Review Article
iDarwin Volume 1, pp. 3-36
Published on February 12, AS 0021 (2021 AD)

Conserved noncoding sequences: Evolving puzzles

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Abstract Majority of the molecular evolutionary studies in the pre-genomic era were focused on the protein-coding parts which account for less than 2% of the human genome and are mostly evolutionarily conserved. However, the abundance and the properties of noncoding functional sequences were a puzzle. With the advent of sequencing technologies and the release of vertebrate genome sequences, it became obvious that certain noncoding parts of the genomes tend to be evolutionarily conserved over hundreds of millions of years. These regions of the genome with specific properties, termed conserved noncoding sequences (CNSs), are usually computationally discovered. Unlike genes with specific transcription units, the definitions of CNSs are vague and thresholds are often set to delimit the regions under conservations and other regions that are neutrally evolving. Thought to be regulatory elements, many of these sequences have been shown to be transcribed and deletions have occasionally had no observable effects. The puzzles surrounding CNSs are evolving. In this review, I discuss the various definitions of CNSs and the computational approaches available for discovering them with the accompanied puzzles. The reported features of the CNSs and the reported functionalities are discussed. I conclude this review by discussing the current puzzles in the studies of CNSs.

Keywords: Conserved noncoding sequences; computational search; junk; regulatory elements; gene deserts

Introduction

Numerous studies reported in the pre-genomic eras have suggested that genomic functional sequences might reside outside the protein-coding regions (Dawson et al. 1995; Hezroni et al. 2017; King and Wilson 1975; Lou et al. 1995; Oeltjen et al. 1997a). If the sequences are functionally important, they would not evolve neutrally (Kimura 1983). Rather, obvious signatures of evolutionary constraints would be expected (Kimura and Ohta 1974). The regions outside the protein coding parts of the genomes that show detectable signatures of evolutionary constraints are referred to as conserved noncoding sequences, abbreviated as CNS (Hardison 2000; Loots et al. 2000) or conserved noncoding elements abbreviated as CNEs (McEwen et al. 2006; Vavouri et al. 2007). Some of the other variants of the name (**Table 1**) include ultraconserved elements or UCEs (Bejerano et al. 2004; Siepel et al. 2005), long conserved noncoding sequences or LCNSs (Sakuraba et al. 2008), highly conserved noncoding regions or HCNRs (De La Calle-Mustienes et al. 2005). Each of the names is often defined by the percent identity and/or length thresholds (**Table 1**). For some studies (Lowe et al. 2011; Siepel et al. 2005), there is no specified constant thresholds. Rather, definition of constraint is based on models that are able to distinguish functional regions from neutrally evolving ones (**Table 1**). In cases with variable thresholds, the studies used specific models to delimit conserved regions from neutrally evolving regions. Such models might have no rigid and constant identity and/or length thresholds. For Siepel et al. (2005), sliding window of 5 bp was used.

Unlike mRNA, lncRNAs or transcribed enhancers, CNSs are not necessarily transcribed but are rather computationally discovered. Using various computational strategies, conserved noncoding regions have been reported in numerous taxonomic groups such as mammals (Babarinde andSaitou 2013, 2016; Bejerano et al. 2004; Loots et al. 2000; Mahmoudi Saber and Saitou 2017; Saber et al. 2016), insects (Brody et al. 2020; Glazov et al. 2005), plants (Hettiarachchi et al. 2014; Van de Velde et al. 2016) and other species (Siepel et al. 2005; Vavouri et al. 2007).

Table 1: Representative names for the conserved noncoding part of the genomes

Name	Identity constraint (%)	Length threshold (bp)	Representative reference
Ultraconserved element (UCE)	100 (human, mouse and rat)	200	(Bejerano et al. 2004; Glazov et al. 2005)
Ultraconserved noncoding element (uCNE)	95 (human and chicken)	200	(Dimitrieva and Bucher 2013)
Conserved noncoding sequence (CNS)	70 (human, mouse)	100	(Hardison 2000; Loots et al. 2000)
	variable	100	(Babarinde and Saitou 2013, 2016)
Conserved noncoding element (CNE)	variable	variable	(Siepel et al. 2005)
Conserved nonexonic elements (CNEEs)	variable	variable	(Lowe et al. 2011)
Long conserved noncoding sequences (LCNS)	95 (mouse, human)	500	(Janes et al. 2011; Sakuraba et al. 2008)
Highly conserved noncoding sequence (HCNS)	variable	100	(Saber et al. 2016; Takahashi and Saitou 2012)
Highly conserved noncoding regions (HCNR)	75 (in vertebrates)	100	(De La Calle-Mustienes et al. 2005)
Noncoding highly conserved elements (noncoding HCE)	variable	5	(Siepel et al. 2005)

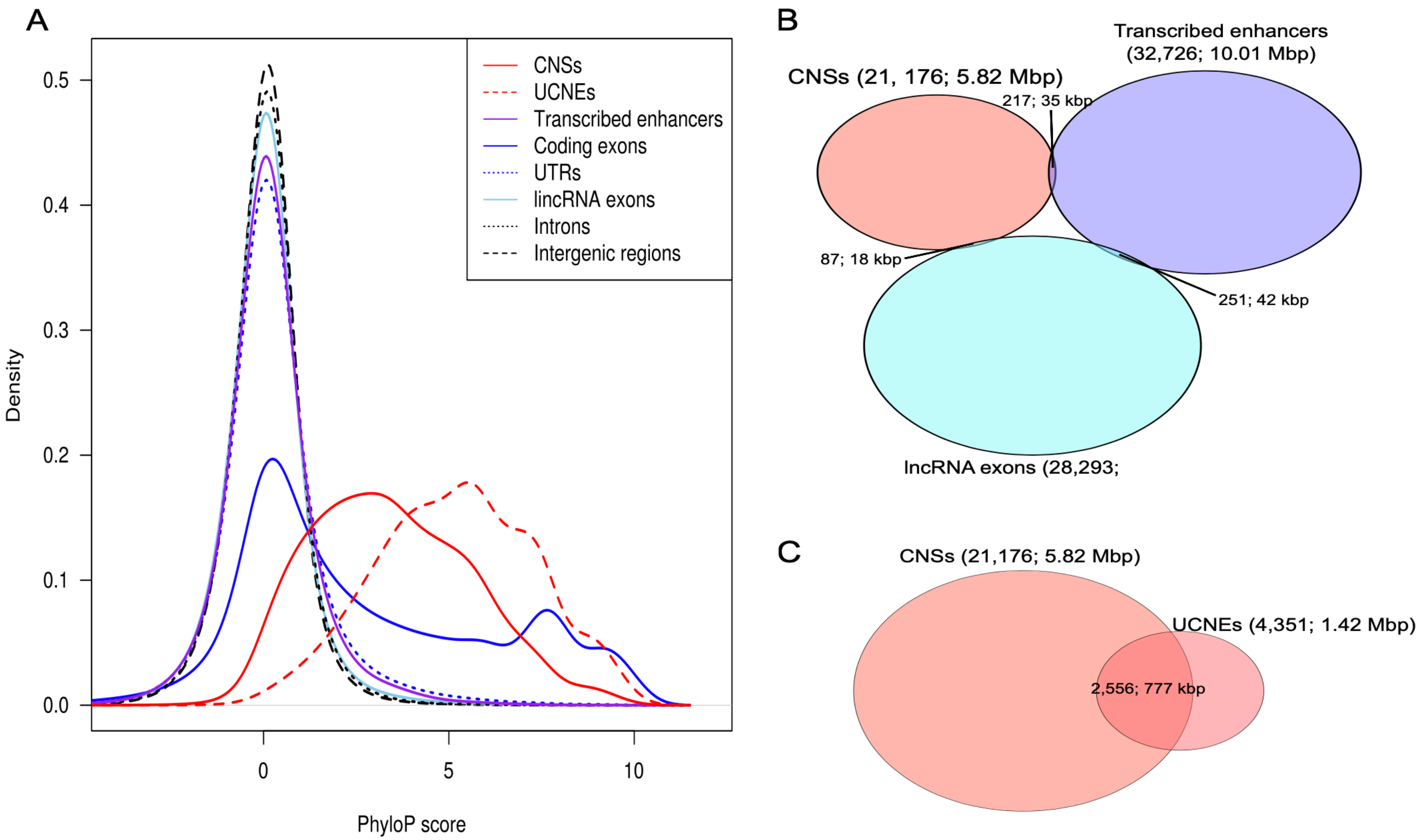
Definitions of CNSs

By definition, any region of the genome that is under evolutionary constraint but does not code for protein is defined as CNS (Hardison 2000; Kellis et al. 2014). Although the definition is grammatically simple, the actual identification of the CNSs in a genome is not such a simple task and different approaches have been made to get the task done. The first task is the definition of “coding”. In the pre-genomic era, “coding” was mostly used to represent protein-coding part of the genome (Poon et al. 1978). However, it is clear that not all the parts of protein-coding genes code for amino acids. Therefore, intron should be considered as noncoding (Hardison 2000). Furthermore, not all exons code for amino acid. Therefore, UTR might be considered noncoding, if the definition of “coding” is protein-coding part of the genome. With increasing sequencing power, many more noncoding RNAs are now being discovered (Iyer et al. 2015; Zhao et al. 2016). In fact, majority of human genome has been reported to be “biochemically active”, an expression used to describe transcribed regions or regions bound or modified by proteins or other elements (ENCODE Project Consortium 2012). Although these transcripts exist, they mostly do not code for amino acids (Iyer et al. 2015; Zhao et al. 2016), and so can be referred to as noncoding. For simplicity, this review defines “noncoding” as “genomic regions that do not code for protein”. Therefore, “noncoding” can include intergenic regions, intronic regions and untranslated regions (UTRs), irrespective of transcription. This represents a proportion of the genomic sequences that are not translated. Of course, each part of the genome is operating under different intensities of evolutionary forces (**Figure 1A**). CNSs and UCNEs show conservation level higher than those of the UTR, intronic and intergenic regions. Comparison of three noncoding sequences shown in **Figure 1B** shows very little overlaps among transcribed enhancers, lincRNAs and CNSs.

The second aspect of the definition of CNS implies that the region must be under evolutionary constraints stronger than those operating on neutrally evolving regions (Babarinde and Saitou 2013). Then the next task is defining neutrally evolving regions. The classic definition of neutrally evolving regions includes intron and intergenic regions (Kimura 1983). These are the exact regions where CNSs are located. And the presence of transposable elements in these regions can make the expectation under neutral evolution much more complicated. A number of studies (Babarinde and Saitou 2013; Hettiarachchi and Saitou 2016; Saber et al. 2016; Takahashi and Saitou 2012) masked repeats in the genome, effectively shifting the focus away from transposable elements, which might be functionally important (Hutchins and Pei 2015; Kim et al. 2012; Ramsay et al. 2017).

Figure 1. Functional constraint and genomic overlap of noncoding sequences in human genome. A. The PhyloP scores were obtained for CNSs (Babarinde and Saitou 2016), UCNEs (Dimitrieva and Bucher 2013), transcribed enhancers (Andersson et al. 2014), coding exons, UTRs, lincRNAs, introns and intergenic regions. The vertical axis represents the proportion of the distribution. **B**. CNSs, transcribed enhancers and lincRNA exons have small overlaps. **C**. Different criteria give imperfect but significant overlaps. Assuming 51.4% unique genomic regions (Hutchins

and Pei 2015), this much overlap is less likely to be obtained by chance (binomial p value $< 2.2 \times 10^{-308}$). Unless otherwise stated, the genomic coordinates were obtained from version 96 of Ensembl database. For **B** and **C**, the first values were the numbers of sequences while the second values were the numbers of base pairs covered.



In identifying genomic regions under evolutionary constraints, statistical tests are often conducted to separate regions of interest from neutrally evolving regions. Two parameters are often checked. The first parameter is sequence conservation. These are sometimes estimated as percent identity (Babarinde and Saitou 2013; Hettiarachchi and Saitou 2016; Kellis et al. 2014; Takahashi and Saitou 2012). However, conservation score per site can also be calculated by phyloP (Pollard et al. 2010), PhastCons score (Siepel et al. 2005), GERP (Pollard et al. 2010) or SiPhy (Garber et al. 2009). The second parameter is length. Evolutionary strength is often estimated as sequence constraint over certain length. For example, UCE (Bejerano et al. 2004) requires 100% sequence identity over >=200bp between human and mouse. LCNSs (Sakuraba et al. 2008) have at least 95% identity over >=500bp. The sequence identity thresholds are often arbitrary. That is why Babarinde and Saitou (2013) proposed thresholds that were informed by protein-coding gene properties. Examples of the definitions of CNSs are presented in **Table 1**. Various definitions of criteria make overlap between regions reported from different studies not perfect, but still significantly more than random expectations (binomial p value $< 2.2 \times 10^{-308}$, **Figure 1C**). The binomial test was conducted to find the probability of having at least that many nucleotide overlaps under the assumption that the sequences come from the 51.4% unique part of human genome (Hutchins and Pei 2015).

Digging into the junk: Computational tools for the discovery of CNSs

The search for CNSs starts with the acquisition of genome sequence and annotation data. The search can be restricted to certain genomic region (Bulger et al. 1999; Hardison et al. 1997; Loots et al. 2000; Oeltjen et al. 1997b; Poon et al. 1978) based on gene neighborhood (Bush and Lahn 2005; Jareborg et al. 1999) or genome wide (McEwen et al. 2006). Many studies (Babarinde and Saitou 2013; Hettiarachchi and Saitou 2016; Saber et al. 2016; Takahashi and Saitou 2012) use repeat-masked genome sequences as the repeat sequences are usually complex to analyze. The annotation data are usually used to mask the coding parts of the genome such that only the noncoding part of the genome is searched (Babarinde and Saitou 2013). The search for conserved regions can be initiated from each nucleotide position or genomic regions. Genomic position scores such as GERP (Pollard et al. 2010) and phyloP (Pollard et al. 2010) can be used to identify the conservation level of each genomic position. Other scores such as phastCons (Pollard et al. 2010) and SiPhy (Garber et al. 2009) can also be used, and are also available for short genomic regions. The scores for each genomic position can be easily obtained from UCSC database (Haeussler et al. 2019) or other sources like PhastWeb (Ramani et al. 2019). Using sliding window analyses, regions that are evolving significantly below neutral expectations can be retrieved. This approach was employed by some previous studies (e.g. Siepel et al. 2005).

Another approach is to download the alignment files from databases like UCSC (Haeussler et al. 2019). Using sliding window analyses, regions that satisfy specified percent identity and length thresholds are then extracted as done by Takahashi and Saitou (2012). Another common way is to use pairwise searches with local alignment tools such as BLAST (Altschul et al. 1997) or BLAT (Kent 2002) to identify regions conserved between two species at specified percent identity and length thresholds. In this approach, the species divergence should be considered to effectively delimit neutrally evolving sequences from functional ones. If species are phylogenetically too close, more stringent conditions should be applied (Saber et al. 2016; Takahashi and Saitou 2012). However, less stringent conditions can be applied if the species are significantly divergent (McEwen et al. 2006; Siepel et al. 2005; Van Hellemont et al. 2005; Woolfe et al. 2004). This consideration also applies to overall evolutionary rates of the species being considered (Babarinde and Saitou 2013; Takahashi and Saitou 2012) as the heterogeneity of evolutionary rates across species has been established (Babarinde and Saitou 2020). Using a reference species, regions that are conserved across multiple species can then be retrieved. This approach has been employed in multiple studies (Babarinde and Saitou 2016; Hettiarachchi and Saitou 2016; Mahmoudi Saber and Saitou 2017; Saber et al. 2016). One major challenge with pairwise search is the false discovery rates of short regions (Kamoun et al. 2013; Lai et al. 2017). To circumvent this issue, STAG-CNS (Lai et al. 2017) was published with the ability to identify CNSs as short as 9bp. CNEFinder (Ayad et al. 2018) offers the users the opportunity to input several thresholds to retrieve CNSs. Attempts have been made not only to discover CNSs, but also to prepare accessible databases of the discovered CNSs (Dousse et al. 2016; Inoue and Saitou 2020; Persampieri et al. 2008; Woolfe et al. 2007).

Identified features of CNSs

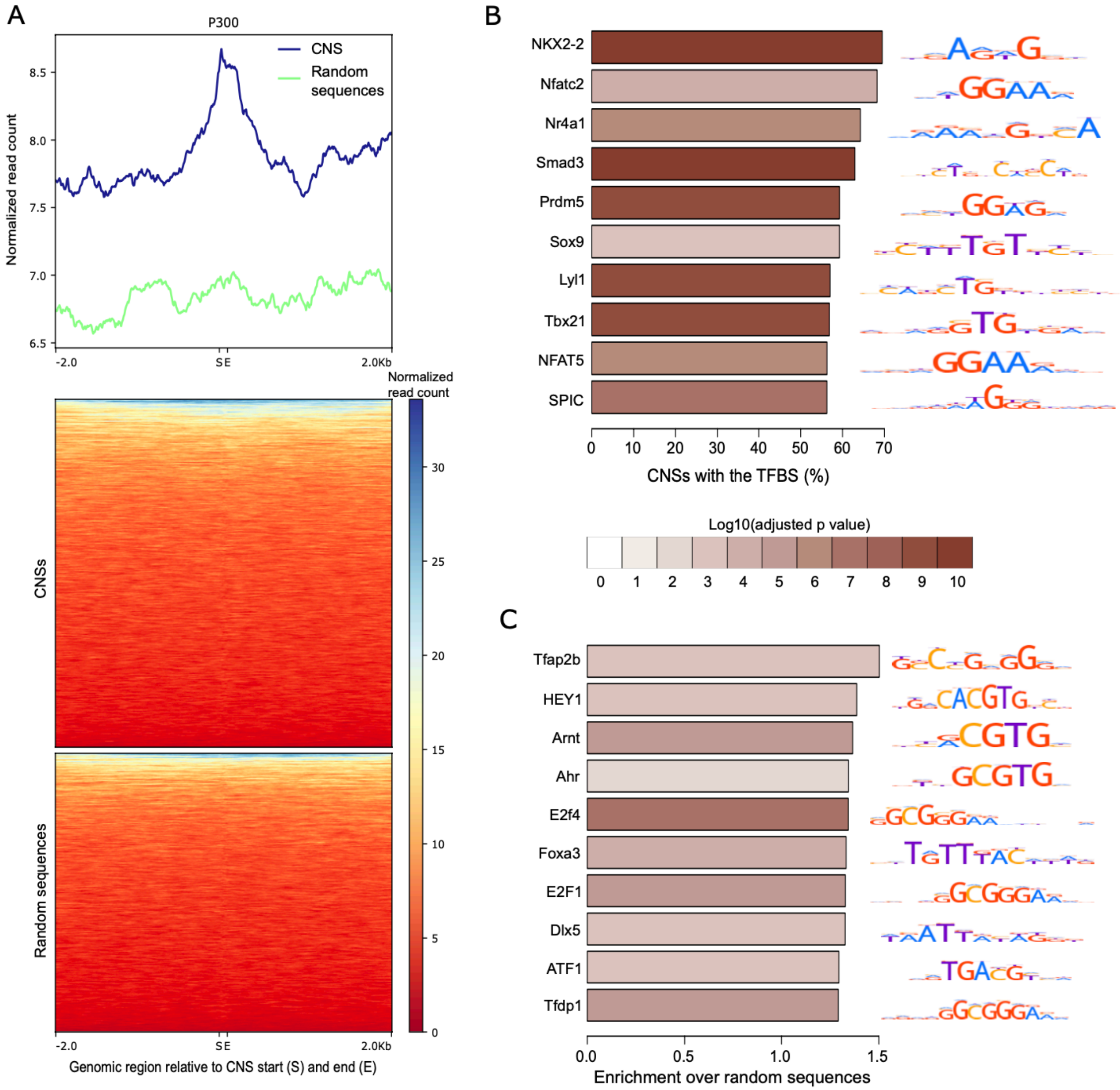
CNSs are known to have genomic distribution that are different from random expectation. First, they tend to occur in clusters close to certain types of genes (Matsunami et al. 2010; Mignone et al. 2008; Takahashi and Saitou 2012). Their closeness to genes involved in processes such as developmental, transcription and nervous systems (Babarinde and Saitou 2013) suggests that they might regulate the expression of such genes. Compared to lincRNAs and random intergenic sequences, CNSs found in intergenic regions tend to be located farther from transcription start sites or TSSs (Babarinde and Saitou 2016). This suggests that distance might not be a barrier for the functionality of CNSs.

In fact, Visel et al. (2009) reported that ChIP-Seq can accurately predict regulatory elements. **Figure 2A** shows that the enrichment pattern of p300 gene which marks enhancer activity (Raisner et al. 2018; Visel et al. 2009) is different between intergenic CNSs and randomly selected genomic sequences. Specifically, CNSs tend to be located in the peaks of p300 ChIP-Seq in mouse embryonic forebrain (Visel et al. 2009) suggesting the CNS activities at this stage. Two things stand out in **Figure 2A**. First, average ChIP-Seq outside CNSs is higher than random averages, suggesting that CNSs and ChIP-Seq do not share the same boundary.

Second, only a subset of CNSs are enriched in mouse embryonic forebrain p300 binding, highlighting CNS tissue specificity as previously reported (Babarinde and Saitou 2016). This p300 enrichment and enrichment of genes involved in transcription suggest that CNSs might be enriched in certain transcription factor binding motifs (TFBM). Indeed, the overrepresentation of certain motifs has been reported (Babarinde and Saitou 2016). For example, TFBM analyses using AME (McLeay and Bailey 2010) in MEME Suite (Bailey et al. 2009) with default settings returned 217 transcription factors from human and mouse full HOCOMOCO database (Kulakovskiy et al. 2018). The results indicated that about 70% of the intergenic CNSs from Babarinde and Saitou (2016) have NKX2-2 binding motifs. The top 10 most represented transcription factors are shown in **Figure 2B**. It is important to note that the number of CNSs with TFBMs for each transcription factor is significantly higher than the corresponding number in randomly selected genomic sequences (adjusted p value of Fisher exact test is shown in **Figure 2B**). For each TFBM, the enrichment was calculated as the number of CNSs with the motif divided by the number of randomly selected genomic sequences with the motif. The top 10 enriched TFBMs which include Tfap2b, HEY and Arnt are shown in **Figure 2C**.

Figure 2. CNS binding motif enrichments

A. CNSs are found at the peak of P300 bound regions. The short reads sequences, obtained from embryonic forebrain (Visel et al. 2009) were aligned with BWA (Li and Durbin 2010). Deeptools (Ramírez et al. 2016) was then used to plot the profile and heatmaps of intergenic CNSs with 2kbp flanking regions. Similar analyses were also conducted for random sequences of the similar number and lengths. The top panel represents the average normalized read count over 20 bp windows. The horizontal axis in the top and bottom panels are the positions, relative to CNSs. Each row in the lower panel represents each genomic region. **B**. The top 10 transcription factors with the most represented TFBM in CNSs are presented. Mouse intergenic CNSs and randomly selected intergenic sequences were analyzed using AME package (McLeay and Bailey 2010) in MEME Suite (Bailey et al. 2009). Human and mouse (HOCOMOCO v11 FULL) motif database (Kulakovskiy et al. 2018) was used. For other parameters, default values were used. **C**. The enrichment scores of the top 10 TFBMs. Enrichments were computed by dividing the number of motif-containing CNSs by the number of motif-containing random sequences. The colors of the bars in B and C correspond to the adjusted Fisher exact test p values reported in AME.



Associating a CNS to the closest TSS, **Figure 3** shows the enrichment of 1,385 genes with at least four associated CNSs. As previously reported (Elgar and Vavouri 2008; Hettiarachchi and Saitou 2016; Ishibashi et al. 2012; Matsunami et al. 2010), there is higher enrichment in genes associated with development. The topmost associated gene ontology terms include head development, embryonic morphogenesis and others (**Figure 3**). These gene ontology terms are heavily connected in gene networks (**Figure 4**) suggesting interconnections in activities.

Figure 3. Gene ontology enrichment analysis of CNS-associated genes

CNSs tend to cluster around genes associated with development. CNSs in mouse genomes reported by Babarinde and Saitou (2016) were associated to the genes with the closest TSS. The top 1385 genes with at least four associated CNSs were then analyzed using Metascape (Zhou et al. 2019).

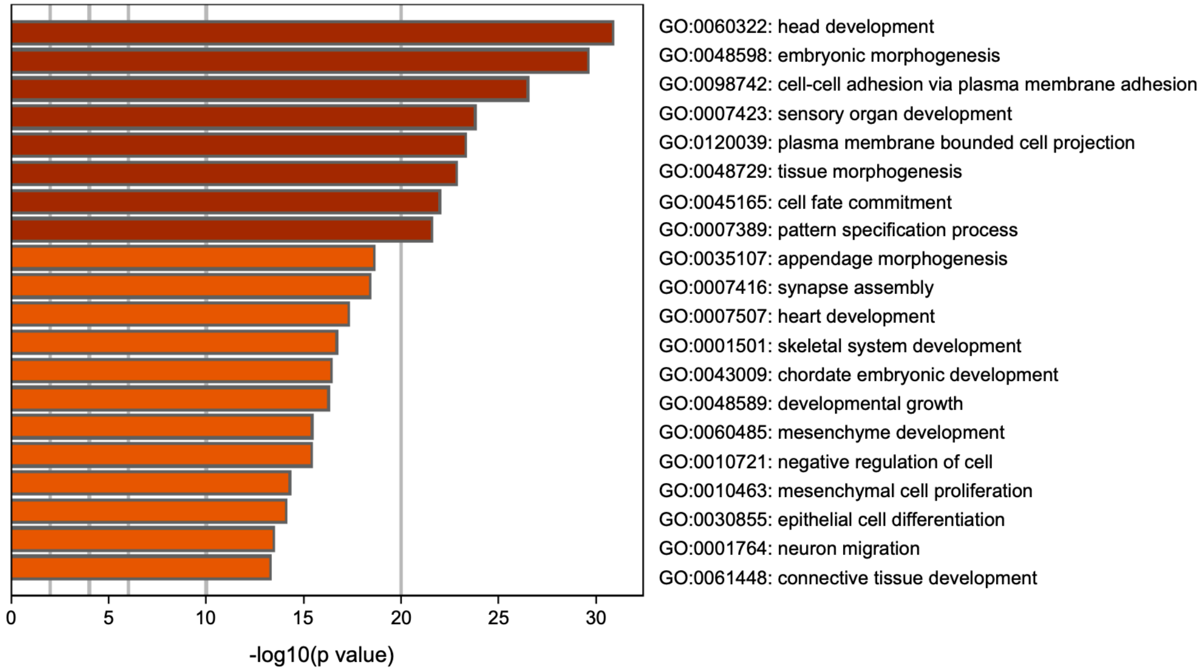
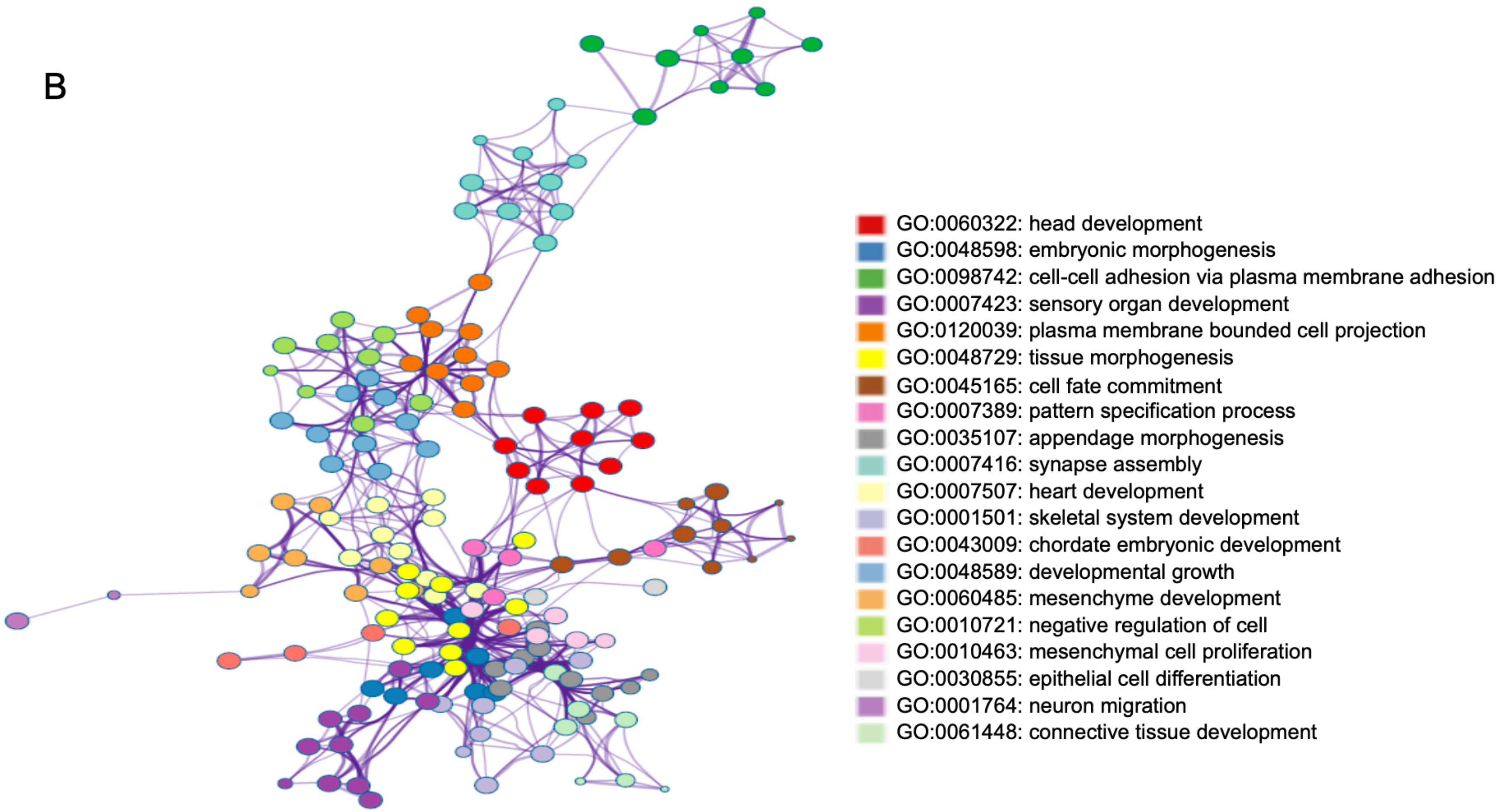


Figure 4. Network analysis of CNS-associated genes

The top 1000 CNS-enriched genes are heavily connected in functions. The network presented was produced with Metascape (Zhou et al. 2019).



Associated gene enrichment pattern suggests that CNS activities might be specific to certain stage and tissues. For example, enrichment of genes involved in development and nervous system implies that CNSs might be more active in embryonic brain (Dickel et al. 2018; Saber et al. 2016). Indeed, we previously found evidence of CNS activities in mouse embryonic brain (Babarinde and Saitou 2016). The evidence involved ChIP-Seq signal and the conservation in expression. Conversely, genes associated with defense and immunity and response to stimulus were found to be underrepresented in CNSs (Babarinde and Saitou 2016; Mahmoudi Saber and Saitou 2017). Further, genes expressed in testes or housekeeping genes with ubiquitous expressions were found to be underrepresented in CNSs (Babarinde and Saitou 2016). These observations reveal the specificity of CNS activities. Another nature of CNSs is the GC content heterogeneity (Babarinde and Saitou 2013; Hettiarachchi and Saitou 2016). We previously reported that tetrapod CNSs of different evolutionary ages, associated with recently acquired or more ancestral functions, tend to have different GC contents (Babarinde and Saitou 2013). Other studies (Hettiarachchi et al. 2014; Hettiarachchi and Saitou 2016) also showed that the heterogeneity exists also in non-tetrapod species. Interestingly, the GC contents of CNSs tend to be different from those of the flanking regions (Hettiarachchi and Saitou 2016). This GC content patterns have been associated with nucleosome occupancy (Dekker 2007; Hettiarachchi and Saitou 2016; Zhu et al. 2011).

Another interesting feature of CNSs is the conservation of CNS-TSS distance. The distance conservation was reported by (Babarinde and Saitou 2016). Comparing CNS-TSS distance in human and mouse, the study showed that genes associated with conserved CNS-TSS distance in human and mouse tend to have higher expression correlation, suggesting more stable expression across evolutionary timescales. The evolutionary analyses suggested that proper CNS function is dependent not only on the nucleotide sequences, but also on the genomic location of the CNSs. Later, Bagadia et al. (2019) further explored this possibility and found that indeed, evolutionary loss of genomic proximity of CNSs impacts expression dynamics during mammalian brain development. They reported that CNS-gene proximity interrupted by mechanisms such as chromosomal rearrangements could cause brain abnormality of germ line origin. These studies reveal another level of the functionality of CNSs.

Functionality of CNSs

It is known that different genomic regions have different evolutionary rates (Kimura 1983). Therefore, high cross-species sequence similarity found in genomes could be due to mutation cold spot or actual evolutionary constraint. However, derived allele frequency analyses showed that CNSs are under evolutionary constraint (Asthana et al. 2007; Drake et al. 2006; Ishibashi et al. 2012; J. Xie et al. 2018). Therefore, high sequence conservation in CNSs indicates functionality but does not necessarily indicates what type of functions they perform. However, since the discovery of abundant CNSs in vertebrate genomes, many studies have attributed CNSs to regulatory functions (Fishilevich et al. 2017; Frankel et al. 2010; Ishibashi et al. 2012; Osterwalder et al. 2018; Sumiyama et al. 2012; Visel et al. 2007). CNSs tend to cluster around specific genes (**Figures 3 and 4**), and there is an overrepresentation of certain TFBSs (**Figures 2B and C**). Furthermore, ChIP-Seq analyses show that many CNSs function as, or at least overlap, regulatory sequences (Visel et al. 2009). In a previous study (Babarinde and Saitou 2016), we demonstrated that CNSs have substantial signatures of regulatory functions and genes associated with more CNSs tend to have more stable expression patterns as indicated by expression correlations between mouse and human. Although many of these studies are computationally conducted, a good number of studies have experimentally validated regulatory functions of CNSs through *in vitro* enhancer assay. Furthermore, phenotypic effects of the genomic deletions of some CNSs have been reported. For example, a number of studies (Furniss et al. 2008; Lettice et al. 2002; Sagai et al. 2005) have attributed abnormal limb deformity and polydactyly to loss of certain CNSs. Vista (Visel et al. 2007), CONDOR (Woolfe et al. 2007) and Genehancer (Fishilevich et al. 2017) are databases of CNSs, including those with experimentally confirmed enhancer activities. CNSs have also been reported to have silencing functions (Hermann and Heckert 2005; Mahmoudi Saber and Saitou 2017). Consequently, many CNSs are believed to be involved in regulating proximal gene expression (Babarinde and Saitou 2016; Nelson et al. 2013).

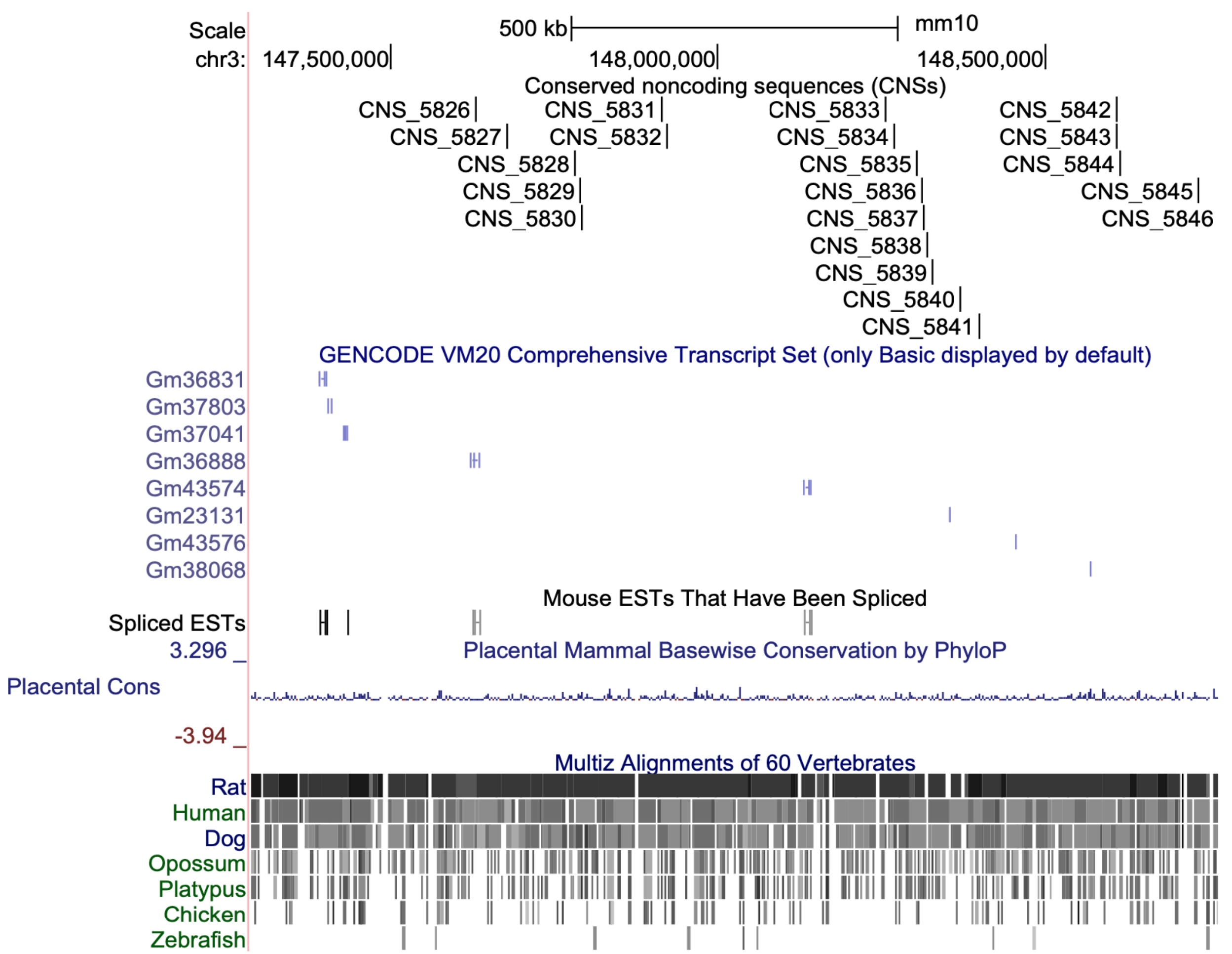
There are other reported possibilities of CNS functions. For example, Hezroni et al. (2017) reported that some pseudogenes are conserved across species. The conservation of promoters of these pseudogenes is particularly reported to be relatively high. Whether the conserved sequences of these pseudogenes acquired new functions after pseudogenization or they had another function before pseudogenization is often not very clear. Also, recent improvement in sequencing technologies has made it possible to detect even lowly expressed transcripts. This has led to the annotation of tens of thousands of lncRNAs (Harrow et al. 2012; Lagarde et al. 2017; Uszczynska-Ratajczak et al. 2018; C. Xie et al. 2014). Low expression level and extremely high tissue and stage specificity of lncRNAs (Mattioli et al. 2019; Necsulea et al. 2014; Talyan et al. 2018) make their detection difficult. Cheaper and better sequencing technologies however have made it possible to detect more lncRNAs. Therefore, there is a possibility that some of the CNSs might correspond to lncRNAs or other classes of RNAs. Indeed some ultraconserved elements have been reported to be transcribed in human cancers (Peng et al. 2013). However, there is a little overlap between GENCODE lincRNA exons and CNSs (**Figure 1B**). Also, ENCODE project (ENCODE Project Consortium 2012) ascribed some "biochemical functions" to about 80% of the human genome. Some of these functions were linked to the ChIP-Seq results. Specifically, data on DNA binding, transcription, DNA accessibility and DNA methylation would give relevant insights about the functions of specific genomic regions. Thus, overlap with ENCODE data might give insights into other likely functions of CNSs. Finally, roadmap epigenome data (Bernstein et al. 2010) might be valuable as some histone modification marks have been associated with enhancer activities (Babarinde and Saitou 2016; Creyghton et al. 2010).

Unexpected switch: No obvious effect of CNS deletion on mutated individuals

With the extremity of CNS conservation at nucleotide level, the evolutionary constraint is believed to be extremely high. Therefore, deletion of such sequences is expected to result in seriously affected phenotype. In a surprising turn, no obvious phenotypic change was found when 1.5 Mbp CNS-rich gene desert in chromosome 3 (**Figure 5**) and another 845 kbp gene desert in chromosome 19 were deleted in mouse (Nóbrega et al. 2004). The 1.5 Mbp region contains 21 CNSs identified in (Babarinde and Saitou 2016). Similarly, deletion of ultraconserved elements have also been reported to yield viable mice (Ahituv et al. 2007). These reports were puzzling, considering the level of constraints on CNSs. A number of studies have shed some lights into the puzzle. First, the laboratory environment under which the mice were raised might be very different from real environment in which the animals live. This possibility is reiterated by a *Drosophila* experiment in which the deletion of an enhancer element led to an observable phenotype only at a specific temperature range (Frankel et al. 2010).

Figure 5. UCSC genome view of the mouse region deleted with no obvious phenotype

The genome view, captured from https://www.genome.ucsc.edu/ on 12th September, 2019, shows MMU3 of Nóbrega et al. (2004) which corresponds to chr3:147287066-148764954 of mm10 build of mouse genome. This region contains 21 CNSs identified in Babarinde and Saitou (2016).



From the population genetics point of view, the controlled environment and the smaller effective population size could greatly reduce the impact of evolutionary force especially under small selection coefficient (Kousathanas et al. 2011; Mueller et al. 2013). Also, it is possible that there is redundancy in CNS function. Such enhancer redundancy has been reported to provide phenotypic robustness in mammalian development (Osterwalder et al. 2018). Another thing to consider is the ability to measure phenotypic changes. This greatly impacts the observable phenotypic changes. For example, Dickel et al. (2018) reported that mice with deletions in each or pairs of the studied ultraconserved elements were viable and fertile as previously reported (Ahituv et al. 2007; Nóbrega et al. 2004). However, further analyses revealed that in nearly all cases, there were neurological or growth abnormalities (Dickel et al. 2018). These abnormalities included alterations of neuron populations and structural brain defects. They concluded that some of the phenotypic changes induced by deletion of ultraconserved elements are real but might be too subtle to be discovered in normal laboratory settings (Dickel et al. 2018). Therefore, inability to discover the phenotypic effect of a CNS might not imply lack of function; it might just reflect the limited experimental ability to discover such phenotypic effects.

Current puzzles: unanswered questions

While some CNSs have strong phenotypic effects, others have rather subtle effects that are often difficult to observe. However, not much is known about the characteristic differences of these classes of CNSs. As numerous properties of CNSs have been enumerated, one of these properties might be different depending on the phenotypic effect. Another puzzle that has attracted less attention is the reason for the length of CNSs. As an enhancer element which binds a transcription factor, the typical TFBS can be shorter than 10 bp. However, many CNSs spans more than 100bp (Table 1). What differentiates longer CNSs from shorter CNSs at the functional level is not yet fully understood despite the understanding of super enhancers (Hnisz et al. 2013; Pott and Lieb 2015; Whyte et al. 2013). With the rapidly falling sequencing cost, it is now possible to detect new transcripts with tissue-specific expression. It is important to understand how overlapping the transcripts and CNSs are, in a hypothetical scenario of monitoring expressions across all stages and tissues as well as cell types. Finally, there are other epigenomic signatures (Tsankov et al. 2015; W. Xie et al. 2013) of regulatory elements including transcribed enhancers (Andersson et al. 2014), protein-DNA interaction (Landt et al. 2012; Visel et al. 2009) and histone methylation or acetylation (Barski et al. 2007; Creyghton et al. 2010) investigated by ChIP-Seq technologies as well as DNA methylation data (Sharifi-Zarchi et al. 2017). It would be interesting to dissect the activities of these sequences to unravel the functional dynamics of the sequences as they relate to gene expression regulation.

Conclusion

The studies of CNSs have undergone dramatic evolution of puzzles. In the pre-genomic eras, abundance and the nature of CNSs were a puzzle. After the release of multiple genomes, the puzzles have morphed into those of functions. The inability to observe a phenotypic change when CNSs were deleted was puzzling. However, new studies are illuminating the puzzle of seemingly intact phenotypes in mutants despite the extreme conservation of CNSs. Therefore, the puzzle is gradually evolving to another form. Now, the puzzle is how to extensively link the properties of these sequences to their functional importance. Although a lot of puzzles have been solved about CNSs, it appears that the puzzles are not static. The puzzles are evolving and as they are evolving, so is the biological knowledge of properties and functions of these sequences.

List of abbreviations

BLAST: Basic Local Alignment Search Tool
BLAT: Blast-Like Alignment Tool
ChIP-Seq: Chromatin ImmunoPrecipitation Sequencing
CNEE: Conserved NonExonic Element
CNS: Conserved Noncoding Sequence
CONDOR: COnserved Non-coDing Orthologous Region
ENCODE: ENCyclopedia Of DNA Element
GERP: Genomic Evolutionary Rate Profiling
HCE: Highly Conserved Element
HCNR: Highly Conserved Noncoding Region
HCNS: Highly Conserved Noncoding Sequence
LCNS: Long Conserved Noncoding Sequence
lncRNA, lincRNA: long noncoding RNA, long intergenic noncoding RNA
RNA, mRNA: RiboNucleic Acid, messenger RNA

STAG-CNS: Suffix Tree Arbitrary Gene number: Conserved Noncoding Sequence
TFBM: Transcription Factor Binding Motif
TSS: Transcription Start Site
UCE: UltraConserved Element
UCNE: UltraConserved Noncoding Element
UTR: UnTranslated Region

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Publication history of this article

November 18, AS 0020: review article was submitted from Dr. Isaac BABARINDE to iDarwin (handled by SAITOU Naruya)

December 11, AS 0020: review result (one associate editor and SAITOU reviewed this manuscript) was sent to author

December 31, AS 0020: revision was sent from author

January 5, AS 0021: this revision was accepted for iDarwin

January 11, AS 0021: first proof of this review article was sent to author

January 15, AS 0021: reply to first proof was sent from author

January 17, AS 0021: second proof of this review article was sent to author

January 31, AS 0021: third proof of this review article was sent to author

February 12, AS 0021: this review article is published in iDarwin

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