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Gene prediction in the immunoglobulin loci

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Abstract

The V(D)J recombination process rearranges the variable (V), diversity (D), and joining (J) genes in the immunoglobulin loci to generate antibody repertoires. Annotation of these loci across various species and predicting the V, D, and J genes (IG genes) is critical for studies of the adaptive immune system. However, since the standard gene finding algorithms are not suitable for predicting IG genes, they have been semi-manually annotated in very few species. We developed the IGDetective algorithm for predicting IG genes and applied it to species with the assembled IG loci. IGDetective generated the first large collection of IG genes across many species and enabled their evolutionary analysis, including the analysis of the "bat IG diversity" hypothesis. This analysis revealed extremely conserved V genes in evolutionary distant species indicating that these genes may be subjected to the same selective pressure, e.g., pressure driven by common pathogens. IGDetective also revealed extremely diverged V genes and a new family of evolutionary conserved V genes in bats with unusual non-canonical cysteines. Moreover, in difference from all other previously reported antibodies, these cysteines are located within complementarity-determining regions. Since cysteines form disulfide bonds, we hypothesize that these cysteine-rich V genes might generate antibodies with non-canonical conformations and could potentially form a unique part of the immune repertoire in bats. We also

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analyzed the diversity landscape of the recombination signal sequences and revealed their features that trigger the high/low usage of the IG genes.

Introduction

Antibodies (or *immunoglobulins*) are the key components of the immune system of jawed vertebrates that provide adaptive immune response by recognizing and neutralizing *antigens.* Antibodies are not encoded in the germline genome but rather result from somatic *VDJ recombinations* (Tonegawa, 1983). This process affects an immunoglobulin (IG) loci containing the families of the *variable* (V), *diversity* (D), and *joining* (J) genes (referred to as *IG genes*) by selecting one V, one D gene, and one J gene, and concatenating them together to generate one of the antibody chains. Antibodies are further diversified by *somatic hypermutations* (Dudley et al. 2005).

The diversity of the IG loci is driven by the variety of antigens: different species encounter different antigens and develop their unique ways to fight them through mutations in IG genes. As a result, the IG loci have rapidly evolved independently in different species and resulted in a diverse collection of IG genes that remain largely unknown since both sequencing highly-repetitive IG loci and predicting IG genes in these loci are challenging tasks (Das et al., 2012, Pettinello and Dooley, 2014).

Mammalian genomes have three IG loci: *heavy chain* (*IGH*), *kappa light chain* (*IGK*), and *lambda light chain* (*IGL*) as well as four *T-cell antigen receptor* (*TCR*) loci (TRA, TRB, TRG, and TRD). In this work, we mainly focus on the IGH locus. The V, D, and J genes in the IGH locus (and the fragments of the IGH locus containing these genes) are also referred to as IGHV, IGHD, and IGHJ, respectively.

Diversity of immunoglobulin genes. Studies of adaptive immune responses across various vertebrate species open new therapeutics opportunities (Muyldermans and Smider, 2016). For example, studies of single-chain camelid antibodies led to the development of *nanobodies* that are able to diagnose cancer (Rashidian et al., 2015; Keyaerts et al., 2016), while studies of ultralong cow antibodies

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revealed that they recognize various HIV strains (Sok et al., 2017). Understanding the diversity of the adaptive immune systems across various vertebrates can also contribute to analyzing the spread of newly emerged zoonotic pathogens. It is particularly important for bat species that possess a unique immune system capable of neutralizing viruses that are often lethal to other mammals, such as rabies, Ebola, SARS-CoV, MERS-CoV, and SARS-CoV-2 (Teeling et al., 2018).

Schountz et al., 2017 formulated a “bat IG diversity” hypothesis that argues that, since bats have a greater combinatorial diversity of IG genes than other mammals, their large naive antibody repertoires do not need substantial affinity maturation to successfully neutralize antigens. Indeed, while the human IGH locus has only 55 functional V genes, the little brown bat is estimated to have at least 200 V genes (Bratsch et al., 2011), suggesting that bats may be better equipped for clonal selection of B cells responding to viral antigens. However, the IG genes in bats remain poorly characterized, moreover, the only support for the “bat IG diversity” hypothesis comes from a probabilistic model (Bratsch et al., 2011) rather than an annotated IG loci in a well-assembled bat genome.

Annotation of immunoglobulin genes. Annotation of the IG loci (i.e., predicting IG genes) is a prerequisite for most follow-up immunogenomics studies. Since assembly and annotation of the IG loci for novel species is complicated by their highly-repetitive structure (Matsuda et al., 1998; Watson et al., 2013; Rodriguez et al., 2020), the sequences of the IG loci are only known for a few species. However, recent advancements in long-read sequencing enabled the first contiguous assemblies of highly-repetitive genomic regions and the Vertebrate Genome Project (VGP) now aims to generate high-quality reference genomes for all vertebrate species (Rhie et al., 2020). So far, the VGP has generated well-assembled genomes of nearly 150 vertebrate species.

Although many IG loci have been assembled in the last two years, their automated annotation remains an open problem. Previous studies of IG genes combined a time-consuming experimental approach with semi-manual computational analysis, such as in the studies of the platypus (Gambon-Deza et al., 2009), the cow (Ma et al., 2016), and the ferret (Wong et al., 2020). These studies used the human IG genes for a similarity-based detection of IG genes in the novel species and may have missed diverged

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IG genes. For example, since most (if not all) V genes in mammalian species were predicted based on their similarities with human V and J genes, it remains unclear whether there exist still unknown families of V genes that are highly diverged from the human IG genes.

Annotation of recombination signal sequences. De novo prediction of IG genes relies on identifying conserved *recombination signal sequences* (*RSSs*) that flank IG genes and enable the VDJ recombination. Since short RSSs have many spurious RSS-like occurrences in the genome (*cryptic RSSs*), their prediction and downstream IG gene annotation is a challenging problem. It is further complicated by the fact that some cryptic RSSs are implicated in unusual genomic rearrangements outside the IG and TCR loci (Messier et al., 2003) and sometimes play an important role in antibody generation. For example, cryptic RSSs flanking the human *LAIR1* gene participate in the off-target VDJ recombination and generates a new type of antibodies (where *LAIR1* represents an additional domain of an unusual VDJ region) that broadly neutralize *Plasmodium* parasites (Tan et al., 2016). Although Teng et al., 2016 analyzed the impact of off-target VDJ recombinations on lymphocyte genomes, no attempts to analyze the landscape of cryptic RSSs within the IG loci have been made yet.

The problem of identifying RSSs in a genomic sequence was considered by Merelli et al., 2010 (RSSsite tool) and Olivieri et al., 2013 (VgeneExtractor tool). VgeneExtractor further used its RSS predictions for detecting V genes in various species. However, this method does not account for the variations in the RSSs within species and does not report D and J genes. Safonova and Pevzner, 2020 recently benchmarked RSSsite and demonstrated that it results in a high false-positive rate. Even though they developed a more accurate SEARCH-D algorithm for detecting RSSs of D genes, the problem of detecting RSSs for all types of IG genes and the follow-up evolutionary analysis of IG genes across multiple species remains open.

Role of cysteines in immunoglobulins. Cysteines are structurally important amino acids that form *disulfide bonds* in immunoglobulins (Frangione et al., 1969). Conserved cysteines of human immunoglobulins are referred to as *canonical* cysteines (Tonegawa, 1983). Non-canonical cysteines are common in some biomedically important species, such as llamas and cows (de Los Rios, 2015;

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Prabakaran and Chowdhury, 2020). The current consensus is that the additional disulfide bonds in immunoglobulins increase their stability and enrich the complexity of antigen-binding site topology (Wu et al., 2012). Since cysteine patterns represent a structurally important feature of antibodies, we analyzed non-canonical cysteines in the newly identified IG genes.

IGDetective tool. We describe the IGDetective tool for predicting IG genes, apply it for annotating IG loci in the recently assembled mammalian genomes, generate the largest set of IG genes to date, and reveal surprising diversity of IG genes across multiple species, such as a new family of unusual cysteine-rich V genes in bats.

Since all previous attempts to annotate V (J) genes in newly sequenced species relied on their similarities with known human IG genes, it remains unclear whether there exist V (J) genes that are not similar to the human ones. In addition to a similarity-based search for IG genes (IterativeIGDetective mode), IGDetective has a BlindIGDetective mode for annotating IG genes in the absence of any prior knowledge about the sequences of IG genes in other species. We benchmark BlindIGDetective on well-annotated human, mouse, and cow genomes, demonstrate that it automatically derives nearly all known IG genes in these species in a blind fashion, apply it to newly-sequenced genomes to reveal highly-diverged IG genes, and reveal new families of highly diverged V genes in bats that evade the similarity-based approach to predicting IG genes.

Results

From predicting RSSs to predicting IG genes. Sequences of human IG genes were inferred as the result of painstaking manual analysis at the dawn of the DNA sequencing era (Li et al., 2002). Consequently, sequences of all non-human IG genes were inferred based on similarities with human IG genes (Sitnikova and Su, 1998). IterativeIGDetective automates this approach and iteratively extends it by predicting more and more distant IG genes at each iteration. In contrast, BlindIGDetective is designed to predict IG genes even if they share no similarities with currently known IG genes.

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Given a newly assembled genome, both IterativeIGDetective and BlindIGDetective start from predicting RSSs in this genome. Each RSS consists of a conserved *heptamer* followed by a non-conserved spacer (12 nt long in D genes and 23 nt long in V and J genes in the IGH locus), and a conserved *nonamer* (**Figure 1**A). Each V (J) gene has an RSS at the 3' (5') end and each D gene is flanked by RSSs on both ends. We henceforth refer to the 3' (5') flanking signal of a V (J) gene as RSSV (RSSJ) and the 5' (3') flanking signals of a D gene as $RSSD_{left}$ ($RSSD_{right}$).

Since RSS motifs are highly conserved across all mammals, the human RSS motif (**Figure 1**A) can be used for predicting RSS motifs in other mammalian species. IGDetective forms a *motif profile* from all known RSSs in a reference genome and uses this profile to evaluate the *likelihood ratio* of an arbitrary string from a target genome to decide whether this string represents a putative RSS (see Methods). For each genomic position, it computes the likelihood ratio that there is an RSS flanking this position and classifies a position as a candidate RSS if this ratio exceeds a *likelihood threshold* (see Methods).

IterativeIGDetective pipeline. IterativeIGDetective predicts IG genes in the *target* genome by leveraging the knowledge of IG genes in well-studied *reference* genomes (human, mouse, and cow). Although the highly-repetitive IGH loci in a few other genomes have been semi-manually assembled and annotated (e.g., IG loci in pig (Eguchi-Ogawa et al. 2010), goat (Du et al. 2018), and rabbit (Gertz et al. 2013)), it remains unclear how accurate these short-read assemblies are since it is difficult to assemble the IG loci even from long reads (Bankevich and Pevzner, 2020), let alone from short reads. In the absence of accurate IG annotation tools, it is also unclear what are the false positive/false negative rates of the manually predicted IG genes in these assemblies.

Figure 1A illustrates the IterativeIGDetective pipeline with emphasis on detecting V genes. It starts by identifying a contig (or multiple contigs) containing the IGH locus in the target species and finding candidate RSSs in this locus based on their similarity to the known RSSs in the reference species. Afterward, it analyzes the genomic region flanked by the found RSSs to predict the IG genes themselves. In the case of V and J genes (that are longer and more conserved than D genes), it

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classifies a region preceding/following the identified RSS as a novel V/J gene if its similarity with a known V/J gene exceeds a *similarity threshold* determined by percent identity and shared *k*-mers (See Supplemental Method "Identification of candidate V and J genes" for parameters specifying similarity thresholds).

IterativeIGDetective extends the *non-iterative mode* described above (that ends with the similarity-based identification of V/J genes), by the *iterative mode* for identifying novel V/J genes whose similarity with known V/J genes does not exceed the similarity threshold. The iterative mode extends the set of known V/J genes by the newly identified V/J genes and uses this extended set to iteratively repeat the non-iterative mode until no novel V/J genes are found (**Figure 1**A**)**.

The challenge of identifying highly-divergent IG genes. Although IterativeIGDetective identifies many candidate IG genes in target species, it is not capable of detecting distant IG genes that significantly deviate from all canonical human IG genes, e.g., IG genes from a distant family that has not been discovered yet. Reducing the similarity threshold in IterativeIGDetective increases its false positive rate and does not necessarily lead to identifying distant IG genes.

We hypothesize that, similarly to IG genes in known families, highly-diverged IG genes from a novel family should (i) display some degree of pairwise similarity to all other IG genes from the same family, and (ii) be located within a relatively short region of the genome. Based on these two "IG family" criteria, BlindIGDetective identifies novel IG genes which do not resemble known human IG genes by constructing the *similarity graph* described below.

Similarity graph. Given a position *s* in a genome, a parameter *direction* (downstream "-" or upstream "+") and an integer *segment-length* (*L*), we define the *s-fragment* as the segment of length *L* either downstream or upstream from this position depending on the parameter *direction*. We define the *coding length* of an *s*-fragment as the length of the longest out of three *open reading frames* ending at position *s* (in the case *direction*="-") or starting at position *s* (in the case *direction*="+"). For simplicity, below we assume that *direction*="-".

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Given a set *S* of positions in a genome, parameters *direction*, and parameter *segment-length*, we define an edge-weighted *similarity graph* (referred to as the *S-graph*) as follows. Each vertex in this graph corresponds to a position in the set *S* and each edge connects *similar s*-fragments, where similarity is established based on percent identity between *s*-fragments. All isolated vertices are removed from the similarity graph. The edge-weight of an edge (*s,s'*) is defined as the *percent identity* between the *s*-fragment and the *s'*-fragment. Given a parameter *span* (default value 0.5 Mb), vertices in the same connected component of the similarity graph are classified as *co-located* if they are separated by less than *span* nucleotides in the genome.

Given a vertex *v* in a connected component, its *clump* is defined as the set of all vertices co-located with *v* after removing all vertices whose percent identity with any other single vertex in the clump does not exceed the similarity threshold (default value 70%). A vertex with a maximum-size clump in a given connected component is called the *center* of this component (ties are resolved randomly). Two clumps are *linked* if the distance between their center vertices does not exceed a distance threshold (default value 1 Mbp). BlindIGDetective constructs *clusters* using single linkage clustering of linked clumps and analyzes the constructed *large clusters* (of size larger than the default value *smallSize*=3) as putative IG genes within a single IG locus. Clusters are further analyzed as candidates for new IG families as they satisfy the "IG family" criteria specified above.

BlindIGDetective pipeline. Given a position-set *S*, parameter *direction*, and parameter *segment-length*, BlindIGDetective constructs the *S*-graph (**Figure 1**B**).** Below, we limit attention to the case when *S* is the set of starting positions of RSSVs predicted by IGDetective, *direction*= "–" and *segment-length*= 350 bp. Since this setting models the search for V genes, we refer to the resulting *S*-graph (*s*-fragments) as the *V-graph* (*v-fragments*). The default parameters *direction* and *segment-length* will need to be modified for D-graphs and J-graphs since they depend on the position of the gene (5' or 3' end) with respect to the RSS and the typical length of the gene. Since IG genes in known genomes are located within relatively short regions (e.g., human IGHV genes are located within 850 kbp long IGHV locus), a clump of co-located vertices within a connected component of

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the V-graph may reveal a family of V genes in a newly sequenced genome. To generate such clumps for each connected component, BlindIGDetective forms a clump of the center vertex in this component, removes vertices of the constructed clump from this connected component, and iterates until the component has no vertices left. It further combines clumps into clusters as described above.

We benchmark BlindIGDetective on the entire human genome and demonstrate that it reveals the vast majority of human V (J) genes, for example, it finds 52 out of 57 known human IGHV genes without any prior information about their sequences, while limiting possible false positives (with respect to known human V genes) to 7. Some of these false positives may represent novel candidate V genes in the human genome (see section "BlindIGDetective reveals novel candidate V genes in the human genome"). Although BlindIGDetective missed a small number (5) of 57 human IGHV genes with "weak" RSSs, these genes can be easily identified by slightly reducing the likelihood threshold with follow-up similarity search against 52 identified IGHVs. In addition to finding IGHV genes, BlindIGDetective finds V genes from other IG and TR loci and even *orphan* V genes on Chromosomes 15 and 16 (**Supplemental Table S1**) that resulted from segmental duplications of the human IGH locus (Nagaoka et al., 1994). We thus argue that applying this approach to any mammalian species would reveal most V genes in this species, including highly-diverged V genes missed by IterativeIGDetective as well as unusual genes that are affected by off-target VDJ recombinations.

[FIGURE 1]

Figure 1. IterativeIGDetective (A) and BlindIGDetective (B) pipelines. (A) IterativeIGDetective iteratively extends the set of identified IGHV genes. "Known V genes" box represents known V genes in a reference genome. "RSSV motif" box represents a profile formed by the reference RSSVs for human V genes. (A1) After identifying a contig containing the IGH locus in the target genome, IterativeIGDetective identifies candidate RSSs for V genes in this contig based on similarities with the human RSS motif. A region preceding a true positive RSS represents a V gene while a region preceding a false positive RSS does not. (A2) A region preceding a candidate RSS is classified as a human-like V gene if its similarity with a known human V gene

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exceeds a similarity threshold. (A3) Target-like V genes in the target genome are identified based on similarities with human-like V genes detected in step (A2). (A3*) Target-like V genes are iteratively identified based on previously detected target-like V genes, until no new genes are identified. **(B)** BlindIGDetective constructs the V-graph, analyzes connected components in this graph, finds clumps of co-localized fragments in each connected component of this graph, and combines the found clumps into clusters that represent candidate families of V genes. (B1) Candidate RSSVs in the entire target genome are identified based on similarities with the human RSSV motif for V genes formed by the reference human RSSVs (represented by the "RSSV motif" box). (B2) The V-graph is constructed on the vertex-set of all RSSVs. Two vertices (RSSVs) in the V-graph are connected by an edge if fragments preceding these RSSVs are similar. True (false) positive RSSVs form large (small) connected components in the V-graph. (B3) Each connected component in the V-graph is partitioned into clumps of co-located genes. (B4) Non-trivial clumps (containing multiple RSSs from the same connected component and clustered within a short region of the genome) represent putative V genes within a putative IG loci. Note that a vertex is not included in a clump if it is not similar to all other vertices in this clump (like light green vertex in the rightmost clump). At the final step (not shown), BlindIGDetective combines the identified clumps into clusters to reveal IG genes.

Datasets. We extracted known IG genes from the IMGT database (Lefranc et al., 2015) for the three reference genomes and mapped them to the IGH locus of the same species as described in **Supplemental Method "Annotating IG genes in reference genomes", Supplemental Table S2. Table 1** presents information about the mapped IG genes in the reference species that we refer to as *canonical* IG genes. We also generated a *combined set* of IG genes by combining human, mouse, and cow IG genes, thus adding one more "reference" to the human, mouse, and cow references. We refer to the profile of all RSSs in this set (for each type of IG gene) as the *combined RSS* and denote it as *RSS**.

We selected twenty target mammalian species for prediction of IG genes: three great ape species (Kronenberg et al., 2018) and seventeen species assembled by the VGP consortium (Rhie et al., 2020) that represent a wide range of biological orders (**Figure 2**A, **Supplemental Table S3**). For each target species, we identified contigs containing fragments of the IGH loci (*IGH-contigs*) as described in the

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Supplemental Method "Identifying putative IGH-contigs in a genome assembly", Supplemental Table S4.

Benchmarking IterativeIGDetective. We benchmark IterativeIGDetective on the IG loci of the human, mouse, and cow genomes by assuming that one of them represents a reference genome and one of the remaining ones represents a target genome. Since the IG genes and RSSs in these species are known, we evaluate IterativeIGDetective based on (i) maximizing the number of the known RSSs and IG genes predicted by IterativeIGDetective and (ii) minimizing the number of false RSSs and IG genes predicted by IGDetective. To this end, we attempt to maximize the True Positive Rate (TPR) while minimizing the False Discovery Rate (FDR).

We tabulated heptamer and nonamer likelihood thresholds (see Methods) (**Supplemental Table S5**) and visualized the percentage of RSSs and heptamers/nonamers passing the human reference L_{min} likelihood thresholds (**Supplemental Figure S1**). The vast majority of the heptamers and nonamers in the genome have very low likelihood ratios.

Given the identified likelihood thresholds for each reference (human, mouse, cow, or combined), we first launched IterativeIGDetective with these thresholds on the same species by considering them as target species. We then tabulated the number of detected candidate signals in the human IGH locus (in four cases that represent identifications of putative human RSSs using RSS profiles in human, cow, mouse and combined) and computed true positives (candidate signals representing canonical signals), false positives (candidate signals that are not canonical signals), and false negatives (canonical signals that are not candidate signals) in **Supplemental Table S6**. Afterward, we tabulated the RSS detection statistics of IGDetective on all combinations of four references and four targets in **Supplemental Method "Extended benchmarking of IterativeIGDetective", Supplemental Table S7**. Finally, we extracted the V, D, and J genes from the candidate RSSs determined by launching IterativeIGDetective in non-iterative mode (see section "Identification of candidate IG genes" in Methods) with the RSS profile based on the combined reference. **Table 1** provides information about the results of IterativeIGDetective on the reference species and using the combined RSS* profile.

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V genes						
species	**# of canonical genes**	**# of predicted genes**	**# of true positive genes**	**FDR**	**TPR**	**F1**
human	70	57	50	0.12	0.71	0.78
cow	36	23	21	0.08	0.58	0.71
mouse	154	97	92	0.05	0.59	0.73
combined	260	177	162	0.08	0.62	0.74
D genes						
species	**# of canonical genes**	**# of predicted genes**	**# of true positive genes**	**FDR**	**TPR**	**F1**
human	25	17	15	0.11	0.6	0.71
cow	14	12	9	0.30	0.64	0.66
mouse	15	9	8	0.11	0.53	0.66
combined	54	38	32	0.17	0.59	0.68
J genes						
species	**# of canonical genes**	**# of predicted genes**	**# of true positive genes**	**FDR**	**TPR**	**F1**
human	6	6	5	0.16	0.83	0.83
cow	12	4	4	0	0.33	0.5
mouse	4	2	2	0	0.5	0.66
combined	22	12	11	0.08	0.5	0.64

Table 1. Information about IG genes predicted by IterativeIGDetective based on the *combined* RSS* profile. Rows refer to the species in which IterativeIGDetective predicts IG genes based on the RSS* profile. "FDR", "TPR" and "F1" columns represent false discovery rate, true positive rate, and F1 score, respectively (see Methods). Since the same candidate D gene could potentially be reported twice on both the forward and reverse strands, such a D gene is considered a true positive if either reported D gene's start and end index matches a reference gene's start and end index. Some of the true positive predictions represent pseudogenes that either have an in-frame stop codon or do not participate in VDJ recombination. We classify a detected gene as a true positive if (i) its end index is the same as the corresponding reference gene's end index, and (ii) its start index is within 3 nucleotides towards the 5' direction of the corresponding reference gene's start index. This ensures that the gene is predicted with an offset of at most +1 amino acid.

Detecting IG genes in target genomes. We applied IterativeIGDetective using the combined RSS* profile to IGH-contigs of all target species (**Supplemental Method "Analysis of RSSs in reference and target species", Supplemental Table S8**). Since the identified IGH-contigs are usually longer than the IGH loci, the predicted RSSs may include many false positives. For example, the number of predicted RSSV candidates for a single species varies from 69 to 7027 with the median value 995 (**Supplemental Table S3**). However, further similarity-based filtering (described in Supplemental Method "Identification of candidate V and J genes") of regions flanking these candidate RSSVs greatly reduces the number of false positive predictions, resulting in 3–64 V genes per species (the

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298 median value is 34) (**Supplemental Table S3**). In total, IterativeIGDetective found 1021 candidate V
299 genes across twenty target species, including 50 target-like V genes (**Supplemental Table S9**). 34 out
300 of 50 target-like V genes share at least 80% percent identity with other V genes identified at the
301 previous iteration (**Supplemental Table S9**). After filtering candidates with stop codons in the open
302 reading frame, the number of candidate V genes was reduced to 581.

303 The number of predicted RSSJs varies from 54 to 3911 and from 4 to 21 (with the median value 8)
304 after similarity-based filtering, resulting in a total of 174 candidate J genes. After applying the
305 additional filters based on the conservation of the tryptophan-encoding TGG codon in the candidate J
306 genes (**Supplemental Method "Comparative analysis of IGHJ gene candidates", Supplemental**
307 **Figure S2**), the number of candidate J genes was reduced to 60.
308
309 The number of predicted RSSDs varies from 1 to 17 with a median value of 4. IterativeIGDetective
310 identified a total of 137 candidate D genes which were extracted as short regions flanked by the
311 predicted RSSDs (without any filtering). After redefining the boundaries (see **Supplemental Method**
312 **"Computing boundaries of the IGH loci using predicted IG genes"**) of the IGH loci, we discarded
313 45 candidate D genes located outside these loci (to minimize the number of false positive D genes),
314 resulting in a set of 92 candidate D genes.
315
316 For each target species, we also found positions of *constant* (*C*) immunoglobulin genes in its
317 assembly by aligning highly-conserved human IGHC genes using Bowtie2 (Langmead and Salzberg,
318 2012). The number of IGHC genes per species varies from 2 to 19 with the median value 7, resulting
319 in 149 IGHC gene candidates.
320
321 **The IGH loci widely vary in length across mammalian species.** We analyzed the positions of the
322 candidate V, D, J, and C genes within the assembled genome in order to identify the boundaries of the
323 IGH loci and their lengths assuming the standard V→D→J→C ordering (see Methods). Long repeats

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within the IGHV locus often break its assembly into multiple contigs, with one of the contigs containing the first *i* V genes (referred to as the *IGH-start*) and another contig (referred to as an *IGH-end*) containing all (or some of) the remaining V genes as well as D, J, and C genes (**Figure 2**B**).** See **Supplemental Method "Unusual IGH locus in the sloth genome", Supplemental Figure S3**.

The sloth and the spear-nosed bat have the longest IGH loci among analyzed species (6.7 and 4.6 Mbp, respectively), while aquatic animals (vaquita, blue whale, platypus, sea lion, and dolphin) have the shortest IGH loci, varying from 311 kbp for the vaquita to 607 kbp for the dolphin.

Our estimate of the length of the platypus IGH locus (457 kbp) is higher than the previous estimate by Gambon-Deza et al., 2009 (271 kbp). The analysis of the IGH-start contig (containing V genes only) and IGH-end contig (containing V, D, J and C genes) in platypus revealed an unusual feature. It turned out that, the IGH-end contig contains the entire IGH locus in platypus, while the IGH-start contig contains V genes from the *TCRμ* locus, a unique T-cell receptor locus found only in marsupials and monotremes (Miller, 2010). Since previous studies demonstrated that V genes from this locus are more similar to immunoglobulin V genes than TCR V genes (Miller, 2010), this finding illustrates that IGDetective is capable of detecting unusual TCR genes. Nevertheless, to limit analysis to the IGH loci only, we discarded V genes from the platypus TCRμ locus from further analyses.

[FIGURE 2]

Figure 2. Information about the IGH loci in twenty target mammalian species. (A) The phylogenetic tree formed by twenty target and three reference species. The tree was subsampled from the Tree of Life constructed in Hedges et al., 2015. Each species is shown by its common name and a VGP identifier specified in the parenthesis if available. Each species is encoded by a unique color (left vertical color panel) and a color representing its order (right vertical color panel). The list of orders is shown in the upper left corner. Here and below visualization was performed using the BioRender and Iroki (Moore et al., 2020) tools. **(B)** Information about the IGH loci of twenty target species. Each line corresponds to a target species and shows fragments of the IGH locus with positions of candidate V (blue), D (orange), J (green), and C (red) genes. For six out of twenty species, the IGH-end covers less than 80% of the predicted IGH locus length (lynx, blue whale, mastiff

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bat, horseshoe bat, chimpanzee, and grey squirrel). We showed both the IGH-start and the IGH-end for these six species and only the IGH-end for the remaining species. For a better visualization, all IGH loci are shown as having the same length that does not reflect their real lengths (the bar plots next to the IGH loci show the predicted lengths). The map on the right shows the counts of the productive V, D, J, and C genes identified in the IGH locus. A C gene is classified as productive if its translated regions (defined by the closest human C gene) does not contain stop codons. Non-zero counts are shown in green. The green triangles indicate partially found and (likely) partially missing D and J genes in the sloth IGH locus. Although IterativeIGDetective did not identify any J genes in two species (chimpanzee and spear-nosed bat) within the boundaries of the IGH loci, it found a highly conserved candidate J gene in a short contig in the chimpanzee assembly (denoted as 1* in the map of the right). IterativeIGDetective did not identify any candidate J genes in the spear-nosed bat, presumably because all its RSSJs did not pass the likelihood threshold.

Comparative analysis reveals highly-similar V genes in evolutionary distant species. We combined the candidate V genes (referred to simply as V genes) across all target species with known V genes in reference species and constructed a phylogenetic tree on their amino acid sequences using Clustal Omega (Sievers et al., 2011). **Figure 3**A shows the computed tree where leaves (representing V genes) are colored according to the species they belong to and the order of the species. "Cutting" this tree by a horizontal line at a *height threshold* results in subtrees formed by clusters of similar V genes. We classified a cluster as *large* if it contains more than 5 V genes and as *multi-species* (multi-order) if it includes V genes from multiple species (orders). Since large multi-species clusters are the main focus of the comparative analysis, we selected the height threshold 1.12 maximizing the number of these clusters. The resulting 219 clusters include 43 large clusters, 25 large multi-species clusters, and 7 large multi-order clusters (**Supplemental Figure S4**).

For each large multi-species cluster formed by V genes $g_1,...,g_n$ from species $s_1,...,s_m$, we computed its *gene distance* and *species distance*. The gene distance is computed as the $max\{GeneDist(g_i, g_j)\}$ for all pairs of genes $g_1,...,g_n$, where $GeneDist(x, y)$ represents the fraction of non-matching positions in the alignment between genes x and y. The species distance is computed as $max\{SpeciesDist(s_i, s_j)\}$ for all pairs of species $s_1,..., s_m$, where $SpeciesDist(x, y)$ is the distance between species x and y in the tree

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shown in **Figure 2**A. **Figure 3**B illustrates correlation between species distances and gene distances for 25 large multi-species clusters (Pearson's correlation r=0.77, P-value=7.15×10^{-6}). Seven multi-order clusters (referred to as C1-C7) are represented in blue in **Figure 3**B, and, for all of them but one (cluster C7 shown as a blue asterisk), the gene distance is either well-estimated or under-estimated by the regression line. The unusual C7 cluster is formed by V genes from six target species (chimp, gorilla, orangutan, marmoset, red squirrel, and horseshoe bat) that are similar to human genes IGHV3-30, IGHV3-30-3, IGHV3-30-5, and IGHV3-33 (**Figure 3**C). This cluster reveals a surprising conservation of V genes across distant species, e.g., the amino acid sequence of the gene HS_bat_58 in the horseshoe bat has even fewer differences (8) with the human V genes in this cluster than some chimpanzee V genes (9). We thus conjecture that V genes in this cluster are subjected to the same selective pressure, e.g., driven by common pathogens that are faced by the species in this cluster.

[FIGURE 3]

Figure 3. Comparative analysis of mammalian IGHV genes. (A) A phylogenetic tree of IGHV genes in twenty target (581 V genes) and three reference (310 V genes) species. Edges corresponding to clusters C1–C7 described in **(B)** are shown in blue. The scale is shown on the left. The upper and lower horizontal bars show colors of species and their orders, respectively. List of species and their colors are specified below the tree (species from the same order are shown by a colored vertical bar on the left). **(B)** The plot on the left shows the species distance (x-axis) and the gene distance (y-axis) for 25 large multi-species clusters of V genes. Red and blue dots correspond to single-order and multi-order clusters, respectively. The linear regression line is shown in grey. The Pearson's correlation (r) and P-value (P) are shown on the top of the plot. Each of seven multi-order clusters C1–C7 is represented as a pie-chart on the right. An inner (outer) wedge in each pie-chart corresponds to a species (an order) and the wedge size is proportional to the number of V genes it contains. **(C)** Multiple alignment of 21 V genes from the cluster C7. Four human V genes from this cluster are shown on the top. Non-human genes are denoted according to the short names of species, and "HS_bat" refers to the horseshoe bat. A position in a non-human V gene is shown as "." if the amino acid at this position matches the corresponding amino acid in one of four human V genes and by the corresponding amino acid otherwise. Red rectangles show positions of CDR1 and CDR2 according to the IMGT notation. Green bars show positions of two conserved cysteines (one located close to the start of CDR1 and another located close to the end of the V gene).

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A new family of cysteine-rich IGHV genes. 254 out of 309 canonical human V genes (82%) listed in the IMGT database with productive amino acid sequences (including allelic variants) have two *canonical cysteines* located at conserved positions (**Figure 3**C). We classify a V gene as *cysteine-rich* if it contains four or more cysteines and analyze cysteine-rich V genes among all V genes shown in **Figure 3**A. There are no human cysteine-rich V genes and only 2 (1) mouse (cow) cysteine-rich V genes. It remains unclear whether the mouse and cow cysteine-rich V genes represent pseudogenes rather than functional genes, e.g., the cysteine-rich V gene in cow does not contribute to antibody repertoires (Safonova et al., 2022).

715 out of 891 V genes (80%) from both reference and target species have 2 cysteines and only 61 (7%) are cysteine-rich. Cysteine-rich V genes are grouped together in the phylogenetic tree (shown in dark green in **Figure 4**A) and appear only in 2 out of 25 identified large multi-species clusters, including a multi-order cluster C4 (**Figure 3**B) and a single-order cluster that we refer to as C*. Cluster C4 contains 27 V genes from six species: dolphin, blue whale, sloth, horseshoe bat, spear-nosed bat, and mastiff bat (**Figure 4**B). Cluster C* consists of 12 V genes from grey and red squirrels (**Figure 4**B). 25 out of 27 V genes in cluster C4 are cysteine-rich and 11 out of 12 V genes in cluster C* are cysteine-rich.

Figure 4C shows that, in addition to two canonical cysteines at conserved positions, most V genes from clusters C4 and C* have two other cysteines, also at conserved positions (one cysteine in CDR1 and another in CDR2). While several antibodies with a single cysteine in either CDR1 or CDR2 were reported before (Wu et al, 2012; Prabakaran and Chowdhury, 2020), antibodies with cysteines in both CDR1 and CDR2 have not been reported yet. Since cysteines form disulfide bonds, we hypothesize that cysteine-rich V genes might generate unusual antibodies with non-canonical conformations and could potentially form a unique part of bat immunity against the great variety of viruses they host.

The cysteine-rich cluster C4 includes a sloth V gene (denoted as “sloth_38” in **Figure 4**C) with the unusual 6 aa long suffix CVLLCE classified as the beginning of CDR3. The vast majority of known V genes have the conserved CAR suffix and thus contribute to at most three first amino acids of

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CDR3s. Two known exceptions from this rule are the cattle IGHV1-7 gene that contributes to ultralong antibodies in combination with ultralong D gene IGHD8-2 (Wang et al., 2013; Safonova et al., 2022) and the platypus IGHV1-20 gene with unknown functions (Gambon-Deza et al., 2009). We hypothesize that, similar to the cattle IGHV1-7 gene contributing to generation of ultralong antibodies, both sloth mChoDid1_38 and platypus IGHV1-20 V genes with long CDR3 prefixes may generate (alone or in combination with D genes) antibodies with non-canonical structural features. Below we analyze unusual candidate D genes in these species and perform comparative analysis of all detected D genes.

[FIGURE 4]

Figure 4. Two novel cysteine-rich clusters of mammalian IGHV genes. (A) A phylogenetic tree of IGHV genes colored according to the number of cysteines in their amino acid sequences. **(B)** Only 2 out of 25 large multi-species clusters of V genes contain cysteine-rich V genes. The description of pie plots is provided in the caption to **Figure 3**B. The number within the parenthesis next to the species name indicates the number of V genes from this species. **(C)** Multiple alignment of genes from two clusters shown in **(B)**. Three non-cysteine-rich V genes are marked with a grey circle on the left. The gene on top of each cluster is chosen as the sequence of a V gene with the minimum average distance from other genes in the cluster. Green (purple) bars show positions of canonical (non-canonical) cysteines. Some proteins contain cysteines (that are shown in purple) outside these positions. “HS_bat”, “M_bat”, and “SN_bat” in the top alignment refer to V genes of the horse-shoe bat, the mastiff bat, and spear-nosed bat, respectively. “Red_sq” and “grey_sq” in the bottom alignment refer to V genes of the red squirrel and the grey squirrel, respectively.

Comparative analysis of mammalian D genes. IGDetective identified 92 candidate D genes in target species (**Figure 5**A). For the comparative analysis, we combined these D gene candidates with 81 known D genes of three reference species (27 human, 31 mouse, and 23 cow D genes), resulting in a set of 173 D genes. For the sake of simplicity, we refer to both reference and candidate D genes as simply D genes.

Figure 5B shows that only five D genes are shared among two or more species. Since, in difference from V and J genes, D genes are short and very diverse, it remains unclear whether there exist specific

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features of D genes that are shared among nearly all mammalian species. To reveal such features, we searched for common substrings in all 173 D genes. We translated each D gene into three reading frames and extracted all its 4-mers in the amino acid alphabet. In total, we collected 738 4-mers, with 128 of them appearing in multiple species. The maximum number of species represented by a single 4-mer is 6.

We constructed the *Hamming graph* on 128 shared 4-mers by connecting two 4-mers by an edge if they differ in a single amino acid (Safonova et al., 2015). It turned out that all connected components in this graph are small (less than 5 vertices) with exception of two components consisting of 43 and 42 4-mers and covering 68% of all 4-mers appearing in multiple species (**Figure 5**C). These two components cover all highly abundant 4-mers (4-mers that are present in 3–6 species). Below we focus on the first component since the second component represents the same substrings of D genes as the first component but translated in a different reading frame.

The 4-mers in the first component represents 16 out 23 analyzed species and are mostly formed by amino acids G, S, and Y. We refer to D genes that encode these 4-mers as *G/S/Y-rich* D genes. A half of all possible single-nucleotide mutations of cysteine-encoding codons (TGT and TGC) result in codons GGT, AGT, TAT, and TCT encoding amino acids G, S, S, and Y, respectively. These three amino acids are extremely frequent in the longest cattle D gene IGHD8-2 (**Figure 5**C) where they play a special *cysteine-triggering* role in ultralong cattle antibodies: somatic hypermutations create new cysteines from these amino acids, forming new disulfide bonds, and reshaping the resulting antibody (Wang et al., 2013). We conjecture that the G/S/Y-rich D genes may play a similar cysteine-triggering role as the IGHD8-2 gene in cows.

Unusual cysteine-rich D genes in the platypus genome. Platypus and sloth genomes have two V genes with long non-canonical suffixes that represent the beginnings of CDR3s: the platypus gene IGHV1-20 with suffix LAAELLYCR and the sloth gene “sloth_38” with suffix CVLLCE (**Supplemental Figure S5**). The only other known V gene with a long non-canonical suffix is the cow

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gene IGHV1-7 that plays a special role in generating ultralong CDR3s by recombining with the longest known D gene (IGHD8-2) of length 148 nt (Wang et al., 2013, Safonova et al., 2022). This long D gene is highly unusual: all but three amino acids in its translation are either cysteines or cysteine-triggering amino acids G, S, and Y (**Figure 5**C)**.** We thus searched for D genes with similar properties in the platypus and sloth IGH loci. However, IGDetective, which uses rather stringent parameters for RSS search, did not report any unusual (long or C/G/S/Y-rich) D genes. In contrast to IGDetective, SEARCH-D (Safonova and Pevzner, 2020) uses relaxed parameters for finding RSSs at the cost of reporting more false positive D gene candidates.

We thus launched SEARCH-D on the platypus (457 kbp long) and sloth (6.7 Mbp long) IGH loci. SEARCH-D reported 45 and 76 D gene candidates (simply referred to as D genes) for the platypus and the sloth, respectively (including 6 platypus and 1 sloth D gene candidates reported by IGDetective). Since the candidate D genes in the sloth are scattered through the entire IGH locus, we were unable to identify the location of the sloth IGHD locus. (**Supplemental Figure S6**). In contrast, 29 out of 45 candidate D genes in platypus form a dense 60 kbp long cluster pointing to a previously unknown location of the IGHD locus (**Figure 5**D,E).

Similarly to other mammalian IGHD loci (Safonova and Pevzner, 2020), the identified 60 kb long fragment harboring 29 candidate D genes in platypus is a tandem repeat (**Figure 5**D,E), reinforcing the conclusion that this region indeed represents the IGHD locus. We classify a D gene as *cysteine-rich* if it contains at least two cysteines in one of its reading frames (only 3 out of 25 human D genes are cysteine-rich). Clustering 29 candidate D genes in platypus revealed 4 groups of similar D genes with percent identity ≥70% (referred as D1–D4) that include many cysteine-rich D genes: 12 out of 15 D genes in these groups are cysteine-rich and all genes in these groups, similarly to the cow D gene IGHD8-2, have many cysteine-triggering amino acids (**Figure 5**E).

Even though these 15 candidate D genes have rather diverged RSSs (**Figure 5**F), the high level of their sequence conservation indicates that they are likely functional. We assume that, similarly to

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human IGHD2 genes, these D genes can be responsible for generating antibodies with a disulfide bond inside CDR3s (Prabakaran and Chowdhury, 2020). The high number of these D genes suggests that the fraction of such antibodies is likely higher in the platypus repertoires as compared to the human repertoires. This finding agrees with a study by Johansson et al., 2002 (that reported an unusually high percentage of cysteine-rich antibodies in platypus antibody repertoires) and extends it by revealing the germline D genes contributing to the cysteine-rich antibodies. Further analysis of platypus Rep-Seq data will help to determine if these cysteine-rich D genes are recombined with IGHV1-20 (with unusual suffix LAAELLYCR) and shed light on their role in antibody repertoires.

[FIGURE 5]

Figure 5. Comparative analysis of D genes. (A) The distribution of the counts and lengths of D genes for 20 target species. **(B)** D genes shared among two or more reference and target species. Green cells show species containing the corresponding D gene candidates. Species from left to right: chimp, gorilla, human, orangutan, mastiff bat, horseshoe bat, and otter. **(C)** The largest connected component of the Hamming graph on amino acid 4-mers of D genes. The component is shown by the subgraph of the Hamming graph (left subpanel) and the amino acid content at each position of the 4-mer (right subpanel). Vertices of the Hamming graph are colored according to the number of species they represent: from 2 (pale green) to 6 (dark green). The amino acid sequence of the G/S/Y-rich cow D gene IGHD8-2 is shown on the bottom of the right subpanel. Panels D–F illustrate the analysis of D genes in the platypus genome. **(D)** Positions of D genes detected by SEARCH-D in the platypus IGH locus. D genes are colored according to their lengths: 50 nt or less (purple), from 51 to 100 nt (green), and from 51 to 150 nt (orange). **(E)** The dotplot on the left shows the alignment of the ≅60 kbp long platypus IGHD locus against itself. Positions and sequences of genes from four D gene families with two cysteines are shown on the right. **(F)** Motif logos of $RSSD_{left}$ heptamer (L7), $RSSD_{left}$ nonamer (L9), $RSSD_{right}$ heptamer (R7), $RSSD_{right}$ nonamer (R9) for families D1–D4. Positions that do not match nucleotides in the consensus RSSs computed using the combined references are highlighted in grey. Consensus RSSs for the combined reference are shown in **Supplemental Figure S7**.

Benchmarking BlindIGDetective. BlindIGDetective constructed the human V-graph from 28394 sites in the human genome that passed the RSSV likelihood threshold. A connected component is

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classified as either small (of size at most *smallSize*), large (of size larger *smallSize* but smaller than *giantSize*), or giant (of size at least *giantSize*) for default values *smallSize*=3 and *giantSize*=500. The vast majority of candidate RSSs in the human genome are false RSSs that often represent isolated vertices or vertices of giant components that likely originated from spurious RSSs within repeated regions. Indeed, the human V-graph contains 6942 isolated vertices and one giant component on 20768 vertices. The vast majority of vertices in the giant component represent spurious repeats that we have ignored in further analysis. **Supplemental Table S10** provides information about the V-graphs for three reference species (human, mouse, and cow) and two selected target species (the spear-nosed bat and the horseshoe bat).

For each vertex in the V-graph, we define the *percent identity*, *coding length*, *annotation index*, and *conservation index* (see **Supplemental Methods "Analyzing connected components in the similarity graph" and "Speeding-up BlindIGDetective"** for details). The conservation index of a vertex is defined as the percent identity between its *v*-fragment and the closest *predicted* gene (predicted genes could either be canonical genes from reference species or candidate genes detected by IterativeIGDetective). A vertex is *annotated* if its conservation is at least $PI_{annotation}$ (default value = 90%). For annotating human (cow, mouse) vertices, we define conservation with respect to human (cow, mouse) canonical V genes. However, for annotating the target species, we use either a conservation threshold of $PI_{annotation}$ = 90% with respect to IG genes in this species predicted by IterativeIGDetective or a conservation threshold of $PI_{annotation}$ = 80% with respect to human canonical V genes.

A vertex in the V-graph is classified as *accordant* if its coding length exceeds the *minimum coding length* threshold (default value *minCL*=200 bp). Since clusters with short coding lengths are likely formed by spurious RSS (for reference species, all V genes, except one, have length exceeding 208 bp), below we focus on *accordant* clusters (clumps) defined as clusters (clumps) with coding lengths exceeding the *minCL* threshold. We note that accordant clusters (clumps) may contain both accordant and non-accordant vertices.

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We launched BlindIGDetective with the RSS profile corresponding to a 23 nt spacer. This setting is aimed at finding V genes in IGH, IGL, TRA, TRB, TRD or TRG loci (that all have RSSs with 23 nt spacer) but not the IGK locus (since RSSs in this locus have a 12 nt rather than a 23 nt long spacer). However, the IGK locus can be easily identified by simply changing a spacer length from 23 to 12 nt in BlindIGDetective.

An annotated vertex in a cluster is said to be part of a specific V gene locus (IGH, IGL, TRA, TRB, or TRG) defined by its most similar (as measured by percent identity) annotated V gene, where the set of annotated V genes can be sourced either from a canonical set of V genes (if available) or predictions made by IterativeIGDetective. A cluster is classified as *annotated* by a specific V gene locus if all its annotated vertices are assigned to this locus.

BlindIGDetective reveals novel candidate V genes in the human genome. BlindIGDetective identified 79 clumps from 179 connected components in the human V-graph on 684 vertices (after removing isolated vertices and the giant component). It further removed clumps with very small spans (below 1 kb) as such clumps are typically formed by multiple candidate (likely spurious) RSSs located within a short region. Afterward, it combined the remaining clumps into clusters as described in the subsection “The similarity graph”, resulting in 11 (8) large (accordant) clusters in the human genome (**Table 2**). 7 out of 8 accordant clusters revealed seven known loci of human IG genes (IGH and IGL loci are represented by the largest clusters of size 59 and 31, respectively).

Table 2 shows that the cluster representing human IGH (IGL) genes is formed by 5 (5) clumps, including 2 (1) unannotated clumps (**Supplemental Table S11**). There are only 2 (7) unannotated fragments contained in IGH (IGL) annotated clumps, including 1 (3) accordant *v*-fragments. Moreover, unannotated fragments within annotated clumps still show high median conservation of 83% for both IGH and IGL clusters.

Since 2 (7) unannotated fragments in IGH (IGL) annotated clumps have large median conservation, we launched an IgBLAST search on them and revealed significant hits with E-values of at most 4e-76

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(1e-60) and percent identities of at least 84 (75) % against human V genes IGHV3-48 and IGHV1-46 (for 2 unannotated fragments in IGHV clumps) and IGLV1-44, IGLV3-22, IGLV3-21, IGLV3-9, IGLV3-31 and IGLV7-46 genes (for 5 unannotated fragments in IGLV clumps). Alignment of 1 (3) out of 2 (7) unannotated and accordant IGH (IGL) *v*-fragments from annotated clusters revealed that they align with their closest human V gene in a reading frame containing a stop codon. We therefore suggest that these accordant unannotated fragments could represent previously undiscovered V genes (or pseudogenes) that may be affected by RAG proteins during VDJ recombination.

The unannotated clumps representing human IGH (IGL) genes accounted for 5 (2) unannotated *v*-fragments in the IGH (IGL) cluster and included one accordant *v*-fragment in the IGH locus (coding length 597 nt) with a low percent identity with known human genes (under 62%). Unannotated clumps retained a low median conservation under 62% in both IGH and IGL clusters. A similar IgBLAST search of these the 5 (2) unannotated *v*-fragments in the IGH cluster revealed hits for only three *v*-fragments against human IGHV genes IGHV3-7, IGHV3-11, and IGHV3-21 with low E-values of 8×10^{-13}, 2×10^{-18}, and 7×10^{-20} and percent identities of 61%, 62%, and 63%, respectively. Although these three *v*-fragments share some (albeit low) similarity with known human V genes, they are missing in the IMGT database. Moreover, all these *v*-fragments are located in the IGH locus within a short distance from the canonical IGHV3 gene (at distance 25 kbp, 11 kbp, and 61 kbp, respectively). We therefore suggest that they represent distant IGHV3 genes missed by earlier methods for annotating V genes.

The remaining 2 (2) *v*-fragments in the IGH (IGL) loci had high E-values exceeding 0.42. **Supplemental Figure S8** shows two alignments between the two pairs of these *v*-fragments (that extends through the entire sequence) and suggests these 2+2 *v*-fragments could represent undiscovered genes (or pseudogenes) that are not similar to known human V genes and therefore would not have been discovered through V gene finding methods reliant on similarity with previously identified genes.

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See **Supplemental Method "BlindIGDetective results on cow and mouse genomes"** and **Supplemental Table 12** for details of BlindIGDetective benchmarking on other reference species.

Human														
cluster ID	cluster size	# clumps / |L.C|	PI	coding length (nt)	cluster density (%)	cluster span (Mb)	center vertex		AI/AI80	conservation wrt species genes		conservation wrt human genes		locus
							chr	coordinate (Mb)		min	med	min	med	
H0	59	5/27	91	342	29	0.93	14	106.34	0.88/0.92	56	100	56	100	IGH
H1	31	5/21	89	348	18	0.85	22	22.43	0.71/0.84	55	100	55	100	IGL
H2	8	4/2	100	201	14	0.58	15	21.72	0.5/0.5	54	83	54	83	IGH*15
H3	8	3/4	99	345	57	1.93	16	33.83	0.75/0.75	53	100	53	100	IGH*16
H4	6	1/6	95	435	100	0.03	7	38.36	0.83/1	88	100	88	100	TRG
H5	6	2/4	83	360	47	0.18	14	21.90	1/1	100	100	100	100	TRA
H6	4	2/2	88	330	33	0.12	7	142.50	0.75/1	88	100	88	100	TRB
H7	4	2/2	87	252	33	0.28	17	3.22	0/0	54	56	54	56	
Spear-nosed bat														
cluster ID	cluster size	# clumps / |L.C|	PI	coding length (nt)	cluster density (%)	cluster span (Mb)	center vertex		AI/AI80	conservation wrt species genes		conservation wrt human genes		locus
							contig	coordinate (Mb)		min	med	min	med	
PD0	130	12/38	94	357	19	1.672	S13	59.11	0/0.67	53	56	54	82	IGL
PD1	56	5/27	90	186	21	0.834	S16	0.97	0.38/0.46	54	86	55	79	IGH
PD2	50	4/27	94	315	30	0.762	S16	2.33	0.66/0.64	68	95	65	81	IGH
PD3	35	9/8	96	327	16	1.401	S3	145.30	0/0.43	53	55	73	80	TRA
PD4	22	3/8	84	192	19	0.387	MS7	0.38	0.86/0.5	72	100	62	80	IGH
PD5	14	3/8	84	195	30	0.163	MS2	0.08	0.36/0.5	53	88	55	81	IGH
PD6	14	3/5	92	177	23	0.127	MS12	0.06	0.79/0.5	76	99	67	80	IGH
PD7	13	3/8	89	126	15	0.206	MS33	0.06	0.92/0.46	84	96	69	72	IGH
PD8	12	4/5	90	369	23	0.052	MS7	0.01	0/0.67	55	56	56	83	IGL
PD9	10	1/10	93	378	49	0.167	S11	85.66	0/0.1	55	57	72	76	TRB
PD10	9	2/5	79	318	31	0.15	S2	133.59	0.89/0.44	85	100	66	79	IGH
PD11	7	2/5	81	177	52	0.283	S5	85.09	0.71/0.5	54	100	54	83	IGH
PD12	4	1/4	83	210	100	0.048	S3	80.22	0.75/1	90	91	84	87	IGH
PD13	7	2/4	99	222	43	0.629	S14	10.95	0/0	52	53	53	54	
PD14	5	1/5	87	2286	100	0.002	S4	208.06	0/0	51	51	52	53	

Table 2. Information about large clusters derived from the human and spear-nosed bat genomes. BlindIGDetective constructed 14 large clusters (8 accordant clusters) in the human genome (only accordant clusters are shown). "L.C" represents the size of the largest clump in the cluster. Annotation index and annotation80 index are abbreviated to "AI " and "AI80". Annotated clusters are highlighted in blue. Clusters are ordered in the decreasing orders of their sizes. Conservation is shown with respect to predicted V genes from the same species as well as canonical human V genes, with annotation index (annotation80 index) defined with respect to the former (latter). Predicted V genes are the canonical V genes for human and are the candidate V genes predicted by IterativeIGDetective for spear-nosed bat. The locus column classifies the annotated clusters

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as one of the families of human IG or TCR genes described in **Supplemental Table S1** (highlighted in blue). All annotated human clusters, except for H2, have coding length greater than 315 nt, consistent with the range of coding lengths in known V genes. IGH orphons on Chromosomes 15 and 16 are noted as IGH*15 and IGH*16. Human cluster H2 with coding length 201 nt (locus IGH*15) has shorter coding length because they contain many short pseudogenes. The table also shows all 13 large annotated spear-nosed bat clusters, 7 of which are accordant. Only accordant unannotated spear-nosed bat clusters are shown. In the spear-nosed bat's "contig" column, "mPhyDis1_scaffold_<N>" in the VGP assembly version "mPhyDis1.pri.cur.20200504" is shortened to "MS<N>".

BlindIGDetective reveals highly diverged candidate V genes in the spear-nosed bat genome. Bratsch et al., 2011 and Schountz et al., 2017 formulated the "bat IG diversity" hypothesis stating that bat's ability to carry many disease-causing pathogens (while themselves being unaffected) could be linked to their large and diverse immunoglobulin gene-set. However, since assembly of IG loci in any species is challenging (Bankevich and Pevzner, 2020), accurately assembled (let alone, annotated) IG loci in bats remained unavailable until recently. In fact, the only support for the claim that bats have a very large number of IG genes comes from a probabilistic model rather than an annotated IG loci in an assembled bat genome: Bratsch et al., 2011 used this model to predict ≅240 V genes in the little brown bat without having access to its genome. IterativeIGDetective results do not support the "bat IG diversity" hypothesis": it reported from only 9, 10, 32 and 63 IGHV genes across four bat species. We thus applied BlindIGDetective to reveal divergent V genes that IterativeIGDetective may have missed. We focused on the spear-nosed bat (only 34 IGHV genes reported by IterativeIGDetective) as the bat species with the longest IGH locus (4.6 Mbp).

BlindIGDetective constructed 72 clusters in the spear-nosed bat genome, including 17 (29) accordant (large) clusters (**Table 2**). Vertices in these clusters were annotated using the candidate IGHV genes predicted by IterativeIGDetective and canonical human genes to annotate V genes from IGL, TRA, TRB, and TRG loci. A total of 13 large, annotated clusters were observed in the spear-nosed bat genome.

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BlindIGDetective identified 9 large IGH clusters (3 of which are accordant) encompassing 27 clumps and 189 *v*-fragments. It also identified 2 large IGL clusters with 142 *v*-fragments, 1 TRA cluster with 35 *v*-fragments, and 1 TRB cluster with 10 *v*-fragments (all these clusters are accordant). In addition to the large clusters, it identified a small IGH cluster containing 2 *v*-fragments. No small clusters were detected for IGL, TRA, and TRB loci. For each *v*-fragment, the opening reading frame and the start position of the gene were computed using alignment to the closest human germline V gene. A *v*-fragment is classified as *productive* if it has an open reading frame that begins at its start position and terminates at its end position. 147 IGH, 95 IGL, 15 TRA, and 1 TRB annotated *v*-fragments were found within the identified clusters; 29 IGH, 58 IGL, 6 TRA, and 0 TRB annotated *v*-fragments were productive and thus classified as V gene candidates.

BlindIGDetective also identified 44, 47, 20, and 9 unannotated *v*-fragments within the IGH, IGL, TRA, and TRB clusters, respectively (including 1 IGH, 20 IGL, 4 TRA, and 7 TRB productive *v*-fragments). The alignments of these productive unannotated v-fragments against the translated human IG and TCR genes revealed high percent identity (in amino acids) varying from 49% to 77% with the average value 68%. To analyze the origin of unannotated candidate V genes, we combined them with productive annotated gene candidates and constructed phylogenetic trees using Clustal Omega (Sievers et al., 2011) for IGH, IGL, and TRA loci. Annotated and unannotated V gene candidates are interspersed in trees for IGH and IGL loci, indicating that unannotated V gene candidates likely represent highly diverged members of the canonical V gene families (**Supplemental Figure S9**). In the TRA locus, unannotated V gene candidates form an independent subtree suggesting that they represent an unknown V gene family.

Prediction of V genes in bats does not support the "bat IG diversity" hypothesis. Our benchmarking of BlindIGDetective revealed nearly all IG genes identified by IterativeIGDetective and more. However, the "bat IG diversity" hypothesis was not supported by our analysis of the spear-nosed bat genome as we identified a much smaller number of IGHV genes than 200+ V genes predicted in Bratsch et al., 2011 using a probabilistic model applied to the little brown bat. There are

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three possible explanations for a discrepancy between our analysis and the "bat IG diversity" hypothesis: (i) spear-nosed bats have relatively few genes as compared to little brown bats; (ii) probabilistic model in Bratsch et al., 2011 does not adequately approximates the number of IGHV genes; and (iii) many IGHV genes in spear-nosed bats have "diverged" RSSs that do not pass the default RSS likelihood threshold. **Supplemental Method "Applying BlindIGDetective to the horseshoe bat genome"** and **Supplemental Table S13** present BlindIGDetective results on the horseshoe bat and also does not support the "bat IG diversity" hypothesis.

Variations in RSSs trigger high/low usage of human D genes. In addition to analyzing variations in IG genes, we also analyzed variations in RSSs and their effect on antibody repertoires.

The usage of a gene in an antibody repertoire is defined as the percentage of antibodies formed by VDJ recombinations in this repertoire that involve this gene. The IG genes have a highly non-uniform usage that may vary by orders of magnitude, e.g., while the most widely used human D gene (IGHD3-10) is used in ~15% of all human antibodies, some human D genes hardly ever contribute to formation of human antibodies (Safonova and Pevzner, 2019). Since the usage of IG genes is likely affected by the sequence of their RSSs, we analyzed the associations between the gene usage of IG genes and their RSSs. **Supplemental Method "Clustering nonamers in RSSVs"** and **Supplemental Figures S10, S11** illustrate that RSSs can be partitioned into subgroups of highly similar signals within the set of all RSSs. By revealing these subgroups of similar RSSs we can shed light on the correlations between RSSs and the usage of the genes they flank.

Below we focus on analyzing correlations between the usage of D genes and their RSSs by analyzing immunosequencing datasets containing rearranged VDJ sequences from 24 donors from the study by Levin et al., 2017. For each such VDJ sequence, we used the IgScout tool (Safonova and Pevzner, 2019) to identify the D gene that contributed to this sequence. The *individual usage* of a gene for a single dataset is defined as the percentage of total VDJ recombinations derived from this gene in this dataset. Although the individual usages vary, they have a rather low variance across various individuals in a given species (Safonova and Pevzner, 2019). The *usage of an IG gene* is defined as

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699 the average of individual usages across all 24 datasets generated in Levin et al., 2017. The *usage of an*
700 *RSS* is defined as the usage of the gene that this signal flanks.

701 Below we analyze N D-genes in a reference species and consider N pairs ($RSSD_{left}$, $RSSD_{right}$)
702 flanking these genes (similar analysis has been conducted for V and J genes). We refer to heptamers
703 (nonamers) in this pair as *l7* and *r7* (*l9* and *r9*) and consider 16-mers *l7l9* and *r7r9* as well as a 32-mer
704 *l9l7r7r9*, resulting in various signal types. For each signal type, we computed the $N \times N$ matrix of
705 Hamming distances between all pairs of signals and performed k-means clustering on N-dimensional
706 vectors formed by rows of this matrix.

707 We launched the k-means clustering algorithm (20 runs with 200 iterations for each run) for various
708 cluster numbers and selected an optimal number of clusters based on the elbow method (Yuan and
709 Yang, 2019) for all signal types. We determined three *l7* and three *l9* clusters as well as two *r7* and
710 three *r9* clusters. Each *l9* (*r9*) cluster can be decomposed into groups of D genes, where each group is
711 a subset of an *l7* (*l9*) cluster and no pair of groups can be merged. For three *l9* clusters, the relative
712 sizes of the largest groups with respect to the cluster size are 100%, 100%, and 70% (**Supplemental**
713 **Table S14**). For three *r9* clusters, the relative sizes of the largest groups are 53%, 75%, and 100%.
714 We hypothesize that clusters *l7* and *l9* (as well *r7* and *r9*) and the high level of overlap between them
715 can trigger variations in usage patterns of D genes.

716 **Figure 6**B illustrates that 12 out of the total 25 human D genes dominate the vast majority (93%) of
717 the usage. We integrate the usage statistics of the D genes into the clustering process. The distribution
718 of usage between the clusters described earlier is recorded for all combinations of the RSSD signals in
719 the **Supplemental Method "Cluster-based usage of RSSDs", Supplemental Table S15.** The
720 Kruskal-Wallis test on the usage of the signals belonging to each cluster revealed statistically
721 significant associations for both $RSSD_{left}$ and $RSSD_{right}$ heptamer clusters (P-values = 0.042 and
722 0.007, respectively). We also found statistically significant associations for clusters *l9l7r7r9*, *l7l9* and
723 *r7r9* (P-values = 0.02, 0.009, 0.028, respectively). These clusters and their significant usage
724 associations are shown in **Figure 6**A,B.

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Analysis of clusters revealed motifs of RSSs associated with high-usage D genes. Among the heptamers, the highest used $RSSD_{left}$ cluster (accounting for over 80 % of usage) has the general pattern **NACTGTG**, where 'N' stands for an arbitrary nucleotide. The most used $RSSD_{right}$ heptamer cluster (accounting for over 94% of usage) has each heptamer equal to **CACAGTG**. The highest used nonamer cluster for $RSSD_{left}$ follows a pattern **GGTTTNNNN**, whereas the $RSSD_{right}$ nonamer cluster follows a pattern **NNNAAAACN**.

Analysis of V and J genes did not reveal any associations between RSS clusters and usage patterns (**Supplemental Method "Finding association between RSSs and usages of V and J genes", Supplemental Tables S16–17, Supplemental Figures S12–16**).

[FIGURE 6]

Figure 6. Clustering and distribution of human RSSDs. (A) Cluster visualization of *l7*, *r7*, *l9l7r7r9*, *l7l9 and r7r9* signals. We shall henceforth refer to the red, blue, green and yellow clusters as clusters 1, 2, 3, and 4, respectively. The consensus of a cluster is noted as the legend label. PC1 and PC2 refer to the first 2 principal components of the clustering performed on the signals, as described in subsection "Variations in RSSs trigger high/low usage of human D genes" in Results. **(B)** Usage of D genes with respect to clusters on *l7*, *r7*, *l9l7r7r9*, *l7l9 and r7r9* signals. The p-value of correlation is depicted on the top right of each panel - P* (P**) represents a p-value less than 0.05 (0.01). (**B:Bottom-right**) usage of human D genes. Each of 12 highly used human D genes (with usage at least 2 %) is represented by a single bar. All remaining low-usage human D genes are represented by a single bar showing their combined usage equal to 7%.

Discussion

IGDetective algorithm. We benchmarked IterativeIGDetective on three well-annotated IGH loci (human, mouse, and cow) and demonstrated that it accurately predicts the known V, D, and J genes in one of these species based on information about RSSs in other species. This observation justifies our comparative immunogenomics approach to annotating the IGH loci in newly sequenced species. Although the IterativeIGDetective analysis in this paper is limited to the IGH loci, we plan to extend it to other IG and TCR loci in a follow-up study. In addition to the three reference species, we applied

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751 IterativeIGDetective to twenty mammalian species with the recently assembled IGH loci and
752 predicted 581, 92, and 60 new putative IGHV, IGHD, and IGHJ genes, respectively.

753 In addition to IterativeIGDetective, we developed BlindIGDetective algorithm for predicting novel
754 genes which have diverged from currently known IG genes. BlindIGDetective detects most IG genes
755 in the absence of any information about IG genes in other species and thus opens a possibility to
756 identify highly divergent IG genes. Application of BlindIGDetective to detect V genes from three
757 reference species and two bat species (spear-nosed bat and horseshoe bat) revealed that
758 BlindIGDetective was sensitive not only to canonical V genes from the IGH locus but also to other V
759 gene loci driven by RAG proteins, such as V genes in the IGL, TRA, TRB, and TRG loci. Moreover,
760 BlindIGDetective identified multiple highly-divergent candidate V genes (or pseudogenes) in various
761 species.

762 We plan to combine IterativeIGDetective and BlindIGDetective to extend this analysis to
763 immunological model organisms such as rabbits and llamas as well as non-mammalian vertebrates
764 with the goal to construct a comprehensive database of predicted IG genes across multiple species.

765 **The diversity of IG genes.** We applied IterativeIGDetective to twenty mammalian species with
766 poorly-studied IGH loci, performed comparative analysis of the detected IGHV genes, and identified
767 a highly conservative cluster that covers highly divergent species ranging from primates to bats. We
768 hypothesize that V genes in this cluster are subjected to selective pressure driven by common
769 pathogens or the genetic organization of IGH loci. Further investigation of this conservative cluster
770 will require repertoire sequencing (Rep-Seq) data.

771 In addition to revealing the diversity of IG genes, we also studied the diversity of RSSs, identified
772 clusters of similar RSSDs in humans, revealed associations between these clusters and the usage of
773 the IGHD gene they flank, and found the RSSDs motifs triggering high usage of human D genes.

774 **Unusual cysteine-rich V genes.** We revealed a new family of unusual cysteine-rich V genes in bats
775 and other species that have cysteines in both CDR1 and CDR2. We hypothesize that cysteine-rich V

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genes might generate unusual antibodies with non-canonical conformations and could potentially form a unique part of bat immunity against the great variety of viruses they host. Further investigation of antibodies derived from these V genes would require both Rep-Seq data and protein structures to shed light on the functions and to estimate the therapeutic potential of such antibodies.

G/S/Y-rich motifs in D genes. We demonstrated that, despite being highly diverse, D genes in various mammalian species share the G/S/Y-rich motifs that are formed by cysteine-triggering codons. In cows, the G/S/Y-rich IGHD8-2 gene plays a special cysteine-triggering role in ultralong cattle antibodies where somatic hypermutations create new cysteines from these three amino acids, forming new disulfide bonds and reshaping the resulting antibody (Wang et al., 2013). We conjecture that found G/S/Y motifs may play a similar cysteine-triggering role in D genes of many mammals.

Limitations and future developments. In the last three decades, gene prediction algorithms have evolved from relatively simple statistical tests to sophisticated machine learning approaches (Mathé et al., 2002). However, since even the state-of-the-art gene prediction algorithms generate some false positive genes, they require validation using complementary experimental approaches, such as transcriptome sequencing (Allen et al., 2004) and mass spectrometry (Tanner et al., 2007). Likewise, since IGDetective is merely the first step toward uncovering the diversity of IG genes across vertebrate species, it has to be complemented by complementary experimental approaches, such as antibody repertoire sequencing. Our next goal is to modify IGDetective for working with both genome assemblies and Rep-Seq data.

IterativeIGDetective is currently based on constructing the profile matrices of known RSSs, detecting novel RSSs using these profile matrices, and follow-up analysis of genomic sequences flanked by these RSSs. In the future, we plan to develop a Hidden Markov Model for joint analysis of RSSs and the flanking genes. We also plan to complement the existing analysis (based on only three reference genomes without re-training) by bootstrapping when the original model is trained on the references only but the set of references is later extended (based on the reliable predictions of IG genes in new species) for further re-training.

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IterativeIGDetective failed to identify any J genes in the spear-nosed bat genome, presumably because all its RSSJ motifs did not pass the default likelihood threshold. Since our current goal is to focus on highly conserved RSSs and minimize the false positive rate, we did not recompute all IG gene predictions in the spear-nosed bat with a lower likelihood threshold. In the future, we plan to develop a version of IterativeIGDetective that iteratively relaxes the RSS search parameters.

BlindIGDetective currently starts from identifying highly-conserved RSS in the entire genome and thus misses all IG genes flanked by less conservative RSSs. Lowering the RSS likelihood threshold leads to explosion of false RSS thus making BlindIGDetective prohibitively slow. We plan to modify BlindIGDetective (with the goal of identifying the missed IG genes with less conservative RSSs) by first identifying the IG-contigs, enriching the set of putative RSSs in these contigs by adding less conservative RSSs, and combining BlindIGDetective and IterativeIGDetective in a single pipeline.

Methods

Gene nomenclature. Followed guidelines of the IMGT nomenclature (https://www.imgt.org/IMGTScientificChart/Nomenclature/IMGTnomenclature.html), we have not italicized genes of immunoglobulin (IG) and T-cell receptor (TCR) genes.

Profiles and likelihood ratio of strings. Given a set of *k*-mers (*k*-nucleotide-long strings), their *profile matrix* is a 4×*k* matrix *Profile* defined below. The j^{th} column in the profile matrix represents the j^{th} position in the *k*-mer and the 4 rows represent the 4 nucleotide bases (A, C, G, and T). *Profile*(*i*,*j*) represents the frequency of occurrence of the i^{th} base at the j^{th} position in the input *k*-mers adjusted for pseudocounts as described in Compeau and Pevzner, 2018. The *consensus string* (referred to as *consensus*=*consensus*(*Profile*)) is a *k*-mer generated by taking a nucleotide with the highest frequency at each position of the profile (ties are broken arbitrarily). As opposed to numerous V and D genes, there are few J genes in the reference species, leading to profile matrices with higher entropies (when compared to V or D gene profiles) due to pseudocounts.

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Given a *k*-mer $S=s_1...s_k$ and a 4×*k* profile *Profile*, the probability that *Profile* generates a string *S* is defined as $prob(S|Profile) = \prod_{j=1,k} Profile(s_j,j)$. Since $prob(S|Profile)$ is maximized when the string *S* is a consensus of *Profile*, we define the *likelihood ratio* $L(S)$ of *S* as

$$L(S) = \frac{prob(S|Profile)}{prob(consensus(Profile)|Profile)}.$$

Given a profile *Profile* and a *likelihood ratio threshold* L_{min}, a *k*-mer *S* is classified as a *candidate* for *Profile* if its likelihood ratio $L(S)$ exceeds L_{min}.

RSS detection algorithm. Given a set of RSSs for each of a reference species (human, mouse, cow, or combined), we compute their profile matrix (with pseudocounts equal to 1) for heptamers and nonamers across all signals (RSSV, $RSSD_{left}$, $RSSD_{right}$, and RSSJ). Given a *profile-pair* (*Profile*,*Profile'*) of 4×7 and 4×9 stochastic matrices and the associated thresholds L_{min} and L'_{min} for a heptamer and a nonamer, a *string-pair* (*heptamer*, *nonamer*) is classified as a *candidate RSS* for a given signal type if both *heptamer* and *nonamer* are candidate strings.

We say that a candidate $RSSD_{left}$ and a candidate $RSSD_{right}$ together form a *paired candidate RSSD* if the $RSSD_{right}$ is located within at most $MaxLength_D$ positions to the right from the $RSSD_{left}$. The condition is important for D genes since they are flanked by $RSSD_{left}$ and $RSSD_{right}$ and since the vast majority of known D genes are short (maximum lengths are 37 nt and 29 nt in human and mouse genomes, respectively). Since the longest currently known D gene is the 148 nt long D gene in cows, we set the default value $MaxLength_D$ =150.

Selection of the likelihood ratio threshold. We select the L_{min} thresholds for heptamers or nonamers based on the reference genome chosen. We launch IterativeIGDetective on the reference species, selecting the heptamer L_{min} and nonamer L'_{min} thresholds for all signal types (RSSV, $RSSD_{left}$, $RSSD_{right}$, and RSSJ) by performing a grid search within a range (0,0.8] in steps of 0.005. We compute the TPR (the fraction of known RSSs in the target species detected by IGDetective), the FDR (the fraction of erroneous RSSs among all RSSs reported by IGDetective), and the F1 score defined as:

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$$F1 = \frac{2TPR(1-FDR)}{TPR+(1-FDR)}.$$

For any given reference, the heptamer L_{min} and nonamer L'_{min} thresholds (determined through grid search) that maximize the F1 score are selected as that references' heptamer L_{min} and nonamer L'_{min}. More details on parameter selection are described in **Supplemental Method "Parameter selection for identifying candidate RSSs''.**

Identification of candidate IG genes. Details of procedures for identification of candidate V, D, and J genes, including selection of parameters of alignments are described in **Supplemental Methods "Identification of candidate V and J genes", "Identification of candidate D genes", "Iterative extension of the set of candidate IG genes", "Parameter selection for identifying candidate IG genes", "Aligning candidate IG genes",** and **"Alternate similarity thresholds for detection of V genes"**.

Software Availability. IGDetective is available as Supplemental Code and at github.com/Immunotools/IgDetective. Sequences of identified IGHV, IGHD, and IGHJ genes are available as **Supplemental Tables S19–21** and at github.com/Immunotools/IgDetective_results.

Competing interest statement. The authors declare no competing financial interests.

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A

IterativeIGDetective pipeline

Known V genes in reference genome

RSSV motif
(Formed by known RSSV in reference genome)

heptamer

nonamer

23 nt

Target genome

(A1)

(A2)

Identified V genes

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(A3)

(A3*)

- RSS

- Human-like V gene

- Target-like V gene

B

BlindIGDetective pipeline

RSSV motif
(formed by known RSSV in reference genome)

heptamer

nonamer

23 nt

Target genome

(B1)

(B2)

V-graph

Co-localized fragments

(B3)

Clumps

(B4)

Candidate V genes

- RSS

- RSS-flanking fragment

A

Orders:
- Artiodactyla
- Carnivora
- Chiroptera
- Monotremata
- Pilosa
- Primates
- Rodentia

stoat (mMusErm1)
otter (mLutLut1)
sea lion (mZalCal1)
lynx (mLynCan4)
vaquita (mPhoSin1)
dolphin (mTurTru1)
blue whale (mBalMus1)
cow
mastiff bat (mMolMol1)
pipistrelle (mPipKuh1)
spear-nosed bat (mPhyDis1)
horseshoe bat (mRhiFer1)
human
chimpanzee
gorilla
orangutan
marmoset (mCalJac1)
lemur (mLemCat1)
red squirrel (mSciVul1)
grey squirrel (mSciCar1)
mouse
sloth (mChoDid1)
platypus (mOrnAna1)

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B

	all predicted IG genes	IGH length	productive IG genes			
stoat			15	2	2	3
otter			29	8	2	4
sea lion			3	7	2	6
lynx			17	4	1	4
vaquita			6	1	2	4
dolphin			27	1	2	5
blue whale			13	1	2	6
mastiff bat			10	8	3	5
pipistrelle			9	1	5	7
spear-nosed bat			32	0	0	5
horseshoe bat			63	4	2	5
chimp			39	2	1*	19
gorilla			47	14	4	13
orangutan			47	12	5	19
marmoset			50	8	5	18
lemur			20	3	5	10
red squirrel			36	1	3	5
grey squirrel			21	5	5	5
sloth			60	2	2	4
platypus			37	8	6	2
	V D J C	0 2 4 6 (Mbp)	V	D	J	C

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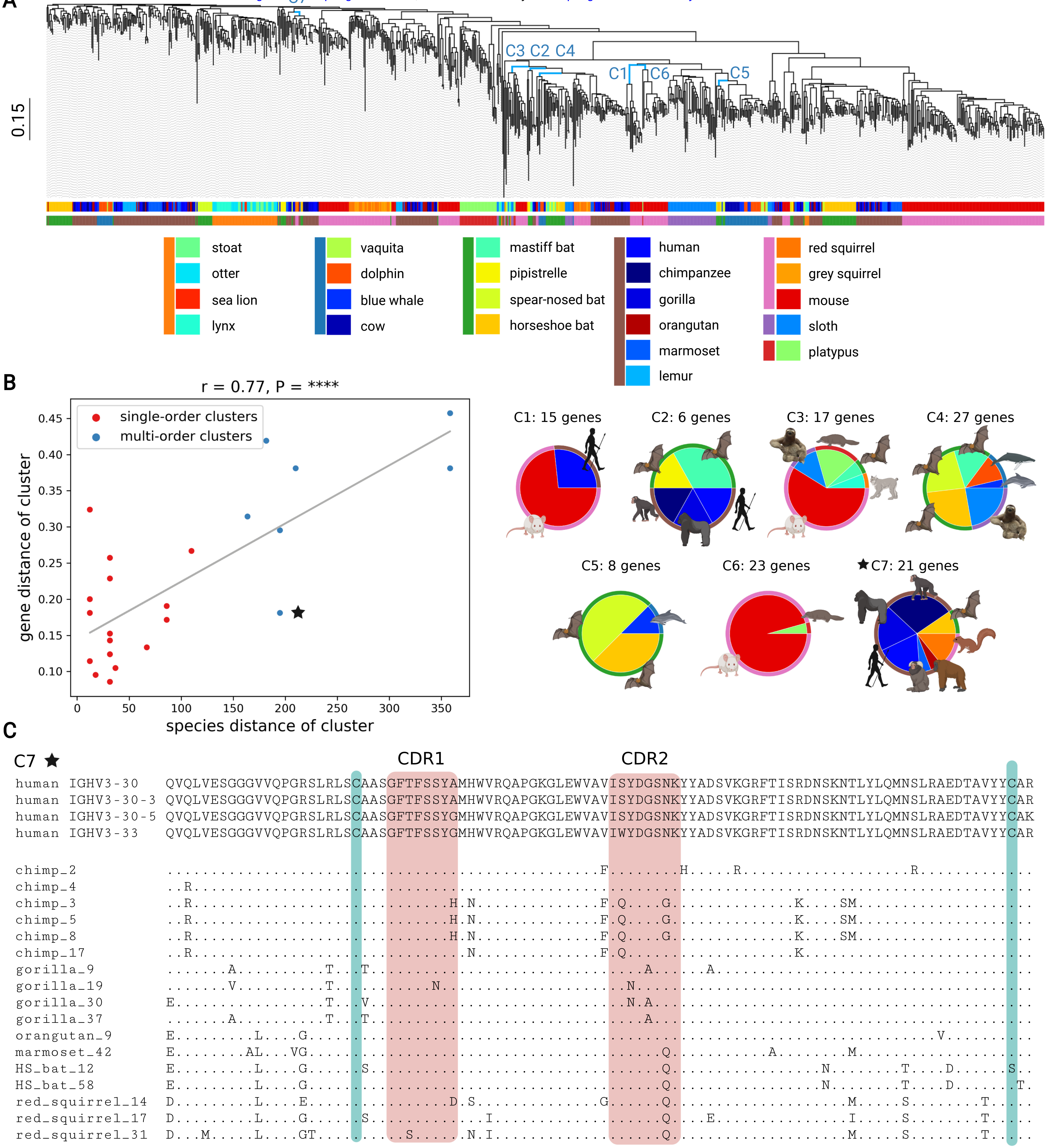

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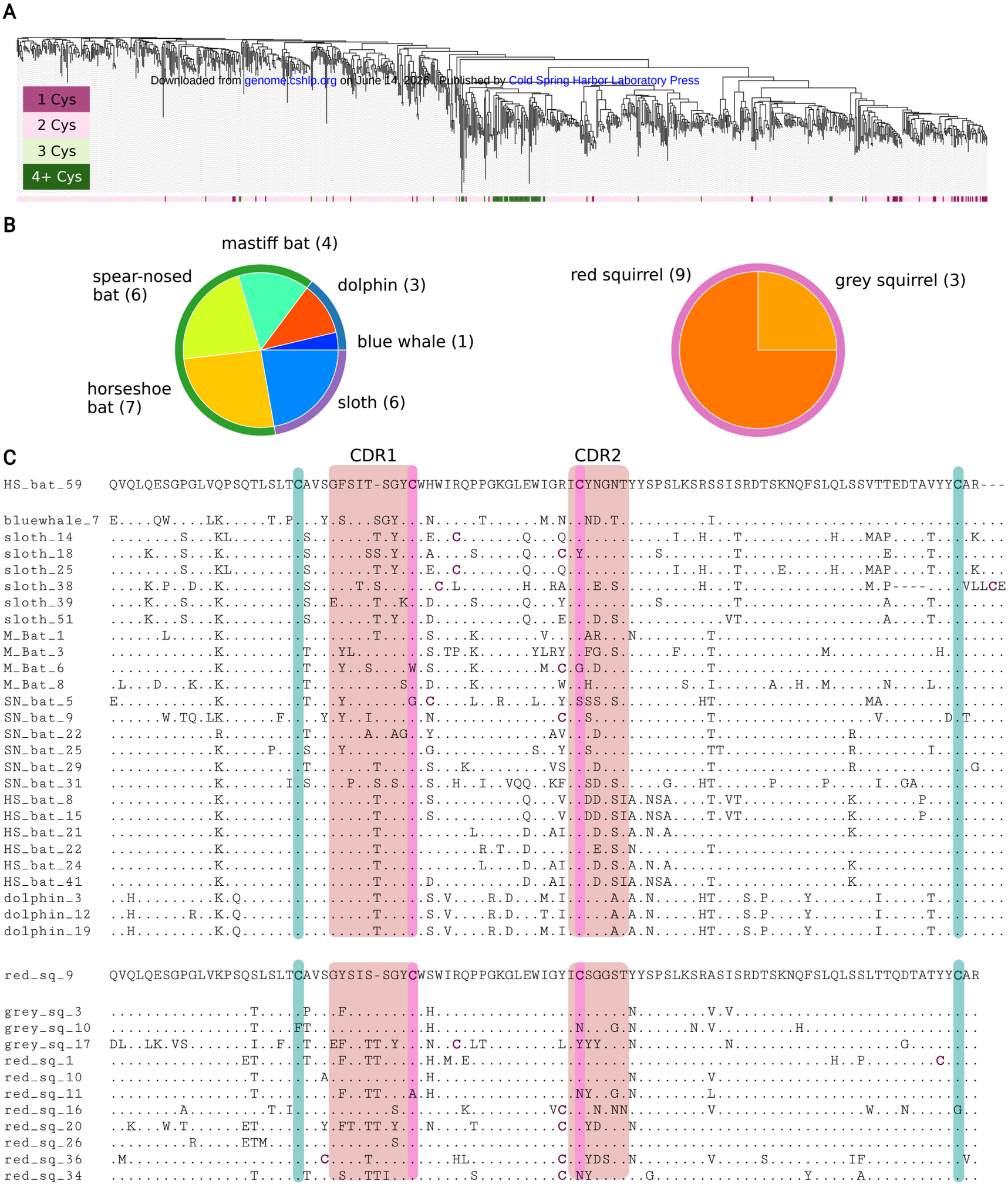

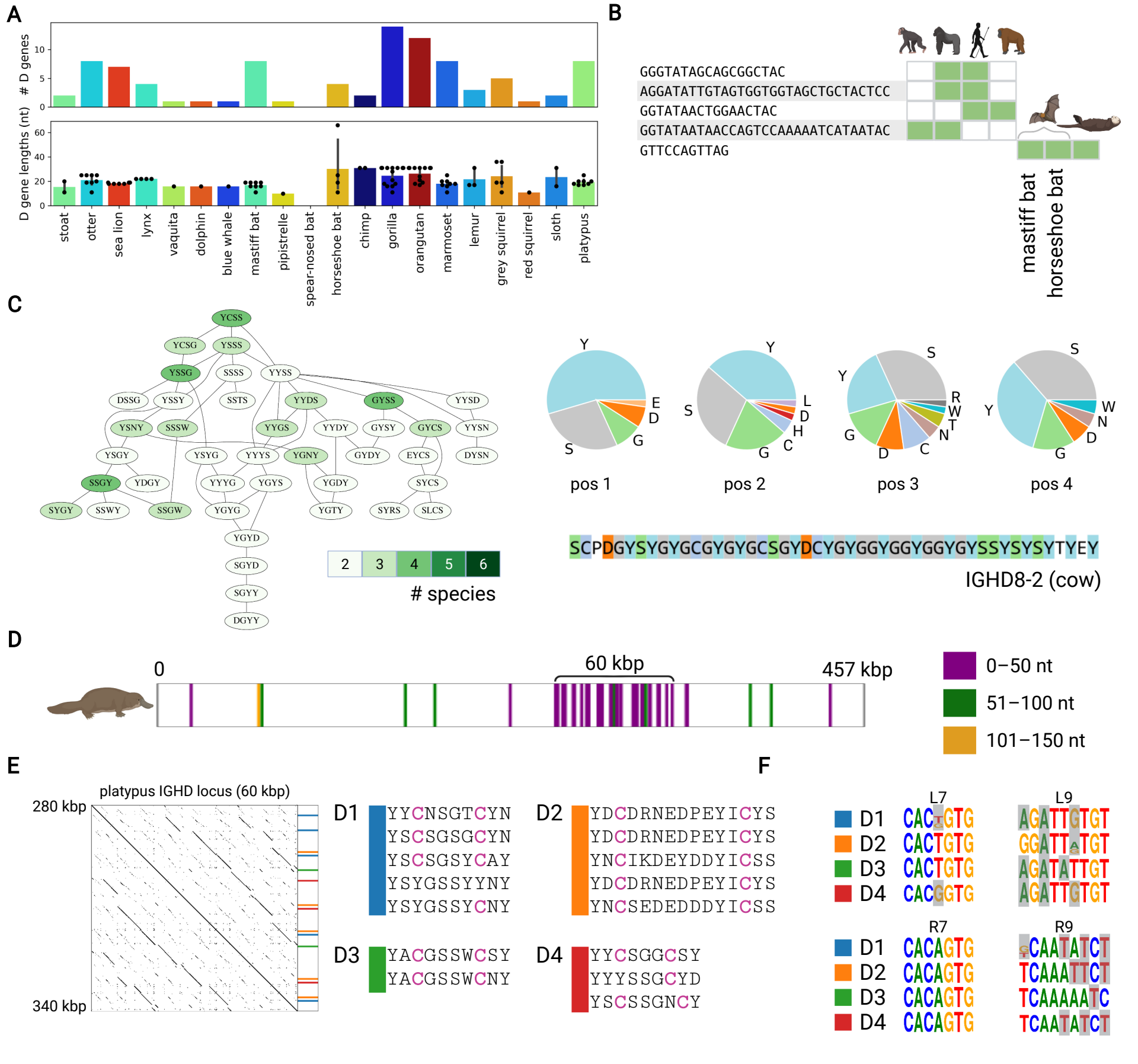
A
D genes
D gene lengths (nt)
stoat
otter
sea lion
lynx
vaquita
dolphin
blue whale
mastiff bat
pipistrelle
spear-nosed bat
horseshoe bat
chimp
gorilla
orangutan
marmoset
lemur
grey squirrel
red squirrel
sloth
platypus
B
GGGTATAGCAGCGGCTAC
AGGATATTGTAGTGGTGGTAGCTGCTACTCC
GGTATAACTGGAACTAC
GGTATAATAACCAGTCCAAAAATCATAATAC
GTTCCAGTTAG
mastiff bat
horseshoe bat
C
YCSS
YCSG
YSSS
YSSG
SSSS
YYSS
DSSG
YSSY
SSTS
YYDS
GYSS
YYSD
YSNY
SSSW
YYGS
YYDY
GYSY
GYCS
YYSN
YSGY
YSYG
YYYS
YGNY
GYDY
EYCS
DYSN
SSGY
YDGY
YYYG
YGYS
YGDY
SYCS
SYGY
SSWY
SSGW
YGYG
YGTY
SYRS
SLCS
YGYD
SGYD
SGYY
DGYY
2
3
4
5
6
species
pos 1
pos 2
pos 3
pos 4
SCPDGYSYGYGCGYGYGCSGYDCYGYGGYGGYGGYGYSSYSYSYTYEY
IGHD8-2 (cow)
D
0
60 kbp
457 kbp
0–50 nt
51–100 nt
101–150 nt
E
platypus IGHD locus (60 kbp)
280 kbp
340 kbp
D1
YYCNSGTCYN
YSCSGSGCYN
YSCSGSYCAY
YSYGSSYYNY
YSYGSSYCNY
D2
YDCDRNEDPEYICYS
YDCDRNEDPEYICYS
YNCIKDEYDDYICSS
YDCDRNEDPEYICYS
YNCSEDEDDDYICSS
D3
YACGSSWCSY
YACGSSWCNY
D4
YYCSGGCSY
YYYSSGCYD
YSCSSGNCY
F
L7
L9
D1
D2
D3
D4
R7
R9

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