INTRODUCTION

Selection can shape the evolution of protein-coding genes by preventing changes in their sequences (purifying selection) or through the fixation of novel adaptive variants (positive selection). Quantifying its nature and strength is a key step to understand the diverse evolutionary histories of orthologous genes across different species and clades. Statistical models of molecular evolution have proven to be fundamental approaches to investigate such processes and can be divided into those based on comparing divergence and segregating polymorphism—such as the MK test and its extensions (McDonald & Kreitman, 1991)—and those based on multi-species
sequence divergence—also known as codon models. The two approaches use different conceptual frameworks and are better applied for analyses at different timescales, with the first approach more suited to investigate recent processes and the latter ones more apt to infer older events (Mugal et al., 2014).

Approaches based on sequence divergence among multiple species are cornerstones in the estimations of patterns of sequence evolution and selection regimes. After the first models were developed to infer the strength of selection on coding sequences globally across their sites and species phylogeny (Goldman & Yang, 1994; Muse & Gaut, 1994), subsequent elaborations allowed for variation across lineages (Yang, 1998), sites (Anisimova et al., 2001; Nielsen & Yang, 1998; Yang et al., 2000), and both (Yang & Nielsen, 2002; Zhang et al., 2005). Pairwise comparisons between models can be performed using likelihood-ratio tests (LRTs) to understand which one better reflects the molecular evolution of a group of orthologous genes (Anisimova et al., 2001). The interpretation of all these models is largely based on the \( dN/dS \) parameter (Kimura, 1977; also known as \( \omega \)), which is calculated as the ratio of nonsynonymous substitution rates (nonsynonymous mutations over nonsynonymous sites; \( dN \)) to synonymous substitution rates ( synonymous mutations over synonymous sites; \( dS \)). This metric is fundamental to investigate the extent to which selection modulates sequence evolution of the protein-coding portions of genes. While \( dS \) are assumed to evolve neutrally, \( dN \) are expected to be exposed to selection, as they change the amino acid structure of proteins. Despite the fact that some of these assumption have been challenged (Davydov et al., 2019; He et al., 2020; Kryazhimskiy & Plotkin, 2008), analyses based on codon models have proved themselves as key approaches in comparative genomics, such as investigating positive selection connected to evolutionary innovations (Li et al., 2014; Parker et al., 2013; Zhang et al., 2014) or testing the relaxation of selective constraints after trait decay (Liu et al., 2019; Policarpo et al., 2020). In other instances, these approaches have been used to observe genome-wide effects linked to events such as shifts in environmental niches or the loss of recombination in asexual species (Bast et al., 2018; Piazza et al., 2017).

Several pieces of software have been developed to infer codon models for coding sequences: Selecton (Stern et al., 2007), HyPhy (Pond et al., 2005), TreeSAAP (Woolley et al., 2003), and the CodeML program in the PAML package (Yang, 2007). The latter program was also subject to several implementations, such as IDEA (Egan et al., 2008), PAMLX (Xu & Yang, 2013), SlimCodeML (Valle et al., 2014), IMPACT_S (Maldonado et al., 2014), LMAP (Maldonado et al., 2016), ete-evol in the ete3 package (Huerta-Cepas et al., 2016), VESPA (Webb et al., 2017), BlastPhyMe (Schott et al., 2019), and EasyCodeML (Gao et al., 2019). With the increment of genomics and transcriptomics studies, it has become rather common to infer selective regimes of thousands of genes for hundreds of species and all of aforementioned CodeML implementations mainly try to overcome its limited ease of use in the context of comparative genomics.

Our focus on developing BASE has been mainly directed to facilitate the integration of an often overlooked—yet incredibly large—portion of genomes in comparative genomics analyses for selection. Genes can differ in many aspects—such as being single-copy or multi-copy—and they can also be either shared by all species considered (i.e., ubiquitous genes) or not found in some of them (i.e., nonubiquitous genes). The latter case can be either due to biological or technical causes, but nonetheless a large number of single-copy genes in comparative genomics datasets consist of nonubiquitous genes. As an exploratory example, we retrieved the proportion of ubiquitous and nonubiquitous genes from 18 published datasets, which have been generated for comparative genomic or phylogenomic purposes. While we tried to consider datasets varying in total gene number and taxonomic level, this overview has to be considered far from comprehensive: The outcome of orthology inferences is rarely included in publications, and thus, we largely relied on authors’ personal communications. Despite the partial nature of this analysis, it shows how comparative genomics datasets consistently include a large portion of nonubiquitous genes (Figure 1). The latter are mostly overlooked in selection analyses—which are typically based only on single copy and ubiquitous genes—due to the lack of automated approaches for their inclusion. Yet, disregarding such a large portion of genes may potentially conceal important evolutionary processes and for this reason we developed a novel workflow intended specifically for this purpose.

## 2 | IMPLEMENTATION

BASE workflow is written in BASH and R and has been tested on Linux operating systems, such as centOS 8. As it extensively leverages GNU utilities, its usage is restricted to Linux distributions. It consists of two main steps: In the first one (“analyze”), evolutionary model parameters are inferred across alignment sites and tree branches for the different genes, while the subsequent step (“extract”) allows to retrieve the different metrics associated with specific branches or clades in the species tree. CodeML provides in large part the statistical and computational framework to perform these analyses and is at the core of the workflow, whose general description is reported in Figure 2.

### 2.1 | “Analyze” step

The inputs required for the “analyze” step are (a) a folder containing protein-coding genes alignments in fasta format; (b) two CodeML control files describing two nested models—where one is a specific case of the other; all the parameters in control files can be customized, supporting branch, site, and branch-site models; moreover, other optional files can be required depending on analysis specifications: (c) a species tree in newick format, which can be multifurcating but has to include all the species present across the different gene alignments; and (d) a labeling scheme when the user wants to analyze models that assume specific clades and/or branch rates. The workflow initially checks the alignments for the presence of stop codons using Transeq of the
EMBOSS package (Rice et al., 2000), excluding from subsequent analysis genes that include any. Either branch length optimization on the species-tree topology or gene-tree inference can be then carried out for each gene alignment, using RAxML with a codon-aware GTR substitutions model (Stamatakis, 2014). Subsequently, the two CodeML analyses configured with the general and the alternative models are performed and compared through a LRT using R (R Core Team, 2013). Replicate analyses can be specified, so that the general and alternative model inferences will be carried out a user-defined number of times and those with the "best" likelihood value will be used for the LRT. The resulting $p$ values are automatically adjusted using a false-discovery-rate (FDR) correction; then, the codeml output relative to the best-fit model is selected for each gene, and LRT outcome is summarized in a table. When leveraging models which assume specific clades and/ or branch rates, the user can specify labels in a custom input file and BASE will automatically use them to annotate the trees using phangorn R package (Schliep, 2011). All the complex labeling schemes possible in CodeML can be replicated in BASE, which allows the use of multiple branch (#) and clade ($) labels in the same analysis. Moreover, also the inference and comparison of two models including labels are supported, as long as the alternative one is nested within the general one. Additional resources on labeling strategies are available in online tutorials. The default behavior of BASE is to process all genes, whether ubiquitous or nonubiquitous, but the user can limit the analysis to just ubiquitous ones. When the analysis is configured to consider also nonubiquitous genes, the species tree will be pruned on the basis of the species present using ape R package (Paradis et al., 2004), prior to species-tree branch length optimization or gene-tree inference.

**FIGURE 1** Comparative genomics datasets consistently include a large number of nonubiquitous genes. The proportion of ubiquitous and nonubiquitous genes was calculated in 18 published datasets, varying in their total number of genes and taxonomic level (from family to phylum). In our analysis, the average percentage of nonubiquitous genes is 73.4% while for ubiquitous ones is 26.6%

| Dataset                        | Ubiquitous | Nonubiquitous |
|-------------------------------|------------|---------------|
| Morani Siniscalchi et al., 2019 | trib (n=759) | phyl (n=258) |
| Burki et al., 2012            |            |               |
| Streicher et al., 2017        |            |               |
| Delsuc et al., 2018           |            |               |
| Lozano-Fernandez et al., 2019 |            |               |
| Burki et al., 2010            |            |               |
| Philippe et al., 2004         |            |               |
| Kuang et al., 2018            |            |               |
| Jebb et al., 2020             |            |               |
| Crawford et al., 2015         |            |               |
| Debray et al., 2019           |            |               |
| Cornetti et al., 2019         |            |               |
| Grummer et al., 2018          |            |               |
| Zhao et al., 2017             |            |               |
| Vasilikopoulos et al., 2019   |            |               |
| Bragg et al., 2018            |            |               |
| Feng et al., 2019             |            |               |
| de Moya et al., 2019          |            |               |
| Li et al., 2019               |            |               |

0% 50% 100%
2.2 | "Extract" step

The "extract" step can be carried out subsequently to the "analyze" one, using as input: (a) the output folder generated by the previous step or a folder containing CodeML outputs generated by means other than BASE; and (b) a list of all branches and/or clades of interest, defined by their associated species. In the first place, this step will annotate internal nodes of each tree to match the output of CodeML and will list all species associated with each branch of the phylogeny. Subsequently, the pipeline will create a table for each branch/clade specified by the user, containing the dN/dS, dN, and dS values relative to the best-fit model for each gene. Equivalent branches/clades can be identified in a phylogeny even in the absence of some species. For example, a clade—and its stem branch—made up of tens of species can be considered to be still present if we subtract a few species from the phylogeny, in either the in-group or the out-group. As such, in BASE "extract" step it is possible to include nonubiquitous genes with two approaches (Figure 3): (A) restrict analyses to genes which are present in all species of the branches/clades of interest but may not be found in external species or (B) also implement a threshold for missing species within the branches/clades of interest. This threshold can be specified by either an absolute number or a proportion (e.g., if 0.8 is specified, at least the 80% of each branch/clade species need to be present in a given gene in order to include it in the output). If a given branch or clade do not meet the selected criteria, this will be stated in the final output and no associated metrics will be reported. The inclusion of nonubiquitous genes should be applied with caution by the user: Too many missing species—both in the branches/clades of interest and/or in the whole tree—could impact evolutionary rates of inference. We suggest to either opt for (A) or to rely on conservative thresholds when opting for (B) (e.g., 90% of species present; van Kruistum et al., 2021).

2.3 | Additional implementations

Even if the focal feature is the integration into selection analyses of single-copy genes which are not found across all considered species, BASE also includes two other major technical implementations. The first one concerns analyses with a high number of
parameters and which may encounter local likelihood optima: A good—yet often overlooked—practice is to run analyses multiple times with varying starting values in order to obtain the global optimum. BASE allows to seamlessly carry out a user-specified number of replicate analyses, incorporating random omega starting values: The replicate which has the “best” likelihood value will be then used for further analyses. A second issue concerning phylogeny-based selection analyses is the possibility of discordance between gene trees and species tree. This circumstance can underlie a wide range of technical and biological phenomena—such as sequence misalignment, nonorthology, and incomplete lineage sorting—which can ultimately bias evolutionary rate inference. In order to account for such possibility, when a fixed species tree is specified BASE will report its normalized Robinson–Foulds distances with each gene tree, calculated using ete3 (Huerta-Cepas et al., 2016). The user can then decide to exclude analyses where there is a strong conflict between the gene-tree and species-tree topologies or to re-launch them using the gene tree.
This latter possibility can for example be leveraged to account for the artefactual substitution rate variation which occurs when substitutions on discordant gene trees are analyzed in the context of a fixed species tree (Mendes & Hahn, 2016). BASE also provides additional features that ease the inference, comparison, and interpretation of analyses on selection regimes, such as the automatic labeling and/or metrics retrieval for specific branches/clades in large phylogenies, simultaneous batch processing of genes to cut down processing times, and a large number of error messages which can definitely ease the user experience.

3 | CONCLUSIONS

BASE is a workflow for analyses on selection regimes that integrates several popular pieces of software, with CodeML at its core. It has been conceived to allow the integration of nonubiquitous genes into comparative genomics analyses for selection in a straightforward and reproducible manner, yet it also implements many other features and quality-of-life improvements. We hope that BASE proves to be a useful tool for comparative genomics and that it generates some interest toward the frequent exclusion of such a vast portion of genes in selection analyses. BASE is an ongoing project, and we welcome bug reports, feedback, and suggestions for feature implementations. All the documentation, including detailed tutorials to explore BASE functionality, can be found at github.com/for-giobbе/BASE.

CONFLICT OF INTERESTS

Authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Giobbе Forni: Conceptualization (lead); Investigation (lead); Methodology (lead); Software (lead); Writing-original draft (lead); Writing-review & editing (lead). Angelo Alberto Ruggeri: Conceptualization (supporting); Investigation (supporting); Methodology (supporting); Software (supporting); Validation (supporting); Writing-original draft (supporting); Writing-review & editing (supporting). Giovanni Piccinini: Conceptualization (supporting); Investigation (supporting); Methodology (supporting); Software (supporting); Validation (supporting); Writing-original draft (supporting); Writing-review & editing (supporting). Andrea Luchetti: Conceptualization (supporting); Methodology (supporting); Supervision (lead); Writing-review & editing (supporting).

OPEN RESEARCH BADGES

This article has earned an Open Data Badge for making publicly available the data necessary to reproduce its results; BASE along with the relative tutorials are hosted on GitHub: https://github.com/for-giobbе/BASE.

DATA AVAILABILITY STATEMENT

BASE along with the relative tutorials are hosted on GitHub: https://github.com/for-giobbе/BASE.

ORCID

Giobbе Forni https://orcid.org/0000-0003-3669-8693

REFERENCES

Anisimova, M., Bielawski, J. P., & Yang, Z. (2001). Accuracy and power of the likelihood ratio test in detecting adaptive molecular evolution. Molecular Biology and Evolution, 18(8), 1585–1592. https://doi.org/10.1093/oxfordjournals.molbev.a003945

Bast, J., Parker, D. J., Dumas, Z., Jalvingh, K. M., Van Tran, P., Jaron, K. S., Figuet, E., Brandt, A., Galtier, N., & Schwander, T. (2018). Consequences of asexuality in natural populations: Insights from stick insects. Molecular Biology and Evolution, 35(7), 1668–1677. https://doi.org/10.1093/molbev/msy058

Davydov, I. I., Salamin, N., & Robinson-Rechavi, M. (2019). Large-scale comparative analysis of codon models accounting for protein and nucleotide selection. Molecular Biology and Evolution, 36(6), 1316–1332. https://doi.org/10.1093/molbev/msz048

Egan, A., Maharkar, A., Crabtree, J., Badger, J. H., Carlton, J. M., & Silva, J. C. (2008). IDEA: Interactive display for evolutionary analyses. BMC Bioinformatics, 9(1), 524. https://doi.org/10.1186/1471-2105-9-524

Gao, F., Chen, C., Arab, D. A., Du, Z., He, Y., & Ho, S. Y. (2019). EasyCodeML: A visual tool for analysis of selection using CodeML. Ecology and Evolution, 9(7), 3891–3898. https://doi.org/10.1002/ece3.5015

Goldman, N., & Yang, Z. (1994). A codon-based model of nucleotide substitution for protein-coding DNA sequences. Molecular Biology and Evolution, 11(5), 725–736.

He, Z., Chen, Q, Yang, H., Chen, Q., Shi, S., & Wu, C. I. (2020). Two decades of suspect evidence for adaptive DNA-sequence evolution-failure in consistent detection of positive selection. bioRxiv, 417717.

Huerta-Cepas, J., Serra, F., & Bork, P. (2016). ETE 3: Reconstruction, analysis, and visualization of phylogenomic data. Molecular Biology and Evolution, 33(6), 1635–1638. https://doi.org/10.1093/molbev/msw046

Kimura, M. (1977). Preponderance of synonymous changes as evidence for the neutral theory of molecular evolution. Nature, 267(5608), 275–276.

Kryazhimskiy, S., & Plotkin, J. B. (2008). The population genetics of dN/dS. PLoS Genetics, 4(12), 1000304.

Li, C., Zhang, Y., Li, J., Kong, L., Hu, H., Pan, H., Xu, L., Deng, Y., Li, Q., Jin, L., Yu, H., Chen, Y., Liu, B., Yang, L., Liu, S., Zhang, Y., Lang, Y., Xia, J., He, W., … Zhang, G. (2014). Two Antarctic penguin genomes reveal insights into their evolutionary history and molecular changes related to the Antarctic environment. GigaScience, 3(1), 2047–2217. https://doi.org/10.1186/2047-217X-3-27

Liu, A., He, F., Shen, L., Liu, R., Wang, Z., & Zhou, J. (2019). Convergent degeneration of olfactory receptor gene repertoires in marine mammals. BMC Genomics, 20(1), 977. https://doi.org/10.1186/s12864-019-6290-0

Maldonado, E., Almeida, D., Escalona, T., Khan, I., Vasconcelos, V., & Antunes, A. (2016). LMAP: Lightweight multigene analyses in PAML. BMC Bioinformatics, 17(1), 1–11. https://doi.org/10.1186/s12859-016-1204-5

Maldonado, E., Sunagar, K., Almeida, D., Vasconcelos, V., & Antunes, A. (2014). IMPACT_S: Integrated multiprogram platform to analyze and combine tests of selection. PLoS One, 9(10), 96243. https://doi.org/10.1371/journal.pone.0096243
McDonald, J. H., & Kreitman, M. (1991). Adaptive protein evolution at the Adh locus in Drosophila. *Nature*, 351(6328), 652–654.

Mendes, F. K., & Hahn, M. W. (2016). Gene tree discordance causes apparent substitution rate variation. *Systematic Biology*, 65(4), 711–721. https://doi.org/10.1093/sysbio/syw018

Mugal, C. F., Wolf, J. B., & Kaj, I. (2014). Why time matters: Codon evolution and the temporal dynamics of dN/dS. *Molecular Biology and Evolution*, 31(1), 212–231. https://doi.org/10.1093/molbev/msu192

Muse, S. V., & Gaut, B. S. (1994). A likelihood approach for comparing synonymous and nonsynonymous nucleotide substitution rates, with application to the chloroplast genome. *Molecular Biology and Evolution*, 11(5), 715–724.

Nielson, R., & Yang, Z. (1998). Likelihood models for detecting positively selected amino acid sites and application to the HIV-1 envelope Gene. *Genetics*, 148(3), 929–936. https://doi.org/10.1093/genetics/148.3.929

Paradis, E., Claude, J., & Strimmer, K. (2004). APE: Analyses of phylogenetics and evolution in R language. *Bioinformatics*, 20(2), 289–290. https://doi.org/10.1093/bioinformatics/btg412

Parker, J., Tsagkogeorga, G., Cotton, J. A., Liu, Y., Provero, P., Stupka, E., & Rossiter, S. J. (2013). Genome-wide signatures of convergent evolution in echolocating mammals. *Nature*, 502(7470), 228–231. https://doi.org/10.1038/nature12737

Pisci, F., Puccio, G., & Passamonti, M. (2017). Burrowers from the past: Mitochondrial signatures of Ordovician bivalve infaunalization. *Genome Biology and Evolution*, 9(4), 956–967. https://doi.org/10.1093/gbe/evx051

Policarpo, M., Fumej, J., Lafargeas, P., Naquin, D., Thermes, C., Naville, M., Dechaud, C., Volf, J.-N., Cabau, C., Klopp, C., Meller, P. R., Bernatchez, L., García-Machado, E., Rétaux, S., & Casane, D. (2020). Contrasting gene decay in subterranean vertebrates: Insights from cavefishes and fossorial mammals. *Molecular Biology and Evolution*, 38(2), 589–605. https://doi.org/10.1093/molbev/msaa249

Pond, S. L. K., Frost, S. D. W., & Muse, S. V. (2005). HyPhy: Hypothesis testing using phylogenies. *Bioinformatics*, 21, 676–679. https://doi.org/10.1093/bioinformatics/bti079

R Core Team. (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing.

Rice, P., Longden, I., & Bleasby, A. (2000). EMBOSS: The European molecular biology open software suite. *Trends in Genetics*, 16(6), 276–277. https://doi.org/10.1016/S0168-9525(00)02042-2

Schliep, K. P. (2011). phangorn: Phylogenetic analysis in R. *bioRxiv*, 274(4), 592–593. https://doi.org/10.1093/bioinformatics/btq706

Schott, R. K., Gow, D., & Chang, B. S. (2019). BlastPhyMe: A Toolkit for Rapid Generation and Analysis of protein-coding Sequence Datasets. *bioRxiv*, 059881.

Stamatakis, A. (2014). RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, 30(9), 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Stern, A., Doron-Faigenboim, A., Erez, E., Martz, E., Bacharach, E., & Pupko, T. (2007). Selecton 2007: Advanced models for detecting positive and purifying selection using a Bayesian inference approach. *Nucleic Acids Research*, 35, W506–W511. https://doi.org/10.1093/nar/gkm382

Valle, M., Schabauer, H., Pacher, C., Stockinger, H., Stamatakis, A., Robinson-Rechavi, M., & Salamin, N. (2014). Optimization strategies for fast detection of positive selection on phylogenetic trees. *Bioinformatics*, 30(8), 1129–1137. https://doi.org/10.1093/bioinformatics/btt760

van Kruistum, H., Nijland, R., Reznick, D. N., Groenen, M. A., Megens, H. J., & Pollux, B. J. (2021). Parallel Genomic changes drive repeated evolution of placenta in live-bearing fish. *Molecular Biology and Evolution*, 38(6), 2627–2638. https://doi.org/10.1093/molbev/msab057

Webb, A. E., Walsh, T. A., & O’Connell, M. J. (2017). VESPA: Very large-scale evolutionary and selective pressure analyses. *PeerJ Computer Science*, 3, e118. https://doi.org/10.7717/peerj-cs.118

Woolley, S., Johnson, J., Smith, M. J., Crandall, K. A., & McClellan, D. A. (2003). TreeSAAP: Selection on amino acid properties using phylogenetic trees. *Bioinformatics*, 19(5), 671–672. https://doi.org/10.1093/bioinformatics/btg043

Xu, B., & Yang, Z. (2013). PAMLX: A graphical user interface for PAML. *Molecular Biology and Evolution*, 30(12), 2723–2724. https://doi.org/10.1093/molbev/msr179

Yang, Z. (1998). Likelihood ratio tests for detecting positive selection and application to primate lysozyme evolution. *Molecular Biology and Evolution*, 15(5), 568–573. https://doi.org/10.1093/oxfordjournals.molbev.a025957

Yang, Z. (2007). PAML 4: A program package for phylogenetic analysis by maximum likelihood. *Molecular Biology and Evolution*, 24, 1586–1591.

Yang, Z., & Nielson, R. (2002). Codon-substitution models for detecting molecular adaptation at individual sites along specific lineages. *Molecular Biology and Evolution*, 19(6), 908–917. https://doi.org/10.1093/oxfordjournals.molbev.a004148

Yang, Z., Nielsen, R., Goldman, N., & Pedersen, A. M. K. (2000). Codon-substitution models for heterogeneous selection pressure at amino acid sites. *Genetics*, 155(1), 431–449. https://doi.org/10.1093/genetics/155.1.431

Zhang, G., Li, C., Li, Q., Li, B., Larkin, D. M., Lee, C., Storz, J. F., Antunes, A., Greenwold, M. J., Meredith, R. W., & Ødeen, A. (2014). Comparative genomics reveals insights into avian genome evolution and adaptation. *Science*, 346(6215), 1311–1320.

Zhang, J., Nielson, R., & Yang, Z. (2005). Evaluation of an improved branch-site likelihood method for detecting positive selection at the molecular level. *Molecular Biology and Evolution*, 22(12), 2472–2479. https://doi.org/10.1093/molbev/msi237

**How to cite this article:** Forni, G., Ruggieri, A. A., Piccinini, G., & Luchetti, A. (2021). BASE: A novel workflow to integrate nonubiquitous genes in comparative genomics analyses for selection. *Ecology and Evolution*, 11, 13029–13035. [https://doi.org/10.1002/ece3.7959](https://doi.org/10.1002/ece3.7959)