation within PREPACT 2.0 makes it possible to check for potentially erroneous feature locations, translational mismatches and CDS naming issues during subsequent revision where necessary. Flexible regular expression-based search and replace classes scanning the sequence entries for necessary modifications are stored on a per-accession basis to curate the available organellar genomes. When present, annotated editing sites were parsed from the different formats currently present in primary accessions (Fig. 1) into a new PREPACT-internal "RNA_editing" feature (Fig. 2). This process simultaneously checked for consistency and more common mistakes (eg, annotation of the wrong DNA strand), which were resolved automatically, and remaining annotation errors (such as obvious mislabeling of editing positions or misannotation of splicing) were corrected manually. Where no editing was annotated at all (eg, most angiosperm chloroplast [cp] DNA entries) RNA editing annotation was introduced manually into the same modifications database.

An auto-annotation module was created to process organelle genome entries without annotated RNA editing sites, but for which complete sets of cDNA are available; for example, the complex mitochondrial (mt) DNAs of lycophytes *Isoetes engelmannii* and *Selaginella moellendorffii* for which cDNAs exist as primary database entries, or *Vitis vinifera*, where editing information has been stored in REDIdb. The auto-annotation script aligns cDNA sequences to the corresponding CDS feature(s) in the organelle genome entries and automatically creates new "RNA_editing" features for these.

Out of a finally-curated organelle genome all CDS features are extracted, translated into proteins taking all corresponding RNA editing into account and stored as a BLAST database, which can be used for analysis. For genomes not being represented by a single accession (eg, the lycophytes mtDNA mentioned above), various accessions can be combined to a single BLAST database.

Generating BLAST results and "commons"

The nucleotide query sequence is run in all six reading frames by BLASTX against all selected reference databases individually. Results are optionally filtered for only the best local identity to the query. Accordingly, one BLAST hit can rule out another in the same reference database if it has a higher identity to the query in the part overlapping between these two. If at least one of the hits fulfils the limit given by the "filter threshold", the hit that is longer and has higher identity to the reference within a range of same query positions is kept. The remaining (protein) alignments are loaded as edited nucleotide sequences from the internal database and analyzed for RNA editing before the results are displayed in individual tabs. For the calculation of "commons", all predicted editing sites are grouped in rows by their position in the

Table 1. Reference organelle genome and transcriptome data as incorporated in PREPACT 2.0.

Genome/species	Accession	RNA editing events appl.	Reference on RNA editing
Mitochondrial genomes			
Arabidopsis thaliana (thale cress)	NC_001284	430	(38)
Beta vulgaris (sugarbeet)	NC_002511	359	(39)
Brassica napus (rapeseed)	NC_008285	423	(40)
Chaetosphaeridium globosum (green alga)	NC_004118	0	–
Chara vulgaris (green alga, "stonewort")	NC_005255	0	–
Citrullus lanatus (watermelon)	NC_014043	456	(41)
Cucurbita pepo (zucchini)	NC_014050	448	(41)
Isoetes engelmannii (lycophyte, "quillwort")	FJ010859+[a]	3738	(5)
Lotus japonicus (legume)	NC_016743	528	Accession only[e]
Marchantia polymorpha (liverwort)	NC_001660	0	–
Millettia pinnata (pongam oiltree)	NC_016742	510	Accession only[f]
Naegleria gruberi (heterolobosean protist)	NC_002573	2	(7)
Nicotiana tabacum (tobacco)	BA000042[c]	536	Accession only[g]
Oryza sativa (rice)	NC_011033	481	(42)
Physcomitrella patens (moss)	NC_007945	11	(12)
Reclinomonas americana (jakobid protist)	NC_001823	0	–
Selaginella moellendorffii (lycophyte, "spikemoss")	JF338143+[b]	2139	(4)
Silene latifolia (white campion)	NC_014487	286	(43)
Vitis vinifera (grapevine)	NC_012119	411	(44)
Chloroplast genomes			
Adiantum capillus veneris (fern)	AY178864[d]	343	(45)
Anthoceros formosae (hornwort)	NC_004543	970	(6)
Arabidopsis thaliana (thale cress)	NC_000932	41	(46)
Atropa belladonna (deadly nightshade)	NC_004561	40	(37)
Chaetosphaeridium globosum (alga)	NC_004115	0	–
Chara vulgaris (alga, "stonewort")	NC_008097	0	–
Hevea brasiliensis (rubber tree)	NC_015308	56	(47)
Marchantia polymorpha (liverwort)	NC_001319	0	–
Nicotiana tabacum (tobacco)	NC_001879	43	(37;48;49)
Oryza sativa (rice)	NC_001320	35	(50;51)
Physcomitrella patens (moss)	NC_005087	1	(22;23)
Pisum sativum (pea)	NC_014057	26	(50)
Zea mays (maize)	NC_016666	32	(52;53)

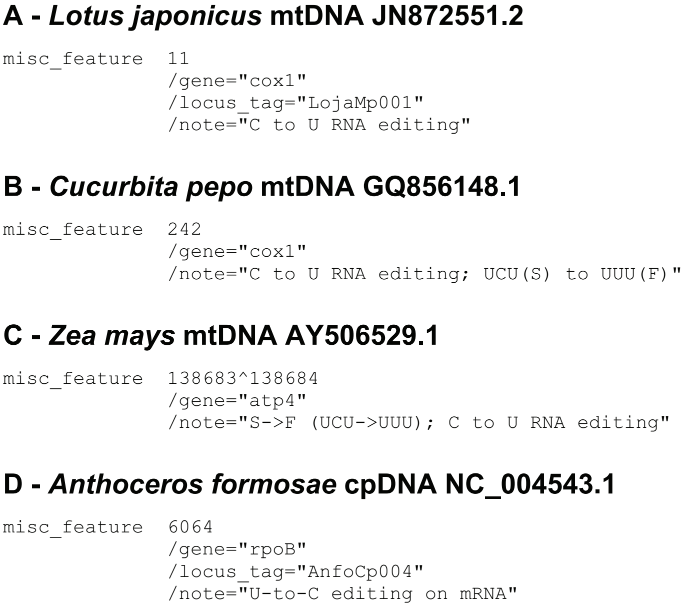
Notes: Annotation of RNA editing relied mostly on the reference genome accessions (NC_x) revised by the NCBI. Exceptions are the complex network-like recombining mtDNAs of the lycophytes [a]*Isoetes engelmannii* (FJ010859, FJ176330, FJ390841, FJ536259 and FJ628360) and [b]*Selaginella moellendorffii* (JF338143, JF338144, JF338145, JF338146 and JF338147) which at present rely on five separate original sequence accessions each and the [c]*Nicotiana tabacum* mtDNA and the [d]*Adiantum capillus-veneris* cpDNA given that RNA editing information is absent in the corresponding organelle genome entries NC_006581 and NC_004766, respectively. No formal publications are presently available on the mitochondrial editomes of [e]*Lotus japonicus*, [f]*Millettia pinnata* and [g]*Nicotiana tabacum*. Dashes (–) indicate that RNA editing is hitherto not identified and assumed to be entirely absent in algae, marchantiid liverworts and protists like *Reclinomonas americana*. Note that due to gene duplications (eg, due to IRs in cpDNAs or redundancy in the recombining mtDNAs) the numbers of "applied RNA editings" in the reference sequences may be significantly larger than the number of actual C/U and U/C differences as reported in the literature.

query sequence and labeled according to the chosen numbering scheme. If no editing is present in a reference database, it is checked whether there is actually no alignment covering this single position ('-') or if the alignment does not predict an editing event ('0'). If an editing prediction matches a position in the reference database that has been edited for generation of the protein BLAST database, these sites are highlighted in red.

Results

RNA editing site annotation

The bioinformatic processing of RNA editing is currently impeded by lack of a standard for annotation of editing sites in the primary database sequence accessions. When RNA editing sites are indicated at all, their annotation so far relies on the multi-purpose "miscellaneous" feature ("misc_feature") and variable free-text comments in "note" qualifiers in sequence



A - *Lotus japonicus* mtDNA JN872551.2

```
misc_feature   11
               /gene="cox1"
               /locus_tag="LojaMp001"
               /note="C to U RNA editing"
```

B - *Cucurbita pepo* mtDNA GQ856148.1

```
misc_feature   242
               /gene="cox1"
               /note="C to U RNA editing; UCU(S) to UUU(F)"
```

C - *Zea mays* mtDNA AY506529.1

```
misc_feature   138683^138684
               /gene="atp4"
               /note="S->F (UCU->UUU); C to U RNA editing"
```

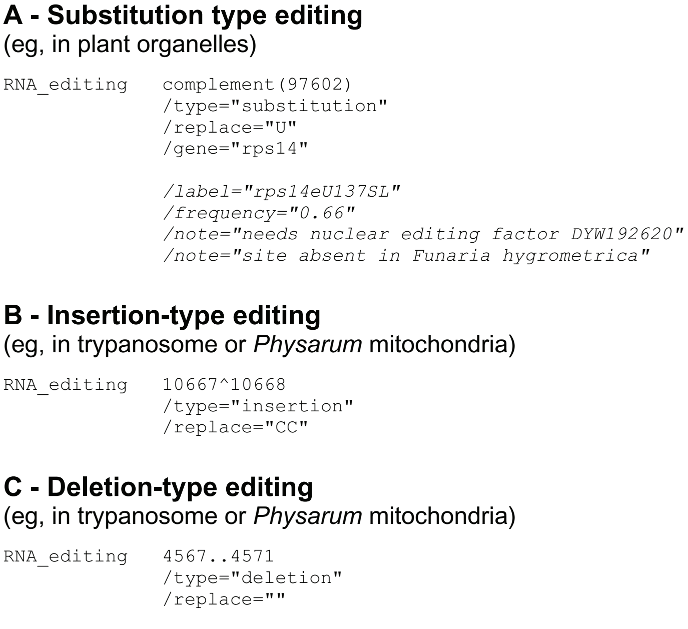
D - *Anthoceros formosae* cpDNA NC_004543.1

```
misc_feature   6064
               /gene="rpoB"
               /locus_tag="AnfoCp004"
               /note="U-to-C editing on mRNA"
```

Figure 1. Four examples (**A**–**D**) of different free-text annotations of RNA editing in primary database plant organelle genome entries.
If indicated at all, RNA editing sites are mostly annotated using the miscellaneous feature ("misc_feature") with additional information given in various ways in the "note" qualifier, which necessitates a parsing into a common standard for bioinformatic processing.

accessions. Some examples in current GenBank entries are shown in Figure 1. Occasionally, such annotations are strangely ambiguous and particularly difficult for bioinformatic processing, for example, when the caret (^), normally used as the standard "in between" location indicator for database entries, is used to designate substitutional editing of individual nucleotides (Fig. 1C). Moreover, it frequently remains ambiguous whether protein translations provided by the submitter are based on DNA or on edited RNA sequences. Alternatively, in many cases, these translations are entirely hypothetical or a mixture of the two, which only took into account the necessary removals of stop codons and the introductions of start or stop codons to create intact open reading frames.

We here suggest a dedicated novel sequence feature "RNA_editing" that is compatible with the grammar of the GenBank flat file format and might hence be considered for future adaptations by the primary sequence databases (Fig. 2). For the purposes of PREPACT 2.0, the differently-formatted information on editing sites and de novo annotation of RNA editing sites in organelle genome sequences (Table 1) were parsed into this new standard. We suggest two mandatory qualifiers "type" and "replace" for the new RNA_editing feature to clearly describe the respective type of editing taking place and the resulting sequence changes introduced in the respective mature RNA (Fig. 2). The obligatory "type" qualifier has one of three alternative qualities: "substitution", "insertion" or "deletion". The second mandatory qualifier "replace" indicates the string of RNA nucleotides that are newly created by the editing event. The "replace" value will indicate the nucleotides newly introduced by an editing event or will be empty ("") for the deletion-type of editing (Fig. 2C). The locations of the RNA_editing feature are given following database standards as individual positions or nucleotide ranges and are indicated as "complement()" when the affected transcript runs opposite to the sequence orientation of the sequence accession (Fig. 2A). The "in between" annotation using the caret (^) will only be used for the insertion-type of editing (Fig. 2B). As the RNA_editing annotations are not directly associated with a reading frame, the use of a "gene" qualifier to assign a certain editing event to a CDS with the same



A - Substitution type editing
(eg, in plant organelles)

```
RNA_editing    complement(97602)
               /type="substitution"
               /replace="U"
               /gene="rps14"

               /label="rps14eU137SL"
               /frequency="0.66"
               /note="needs nuclear editing factor DYW192620"
               /note="site absent in Funaria hygrometrica"
```

B - Insertion-type editing
(eg, in trypanosome or *Physarum* mitochondria)

```
RNA_editing    10667^10668
               /type="insertion"
               /replace="CC"
```

C - Deletion-type editing
(eg, in trypanosome or *Physarum* mitochondria)

```
RNA_editing    4567..4571
               /type="deletion"
               /replace=""
```

Figure 2. Suggested new feature "RNA_editing" for annotation of editing events. The mandatory "type" qualifier distinguishes the different known cases of RNA editing by (**A**) substitution, (**B**) insertion or (**C**) deletion. A second obligatory qualifier "replace" gives the string of nucleotides to be replaced at the sequence location indicated for the RNA_editing feature. Its value is necessarily empty only for the deletion type of editing (**C**) but gives individual or stretches of nucleotides for the other two types of editing (**A** and **B**). As for the respective affected genetic locus, the "complement" function is to be used (**A**) where necessary to correctly refer to the DNA strand that is co-linear with the respective transcript. The "gene" qualifier is mandatory in case of overlapping CDS features, otherwise its use is encouraged for clarification.
Additional qualifiers may be optionally used (A, examples shown in italics) to introduce RNA editing site labels ("label"), indicate partial editing ("frequency") or to convey additional biological information ("note").

name is encouraged or even mandatory when there are overlapping reading frames present.

Further to the two mandatory qualifiers "type" and "replace" we suggest optional additional qualifiers to convey useful supplementary information (Fig. 2A). Several RNA editing events, for example in plant mitochondria, are realized only partially in a steady-state transcript population. We propose use of the already available qualifier "frequency" to characterize such partial editing events with a decimal value between 0 and 1 to reflect the fraction of editing revealed in a cDNA population. The qualifier "label" is suggested to indicate an informative label for the respective editing site. We have recently suggested such a nomenclature for substitutional editing events,[9,12] which we also use to clearly identify editing sites in comparative studies and for analysis and output in PREPACT. Briefly, this label for RNA editing events consists of the respective gene name, followed by an 'e', the RNA nucleotide identity after editing (ie, U or C in plant organelle editing), the affected nucleotide position in the mature reading frame counting from position 1 of the start codon and the resulting amino acid change (see Fig. 2A).

Restructuring and extending the PREPACT 2.0 reference database

For the automatic ab initio prediction of coding sequences and RNA editing sites in a nucleotide query, PREPACT originally relied on BLASTX hits in manually-assembled collections of reference protein sequences.[9] The reference data set has been completely reorganized and largely extended for PREPACT 2.0. Coding sequences (CDS) are now dynamically extracted from reference organelle genome entries with RNA editing sites automatically taken into account for protein translations, where applicable. To this end, RNA editing as currently documented in its variable ways (Fig. 1) or extracted from the literature or occasionally from other sources[13] was parsed into the standard described above (Fig. 2). Numerous obvious errors in gene annotations (eg, CDS extensions, splice sites, strand complementarity), which occur to variable degrees in most database accessions, were corrected for internal representation in PREPACT.

Organelle gene naming issues

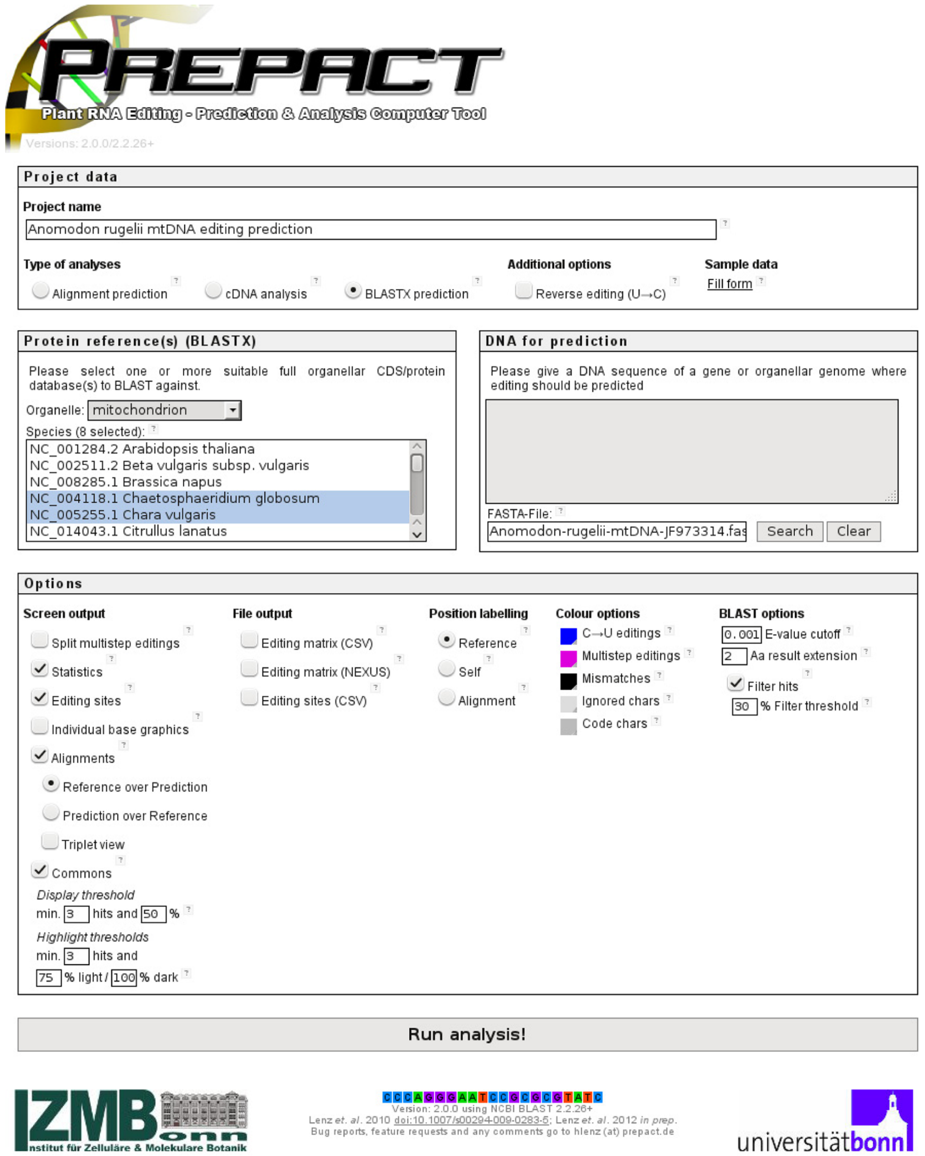
Our manual curation of reference genomes also included de novo annotation of previously non-annotated coding sequences (eg, *chlN* in the *Marchantia polymorpha* cpDNA) and the replacement of non-informative old gene labels with consensus names (eg, "*orfB*" with "*atp*8" or "*orf25*" with "*atp*4"). In other cases we used internal synonymising for different names that are currently in parallel use for orthologous genes, eg, *mttB* and *tatC* for the twin-arginine translocase subunit or *ccb*, *yej* and *ccm* for the suite of cytochrome c maturation genes in mitochondria to appropriately identify orthologous editing sites, as we will exemplarily demonstrate below. Such synonymising can also be advantageous for entirely biological reasons. A prominent example is the mitochondrial *ccmF* locus which in the course of plant evolution got disrupted differently into separate reading frames that were labeled in numerous different ways.[14]

The reference data

Altogether, PREPACT 2.0 currently offers 19 mitochondrial and 13 chloroplast genome sequences as references for comparison. We included several exhaustively analyzed transcriptomes and organelle genomes supposedly devoid of RNA editing as summarized in Table 1. The latter group also includes the mitochondrial genome of the jakobid protist *Reclinomonas americana,* which has a particularly rich gene complement.[15] To avoid misleading results, however, we did not include those organelle genomes where transcriptome analyses are clearly only partial so far, such as for the chloroplast genome of *Pinus thunbergii*[16] or the mitochondrial genomes of *Cycas taitungensis*[17] and *Phoenix dactylifera*.[18] Likewise, we did not include the maize (*Zea mays)* chondrome with ca. 100 RNA editing events annotated in database entry NC_007982 since it is likely that at least 300 additional ones were missing according to our estimate.

Querying PREPACT 2.0 in BLASTX mode: a complete mtDNA example

The "commons" function is a new core feature of PREPACT 2.0. It allows scanning for candidate RNA editing sites against multiple references simultaneously and prepares a comparative output for user evaluation. In describing the new features of PREPACT 2.0 we will here focus on the major enhancements for its BLASTX mode (Fig. 3), which essentially allows analysis of organelle sequences, largely without prior analyses by the user. In fact, the PREPACT 2.0 program code and server setup has been adapted to even allow querying of entire organelle genome sequences with hundreds of kilobases against multiple reference targets within minutes. A status window reflects progress of the analysis (Supplemental Fig. 1). Very long query sequences and simultaneous selection of very many references and a particularly detailed output including full alignments may require computation times of several minutes and slow down local browser display



Project data

Project name
Anomodon rugelii mtDNA editing prediction

Type of analyses
○ Alignment prediction ○ cDNA analysis ● BLASTX prediction

Additional options
□ Reverse editing (U→C)

Sample data
Fill form

Protein reference(s) (BLASTX)

Please select one or more suitable full organellar CDS/protein database(s) to BLAST against.

Organelle: mitochondrion

Species (8 selected):
NC_001284.2 Arabidopsis thaliana
NC_002511.2 Beta vulgaris subsp. vulgaris
NC_008285.1 Brassica napus
NC_004118.1 Chaetosphaeridium globosum
NC_005255.1 Chara vulgaris
NC_014043.1 Citrullus lanatus

DNA for prediction

Please give a DNA sequence of a gene or organellar genome where editing should be predicted

FASTA-File: Anomodon-rugelii-mtDNA-JF973314.fas

Options

Screen output
□ Split multistep editings
☑ Statistics
☑ Editing sites
□ Individual base graphics
☑ Alignments
● Reference over Prediction
○ Prediction over Reference
□ Triplet view
☑ Commons
Display threshold
min. 3 hits and 50 %
Highlight thresholds
min. 3 hits and
75 % light / 100 % dark

File output
□ Editing matrix (CSV)
□ Editing matrix (NEXUS)
□ Editing sites (CSV)

Position labelling
● Reference
○ Self
○ Alignment

Colour options
🟦 C→U editings
🟪 Multistep editings
■ Mismatches
▨ Ignored chars
▨ Code chars

BLAST options
0.001 E-value cutoff
2 Aa result extension
☑ Filter hits
30 % Filter threshold

Version: 2.0.0 using NCBI BLAST 2.2.26+
Lenz *et. al*. 2010 doi:10.1007/s00294-009-0283-5; Lenz *et. al*. 2012 *in prep*.
Bug reports, feature requests and any comments go to hlenz (at) prepact.de

Figure 3. The extended PREPACT 2.0 user interface.
Exemplarily shown is a query in BLASTX mode to scan for C-to-U editing candidate sites in the complete mtDNA of the moss *Anomodon rugelii* (104,239 bp, database accession JF973314). Multiple reference databases may be selected with the usual Ctrl-/Shift functionality. Selecting more than one reference automatically opens the "commons" options (bottom left) allowing to set thresholds selecting for editing sites congruently predicted from given fractions of the reference set for listing (preset to 50%) and two levels of highlighting (preset to 75% and 100% reference support) by light and dark shading. Additional integer thresholds may be set for display (preset ≥3) and highlighting (preset to ≥3) of hits present in only few or individual taxa. The labeling of editing sites may be chosen according to the proposed standard nomenclature for references (preset) or alternatively for query or alignment sequence positions when appropriate. BLAST search sensitivity may be adjusted (bottom right) and a newly introduced filter function allows for exclusion of secondary hits in protein paralogs according to length and similarity for overlapping hits (with "0%" excluding and "100%" allowing all overlaps). A number of codons (preset = 2) may be given by which alignments may be extended to possibly identify editing of start or stop codons when these are not included in BLASTX hits against the reference(s).

because of high memory consumption or even exceed server resource limits (approximately with queries > 500 kbp and >10 references). The recently determined complete mtDNA (104,239 bp) of the moss *Anomodon rugelii*[19] is used here to exemplarily demonstrate the extended PREPACT 2.0 functionality, its query interface (Fig. 3) and the output (Figs. 4 and 5) and to discuss results and important considerations.

Improving BLASTX hit analyses: Filtering paralogs

A major earlier problem for BLASTX-based gene identification has been multiple hits due to similarities among the paralogous core subunits of chloroplast thylakoid membrane subunits (*atpA-atpB, ndhB-ndhD-ndhF, psbA-psbD, psbB-psbC*) and among mitochondrial respiratory chain complex I subunits (*nad*5-*nad*2-*nad*4). This issue has been successfully solved with a new filtering function that excludes secondary hits depending on extension and similarity of the overlap relative to a better primary hit according to an adjustable threshold preset to 30% (Fig. 3). Decreasing the filtering threshold may rarely be necessary to suppress identification of paralogs identified across large phylogenetic distances ('0%' would exclude all weaker hits conflicting with the top-scoring hit). Conversely, increasing its value ('100%' would suppress secondary hits only in case of complete overlapping with the top-scoring hit) promotes display of additional secondary hits, which may be interesting in exceptional cases (such as the

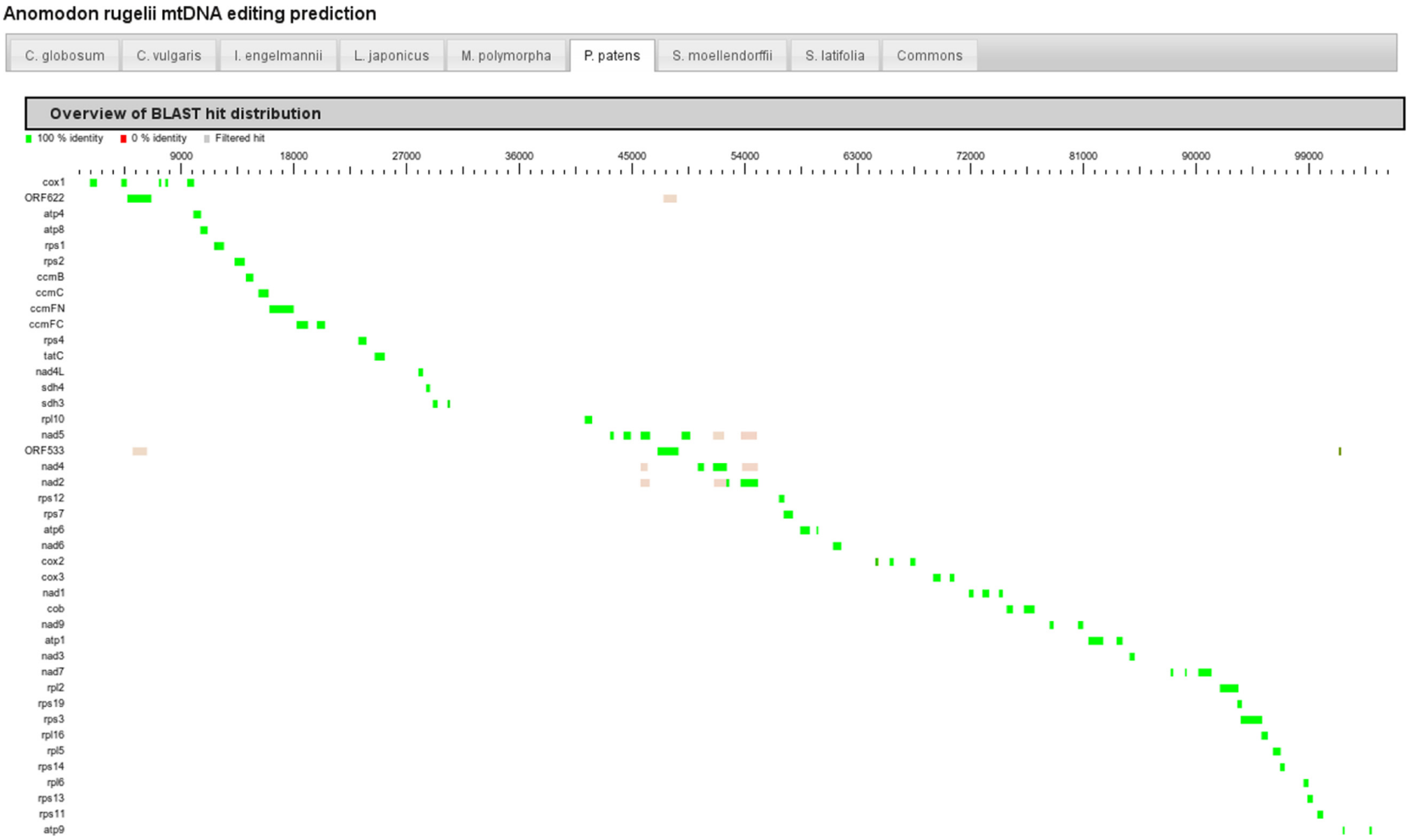
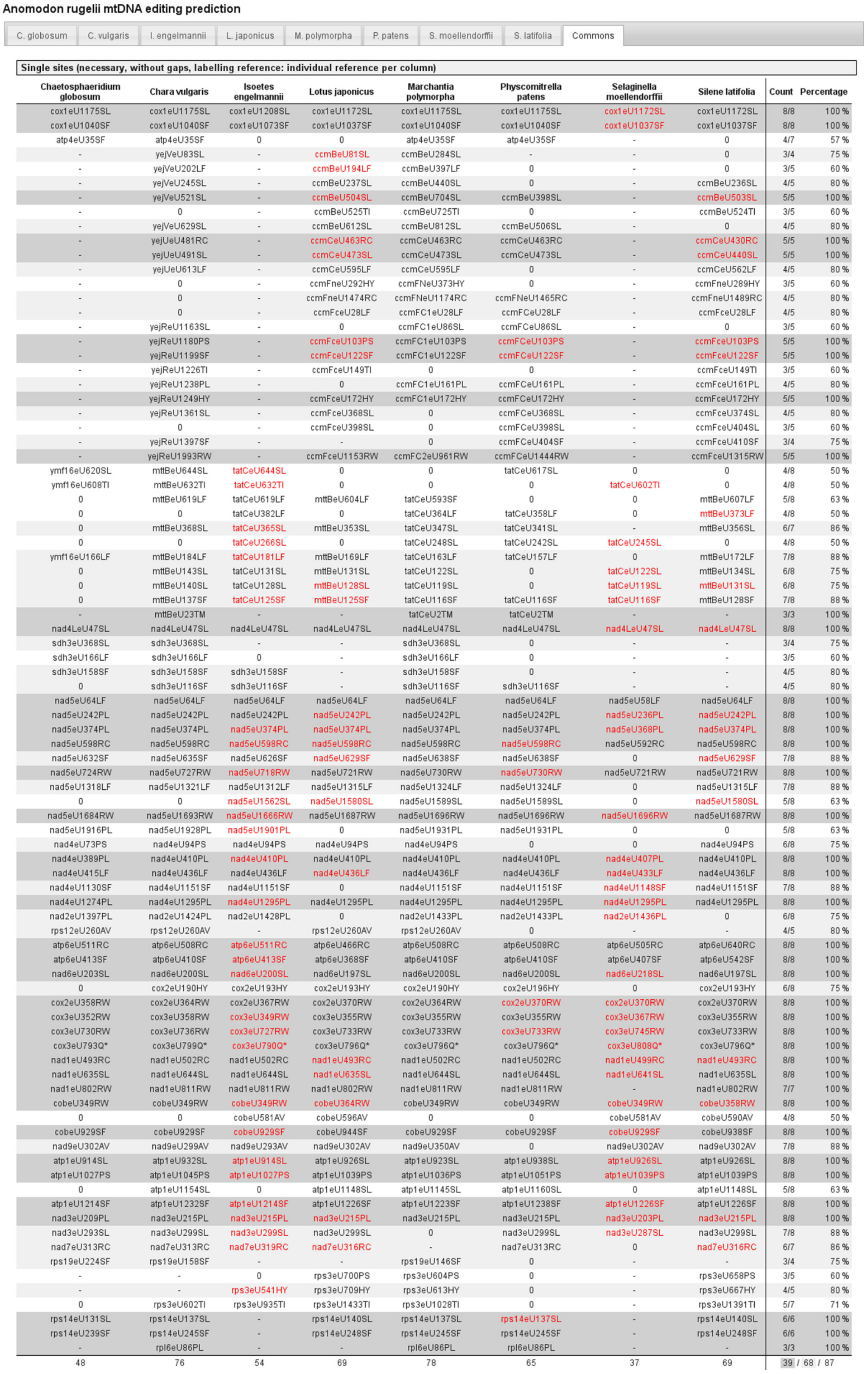


Figure 4. PREPACT output in BLASTX mode: overview on hits in individual references.
The output is organized into tabs with results for the individual references that can be selected for display in the WWW browser. BLAST hits are displayed graphically on top of each reference tab, as exemplarily shown for the *Physcomitrella patens* reference. A very high mitochondrial gene synteny between the two moss mtDNAs is obvious for the *Anomodon rugelii* mtDNA query. Disrupted homologies along the horizontal lines suggest the presence of introns. Individual hits give short information on mouse-over and are directly linked to the respective analysis details included further down in the output as optionally selected in the query form (see Fig. 3), eg, of sequence alignments (see Fig. 6) or of statistics for sequences and BLAST hits, lists of editing sites and individual editing site graphics (not shown). BLAST hits are colored with decreasing similarities from bright green to dark red. The filtering of overlapping BLAST hits for exclusion in editing predictions is visualized by secondary hits in faded coloring, as here becomes obvious for the distantly related complex I paralogs *nad2, nad4* and *nad5* and the intron maturases orf533 and orf622.



Anomodon rugelii mtDNA editing prediction

Single sites (necessary, without gaps, labelling reference: individual reference per column)

Chaetosphaeridium globosum	Chara vulgaris	Isoetes engelmannii	Lotus japonicus	Marchantia polymorpha	Physcomitrella patens	Selaginella moellendorffii	Silene latifolia	Count	Percentage
cox1eU1175SL	cox1eU1175SL	cox1eU1208SL	cox1eU1172SL	cox1eU1175SL	cox1eU1175SL	cox1eU1172SL	cox1eU1172SL	8/8	100 %
cox1eU1040SF	cox1eU1040SF	cox1eU1073SF	cox1eU1037SF	cox1eU1040SF	cox1eU1040SF	cox1eU1037SF	cox1eU1037SF	8/8	100 %
atp4eU35SF	atp4eU35SF	0	0	atp4eU35SF	atp4eU35SF	-	0	4/7	57 %
-	yejVeU83SL	-	ccmBeU81SL	ccmBeU284SL	-	-	0	3/4	75 %
-	yejVeU202LF	-	ccmBeU194LF	ccmBeU397LF	0	-	0	3/5	60 %
-	yejVeU245SL	-	ccmBeU237SL	ccmBeU440SL	0	-	ccmBeU236SL	4/5	80 %
-	yejVeU521SL	-	ccmBeU504SL	ccmBeU704SL	ccmBeU398SL	-	ccmBeU503SL	5/5	100 %
-	0	-	ccmBeU525TI	ccmBeU725TI	0	-	ccmBeU524TI	3/5	60 %
-	yejVeU629SL	-	ccmBeU612SL	ccmBeU812SL	ccmBeU506SL	-	0	4/5	80 %
-	yejUeU481RC	-	ccmCeU463RC	ccmCeU463RC	ccmCeU463RC	-	ccmCeU430RC	5/5	100 %
-	yejUeU491SL	-	ccmCeU473SL	ccmCeU473SL	ccmCeU473SL	-	ccmCeU440SL	5/5	100 %
-	yejUeU613LF	-	ccmCeU595LF	ccmCeU595LF	0	-	ccmCeU562LF	4/5	80 %
-	0	-	ccmFneU292HY	ccmFNeU373HY	0	-	ccmFneU289HY	3/5	60 %
-	0	-	ccmFneU1474RC	ccmFNeU1174RC	ccmFNeU1465RC	-	ccmFneU1489RC	4/5	80 %
-	0	-	ccmFceU28LF	ccmFC1eU28LF	ccmFCeU28LF	-	ccmFceU28LF	4/5	80 %
-	yejReU1163SL	-	0	ccmFC1eU86SL	ccmFCeU86SL	-	0	3/5	60 %
-	yejReU1180PS	-	ccmFceU103PS	ccmFC1eU103PS	ccmFCeU103PS	-	ccmFceU103PS	5/5	100 %
-	yejReU1199SF	-	ccmFceU122SF	ccmFC1eU122SF	ccmFCeU122SF	-	ccmFceU122SF	5/5	100 %
-	yejReU1226TI	-	ccmFceU149TI	0	0	-	ccmFceU149TI	3/5	60 %
-	yejReU1238PL	-	0	ccmFC1eU161PL	ccmFCeU161PL	-	ccmFceU161PL	4/5	80 %
-	yejReU1249HY	-	ccmFceU172HY	ccmFC1eU172HY	ccmFCeU172HY	-	ccmFceU172HY	5/5	100 %
-	yejReU1361SL	-	ccmFceU368SL	0	ccmFCeU368SL	-	ccmFceU374SL	4/5	80 %
-	0	-	ccmFceU398SL	0	ccmFCeU398SL	-	ccmFceU404SL	3/5	60 %
-	yejReU1397SF	-	-	0	ccmFCeU404SF	-	ccmFceU410SF	3/4	75 %
-	yejReU1993RW	-	ccmFceU1153RW	ccmFC2eU961RW	ccmFCeU1444RW	-	ccmFceU1315RW	5/5	100 %
ymf16eU620SL	mttBeU644SL	tatCeU644SL	0	0	tatCeU617SL	0	0	4/8	50 %
ymf16eU608TI	mttBeU632TI	tatCeU632TI	0	0	0	tatCeU602TI	0	4/8	50 %
0	mttBeU619LF	tatCeU619LF	mttBeU604LF	tatCeU593SF	0	0	mttBeU607LF	5/8	63 %
0	0	tatCeU382LF	0	tatCeU364LF	tatCeU358LF	0	mttBeU373LF	4/8	50 %
0	mttBeU368SL	tatCeU365SL	mttBeU353SL	tatCeU347SL	tatCeU341SL	-	mttBeU356SL	6/7	86 %
0	0	tatCeU266SL	0	tatCeU248SL	tatCeU242SL	tatCeU245SL	0	4/8	50 %
ymf16eU166LF	mttBeU184LF	tatCeU181LF	mttBeU169LF	tatCeU163LF	tatCeU157LF	0	mttBeU172LF	7/8	88 %
0	mttBeU143SL	tatCeU131SL	mttBeU131SL	tatCeU122SL	0	tatCeU122SL	mttBeU134SL	6/8	75 %
0	mttBeU140SL	tatCeU128SL	mttBeU128SL	tatCeU119SL	0	tatCeU119SL	mttBeU131SL	6/8	75 %
0	mttBeU137SF	tatCeU125SF	mttBeU125SF	tatCeU116SF	tatCeU116SF	tatCeU116SF	mttBeU128SF	7/8	88 %
-	mttBeU23TM	-	-	tatCeU2TM	tatCeU2TM	-	-	3/3	100 %
nad4LeU47SL	nad4LeU47SL	nad4LeU47SL	nad4LeU47SL	nad4LeU47SL	nad4LeU47SL	nad4LeU47SL	nad4LeU47SL	8/8	100 %
sdh3eU368SL	sdh3eU368SL	-	-	sdh3eU368SL	0	-	-	3/4	75 %
sdh3eU166LF	sdh3eU166LF	0	-	sdh3eU166LF	0	-	-	3/5	60 %
sdh3eU158SF	sdh3eU158SF	sdh3eU158SF	-	sdh3eU158SF	0	-	-	4/5	80 %
0	sdh3eU116SF	sdh3eU116SF	-	sdh3eU116SF	sdh3eU116SF	-	-	4/5	80 %
nad5eU64LF	nad5eU64LF	nad5eU64LF	nad5eU64LF	nad5eU64LF	nad5eU64LF	nad5eU58LF	nad5eU64LF	8/8	100 %
nad5eU242PL	nad5eU242PL	nad5eU242PL	nad5eU242PL	nad5eU242PL	nad5eU242PL	nad5eU236PL	nad5eU242PL	8/8	100 %
nad5eU374PL	nad5eU374PL	nad5eU374PL	nad5eU374PL	nad5eU374PL	nad5eU374PL	nad5eU368PL	nad5eU374PL	8/8	100 %
nad5eU598RC	nad5eU598RC	nad5eU598RC	nad5eU598RC	nad5eU598RC	nad5eU598RC	nad5eU592RC	nad5eU598RC	8/8	100 %
nad5eU632SF	nad5eU635SF	nad5eU626SF	nad5eU629SF	nad5eU638SF	nad5eU638SF	0	nad5eU629SF	7/8	88 %
nad5eU724RW	nad5eU727RW	nad5eU718RW	nad5eU721RW	nad5eU730RW	nad5eU730RW	nad5eU721RW	nad5eU721RW	8/8	100 %
nad5eU1318LF	nad5eU1321LF	nad5eU1312LF	nad5eU1315LF	nad5eU1324LF	nad5eU1324LF	0	nad5eU1315LF	7/8	88 %
0	0	nad5eU1562SL	nad5eU1580SL	nad5eU1589SL	nad5eU1589SL	0	nad5eU1580SL	5/8	63 %
nad5eU1684RW	nad5eU1693RW	nad5eU1666RW	nad5eU1687RW	nad5eU1696RW	nad5eU1696RW	nad5eU1696RW	nad5eU1687RW	8/8	100 %
nad5eU1916PL	nad5eU1928PL	nad5eU1901PL	0	nad5eU1931PL	nad5eU1931PL	0	0	5/8	63 %
nad4eU73PS	nad4eU94PS	nad4eU94PS	nad4eU94PS	nad4eU94PS	0	0	nad4eU94PS	6/8	75 %
nad4eU389PL	nad4eU410PL	nad4eU410PL	nad4eU410PL	nad4eU410PL	nad4eU410PL	nad4eU407PL	nad4eU410PL	8/8	100 %
nad4eU415LF	nad4eU436LF	nad4eU436LF	nad4eU436LF	nad4eU436LF	nad4eU436LF	nad4eU433LF	nad4eU436LF	8/8	100 %
nad4eU1130SF	nad4eU1151SF	nad4eU1151SF	0	nad4eU1151SF	nad4eU1151SF	nad4eU1148SF	nad4eU1151SF	7/8	88 %
nad4eU1274PL	nad4eU1295PL	nad4eU1295PL	nad4eU1295PL	nad4eU1295PL	nad4eU1295PL	nad4eU1295PL	nad4eU1295PL	8/8	100 %
nad2eU1397PL	nad2eU1424PL	nad2eU1428PL	0	nad2eU1433PL	nad2eU1433PL	nad2eU1436PL	0	6/8	75 %
rps12eU260AV	rps12eU260AV	-	rps12eU260AV	rps12eU260AV	0	-	-	4/5	80 %
atp6eU511RC	atp6eU508RC	atp6eU511RC	atp6eU466RC	atp6eU508RC	atp6eU508RC	atp6eU505RC	atp6eU640RC	8/8	100 %
atp6eU413SF	atp6eU410SF	atp6eU413SF	atp6eU368SF	atp6eU410SF	atp6eU410SF	atp6eU407SF	atp6eU542SF	8/8	100 %
nad6eU203SL	nad6eU200SL	nad6eU200SL	nad6eU197SL	nad6eU200SL	nad6eU200SL	nad6eU218SL	nad6eU197SL	8/8	100 %
0	cox2eU190HY	cox2eU193HY	cox2eU193HY	cox2eU190HY	cox2eU196HY	0	cox2eU193HY	6/8	75 %
cox2eU358RW	cox2eU364RW	cox2eU367RW	cox2eU370RW	cox2eU364RW	cox2eU370RW	cox2eU370RW	cox2eU370RW	8/8	100 %
cox3eU352RW	cox3eU358RW	cox3eU349RW	cox3eU355RW	cox3eU355RW	cox3eU355RW	cox3eU367RW	cox3eU355RW	8/8	100 %
cox3eU730RW	cox3eU736RW	cox3eU727RW	cox3eU733RW	cox3eU733RW	cox3eU733RW	cox3eU745RW	cox3eU733RW	8/8	100 %
cox3eU793Q*	cox3eU799Q*	cox3eU790Q*	cox3eU796Q*	cox3eU796Q*	cox3eU796Q*	cox3eU808Q*	cox3eU796Q*	8/8	100 %
nad1eU493RC	nad1eU502RC	nad1eU502RC	nad1eU493RC	nad1eU502RC	nad1eU502RC	nad1eU499RC	nad1eU493RC	8/8	100 %
nad1eU635SL	nad1eU644SL	nad1eU644SL	nad1eU635SL	nad1eU644SL	nad1eU644SL	nad1eU641SL	nad1eU635SL	8/8	100 %
nad1eU802RW	nad1eU811RW	nad1eU811RW	nad1eU802RW	nad1eU811RW	nad1eU811RW	-	nad1eU802RW	7/7	100 %
cobeU349RW	cobeU349RW	cobeU349RW	cobeU364RW	cobeU349RW	cobeU349RW	cobeU349RW	cobeU358RW	8/8	100 %
0	0	cobeU581AV	cobeU596AV	0	0	cobeU581AV	cobeU590AV	4/8	50 %
cobeU929SF	cobeU929SF	cobeU929SF	cobeU944SF	cobeU929SF	cobeU929SF	cobeU929SF	cobeU938SF	8/8	100 %
nad9eU302AV	nad9eU299AV	nad9eU293AV	nad9eU302AV	nad9eU350AV	0	nad9eU302AV	nad9eU302AV	7/8	88 %
atp1eU914SL	atp1eU932SL	atp1eU914SL	atp1eU926SL	atp1eU923SL	atp1eU938SL	atp1eU926SL	atp1eU926SL	8/8	100 %
atp1eU1027PS	atp1eU1045PS	atp1eU1027PS	atp1eU1039PS	atp1eU1036PS	atp1eU1051PS	atp1eU1039PS	atp1eU1039PS	8/8	100 %
0	atp1eU1154SL	0	atp1eU1148SL	atp1eU1145SL	atp1eU1160SL	0	atp1eU1148SL	5/8	63 %
atp1eU1214SF	atp1eU1232SF	atp1eU1214SF	atp1eU1226SF	atp1eU1223SF	atp1eU1238SF	atp1eU1226SF	atp1eU1226SF	8/8	100 %
nad3eU209PL	nad3eU215PL	nad3eU215PL	nad3eU215PL	nad3eU215PL	nad3eU215PL	nad3eU203PL	nad3eU215PL	8/8	100 %
nad3eU293SL	nad3eU299SL	nad3eU299SL	nad3eU299SL	0	nad3eU299SL	nad3eU287SL	nad3eU299SL	7/8	88 %
nad7eU313RC	nad7eU313RC	nad7eU319RC	nad7eU316RC	-	nad7eU313RC	0	nad7eU316RC	6/7	86 %
rps19eU224SF	rps19eU158SF	-	-	rps19eU146SF	0	-	-	3/4	75 %
-	-	0	rps3eU700PS	rps3eU604PS	0	-	rps3eU658PS	3/5	60 %
-	-	rps3eU541HY	rps3eU709HY	rps3eU613HY	0	-	rps3eU667HY	4/5	80 %
0	rps3eU602TI	rps3eU935TI	rps3eU1433TI	rps3eU1028TI	0	-	rps3eU1391TI	5/7	71 %
rps14eU131SL	rps14eU137SL	-	rps14eU140SL	rps14eU137SL	rps14eU137SL	-	rps14eU140SL	6/6	100 %
rps14eU239SF	rps14eU245SF	-	rps14eU248SF	rps14eU245SF	rps14eU245SF	-	rps14eU248SF	6/6	100 %
-	rpl6eU86PL	-	-	rpl6eU86PL	rpl6eU86PL	-	-	3/3	100 %
48	76	54	69	78	65	37	69	39 / 68 / 87	

Figure 5. The "commons" output in PREPACT 2.0 BLASTX mode.
The "commons" tab summarizes candidate RNA editing sites congruently predicted from the fractions of references as selected according to the thresholds chosen in the query form (see Fig. 3). The full list includes only sites fulfilling the display thresholds (here: minimally 50% and identification in ≥3 references). Light and dark gray shading highlights edits predicted for the indicated fractions of references (here: 75% and 100%, respectively) in an indicated minimum total number of references (here: 3). RNA editing events identified in the references are indicated in red. Symbols '-' and '0' indicate missing BLASTX homologies for a given region or no prediction for editing, respectively.

multiple group II intron maturase paralogs in different organelle introns).

References and thresholds in the new "commons" function

Multiple references for identification of genes and editing sites may be selected from the choice of reference genomes/transcriptomes implemented in PREPACT (Table 1). In our example, we included the two green algae related to the plant lineage and the liverwort *Marchantia polymorpha* in all of which no RNA editing has been identified. Additionally, we here selected five highly diverse plant reference taxa with analyzed mitochondrial transcriptomes: the moss *Physcomitrella patens* with very rare RNA editing, the lycophytes *Isoetes engelmannii* and *Selaginella moellendorffii* with thousands of documented RNA editing sites as well as the "low-editing" *Silene vulgaris* and the comparatively "high-editing" *Lotus japonicus* flowering plant mtDNA references (Table 1).

The "commons" option allows restricting of the listing of RNA editing candidate sites to those that are congruently predicted from a given fraction of references (display threshold, preset to 50%). Two further thresholds (preset to 75% and 100%, respectively) may be adjusted for selective highlighting (Fig. 3). Additional filters are available to restrict candidate sites in "rare" genes (eg, maturases, ribosomal proteins) according to minimum numbers of parallel hits in the set of references for display or highlighting (both preset to ≥3) in the output.

The PREPACT 2.0 output for BLASTX mode

The PREPACT 2.0 output using the above example settings is shown in Figures 4 and 5. Results on gene identification and the prognoses for RNA editing are available as individual tabs for each reference selected in the query form (Figs. 4 and 5). Users may thus jump between reference tabs to inspect outputs in detail (eg, individual sequence alignments when needed, not shown) as selected in the query options (Fig. 3). A graphic overview of BLASTX hits along the query is displayed on top for each individual reference, as exemplarily displayed here for the top part of the output in the *Physcomitrella patens* reference tab (Fig. 4). Exon-intron gene structures in the query become immediately apparent with horizontally-arranged separate exon hits against the continuous protein reference targets in the BLASTX searches. Moreover, the sorting of matches between the query (horizontally) against the reference (vertically) also allows immediate recognition of gene syntenies in the organelle DNAs, as becomes very obvious for the two moss chondromes in our example (Fig. 4). Hits are colored from bright green to dark red according to increased expectancy for chance similarities. Secondary hits overlapping others with higher and/or more extended similarity that have been excluded for editing prognosis according to the filter settings are displayed with light shading. In the example shown this becomes clearly apparent for the *nad*5-*nad*4-*nad*2 cluster where similarities with the respective paralogs are appropriately excluded (Fig. 4). For convenient navigation in the output, BLASTX hits are actively linked to individual hit statistics, lists of editing sites and/or the individual alignments optionally displayed further down in the output as selected in the query form.

An additional tab "commons" (Fig. 5) summarizes the editing site predictions from the individual tabs according to the threshold settings in the query form. PREPACT 2.0 now distinguishes the absence of an editing prediction (0) from the absence of a BLASTX hit for a given position (-). This distinction becomes highly useful for genes that are absent (mostly due to nuclear gene transfer) in some of the reference genomes, thus allowing a smaller number of references to be taken into account for the threshold calculations. The absence of the entire suite of *ccm* genes (*ccmB, ccmC* and *ccmF*) from the chondromes of *Chaetosphaeridium globosum, Isoetes engelmannii* and *Selaginella moellendorffii* is an obvious bona fide case along those lines in the example output (Fig. 5). At the same time the *ccm* gene example also reflects the new gene synonymising functionality. The orthologous candidate RNA editing sites referring to identical query positions are given on the same line in spite of different gene labels in the reference database entries. Using the reference numbering for RNA editing site labeling, as chosen in our query example (Fig. 3), immediately allows recognition of amino-terminal length variation or indels in the homologous genes.

An advantage of the new reference data setup relying on edited coding sequences is that actual editing events previously identified in reference transcriptomes can be distinguished from "pre-edited" sites, ie, the edited versions of the affected pyrimidine, on the DNA level in others. We use red font for true editing events identified in a reference versus pre-edited sites on the DNA level in others given in black.

In our example querying the *Anomodon rugelii* mtDNA against the eight selected references using the "commons" default threshold settings as given in Figure 3, a total of 39 sites are consistently predicted against all reference genomes (100%, with minimally 3 hits total) as summarized in the bottom line of the output (Fig. 5). Most of these sites (27) are predicted by all 8 of 8 references, whereas others are also unequivocally predicted, but homologues for the respective loci are lacking in some references (2x 6/6 in *rps14*, 7x 5/5 in the suite of *ccm* genes, 2x 3/3 in *rpl6* and for the start codon editing prediction in *mttB*/*tatC* and 7/7 for one site in *nad*1 lacking homology in *Selaginella*).

Additional support for the very strongly predicted editing sites as well as for the extended set included within the 75% threshold comes from the fact that the overwhelming majority of these sites are identified as being RNA editing positions (red) in at least one or more of the reference taxa. In fact, nad5eU64LF is the only example of a candidate site in the 8/8 subset that is consistently pre-edited in all reference taxa (Fig. 5). Similarly, RNA editing sites are also identified in the references for several other candidate sites that are included among the lower thresholds. Highly interesting cases are five candidate-editing sites in the *tatC* (or *mttB*) gene, all of which are independently corroborated by homologous editing in the reference taxa. However, two of them that would comparably be predicted for *Physcomitrella patens* have not been confirmed in cDNA analysis of that moss ("0"). In our example using the primary display threshold of ≥3 hits, a few candidate sites were not included in the output list since they were identified in only two homologues among the references (eight sites in maturase-like ORFs and one site each in *rpl2* and *rps7*). No editing events have previously been documented for those positions in any of the references.

Note that our editing site nomenclature allows for quick identification of certain types of editing events also in long outputs (eg, by searching for a particular gene or for changes converting two particular codon identities) using the Ctrl-F search functionality of the WWW browser.

Re-checking the references

No RNA editing has so far been described for the organelle genomes of green algae like *Chara vulgaris* and *Chaetosphaeridium globosum* and the liverwort *Marchantia polymorpha,* which were accordingly here included as mtDNA and cpDNA references. The absence of RNA editing in the latter is most likely due to a loss of RNA editing in the entire clade of marchantiid, ie, complex-thalloid liverworts,[20] recently further supported by an extended taxon sampling for selected mitochondrial loci.[21] On the other hand, the recent discovery of rare RNA editing in the mtDNA of the protist *Naegleria gruberi*[7] suggests that one should be careful about assuming that there is a general absence of RNA editing in certain organelle genomes.

We re-inspected the algal and *Marchantia* plastome and chondrome sequences using the new PREPACT 2.0 possibilities. To this end we replaced them individually using the monocot *Oryza sativa* as a reference but otherwise used the same stringent parameters as in our above example scan for candidate RNA editing sites in the *Anomodon rugelii* mtDNA. This search strategy indeed revealed no strongly-predicted mitochondrial RNA editing in *Chaetosphaeridium globosum*. However, two sites of mitochondrial RNA editing were consistently predicted for *Chara vulgaris* and four were consistently predicted for *Marchantia polymorpha,* all of which are edited in at least one of the reference taxa. This finding strongly warrants eventual re-inspecting of at least those loci on the cDNA level in the alga and in the liverwort.

Along similar lines we investigated the chloroplast reference set and likewise found that checking on cDNA level of some candidate sites may be warranted for the algae (eg, rps2eU463HY, psaAeU244LF, ndhFeU503SF and ndhDeU1450PS in *Chaetosphaeridium globosum*). Notably, only one codon-changing edit was reported for the *Physcomitrella patens* cpDNA[22,23] but one particular striking candidate RNA editing site was additionally suggested in our PREPACT 2.0 analyses: atpBeU1322SF. Indeed, this position turned out to be a sequence error in the database accession to be corrected into a genomic thymidine soon (Dr. M. Sugita, Nagoya, personal communication).

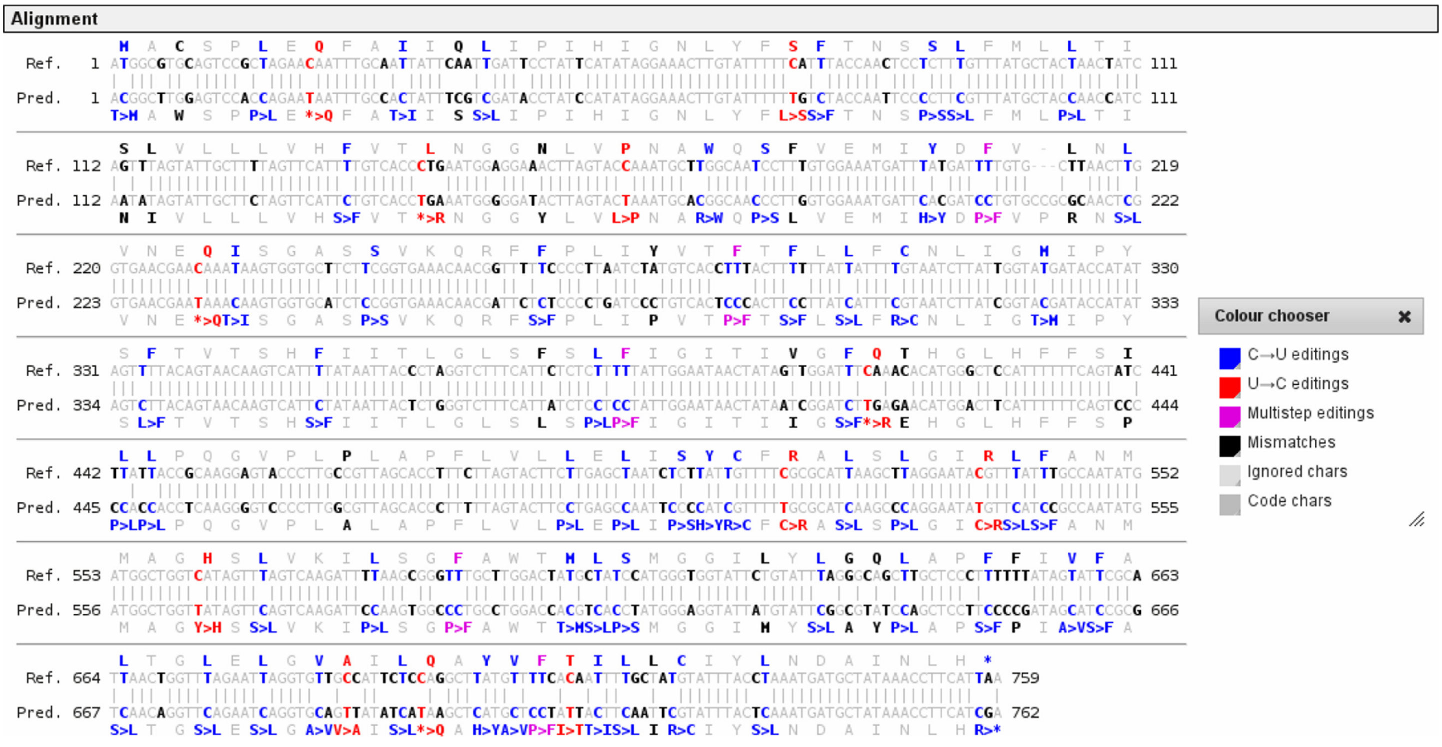
Improving start and stop codon editing predictions in both directions

A previous limitation of the BLASTX approach implemented in PREPACT had been that start and stop codons, which would need to be established by C-to-U editing, would be missed because T versus M and Stop versus Q or R mismatches were not included at the ends of BLASTX hits. This issue is now successfully addressed in PREPACT 2.0: An adjustable number of codons (preset to 2, see Fig. 3) flanking the BLASTX hits is re-investigated a posteriori to scan for additional possibilities of improving similarities towards the ends of the reference protein sequences.

Conversely, removal of in-frame stop codons has previously only been suggested when arginine or glutamine codons conserved in the reference could be reconstituted by candidate reverse editing. A novel "always edit stop" option may be activated when U-to-C prediction of editing is switched on (see Supplemental Fig. 2), which always suggests the conversion of in-frame stop codons, even when other codon identities are present in the references.

Here we demonstrate the capabilities of these new features using the mitochondrial *atp*6 gene of *Isoetes engelmannii* since this extremely edited locus combines the requirements of introducing start and stop codons with multiple stop removals of in-frame stops.[5] The RNA editing prediction is exemplarily shown in an alignment with the *Marchantia polymorpha* reference (Fig. 6). The introduction of start and stop codons by C-to-U editing is now reliably predicted, given the codon extension option. Conversely, the "always edit stop" option suggests editing events eC145*R and eC415*R alongside three others removing stops, although a leucine and a glutamine codon are placed opposite the arginines that can be introduced here by reverse U-to-C editing.

In fact, all 74 codon edits as predicted from the alignment with *Marchantia* (and most of the other references) were indeed confirmed by cDNA analysis. Four additional sites of RNA editing detected in *Isoetes*


Alignment

```
           M  A  C  S  P  L  E  Q  F  A  I  I  Q  L  I  P  I  H  I  G  N  L  Y  F  S  F  T  N  S  S  L  F  M  L  L  T  I
Ref.    1 ATGGCGTGCAGTCCGCTAGAACAATTTGCAATTATTCAATTGATTCCTATTCATATAGGAAACTTGTATTTTTCATTTACCAACTCCTCTTTGTTTATGCTACTAACTATC 111
          | ||| || ||||| | |||| ||||||| | ||||   | ||| ||||| ||||||||||||||||||||||  | ||||||| ||| ||| |||||||||||| ||| |||
Pred.   1 ACGGCTTGGAGTCCACCAGAATAATTTGCCACTATTTCGTCGATACCTATCCATATAGGAAACTTGTATTTTTTGTCTACCAATTCCCCTTCGTTTATGCTACCAACCATC 111
          T>M A  W  S  P P>L E *>Q F  A T>I I  S S>L I  P  I  H  I  G  N  L  Y  F L>SS>F T  N  S P>SS>L F  M  L P>L T  I

           S  L  V  L  L  L  V  H  F  V  T  L  N  G  G  N  L  V  P  N  A  W  Q  S  F  V  E  M  I  Y  D  F  V  -  L  N  L
Ref.  112 AGTTTAGTATTGCTTTTAGTTCATTTTGTCACCCTGAATGGAGGAAACTTAGTACCAAATGCTTGGCAATCCTTTGTGGAAATGATTTATGATTTTGTG---CTTAACTTG 219
          | | ||||||||| ||||||||| |||||||   ||||| ||| |||||||| | |||||||  ||||| ||| || ||||||||||| | ||||  ||||   |  |||| |
Pred. 112 AATATAGTATTGCTTCTAGTTCATTCTGTCACCTGAAATGGGGGATACTTAGTACTAAATGCACGGCAACCCTTGGTGGAAATGATTCACGATCCTGTGCCGCGCAACTCG 222
           N  I  V  L  L  L  V  H S>F V  T *>R N  G  G  Y  L  V L>P N  A R>W Q P>S L  V  E  M  I H>Y D P>F V  P  R  N S>L

           V  N  E  Q  I  S  G  A  S  S  V  K  Q  R  F  F  P  L  I  Y  V  T  F  T  F  L  L  F  C  N  L  I  G  M  I  P  Y
Ref.  220 GTGAACGAACAAATAAGTGGTGCTTCTTCGGTGAAACAACGGTTTTTCCCCTTAATCTATGTCACCTTTACTTTTTTATTATTTTGTAATCTTATTGGTATGATACCATAT 330
          ||||||||| ||| |||||||||| ||| |||||||||||||| || |  ||| | ||||  ||||||    ||||  |||| ||||| |||||||||||| |||| |||||||||
Pred. 223 GTGAACGAATAAACAAGTGGTGCATCTCCGGTGAAACAACGATTCTCTCCCCTGATCCCTGTCACTCCCACTTCCTTATCATTTCGTAATCTTATCGGTACGATACCATAT 333
           V  N  E *>QT>I S  G  A  S P>S V  K  Q  R  F S>F P  L  I  P  V  T P>F T S>F L S>L F R>C N  L  I  G T>M I  P  Y

           S  F  T  V  T  S  H  F  I  I  T  L  G  L  S  F  S  L  F  I  G  I  T  I  V  G  F  Q  T  H  G  L  H  F  F  S  I
Ref.  331 AGTTTTACAGTAACAAGTCATTTTATAATTACCCTAGGTCTTTCATTCTCTCTTTTTATTGGAATAACTATAGTTGGATTTCAAACACATGGGCTCCATTTTTTCAGTATC 441
          ||| ||||||||||||||||||| |||||||||| || ||||||||||||| |||| |  |||||||||||||||||| | |||| |  |  |||||| || ||||||||||||  |
Pred. 334 AGTCTTACAGTAACAAGTCATTCTATAATTACTCTGGGTCTTTCATTATCTCCTCCTATTGGAATAACTATAATCGGATCTTGAGAACATGGACTTCATTTTTTCAGTCCC 444
           S L>F T  V  T  S  H S>F I  I  T  L  G  L  S  L  S P>LP>F I  G  I  T  I  I  G S>F*>R E  H  G  L  H  F  F  S  P

           L  L  P  Q  G  V  P  L  P  L  A  P  F  L  V  L  L  E  L  I  S  Y  C  F  R  A  L  S  L  G  I  R  L  F  A  N  M
Ref.  442 TTATTACCGCAAGGAGTACCCTTGCCGTTAGCACCTTTCTTAGTACTTCTTGAGCTAATCTCTTATTGTTTTCGCGCATTAAGCTTAGGAATACGTTTATTTGCCAATATG 552
            |  ||| ||||| || |||||| |||||||||| || |||||||||| ||||| |||  |  || ||||| |||||| | |||  |||||||| ||| ||| ||  |||||||||
Pred. 445 CCACCACCTCAAGGGGTCCCCTTGGCGTTAGCACCCTTTTTAGTACTTCCTGAGCCAATTCCCCATCGTTTTTGCGCATCAAGCCCAGGAATATGTTCATCCGCCAATATG 555
          P>LP>L P  Q  G  V  P  L  A  L  A  P  F  L  V  L P>L E P>L I P>SH>YR>C F C>R A S>L S P>L G  I C>RS>LS>F A  N  M

           M  A  G  H  S  L  V  K  I  L  S  G  F  A  W  T  M  L  S  M  G  G  I  L  Y  L  G  Q  L  A  P  F  F  I  V  F  A
Ref.  553 ATGGCTGGTCATAGTTTAGTCAAGATTTTAAGCGGGTTTGCTTGGACTATGCTATCCATGGGTGGTATTCTGTATTTAGGCCAGCTTGCTCCCTTTTTTATAGTATTCGCA 663
          ||||||||| |||||| |||||||||||  ||| ||   ||| ||||| || | |  | | ||||| |||||| ||||||||  |  | |  ||||| |     |||| || ||| 
Pred. 556 ATGGCTGGTTATAGTTCAGTCAAGATTCCAAGTGGCCCTGCCTGGACCACGTCACCTATGGGAGGTATTATGTATTCGGCGTATCCAGCTCCTTCCCCGATAGCATCCGCG 666
           M  A  G Y>H S S>L V  K  I P>L S  G P>F A  W  T T>MS>LP>S M  G  G  I  M  Y S>L A  Y P>L A  P S>F P  I A>VS>F A

           L  T  G  L  E  L  G  V  A  I  L  Q  A  Y  V  F  T  I  L  L  C  I  Y  L  N  D  A  I  N  L  H  *
Ref.  664 TTAACTGGTTTAGAATTAGGTGTTGCCATTCTCCAGGCTTATGTTTTCACAATTTTGCTATGTATTTACCTAAATGATGCTATAAACCTTCATTAA 759
          | ||| |||| ||||| ||||  |  ||     | ||| ||| | | |  | ||  |   |  ||||||||||||||||||||||||||||||||  |
Pred. 667 TCAACAGGTTCAGAATCAGGTGCAGTTATATCATAAGCTCATGCTCCTATTACTTCAATTCGTATTTACTCAAATGATGCTATAAACCTTCATCGA 762
          S>L T  G S>L E S>L G A>VV>A I S>L*>Q A H>YA>VP>FI>TT>IS>L I R>C I  Y S>L N  D  A  I  N  L  H R>*
```

Figure 6. Start and stop codon conversion in an extreme example of RNA editing.

The example shows the RNA editing prediction for the *Isoetes engelmannii atp6* coding sequence (bottom), here exemplarily aligned with the *Marchantia polymorpha* reference (top). The extended capabilities of PREPACT 2.0 allow identification of C-to-U start and stop codon editing possibilities not included in the original BLASTX hits. In addition, they allow for the removal of two stop codons alongside three others by editing events atp6eC145*R and atp6eC415*R even though they are juxtaposed with a leucine and a glutamine, respectively, in the reference when the "always edit stop option" is activated. All 74 codon changes shown were confirmed on cDNA level. An updated version of the floating color picker, here shown next to the alignment, allows a yet more comfortable change of differentially coloring alignment positions in the WWW output.

*atp*6 cDNA were not predicted with *Marchantia*. Editing atp6eU430HY is an unexpected event with a histidine consistently conserved in all references and editing event atp6eU212PL is unpredictable, as it affects the inserted codon exclusive to *Isoetes* (Fig. 6). The two remaining editing events are moderately suggested from four in eight of the previous set of references (here again *Oryza* replacing *Isoetes*): atp6eUU280PF and atp6eU653PL.

Testing the predictions of RNA editing in entire organelle genomes

To obtain reasonable estimates for the success rates of C-to-U RNA editing prediction with the new PREPACT commons function, we ran the complete mtDNAs of the high editor *Lotus japonicus* and the low editor *Silene vulgaris* under the same conditions (ie, 75% threshold rate and a minimum of 3 parallel hits) as used above for the *Anomodon rugelii* mtDNA example with unknown editing. Since both chondromes are included in our references (Table 1), we selected *Oryza sativa* as an alternative additional reference, for comparability. These predictions based on the taxonomically wide sampling as used in the *Anomodon* example revealed sensitivities of 84% and 81%, respectively, to correctly identify the verified codon changes introduced by editing in *Silene latifolia* (total 267) and *Lotus japonicus* (total 524) at a rate of 3.7% and 4.2% false positives, respectively. To test how taxonomic proximity influences these results with respect to sensitivity and specificity, we replaced the phylogenetic wide sampling including the algae and non-seed plants with the full selection of available angiosperm chondrome references. In these runs, sensitivities rose to 90% and 92%, respectively, at the cost of only slightly decreased specificities with false positives still remaining below 5%.

We finally tested the functional innovations in PREPACT2 for predicting RNA editing in chloroplast DNA to its full extent, and to this end chose the *Anthoceros formosae* plastome, which is characterized as the most complex chloroplast editome yet published. More than 500 C-to-U RNA editing events and more than 400 U-to-C events have been identified in *A. formosae* chloroplasts, which altogether create 891 codon changes in chloroplast mRNAs.[6] We performed test runs including prognosis of U-to-C editing with liberal stop codon removal, again using the previous 75% commons threshold settings (Supplemental Fig. 2). With this setup run against the set of other plastome references, we faithfully identified 89% of the verified codon-changing events at the cost of including 17% false positives. Hence, we consider the current preset threshold values to identify candidate edits (Fig. 3) a useful balance for sensitivity versus specificity. This is also the case for highly complex organelle transcriptomes showing pyrimidine exchanges in both directions, which include numerous stop codon removals and start and stop codon introductions by RNA editings.

User alignments with multiple references

The BLASTX mode of PREPACT 2.0 as described above will likely be of use to most users wishing to analyze new organelle sequence data. For comparative studies focusing on a single gene, however, the user may wish to provide sequence alignments containing multiple query sequences to be tested against multiple self-defined references. An example is a recent study investigating variable RNA editing frequencies among a wide sampling of liverworts and mosses in two selected mitochondrial loci.[21] Such datasets may be used in the "alignment prediction" mode with reference sequences placed first in the alignment and their number indicated (Supplemental Fig. 3). In addition to the new analogous "commons" functionality as described above for the BLASTX mode, the alignment prediction mode of analysis also allows increased display functionality for highlighting and grouping of sites, as previously described for the differential cDNA analysis mode.[9]

Discussion

A simple hepta-peptide such as "PREPACT" may be encoded in 6,144 different ways by just considering the codon ambiguities of the universal genetic code. Allowing for pyrimidine editing, this number rises to an astounding 1,310,720 possibilities for alternative 21-nucleotide sequences on DNA level. The ambiguities of oligonucleotide primer design for cDNA amplification in heavily editing taxa are an obvious methodological consequence in the laboratory, while another major issue is the bioinformatic processing of editing.

No obvious evolutionary advantage has been associated with the abundant plant organelle RNA editing and as yet no reasonable explanation exists why hundreds or even thousands of edited RNA positions couldn't be encoded as such in the DNA. Indeed, plant organelle RNA editing is currently best explained by a composite neutral evolution (CNE) model, which posits that an ancestral editing activity, functionally extended to operate on polyribonucleotide stretches allows the encoding genes to mutate more freely.[24] Simple causes of genome evolution such as genomic GC or AT drifts may favor certain RNA editing mechanisms to affect more and more sites until they get evolutionarily "locked in" and can hardly be lost again. Frequent independent gains and losses of RNA editing positions in related taxa, mostly with corresponding pyrimidine transitions in the DNA, support the above hypothesis. The wide variability of RNA editing frequency ranging from apparently none in marchantiid liverworts,[21] few sites in mosses, to thousands in lycophytes,[4,5] support the idea of a freely evolving selfish mechanism.

Selfish as organelle RNA editing may seem, the functional implications in the cell are fascinating. The editing machinery has to select its RNA targets very specifically. Particular pentatricopeptide repeat (PPR) RNA-binding proteins are the obvious key players in recognizing the appropriate transcript sequences.[25–27] Impressive steps forward have recently been made to decipher the code for sequence-specific RNA-binding encoded by the array of PPR repeats.[28] Any such predictions of an RNA-binding code will, however, need testing against similar sites in an organelle transcriptome. Conversely, the identification of editing sites will prompt the quest for the appropriate RNA-binding protein factors. Along those lines, we hope that PREPACT may be of help to address this and similar issues, and possibly also reveal more cases of as yet hidden, unexpected RNA editing events like the ones recently elucidated for *Naegleria gruberi*.[7]

Here we have discussed the issues of proper annotation of RNA editing in primary database sequence accessions and have suggested a standard annotation that could be adapted in the primary databases in the future. Such a standard would significantly help with information exchange between the primary sequence repositories and efforts dedicated to the storage and analysis of data on RNA editing, such as PREPACT or other initiatives elsewhere.[13,29–36] RNA editing database initiatives such as REDIdb include data on the investigation of single loci in taxa for which no extensive organelle transcriptome analyses have been performed. Aside from its RNA editing prognosis features, in the future PREPACT may hopefully concomitantly also serve as a database for curated full "editomes", or "editotypes",[37] ie, the full inventories of editing sites for a given organelle genome. Querying PREPACT 2.0 with an organelle genome against its reference transcriptome will obviously reveal all RNA editing sites causing codon changes, and at the same time will allow for comparisons to be made to the editomes of other references.

For PREPACT 2.0 we have currently processed 19 mitochondrial and 13 chloroplast genomes as references in order to comfortably predict coding sequences and RNA editing in organelle sequence data. The major improvements of the new PREPACT 2.0 WWW server are (i) the exclusion of secondary BLASTX hits by filtering to avoid erroneous prediction of RNA editing from paralogs, (ii) the distinction of a lack of RNA editing prognosis from a simple lack of homology in individual references, (iii) the distinction of editing events from pre-edited sites in references through their re-organization as translated coding sequences from edited nucleotide sequences, (iv) the option to scan for potential introduction of start and stop codons by C-to-U editing beyond homologies identified as BLASTX hits, (v) the option of general stop-codon removal to create continuous reading frames irrespective of conserved arginine or glutamine codons in references, and finally (vi) the "commons" function relying on all of the above to summarize RNA editing predictions for fractions of the multiple-reference set according to threshold settings. Test runs with extensively analyzed mitochondrial transcriptomes show that success rates for faithful prognosis of mRNA editing may even exceed 90% at false positive rates below 5% when using the 75% commons threshold, which we currently recommend. Although prognoses benefit from taxonomically closer references, even a phylogenetically wide sampling including algae and non-seed land plants nevertheless reliably detects more than 80% of mRNA editing. Beyond the BLASTX-based functionality, PREPACT users may provide sequence alignments with multiple queries including multiple self-chosen references for predicting editing in individual loci using the same "commons" functionality. We hope to be able to provide timely updates of PREPACT 2.0 with the addition of novel organelle (transcriptome) reference data in the future, and that the PREPACT software will be of use to comfortably catalogue and predict RNA editing events in upcoming organelle sequences.

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Author contributions

HL and VK designed the program features. HL did the programming and debugging. Both authors revised database entries, extracted RNA editing data from the literature and did test runs of the software. VK wrote the manuscript and both authors revised and approved the final manuscript.

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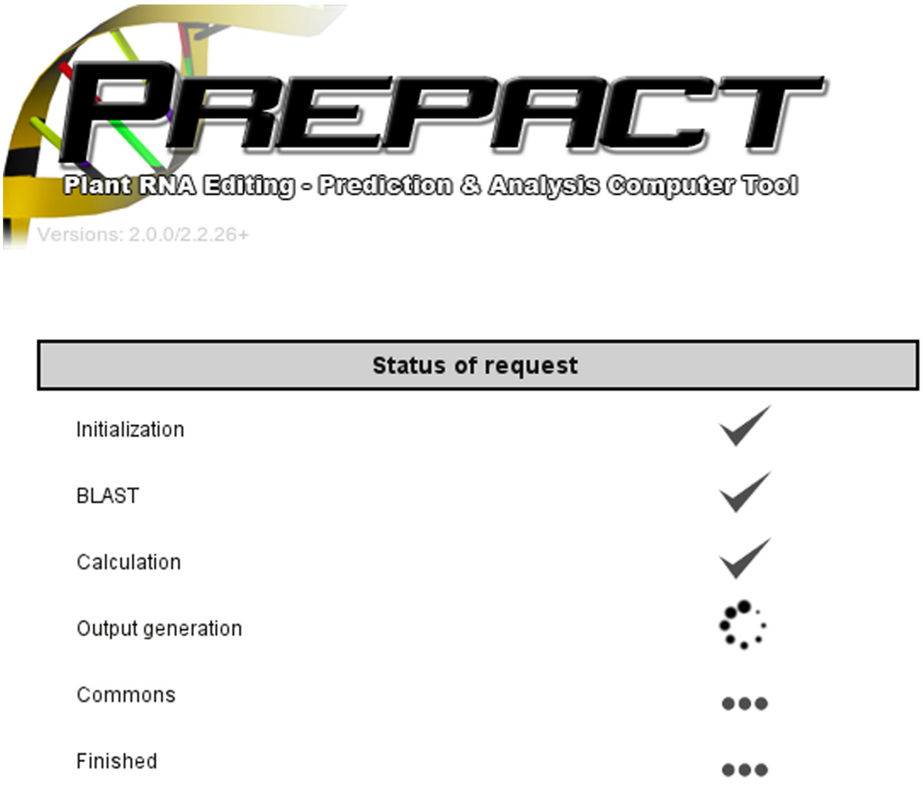
Supplementary Figures



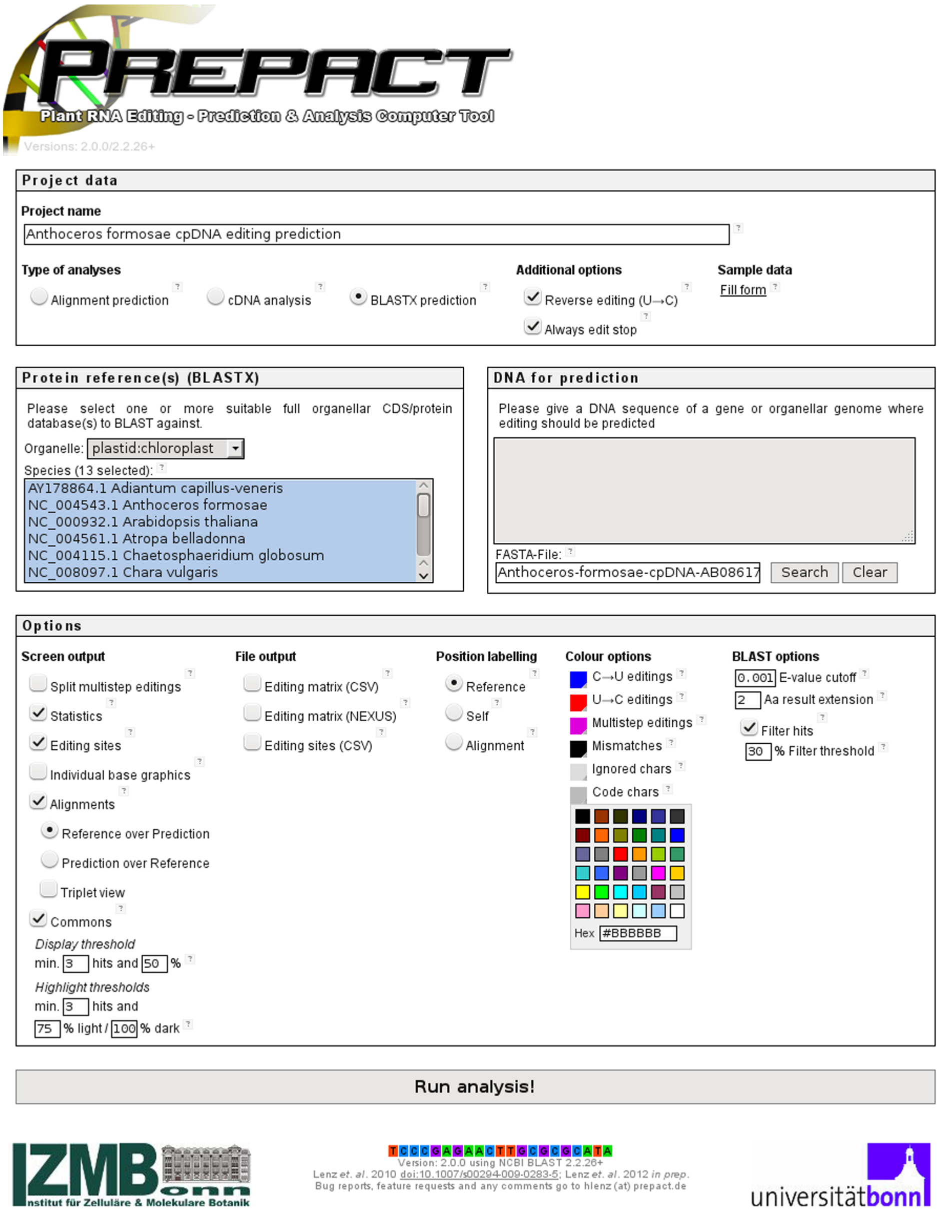
PREPACT

Plant RNA Editing - Prediction & Analysis Computer Tool

Versions: 2.0.0/2.2.26+

Status of request	
Initialization	✓
BLAST	✓
Calculation	✓
Output generation	○
Commons	●●●
Finished	●●●

Figure S1. The request status display indicating progress of an analysis after submission of a query.
Extensive requests employing large sequences and multiple output options may need several minutes for completion of the run and preparation of the output (see Figs. 4 and 5) which is indicated in several steps.



Versions: 2.0.0/2.2.26+

Project data

Project name: Anthoceros formosae cpDNA editing prediction

Type of analyses: ○ Alignment prediction ○ cDNA analysis ● BLASTX prediction

Additional options: ☑ Reverse editing (U→C) ☑ Always edit stop

Sample data: Fill form

Protein reference(s) (BLASTX)

Please select one or more suitable full organellar CDS/protein database(s) to BLAST against.

Organelle: plastid:chloroplast

Species (13 selected):
AY178864.1 Adiantum capillus-veneris
NC_004543.1 Anthoceros formosae
NC_000932.1 Arabidopsis thaliana
NC_004561.1 Atropa belladonna
NC_004115.1 Chaetosphaeridium globosum
NC_008097.1 Chara vulgaris

DNA for prediction

Please give a DNA sequence of a gene or organellar genome where editing should be predicted

FASTA-File: Anthoceros-formosae-cpDNA-AB08617

Options

Screen output:
- □ Split multistep editings
- ☑ Statistics
- ☑ Editing sites
- □ Individual base graphics
- ☑ Alignments
  - ● Reference over Prediction
  - ○ Prediction over Reference
  - □ Triplet view
- ☑ Commons

Display threshold
min. 3 hits and 50 %

Highlight thresholds
min. 3 hits and
75 % light / 100 % dark

File output:
- □ Editing matrix (CSV)
- □ Editing matrix (NEXUS)
- □ Editing sites (CSV)

Position labelling:
- ● Reference
- ○ Self
- ○ Alignment

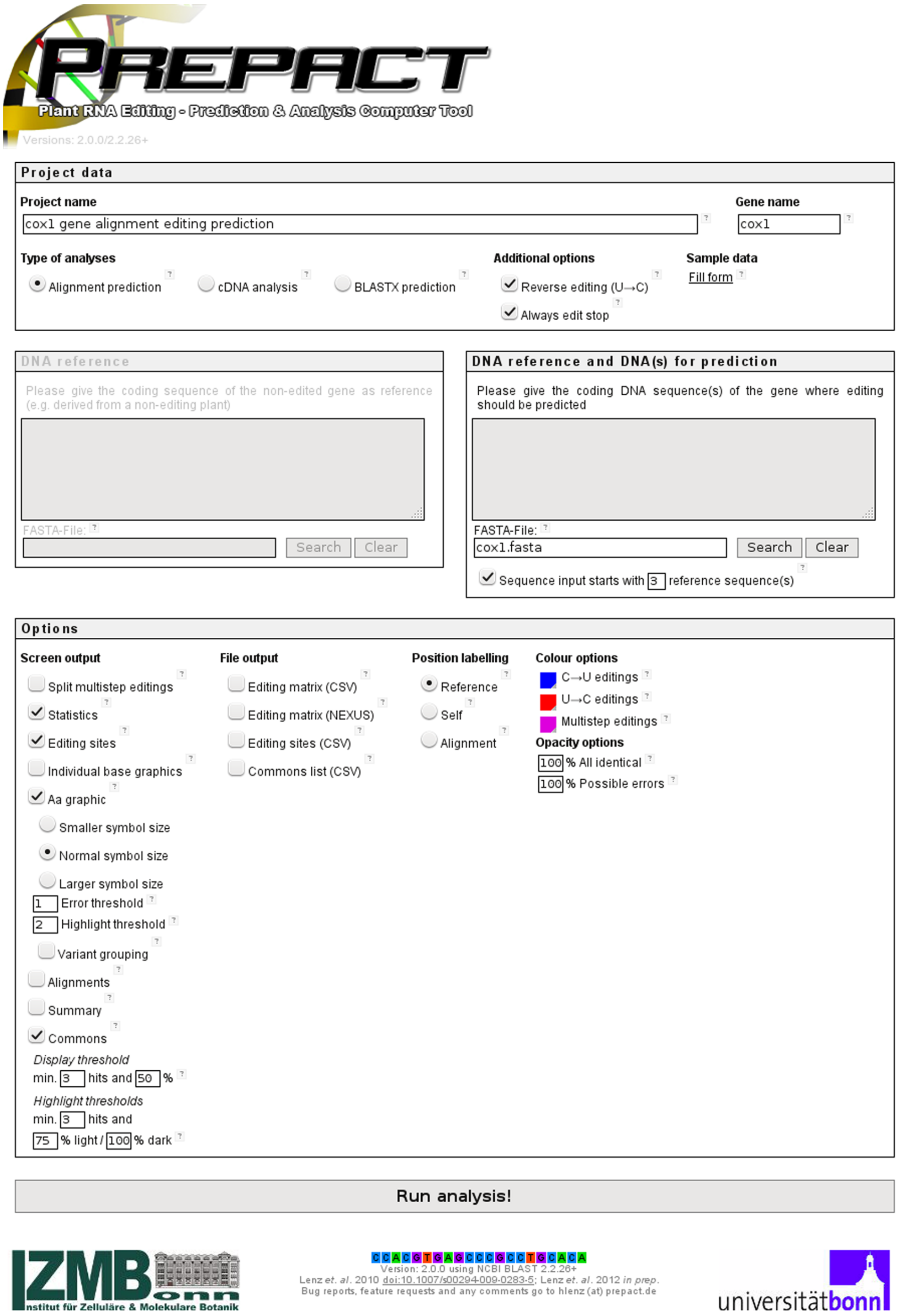
Colour options:
- 🟦 C→U editings
- 🟥 U→C editings
- 🟪 Multistep editings
- ⬛ Mismatches
- ⬜ Ignored chars
- ⬜ Code chars

Hex #BBBBBB

BLAST options:
- 0.001 E-value cutoff
- 2 Aa result extension
- ☑ Filter hits
- 30 % Filter threshold

Version: 2.0.0 using NCBI BLAST 2.2.26+
Lenz *et. al*. 2010 doi:10.1007/s00294-009-0283-5; Lenz *et. al*. 2012 *in prep.*
Bug reports, feature requests and any comments go to hlenz (at) prepact.de

Figure S2. The PREPACT 2.0 query form exemplarily shown for the analysis of the *Anthoceros formosae* plastome.
All available chloroplast references are selected and the "always edit stop" option is included for analysis, which allows for proposal of reverse U-to-C editing to remove stop codons also where no arginine or glutamine codons are conserved in references. Additionally shown is the new quick choice colour selection option in the colour picker popup window for differential colouring of alignment positions (see Fig. 6).



Project data

Project name: cox1 gene alignment editing prediction

Gene name: cox1

Type of analyses: ● Alignment prediction ○ cDNA analysis ○ BLASTX prediction

Additional options: ☑ Reverse editing (U→C) ☑ Always edit stop

Sample data: Fill form

DNA reference

Please give the coding sequence of the non-edited gene as reference (e.g. derived from a non-editing plant)

FASTA-File: Search Clear

DNA reference and DNA(s) for prediction

Please give the coding DNA sequence(s) of the gene where editing should be predicted

FASTA-File: cox1.fasta Search Clear

☑ Sequence input starts with 3 reference sequence(s)

Options

Screen output

- ☐ Split multistep editings
- ☑ Statistics
- ☑ Editing sites
- ☐ Individual base graphics
- ☑ Aa graphic
  - ○ Smaller symbol size
  - ● Normal symbol size
  - ○ Larger symbol size
  - 1 Error threshold
  - 2 Highlight threshold
  - ☐ Variant grouping
- ☐ Alignments
- ☐ Summary
- ☑ Commons

Display threshold
min. 3 hits and 50 %

Highlight thresholds
min. 3 hits and
75 % light / 100 % dark

File output

- ☐ Editing matrix (CSV)
- ☐ Editing matrix (NEXUS)
- ☐ Editing sites (CSV)
- ☐ Commons list (CSV)

Position labelling

- ● Reference
- ○ Self
- ○ Alignment

Colour options

- 🟦 C→U editings
- 🟥 U→C editings
- 🟪 Multistep editings

Opacity options

- 100 % All identical
- 100 % Possible errors

Run analysis!

Version: 2.0.0 using NCBI BLAST 2.2.26+
Lenz *et. al*. 2010 doi:10.1007/s00294-009-0283-5; Lenz *et. al*. 2012 *in prep*.
Bug reports, feature requests and any comments go to hlenz (at) prepact.de

Figure S3. The "Alignment prediction" mode for user-provided alignments of homologous organelle coding sequences in FASTA format. Alignments may as respective first entries on top include any number (to be indicated, here: 3) of self-defined references. Like the differential cDNA analysis mode, the Alignment prediction mode also includes further options for comparative graphic display (bottom left).