STUDY DESIGNS

A beginner's guide to eukaryotic genome annotation

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Abstract | The falling cost of genome sequencing is having a marked impact on the research community with respect to which genomes are sequenced and how and where they are annotated. Genome annotation projects have generally become small-scale affairs that are often carried out by an individual laboratory. Although annotating a eukaryotic genome assembly is now within the reach of non-experts, it remains a challenging task. Here we provide an overview of the genome annotation process and the available tools and describe some best-practice approaches.

Genome annotation
A term used to describe two distinct processes. 'Structural' genome annotation is the process of identifying genes and their intron–exon structures. 'Functional' genome annotation is the process of attaching meta-data such as gene ontology terms to structural annotations. This Review focuses on structural annotation.

RNA-sequencing data
(RNA-seq data). Data sets derived from the shotgun sequencing of a whole transcriptome using next-generation sequencing (NGS) techniques. RNA-seq data are the NGS equivalent of expressed sequence tags generated by the Sanger sequencing method.

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Sequencing costs have fallen so dramatically that a single laboratory can now afford to sequence large, even human-sized, genomes. Ironically, although sequencing has become easy, in many ways, genome annotation has become more challenging. Several factors are responsible for this. First, the shorter read lengths of second-generation sequencing platforms mean that current genome assemblies rarely attain the contiguity of the classic shotgun assemblies of the *Drosophila melanogaster*[1,2] or human genomes[3,4]. Second, the exotic nature of many recently sequenced genomes also presents annotation challenges, especially for gene finding. Whereas the first generation of genome projects had recourse to large numbers of pre-existing gene models, the contents of today's genomes are often terra incognita. This makes it difficult to train, optimize and configure gene prediction and annotation tools.

A third new challenge is posed by the need to update and merge annotation data sets. RNA-sequencing data (RNA-seq data)[5–8] provide an obvious means for updating older annotation data sets; however, doing so is not trivial. It is also not straightforward to ascertain whether the result improves on the original annotation. Furthermore, it is not unusual today for multiple groups to annotate the same genome using different annotation procedures. Merging these to produce a consensus annotation data set is a complex task.

Finally, the demographics of genome annotation projects are changing as well. Unlike the massive genome projects of the past, today's genome annotation projects are usually smaller-scale affairs and often involve researchers who have little bioinformatics and computational biology expertise. Eukaryotic genome annotation is not a point-and-click process; however, with some basic UNIX skills, 'do-it-yourself' genome annotation projects are quite feasible using present-day tools. Here we provide an overview of the eukaryotic genome annotation process, describe the available toolsets and outline some best-practice approaches.

Assembly and annotation: an overview

Assembly. The first step towards the successful annotation of any genome is determining whether its assembly is ready for annotation. Several summary statistics are used to describe the completeness and contiguity of a genome assembly, and by far the most important is N50 (BOX 1). Other useful assembly statistics are the average gap size of a scaffold and the average number of gaps per scaffold (BOX 1). Most current genomes are 'standard draft' assemblies, meaning that they meet minimum standards for submission to public databases[9]. However, a 'high-quality draft' assembly[9] is a much better target for annotation, as it is at least 90% complete.

Although there are no strict rules, an assembly with an N50 scaffold length that is gene-sized is a decent target for annotation. The reason is simple: if the scaffold N50 is around the median gene length, then ~50% of the genes will be contained on a single scaffold; these complete genes, together with fragments from the rest of the genome, will provide a sizable resource for downstream analyses[10,11]. As can be seen in FIG. 1, median gene lengths are roughly proportional to genome size. Thus, if the size of the genome of interest is known, it is possible to use this figure to obtain a rough estimate of gene lengths and hence to obtain an estimate of the minimum N50 scaffold length for annotation. CEGMA[12] provides another,

N50
A basic statistic for describing the contiguity of a genome assembly. The longer the N50 is, the better the assembly is. See box 1 for details.

Long interspersed nuclear elements
(LINEs). Retrotransposons that encode reverse transcriptase and that make up a substantial fraction of many eukaryotic genomes.

Short interspersed nuclear elements
(SINEs). Retrotransposons that do no encode reverse transcriptase and that parasitize LINE elements. *ALU* elements, which are very common in the human genome, are one example of a SINE.

complementary means of estimating the completeness and contiguity of an assembly. This tool screens an assembly against a collection of more or less universal eukaryotic single-copy genes and also determines the percentage of each gene lying on a single scaffold.

Obtaining a high-quality draft assembly is an achievable goal for most genome projects. If an assembly is incomplete or if its N50 scaffold length is too short, we would recommend doing additional shotgun sequencing, as tools are available for the incremental improvement of draft assemblies[13–15].

Annotation. Although genome annotation pipelines differ in their details, they share a core set of features. Generally, genome-wide annotation of gene structures is divided into two distinct phases. In the first phase, the 'computation' phase, expressed sequence tags (ESTs), proteins, and so on, are aligned to the genome and *ab initio* and/or evidence-driven gene predictions are generated. In the second phase, the 'annotation' phase, these data are synthesized into gene annotations (BOX 2). Because this process is intrinsically complicated and involves so many different tools, the programs that assemble compute data (evidence) and use it to create genome annotations are generally referred to as annotation pipelines. Current pipelines are focused on the annotation of protein-coding genes, although Ensembl also has some capabilities for annotating non-coding RNAs (ncRNAs). Tools for annotation of ncRNAs are described in BOX 3.

Step one: the computation phase

Repeat identification. Repeat identification and masking is usually the first step in the computation phase of genome annotation. Somewhat confusingly, the term 'repeat' is used to describe two different types of sequences: 'low-complexity' sequences, such as homopolymeric runs of nucleotides, as well as transposable (mobile) elements, such as viruses, long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs)[16,17]. Eukaryotic genomes can be very repeat rich; for example, 47% of the human genome is thought to consist of repeats[18], and this number is likely to be the lower limit. Also, the borders of these repeats are usually ill-defined; repeats often insert within other repeats, and often only fragments within fragments are present — complete elements are found quite rarely. Repeats complicate genome annotation. They need to be identified and annotated, but the tools used to identify repeats are distinct from those used to identify the genes of the host genome.

Identifying repeats is complicated by the fact that repeats are often poorly conserved; thus, accurate repeat detection usually requires users to create a repeat library for their genome of interest. Available tools for doing so generally fall into two classes: homology-based tools[19–21] and *de novo* tools[22–25] (for an overview, see REFS 26,27). Note, however, that *de novo* tools identify repeated sequences — not just mobile elements — so their outputs can include highly conserved protein-coding genes, such as histones and tubulins, as well as transposon sequences. Users must therefore carefully post-process the outputs of these tools to remove protein-coding sequences. These same outputs probably also contain some novel repeat families. Repeats are interesting in and of themselves, and the life cycles and phylogenetic histories of these elements are growing areas of research[17,28,29]. Adequate repeat annotation should thus be a part of every genome annotation project.

After it has been created, a repeat library can be used in conjunction with a tool such as RepeatMasker[30], which uses BLAST[31–33] and Crossmatch[34] to identify stretches of sequence in a target genome that are homologous to known repeats. The term 'masking' simply means transforming every nucleotide identified as a repeat to an 'N' or, in some cases, to a lower case a, t, g or c — the latter process is known as 'soft masking'[32,35]. The masking step signals to downstream sequence alignment and gene prediction tools that these regions are repeats. Failure to mask genome

Box 1 | Common statistics for describing genome assemblies

Genome assemblies are composed of scaffolds and contigs. Contigs are contiguous consensus sequences that are derived from collections of overlapping reads. Scaffolds are ordered and orientated sets of contigs that are linked to one another by mate pairs of sequencing reads.

Scaffold and contig N50s
By far the most widely used statistics for describing the quality of a genome assembly are its scaffold and contig N50s. A contig N50 is calculated by first ordering every contig by length from longest to shortest. Next, starting from the longest contig, the lengths of each contig are summed, until this running sum equals one-half of the total length of all contigs in the assembly. The contig N50 of the assembly is the length of the shortest contig in this list. The scaffold N50 is calculated in the same fashion but uses scaffolds rather than contigs. The longer the scaffold N50 is, the better the assembly is. However, it is important to keep in mind that a poor assembly that has forced unrelated reads and contigs into scaffolds can have an erroneously large N50. Note too that scaffolds and contigs that comprise only a single read or read pair — often termed 'singletons' — are frequently excluded from these calculations, as are contigs and scaffolds that are shorter than ~800 bp. The procedures used to calculate N50 may therefore vary between genome projects.

Percent gaps
Another important assembly statistic is its percent gaps. Unsequenced regions between mate pairs in contigs and between scaffolds are often represented as runs of 'N's in the final assembly. Thus two assemblies can have identical scaffold N50s but can still differ in their percent gaps: one has very few gaps, and the other is heavily peppered with them. Estimates of gap lengths are often made based on library insert sizes and read lengths; when these are available, the number of 'N's in these gaps usually, but not always, represents the most likely estimate of that gap's size; sometimes, all gaps are simply represented by a run of 50 'N's regardless of their size.

Percent coverage
Percent coverage is used in two senses: genome coverage and gene coverage. The first number, genome coverage, refers to the percentage of the genome that is contained in the assembly based on size estimates; these are usually based on cytological techniques[116,117]. Genome coverage of 90–95% is generally considered to be good, as most genomes contain a considerable fraction of repetitive regions that are difficult to sequence. So it is not a cause for concern if the genome coverage of an assembly is a bit less than 100%. Gene coverage is the percentage of the genes in the genome that are contained in the assembly. Gene and genome coverage can differ from one another, as hard-to-assemble repetitive regions are often gene-poor. As a result, the percentage gene coverage is often substantially larger than the percentage genome coverage for some difficult-to-assemble genomes.

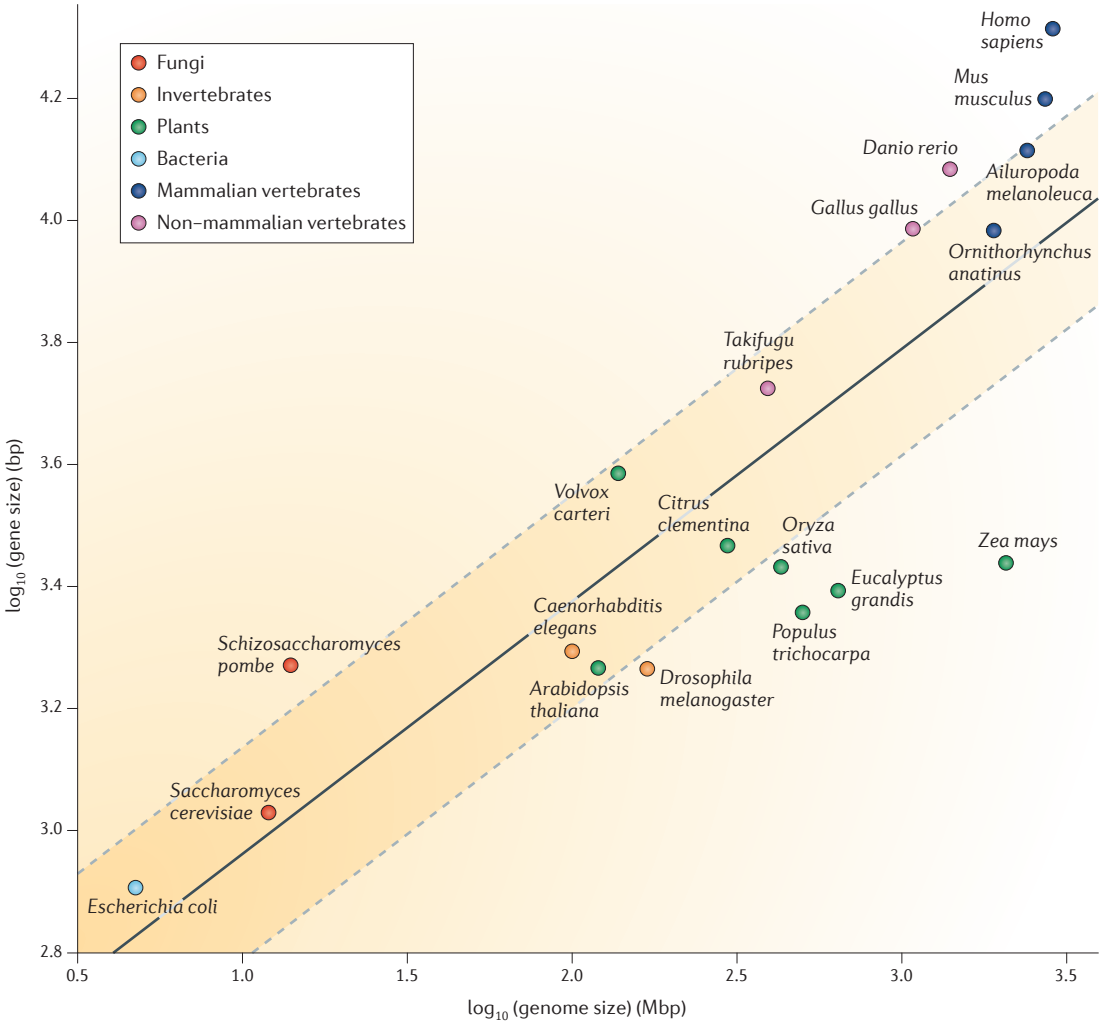


Figure 1 | **Genome and gene sizes for a representative set of genomes.** Gene size is plotted as a function of genome size for some representative bacteria, fungi, plants and animals. This figure illustrates a simple rule of thumb: in general, bigger genomes have bigger genes. Thus, accurate annotation of a larger genome requires a more contiguous genome assembly in order to avoid splitting genes across scaffolds. Note too that although the human and mouse genomes deviate from the simple linear model shown here, the trend still holds. Their unusually large genes are likely to be a consequence of the mature status of their annotations, which are much more complete as regards annotation of alternatively spliced transcripts and untranslated regions than those of most other genomes.

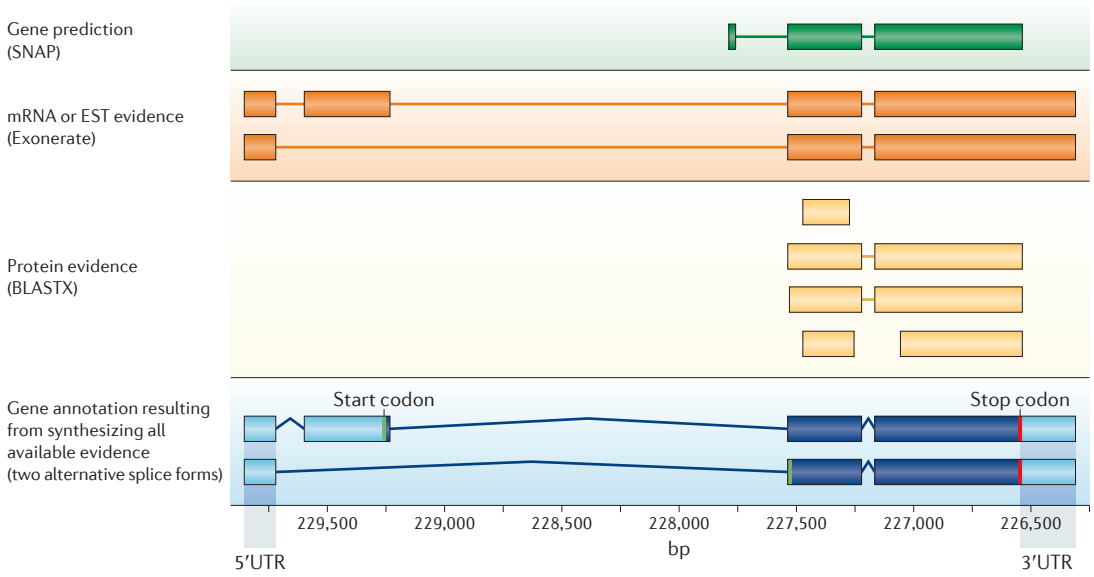
sequences can be catastrophic. Left unmasked, repeats can seed millions of spurious BLAST alignments[32], producing false evidence for gene annotations. Worse still, many transposon open reading frames (ORFs) look like true host genes to gene predictors, causing portions of transposon ORFs to be added as additional exons to gene predictions, completely corrupting the final gene annotations. Good repeat masking is thus crucial for the accurate annotation of protein-coding genes.

Evidence alignment. After repeat masking, most pipelines align proteins, ESTs and RNA-seq data to the genome assembly. These sequences include previously identified transcripts and proteins from the organism whose genome is being annotated. Sequences from other organisms are also included; generally, these are restricted to proteins, as these retain substantial sequence similarity over much greater spans of evolutionary time than nucleotide sequences do. In principle, TBLASTX[31,32,36] can be used to align ESTs and RNA-seq data from phylogenetically distant organisms but, owing to high computational costs, this is only done rarely.

UniProtKB/SwissProt[37–39] is an excellent core resource for protein sequences. As SwissProt is restricted to highly curated proteins, many users might want to supplement this database with the proteomes of related, previously annotated genomes. One easy way to assemble additional protein and EST data sets is to download sequences from related organisms using the NCBI taxonomy browser[40,41].

EST and protein sequence data sets are often aligned to the genome in a two-tiered process. Frequently, BLAST[31,32,36] and BLAT[42] are used to

Box 2 | **Gene prediction versus gene annotation**



Although the terms 'gene prediction' and 'gene annotation' are often used as if they are synonyms, they are not. With a few exceptions, gene predictors find the single most likely coding sequence (CDS) of a gene and do not report untranslated regions (UTRs) or alternatively spliced variants. Gene prediction is therefore a somewhat misleading term. A more accurate description might be 'canonical CDS prediction'.

Gene annotations, conversely, generally include UTRs, alternative splice isoforms and have attributes such as evidence trails. The figure shows a genome annotation and its associated evidence. Terms in parentheses are the names of commonly used software tools for assembling particular types of evidence. Note that the gene annotation (shown in blue) captures both alternatively spliced forms and the 5′ and 3′UTRs suggested by the evidence. By contrast, the gene prediction that is generated by SNAP (shown in green) is incorrect as regards the gene's 5′ exons and start-of-translation site and, like most gene-predictors, it predicts only a single transcript with no UTR.

Gene annotation is thus a more complex task than gene prediction. A pipeline for genome annotation must not only deal with heterogeneous types of evidence in the form of the expressed sequence tags (ESTs), RNA-seq data, protein homologies and gene predictions, but it must also synthesize all of these data into coherent gene models and produce an output that describes its results in sufficient detail for these outputs to become suitable inputs to genome browsers and annotation databases.

identify approximate regions of homology rapidly. These alignments are usually filtered to identify and to remove marginal alignments on the basis of metrics such as percent similarity or percent identity. After filtering, the remaining data are sometimes clustered to identify overlapping alignments and predictions. Clustering has two purposes. First, it groups diverse computational results into a single cluster of data, all supporting the same gene. Second, it identifies and purges redundant evidence; highly expressed genes, for example, may be supported by hundreds if not thousands of identical ESTs.

The term 'polishing' is sometimes used to describe the next phase of the alignment process. After clustering, highly similar sequences identified by BLAST and BLAT are realigned to the target genome in order to obtain greater precision at exon boundaries. BLAST, for example, although rapid, has no model for splice sites, and so the edges of its sequence alignments are only rough approximations of exon boundaries[43]. For this reason, splice-site-aware alignment algorithms, such as Splign[44], Spidey[45], sim4 (REF. 46) and Exonerate[43], are often used to realign matching and highly similar ESTs, mRNAs and proteins to the genomic input sequence. Although these programs take longer to run, they provide the annotation pipeline with much improved information about splice sites and exon boundaries.

Of all forms of evidence, RNA-seq data have the greatest potential to improve the accuracy of gene annotations, as these data provide copious evidence for better delimitation of exons, splice sites and alternatively spliced exons. However, these data can be difficult to use because of their large size and complexity. The use of RNA-seq data currently lies at the cutting edge of genome annotation, and the available toolset is evolving quickly[47]. Currently, RNA-seq reads are usually handled in two ways. They can be assembled *de novo* — that is, independently of the genome — using tools such as ABySS[48], SOAPdenovo[49] and Trinity[50]; the resulting transcripts are then realigned to the genome in the same way as ESTs. Alternatively, the RNA-seq data can be directly aligned to the genome using tools such as TopHat[51], GSNAP[52] or Scripture[53] followed by the assembly of alignments (rather than reads) into transcripts using tools such as Cufflinks[54]. See REF. 55 for guidance on the best way to use TopHat with Cufflinks.

Percent similarity
The percent similarity of a sequence alignment refers to the percentage of positive scoring aligned bases or amino acids in a nucleotide or protein alignment, respectively. The term positive scoring refers to the score assigned to the paired nucleotides or amino acids by the scoring matrix that is used to align the sequences.

Percent identity
The percent identity of a sequence alignment refers to the percentage of identical aligned bases or amino acids in a nucleotide or protein alignment, respectively.

Box 3 | **Non-coding RNAs**

Non-coding RNA (ncRNA) annotation is still in its infancy compared with protein-coding gene annotation, but it is advancing rapidly. The heterogeneity and poorly conserved nature of many ncRNA genes present major challenges for annotation pipelines. Unlike protein-encoding genes, ncRNAs are usually not well-conserved at the primary sequence level; even when they are, nucleotide homologies are not as easily detected as protein homologies, which limits the power of evidence-based approaches.

One common approach is to identify ncRNA genes using conserved secondary structures and motifs. Established examples of these types of tools include tRNAscan-SE[118] and Snoscan[119]. MicroRNA (miRNA) gene finders are also available[120]. A more general approach is first to align nucleotide sequences — genomic, RNA-seq and ESTs — from closely related organisms to the target genome and then search these for signs of conserved secondary structures. This is a complex process, however, and can require substantial computational resources; qRNA is one such tool[121], another is StemLoc[122]. Be aware that these tools have high false-positive rates. RNA sequencing is also greatly aiding ncRNA identification. For example, miRNAs can be directly identified using specialized RNA preps and sequencing protocols[123,124]. Even with such sophisticated tools and techniques, distinguishing between bona fide ncRNA genes, spurious transcription and poorly conserved protein-encoding genes that produce small peptides remains difficult, especially in the cases of long intergenic non-coding RNAs (lincRNAs)[125,126] and expressed pseudogenes[127,128].

Another approach is to annotate possible ncRNA genes liberally and then use Infernal[129] and Rfam[114] to triage and classify these genes based on primary and secondary sequence similarities. Even with these resources, however, many ncRNAs will remain unclassifiable. Currently, ncRNA annotation is cutting edge, and those using ncRNA annotations should bear in mind that ncRNA annotation accuracies are generally much lower than those of their protein-coding counterparts.

Opinions differ as to the best approach for using RNA-seq data, and the most promising avenue will probably heavily depend on both genome biology (for example, gene density) and the contiguity and completeness of the genome assembly. Gene density is an important consideration. If genes are closely spaced in the genome, then tools such as Cufflinks[54] sometimes erroneously merge RNA-seq reads from neighbouring genes. In such cases, *de novo* assembly of the RNA-seq data mitigates the problem; in fact, Trinity[50] is designed to deal with this issue. Several annotation pipelines are now compatible with RNA-seq data: these include PASA[56], which uses inchworm[50] outputs, and MAKER[10], which can operate directly from Cufflinks[54] outputs or can use preassembled RNA-seq data.

Ab initio *gene prediction.* When gene predictors[57–60] first became available in the 1990s (see REF. 61 for an overview), they revolutionized genome analyses because they provided a fast and easy means to identify genes in assembled DNA sequences. These tools are often referred to as *ab initio* gene predictors because they use mathematical models rather than external evidence (such as EST and protein alignments) to identify genes and to determine their intron–exon structures.

The great advantage of *ab initio* gene predictors for annotation is that, in principle, they need no external evidence to identify a gene or to determine its intron–exon structure. However, these tools have practical limitations from an annotation perspective. For instance, most gene predictors find the single most likely coding sequence (CDS) and do not report untranslated regions (UTRs) or alternatively spliced transcripts (BOX 2). Training is also an issue; *ab initio* gene predictors use organism-specific genomic traits, such as codon frequencies and distributions of intron–exon lengths, to distinguish genes from intergenic regions and to determine intron–exon structures. Most gene predictors come with precalculated parameter files that contain such information for a few classic genomes, such as *Caenorhabditis elegans*, *D. melanogaster*, *Arabidopsis thaliana*, humans and mice. However, unless your genome is very closely related to an organism for which precompiled parameter files are available, the gene predictor needs to be trained on the genome that is under study, as even closely related organisms can differ with respect to intron lengths, codon usage and GC content[62].

Given enough training data, the gene-level sensitivity of *ab initio* tools can approach 100%[63,64] (BOX 4). However, the accuracy of the predicted intron–exon structures is usually much lower, ~60–70%. It is also important to understand that large numbers of pre-existing, high-quality gene models and near base-perfect genome assemblies are usually required to produce highly accurate gene predictions[63,65]; such data sets are rarely available for newly sequenced genomes.

In principle, alignments of ESTs, RNA-seq and protein sequences to a genome can be used to train gene predictors even in the absence of pre-existing reference gene models. Although many popular gene predictors can be trained in this way, doing so often requires the user to have some basic programming skills. The MAKER pipeline provides a simplified process for training the predictors Augustus[66,67] and SNAP[62] using the EST, protein and mRNA-seq alignments that MAKER has produced[10,56]. An alternative is to use GeneMark-ES[68,69]: a self-training, but sometimes less-accurate, algorithm[69,70].

Evidence-driven gene prediction. In recent years, the distinction between *ab initio* prediction and gene annotation has been blurred. Many *ab initio* tools, such as TwinScan[71], FGENESH[72], Augustus, Gnomon[73], GAZE[74] and SNAP, can use external evidence to improve the accuracy of their predictions. ESTs, for example, can be used to identify exon boundaries unambiguously. This process is often referred to as evidence-driven (in contrast to *ab initio*) gene prediction. Evidence-driven gene prediction has great potential to improve the quality of gene prediction in newly sequenced genomes, but in practice it can be difficult to use. ESTs and proteins must first be aligned to the genome; RNA-seq data must be aligned too, if they are available. Splice sites must then be identified, and the assembled evidence must be post-processed before a synopsis of these data can be passed to the gene finder. In practice, this is a lot of work, requiring a lot of specialized software. In fact, it is one of the main obstacles that genome annotation pipelines attempt to overcome.

Box 4 | How gene prediction and gene annotation accuracies are calculated

Three commonly used measures of gene-finder performance are sensitivity, specificity and accuracy[130]. Each is measured relative to some standard, usually a reference annotation. Sensitivity (SN) is the fraction of the reference feature that is predicted by the gene predictor. To be more precise, SN = TP / (TP + FN), where TP is true positives and FN is false negatives. By contrast, specificity (SP) is the fraction of the prediction overlapping the reference feature: for example, SP = TP / (TP + FP), where FP is false positives. Note that the definition of SP given here is the one that is commonly used by the gene-finding community[130] but, more correctly, this measure is positive predictive value (PPV) or precision.

Both measures can be calculated for any portion of a gene model, such as genes, transcripts or exons. At the nucleotide level, TP is the number of exonic nucleotides in the reference gene model, FN is the number of these that are not included in the prediction, and FP is the number of exonic nucleotides in the prediction that are not found in the reference gene model. At the exon level, SN is the number of correct exons in the prediction divided by the number of exons in the reference gene model, and SP is the number of correct exons in the prediction divided by the number of exons in the prediction[130]. So-called 'site measures' are also used: for example, the SN and SP for predicting features such as start codons or splice donors. SN and SP are often combined into a single measure called accuracy (AC): for example, AC = (SN + SP) / 2 (see REFS 130–132 for reviews of commonly used accuracy measures).

Panel **A** of the figure shows SN, SP and AC for two different gene models. The reference model is shown in blue, and the two different predictions at the same locus are shown in red. The table on the right gives the values of SN, SP and AC for the two predictions. For the purposes of calculation, exons 1, 2 and 3 of the reference gene model and of prediction 1 have identical start and end coordinates and are 100, 50 and 50 nucleotides long, respectively. In prediction 2, exons are 75 and 50 nucleotides long, respectively, and the start coordinate of its first exon is identical to that of the reference, but its end is not; its second exon is identical to the third exon in the reference.

Numbers in parentheses are the values at the exon level; the others are nucleotide-level values. Note that the values for prediction 2 are lower at the exon level than they are at the nucleotide level. This is because exon-level calculations have an 'all-or-nothing' aspect to them: that is, a model in which the exons each differ by a single nucleotide from the reference will have nucleotide-level SN, SP and AC values near 1; its exon-level SN, SP and AC values, however, will all be 0.

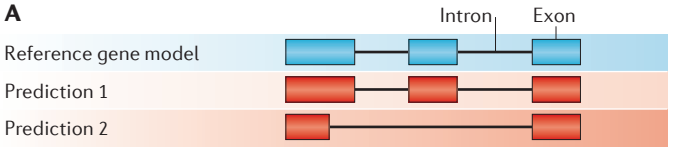
With a few modifications, SN, SP and AC can also be used to compare two annotations to one another. This is the approach taken by the Sequence Ontology Project to calculate annotation edit distance (AED), which can be used to measure the congruence between an annotation and its supporting evidence[96]. AED is calculated in the same manner as SN and SP, but in place of a reference gene model, the coordinates of the union of the aligned evidence (see panel **Ba**) are used instead: AED = 1 – AC, where AC = (SN + SP) / 2.

An AED of 0 indicates that the annotation is in perfect agreement with its evidence, whereas an AED of 1 indicates a complete lack of evidence support for the annotation. More information regarding AED can be found in REF. 96.

Panel **B** illustrates how AED is used. Panel **Ba** shows the protein, expressed sequence tag (EST) and *ab initio* gene predictions that are produced during the computation phase of the annotation process. Panel **Bb** shows two hypothetical annotations based on this evidence. Solid portions of boxes in panel **Bb** delimit coding sequence; note that the two annotations differ at their 3′ untranslated regions (UTRs) as well as their coding-exon coordinates.

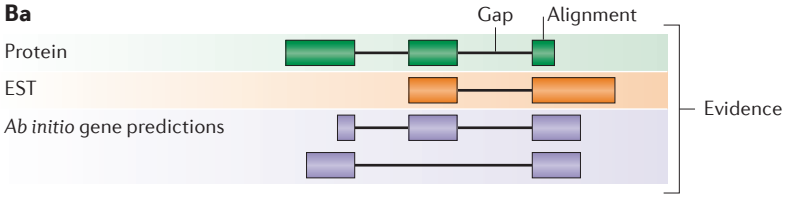
The table on the right in panel **Bb** shows how nucleotide-level AED values can be used to summarize the goodness of fit of an annotation to its overlapping evidence. Annotation 1 has the lower AED (of 0.2), meaning that it is a better fit to the evidence than annotation 2 (with an AED of 0.6) is; thus, bringing annotation 1 into perfect synchrony with the evidence would require fewer manual editing operations than would be required for annotation 2.

A



	SN	SP	AC
Prediction 1	1 (1)	1 (1)	1 (1)
Prediction 2	0.63 (0.33)	1 (0.5)	0.81 (0.42)

Ba



Bb

	AED
Annotation 1	0.2
Annotation 2	0.6

Step two: the annotation phase

The ultimate goal of annotation efforts is to obtain a synthesis of alignment-based evidence with *ab initio* gene predictions to obtain a final set of gene annotations. Traditionally, this was done manually; human genome annotators would review the evidence for each gene in order to decide on their intron–exon structures[75]. Although this results in high-quality annotation[76,77], it is so labour-intensive that, for budgetary reasons, smaller genome projects are increasingly being forced to rely on automated annotations.

There are almost as many strategies for creating automated annotations as there are annotation pipelines, but the common theme is to use evidence to improve the accuracy of gene models, usually through some combination of pre- and post-processing of the gene predictions. FIGURE 2 and TABLE 1 provide an overview of some of the more commonly used approaches.

Automated annotation. The simplest form of automated annotation is to run a battery of different gene finders on the genome and then to use a 'chooser algorithm' (also known as a 'combiner') to select the single prediction whose intron–exon structure best represents the consensus of the models from among the overlapping predictions that define each putative gene locus. This is the process used by JIGSAW[78]. EVidenceModeler (EVM)[79] and GLEAN[80] (and its successor, Evigan[81]) go one step further, attempting to choose the best possible set of exons automatically and to combine them to produce annotations. This is done by estimating the types and frequencies of errors that are made by each source of gene evidence and then choosing combinations of evidence that minimize such errors. Like *ab initio* gene predictors, JIGSAW must be retrained for each new genome, and so it requires a source of known gene models that were not already used to train the underlying *ab initio* gene predictors. EVM allows the user to set expected evidence error rates manually or to learn them from a training set. By contrast, GLEAN and Evigan use an unsupervised learning method to estimate a joint error model, and thus they require no additional training. In a recent gene prediction competition[64], the combiners nearly always improved on the underlying gene prediction models, and JIGSAW, EVM or Evigan performed similarly.

Another popular approach is to feed the alignment evidence to the gene predictors at run time (that is, evidence-driven prediction) to improve the accuracy of the prediction process — a chooser can then be used to identify the most representative prediction. The predictions can also be processed — before or after running the chooser — to attain still greater accuracies by having the annotation pipeline add UTRs as suggested by the RNA-seq and EST data. This is the process used by PASA[56,82], Gnomon[73] and MAKER[10]. The evidence can also be used to inform the choices made by the chooser algorithm — by picking the post-processed gene model that is most consistent with the protein, EST and RNA-seq alignments[83]; EVM, MAKER and PASA all provide methods for doing so (TABLE 1; FIG. 2).

So which approach should you use? Probably the best way to think about the problem is in terms of effort versus accuracy. Simply running a single *ab initio* gene finder over even a very large genome can be done in a few hours of central processing unit (CPU) time. By contrast, a full run by an annotation pipeline such as MAKER or PASA can take weeks, but because these pipelines align evidence to the genome, their outputs provide starting points for annotation curation and downstream analyses, such as differential expression analyses using RNA-seq data. Another factor to consider is the phylogenetic relationship of the study genome to other annotated genomes. If it is the first of its taxonomic order or family to be annotated, it would definitely be preferable to use a pipeline that can use the full repertory of external evidence, especially RNA-seq data, to inform its gene annotations; not doing so will almost certainly result in low-quality annotations[80].

Visualizing the annotation data

Output data: the importance of using a fully documented format. The outputs of a genome annotation pipeline will include the transcript and protein sequences of every annotation, which are almost always provided in FASTA format[84]. Although FASTA files are useful, they only enable a small subset of possible downstream analyses. Visualizing annotations in a genome browser and creating a genome database requires a more descriptive output file. At a bare minimum, output files need to describe the intron–exon structures of each annotation, their start and stop codons, UTRs and alternative transcripts. Ideally, these outputs should go one step further and should include information about the sequence alignments and gene predictions that support each gene model.

Four commonly used formats for describing annotations are the GenBank, GFF3, GTF and EMBL formats. Using a fully documented format is important for three reasons. First, doing so will remove the trouble of writing software to convert outputs into a format that other tools can use. Second, common formats, especially those such as GenBank and GFF3, which use controlled vocabularies and ontologies to define their descriptive terminologies, guarantee 'interoperability' between analysis tools. Third, unless a common vocabulary is used to describe gene models[85], comparative genomic analyses can be frustratingly difficult or downright impossible. In response to these needs, the Generic Model Organism Database (GMOD) project community has developed a series of standards and tools for description, analyses, visualization and redistribution of genome annotations, all of which use the GFF3 file format as inputs and outputs. Leveraging GMOD tools and GFF3 substantially simplifies curation, analysis, publication and management of genome annotations.

GMOD. The GMOD project is an umbrella organization that provides a large suite of tools for creating, managing and using genome annotations, including the analysis, visualization and redistribution of annotation data. Users who have browsed the

Unsupervised learning methods
Refers to methods that can be trained using unlabelled data. One example is a gene prediction algorithm that can be trained without a reference set of correct gene models; instead, the algorithm is trained using a collection of annotations, not all of which might be correct.

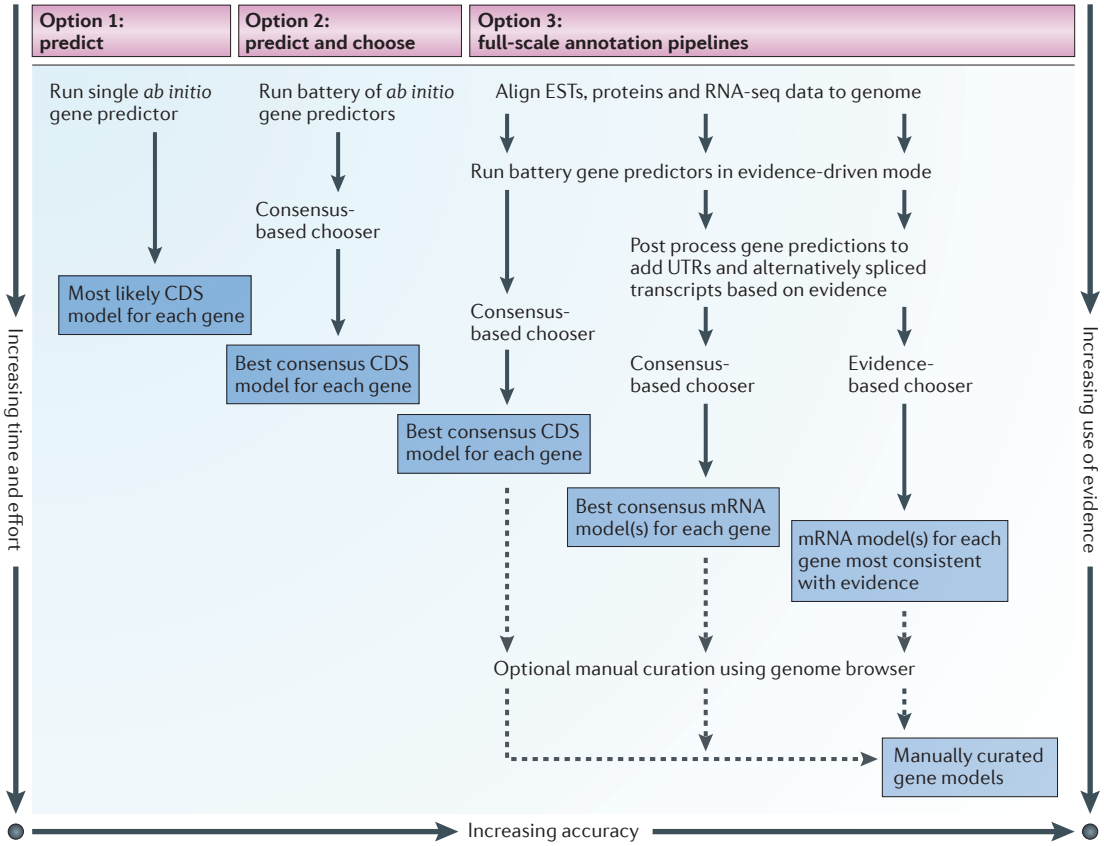


Figure 2 | **Three basic approaches to genome annotation and some common variations.** Approaches are compared on the basis of relative time, effort and the degree to which they rely on external evidence, as opposed to *ab initio* gene models. The *y* axis shows increasing time and effort; the x axis shows increasing use of external evidence and, consequently, increasing accuracy and completeness of the resulting gene models. The type of final product produced by each kind of pipeline is shown in the dark blue boxes. Relative positions in the figure are for summary purposes only and are not based on precisely computed values. See TABLE 1 for a list of commonly used software components. CDS, coding sequence; EST, expressed sequence tag; RNA-seq, RNA sequencing; UTR, untranslated region.

Saccharomyces Genome Database, WormBase, FlyBase, The *Arabidopsis* Information Resource (TAIR) or the University of California Santa Cruz (UCSC) Genome Browser will have used GMOD tools. GMOD tools also aid in creating an online genome database. The key is having annotations and their associated evidence in GFF3 format, which is useable by GMOD tools. Users can directly visualize these files using GBROWSE[86] and JBROWSE[87] to produce views of their data just like those offered at WormBase and UCSC. They can also directly edit the gene models using the Apollo genome browser and JBROWSE. BioPerl[88] also provides a set of database tools for loading GFF3 files into a ready-made Chado[89] database schema with which an online genome database can be rapidly created that contains a genome and its annotations in a 'browse-able' format.

Quality control

Incorrect annotations poison every experiment that makes use of them. Worse still, the poison spreads because incorrect annotations from one organism are often unknowingly used by other projects to help annotate their own genomes. Standard practices for genome annotation have been proposed for bacterial[90], viral[91] and eukaryotic genomes[92], but even when followed, quality control remains an issue. Even the best gene predictors and genome annotation pipelines rarely exceed accuracies of 80% at the exon level[63], meaning that most gene annotations contain at least one mis-annotated exon. Given these facts, assessing how accurately a genome is annotated is an important part of any project.

Over the years, there have been various contests aimed at assessing gene prediction accuracy[63,65] (BOX 4). These contests have played an important part in improving the power and accuracy of gene prediction. However, less progress has been made regarding genome annotations[64]. The heart of the problem is the absence of reference data sets with which to obtain accuracy estimates. The first generation of genome projects — *Saccharomyces cerevisae*, *C. elegans* and *D. melanogaster*, for instance — all had decades of work to draw on when training and measuring the accuracy of gene predictors and annotation pipelines. However, no such data set exists for most of the organisms being sequenced today. Moreover, just because a gene

Table 1 | **Five basic categories of annotation software and some selected examples**

Software	Description	Refs
Ab initio *and evidence-drivable gene predictors*		
Augustus	Accepts expressed sequence tag (EST)-based and protein-based evidence hints. Highly accurate	66,67
mGene	Support vector machine (SVM)-based discriminative gene predictor. Directly predicts 5′ and 3′ untranslated regions (UTRs) and poly(A) sites	133
SNAP	Accepts EST and protein-based evidence hints. Easily trained	62
FGENESH	Training files are constructed by SoftBerry and supplied to users	72
Geneid	First published in 1992 and revised in 2000. Accepts external hints from EST and protein-based evidence	134
Genemark	A self-training gene finder	69,70
Twinscan	Extension of the popular Genscan algorithm that can use homology between two genomes to guide gene prediction	71
GAZE	Highly configurable gene predictor	74
GenomeScan	Extension of the popular Genscan algorithm that can use BLASTX searches to guide gene prediction	135
Conrad	Discriminative gene predictor that uses conditional random fields (CRFs)	136
Contrast	Discriminative gene predictor that uses both SVMs and CRFs	137
CRAIG	Discriminative gene predictor that uses CRFs	138
Gnomon	Hidden Markov model (HMM) tool based on Genscan that uses EST and protein alignments to guide gene prediction	73
GeneSeqer	A tool for identifying potential exon–intron structure in precursor mRNAs (pre-mRNAs) by splice site prediction and spliced alignment	139
EST, protein and RNA-seq aligners and assemblers		
BLAST	Suite of rapid database search tools that uses Karlin–Altschul statistics	31–33
BLAT	Faster than BLAST but has fewer features	42
Splign	Splice-aware tool designed to align cDNA to genomic sequence	44
Spidey	mRNA-to-DNA alignment tool that is designed to account for possible paralogous alignments	45
Prosplign	Global alignment tool that uses BLAST hits to align in a splice-site- and paralogy-aware manner	140
sim4	Splice-aware cDNA-to-DNA alignment tool	46
Exonerate	Splice-site-aware alignment algorithm that can align both protein and EST sequences to a genome	43
Cufflinks	Extension to TopHat. Uses TopHat outputs to create transcript models	54
Trinity	High-quality *de novo* transcriptome assembler	50
MapSplice	Spliced aligner that does not use a model of canonical splice junction	141
TopHat	Transcriptome aligner that aligns RNA sequencing (RNA-seq) reads to a reference genome using Bowtie to identify splice sites	51
GSNAP	A fast short-read assembler	52
Choosers and combiners		
JIGSAW	Combines evidence from alignment and *ab initio* gene prediction tools to produce a consensus gene model	78
EVidenceModeler	Produces a consensus gene model by combining evidence from protein and transcript alignments together with *ab initio* predictions using weights for both abundance and the sources of the evidence	79
GLEAN	Tool for creating consensus gene lists by integrating gene evidence through latent class analysis	80
Evigan	Probabilistic evidence combiner that use a Bayeisan network to weigh and integrate evidence from *ab initio* predictors, alignments and expression data to produce a consensus gene model	81

Table 1 (cont.) | **Five basic categories of annotation software and some selected examples**

Software	Description	Refs
Genome annotation pipelines		
PASA	Annotation pipeline that aligns EST and protein sequences to the genome and produces evidence-driven consensus gene models	56,82
MAKER	Annotation pipeline that uses BLAST and exonerate to align protein and EST sequences. Also accepts features from RNA-seq alignment tools (such as TopHat). Massively parallel	10,83
NCBI	The genome annotation pipeline from the US National Center for Biotechnology Information (NCBI). Uses BLAST alignments together with predictions from Gnomon and GenomeScan to produce gene models	142
Ensembl	Ensembl's genome annotation pipeline. Uses species-specific and cross-species alignments to build gene models. Also annotates non-coding RNAs	107
Genome browsers for curation		
Artemis	Java-based genome browser for feature viewing and annotation. Can use binary alignment map (BAM) files as input	99
Apollo	Java-based genome browser that allows the user to create and edit gene models and write their edits to a remote database	97
JBROWSE	JavaScript- and HTML-based genome browser that can be embedded into wikis for community work. Excellent for Web-based use	87
IGV	Genome browser that supports BAM files and expression data	143

These tools are widely used both as standalone applications and as modular components of genome annotation pipelines. See FIG. 2 for a schematic of the roles of each class of tool in genome annotation.

predictor does well on one genome is no guarantee of a good performance on the next[83]. Assessing annotation quality in the absence of reference genome annotations is a difficult problem. Experimental verification is one solution, but few projects have the resources to carry this out on a large scale.

Approaches for assessing annotation quality. One simple approach for obtaining a rough indication of annotation quality is to quantify the percentage of annotations that encode proteins with known domains using tools such as InterProScan[93] and Pfam[94] or tools such as MAKER, which provides an automated means for carrying out such analyses[83]. Although relative numbers of domains vary between organisms and the expansion and contraction of particular gene families have a well-established role in organismal evolution, among the eukaryotes, the overall percentage of proteins that encode a domain of any sort is reasonably constant[83]. The domain content of the human, *D. melanogaster*, *C. elegans*, *A. thaliana* and *S. cerevisiae* proteomes varies between 57% and 75%[95]. Poorly trained gene finders do not perform nearly this well — 5% to 25% is typical. Thus, a eukaryotic proteome with a low percentage of domains is a warning sign that it could be poorly annotated[83].

Although domain content provides a rough estimate of overall annotation quality, it provides little guidance when trying to judge the accuracy of a given annotation. One approach towards solving this problem is to ask whether the protein, EST and RNA-seq evidence support or contradict the annotated intron–exon structure of the gene. This is fairly straightforward to assess by eye, but performing this task in an automated fashion requires a computable metric. In response, the Sequence Ontology Project[85] has developed several metrics for quality control of genome annotations[96]. Annotation edit distance (AED), for example, measures how congruent each annotation is with its overlapping evidence (BOX 4). AED thus provides a means to identify problematic annotations automatically and to prioritize them for manual curation. AED scores can also be used to measure changes to annotations between annotation runs. The MAKER2 genome annotation pipeline[83] provides some useful tools for automatically calculating AED.

Of course, identifying inaccurate annotations is only half of the problem; errors also need to be corrected. The most direct approach to fixing an erroneous annotation is to edit its intron–exon coordinates manually. The Apollo[97], Argo[98] and Artemis[99] browsers are widely used for this purpose. Gene models can be graphically revised using a series of 'drag-and-drops' and mouse clicks, and the resulting edits are written back to either files or to a remote database connection[89].

Annotation jamborees. Many genome projects choose to manually review and edit their annotation data sets. Although this process is time- and resource-intensive, it provides opportunities for community building, education and training.

Annotation jamborees (a term that was coined by the *D. melanogaster* community to describe the first such gathering[100]) provide a ready means for manual curation and analysis of the data and for putting together a genome paper. The key to hosting a successful jamboree is infrastructure. At a minimum, attendees must be able to search the annotated proteins and transcripts and to view the annotations in a genome browser. Searches can easily be handled by setting up a

BLAST database server coupled with a graphical user interface (GUI) such as a Web browser. The WWW BLAST server package[101] provides an easy means to do so. GBrowse[86,102] and JBrowse[87] can also easily be configured to allow remote users to view the annotated genome, as can the Apollo genome browser, which also provides a means to edit incorrect annotations. As all of these resources can be set up and configured remotely, it is now possible to support a distributed jamboree, in which the community collaborates via the Internet. This model recently proved to be successful for the ant genome community, which organized a distributed jamboree in which investigators and students collaborated to curate and analyse three different ant genomes quickly, all in a distributed manner[103–106].

Making data publicly available

Successful genome annotation projects do not just end with the publication of a paper; they also produce publicly available annotations. Genome annotations fuel the bench work and computational analyses that constitute the day-to-day operations of molecular biology and bioinformatics laboratories worldwide. They also provide an essential resource for other genome annotation projects; the transcripts and proteins produced by one annotation project will probably be used to help annotate other genomes. There are three basic routes to making annotations publicly available: you can build your own genome database and place it online; you can submit your annotations to GenBank and Ensembl; or you can submit them to any of a growing number of theme-based genome databases. We recommend taking all three routes.

Submitting annotations to public databases. One way to make annotations publicly available is to submit them to GenBank. Parties working on vertebrate genomes are also encouraged to contact Ensembl, which continues to incorporate new species at the rate of 5–10 per year in order to create a comprehensive annotation resource for vertebrate genomes, all of which are annotated by its gene build pipeline[107]. Both GenBank and Ensembl have much to offer to smaller genome projects, including powerful data-marts that allow users to browse and download data. Ensembl and GenBank also automatically handle the heavy lifting that is involved in relating gene models to those of other organisms and identifying homologues, paralogues and orthologues. They also provide an easy means to search and browse data; in short, they integrate a data set into the larger landscape of genomics and genome annotations. Best of all, the entire process is free, and submission to these sites in no way abridges the rights of the generators of the data to host and maintain their own genome database. For the research communities of most organisms, members will prefer to visit the specialized genome database for that organism, whereas the larger biological community will tend to access the data through GenBank and Ensembl. In addition to these large sites, intermediate-sized projects that host, manage and maintain sets of annotated genomes that are all related by a common theme are gaining in popularity. Examples include BeeBase[103], Gramene[108], PlantGDB[109], Phytozome[110] and VectorBase[111].

Updating annotations. Many genomes were annotated so long ago that the existing annotations could be dramatically improved using modern tools and data sets such as RNA-seq. In many cases, improved assemblies are possible as well. The question then becomes how to merge, update and improve the existing annotations and, at the same time, to document the process. Like annotation quality control, this is a thorny problem that until recently has garnered little attention, and few published tools yet exist to automate the process. Among existing tools, GLEAN and PASA can be used to report differences between pre-existing gene models and newly created ones. Ensembl has a procedure to merge annotation data sets to produce a consensus, and PASA has one for updating annotations with RNA-seq data. The MAKER annotation pipeline provides an automated toolkit with all of these functionalities and can revise, update and merge existing annotation data sets, as well as map them forwards to new assemblies[10,83].

GenBank provides two avenues for redistributing the results of updates and re-annotation of genomes. If the group that is updating the annotations includes the original authors, the update can simply be submitted; if not, there are two routes for submission. If the work involves substantial improvements to the original assembly, the parties producing them can submit the new annotations to GenBank as primary authors; if not — that is, if the revisions merely improve the original annotations — those producing them can submit their work through the third party submission channel. Ensembl also allows submission of such data, although the process is less formal, and interested parties should contact Ensembl directly.

Conclusions

In some ways, cheap sequencing has complicated genome annotation. As we have explained, the fragmented assemblies and exotic nature of many of the current genome-sequencing projects are part of the reason that this is so, but it is the ever-widening scope of annotation that is presenting the greatest challenges. Genome annotation has moved beyond merely identifying protein-coding genes to include an ever-greater emphasis on the annotation of transposons, regulatory regions, pseudogenes and ncRNA genes[112–115]. Annotation quality control and management are also increasingly becoming bottlenecks. As long as tools and sequencing technologies continue to develop, periodic updates to every genome's annotations will remain necessary. Those undertaking genome annotation projects need to reflect on this fact. Like parenthood, annotation responsibilities do not end with birth. Incorrect and incomplete annotations poison every experiment that makes use of them. In today's genomics-driven world, providing accurate and up-to-date annotations is simply a must.

Data-mart
Provides users with online access to the contents of a data warehouse through user-configurable queries. A data-mart allows users to download data that meet their particular needs: for example, all transcripts from all annotated genes on human chromosome 3.

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Competing interests statement
The authors declare no competing financial interests.

FURTHER INFORMATION

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