CAAStools, a toolbox to identify and test Convergent Amino Acid Substitutions.

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Abstract.

Background.
Upon phylogenetic data, coincidence of Convergent Amino Acid Substitutions (CAAS) with phenotypic convergences allow pinpointing genes that are likely to be associated with trait variation. Such findings can provide useful insights into the genetic architecture of complex phenotypes. Here we introduce CAAStools, a set of bioinformatics tools to identify and validate CAAS in orthologous protein alignments for pre-defined groups of species representing the phenotypic values targeted by the user.

Implementation and availability
CAAStools source code is available at http://github.com/linudz/caastools, along with documentation and examples.

Supplementary information

Supplementary data are available.

Introduction.

Convergent Amino Acid Substitutions (CAAS) can provide important insights into the genetic changes underlying phenotypic variation (Zhang and Kumar, 1997; Ray *et al*., 2015). Recent examples include the identification of genes potentially involved in marine adaptation in mammals (Foote *et al*., 2015), and the convergent evolution of mitochondrial genes in deep-sea fish species (Sheng *et al*., 2019). Notably, in 2018, Muntané *et al*. identified a set of 25 genes involved in longevity in primates (Muntané *et al*., 2018). A few years later, a similar analysis for a wider phylogeny retrieved 996 genes associated with lifespan determination in mammals (Farré *et al*., 2021). While these analyses often need to be adapted for each particular phenotype and phylogeny, the CAAS detection and validation strategies reported in the literature share some common steps (Rey *et al*., 2019). First, researchers select the species to compare for CAAS analysis, and split them into two or more groups according to the phenotype. The criteria to select these groups can be quite diverse. Groups can be formed, for instance, by species having diverging values of a given continuous trait, or by species sharing different adaptations, like terrestrial and marine mammals (Foote *et al.*, 2015). The second step consists in linking amino acid substitutions with each group. For that step, different approaches can be used, such as identifying identical substitutions for the same amino acid (Besnard *et al*., 2009; Chabrol *et al*., 2018), detecting topological incongruencies (Li *et al*., 2008), variations in amino acid profiles (Rodrigue *et al*., 2010; Rey *et al*., 2018), or relying on consistent patterns of groups of amino acids in different groups of species (Zhang et al, 2014; Muntané *et al*., 2018; Farré *et al*., 2021). The third step consists in testing the significance of the results. Molecular convergence is a noisy process, as apparent CAAS may randomly take place under no selective force (Shahoua *et al.*, 2017). To overcome this, researchers have adopted different strategies, mostly based on the idea that adaptive CAAS tend to exceed convergent noise. The delta Site-Specific log-Likelihood Score (ΔSSLS), for instance, is a method that consists in comparing the CAAS likelihood for different phylogenetic topologies (Castoe *et al*., 2009; Wang, Susko and Roger, 2012; Parker *et al*., 2013). Another approach is instead based on bootstrap resampling tests to evaluate whether the number of detected CAAS is larger than expected by chance (Muntané *et al*., 2018; Farré *et al*., 2021). Alternatively, some authors adopted a strategy that consists in quantifying the convergent noise and focus on the detection of Convergence on Conservative Sites (CCS) (Xu *et al.*, 2017; He *et al.*, 2020). In spite of all these contributions, there is still no consensus approach. Some authors question whether phenotypic convergence coincides with genome-wide molecular convergence (Zou and Zhang, 2015b), or that adaptive substitutions outnumber random CAAS (Zou and Zhang, 2015a; Gregg *et al.*, 2015). Further methodological developments can surely benefit from the availability of software tools that are specifically designed to retrieve CAAS, allowing to compare different validation strategies.

Here we present CAAStools, a toolbox to identify and validate CAAS. CAAStools is based on the strategy applied in our previous studies (Muntané et al., 2018; Farré et al., 2021), and implements different testing strategies through bootstrap analysis. CAAStools is designed to be included in parallel workflows and is optimized to allow scalability at proteome level.

Implementation.

CAAStools is a multi-modular python application organized into three tools. The outline of the suite is presented in **Figure 1**. The discovery tool is based on the protocol used in Muntané *et al*., 2018 and Farré *et al*., 2021. This approach identifies CAAS between two groups of species in an amino-acid Multiple Sequence Alignment (MSA) of orthologous proteins. These groups are named Foreground Group (FG) and Background Group (BG). Collectively, the two groups are called Discovery Groups (DG), as they represent the base for CAAS discovery. The CAAS identification algorithm scans each MSA and returns those positions that meet the following conditions: First, the FG and the BG species must share no amino acids in that position. Second, all the species in at least one of the two discovery groups (FG or BG) must share the same amino acid. The combination of these two conditions determines a set of different mutation patterns that the tool identifies as CAAS. Details on these patterns are provided in Supplementary Table 1.

Finally, CAAStools calculates the probability of obtaining a CAAS in a given position compared to randomized DGs, corresponding to the empirical p-value of the predicted CAAS in that position. This p-value represents a quantification of the convergent noise (Shahoua *et al*., 2017) that is associated with a specific position. The details of this calculation are presented in Supplementary section 3. The Resample tool sorts species into *n* virtual DGs (re-samplings) for bootstrap analysis according to different combination strategies. This tool is thought to enable bootstrap analyses based on CAAS excess or likelihood (Muntané et al., 2018; Farré et al., 2021; Castoe *et al*., 2009). In a *Naive* modality, the probability of every species being included in a DG is considered identical and independent. This feature allows for bootstrap analyses aimed at quantifying convergent noise. However, species are phylogenetically related, biasing their probability of sharing a phenotype or amino acid. To address these phylogenetic dependencies CAAStools includes two other testing strategies. In the *Phylogeny-restricted* modality, the randomization can be restricted to some taxonomic orders or defined clades. These clades will match the ones of the species included in the DGs. In the *Brownian motion* modality, resampling is based on Brownian Motion simulations. This latter is inspired by the "permulation" strategy for trait randomization (Saputra et al., 2021), and its implementation relies on the *simpervec()* function from the RERconverge package (Kowalczyk *et al*., 2019). Finally, the *bootstrap* tool determines the iterations returning a CAAS for each position in a MSA to establish the corresponding empirical p-value for the detection of a CAAS in that position. Both the discovery and the bootstrap tools are designed to be launched on single MSAs, in order to allow the user to parallelize the workflow for large protein sets.

Usage and testing

We tested CAAStools on the dataset from Farré *et al*., (2021). The details of this test are reported in Supplementary 3. The full dataset is available in the /test folder within the CAAStools repository.

Figures

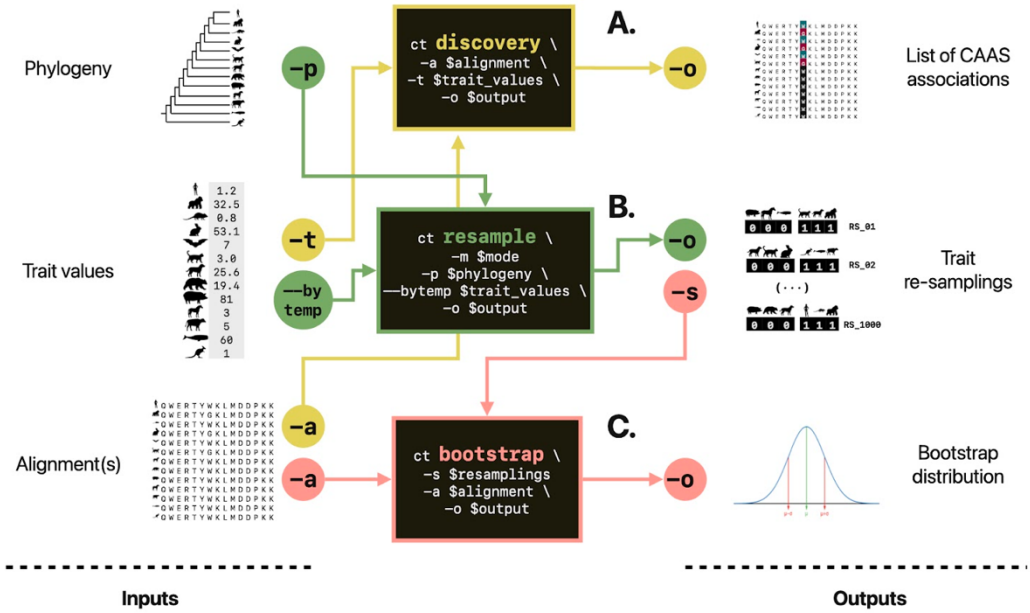

Figure 1. CAAStools layout. The three tools of the CAAStools suite rely on three pieces of information; a phylogenetic tree, the trait information, and an amino acid MSA. The discovery tool (A) detects the CAAS between two groups of species that are defined by the user on the basis of trait values. The resample tool (B) performs *n* trait resamplings in different modalities, on the bases of the phylogeny and the trait value distributions. The output of this resampling is processed by the bootstrap tool (C) that elaborates a bootstrap distribution from the MSA. All the tools can be executed independently.

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Author Contributions

Fabio Barteri (FB) has been in charge of the development of CAAStools. Alejandro Valenzuela (AV) carried on the beta-testing and code debugging. FB and AV have contributed equally to this manuscript. Xavi Farré (XF), Gerard Muntané (GM) and Arcadi Navarro (AN) designed the CAAS identification and validation protocol. XF wrote a set of scripts to identify CAAS that served as template for CAAStools implementation. AN and GM conceptualized the method. AN, David de Juan (DJ) and Borja Esteve-Altava (BEA) - along with GM - participated in the scientific discussion

and supervision of this project. DJ's contribution was particularly helpful for the optimization of CAAStools code.

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CAAStools, a toolbox to identify and test Convergent Amino Acid Substitutions.

Supplementary information.

Supplementary 1. CAAS Discovery algorithm.

Given two Discovery Groups (DGs, Foreground and Background groups, FG and BG, respectively), the discovery tool recognizes as CAAS all those substitutions that meet two requirements. Let *A* be an MSA of *q* sequences of length *t.* We can describe A as an array of *t* positions [1]. Each position (pos_i) will consist of a set of N different amino acids, *a*, with absolute frequency (or count), *f*, where *NS* is the total number of symbols in the alignment [1].

[1] $A = (pos_1\, pos_2\, \dots\, pos_t)\ ;\ pos_i = a_1\, f_1\, a_2\, f_2\, \dots\, a_{NS}\, \dots\, f_{NS}\ ;\ \sum_1^{NS} f = q$

The FG and the BG are formalized as sets of different species s_{FG} and s_{BG}, with no intersection and size l_{FG} and l_{BG} [2].

[2] $s_{FG} = (s_1\, s_2\, \dots\, s_{l_{FG}})\ ;\ s_{BG} = (s_1\, s_2\, \dots\, s_{l_{BG}})$

$s_{FG} \cap s_{BG} = \emptyset$

In each alignment position, s_{FG} and s_{BG} are associated with two sets of amino acids, $fg(pos_i)$ and $bg(pos_i)$, with length w_{FG} and w_{BG}.

[3] $fg(pos_i) = a_1\, f_1\, a_2\, f_2\, \dots\, a_{w_{FG}}\, \dots\, f_{w_{FG}}\ ;\ bg(pos_i) = a_1\, f_1\, a_2\, f_2\, \dots\, a_{w_{BG}}\, \dots\, f_{w_{BG}}$

CAAStools identifies a CAAS when three conditions are met [4]. First, the two groups must share no amino acids. This means that all the species in the FG need to have different AAs than the species in the BG. Second, at least one of the two DGs must share (or "converge to") the same amino acid. Also, the CAAS is detected if at least one amino acid is associated to both DGs

[4] $CAAS_i\{fg(pos_i) \cap bg(pos_i) = \emptyset\ w_{FG} == 1\ or\ w_{BG} == 1\ w_{FG} > 0\ and\ w_{BG} > 0$

The combination of these three rules defines 3 different mutation *patterns*. We define *pattern 1* when the DGs converge to two different amino acids ($w_{FG} = 1;\ w_{BG} = 1$). The *pattern 2* will be verified as the FG converges to one amino acid, but the BG will be associated with different amino acids ($w_{FG} = 1;\ w_{BG} > 1$). *Pattern 3* will consist in the opposite situation, or else when the FG is associated with different amino acids, whilst the BG converges to a single amino acid ($w_{FG} > 1;\ w_{BG} = 1$). **Supplementary Table**

1 summarizes the different mutation patterns and the meeting of requirements for CAAS identification.

Supplementary Table 1. *Mutation patterns and associated program decisions on CAAS assignment.*

Discovery Groups		Difference between DGs	Convergence in		Pattern
FG	**BG**		**FG**	**BG**	
KV	K	NO	NO	YES	Not a CAAS (No difference)
M	TM	NO	YES	NO	Not a CAAS (No difference)
MK	VE	YES	NO	NO	Not a CAAS (No convergence)
K	V	YES	YES	YES	**Pattern 1** (Both convergent)
K	VM	YES	YES	NO	**Pattern 2** (FG convergent, BG multiple)
KE	W	YES	NO	YES	**Pattern 3** (FG multiple, BG convergent)

Supplementary 2. CAAS discovery statistical testing

CAAStools calculates an empirical p-value for each CAAS prediction. This p-value is equal to the probability of obtaining a CAAS with random species, and under the same conditions as the CAAS discovery (size of the DGs, maximum permitted gaps and missing species). Following the MSA description in [1], we'll consider a couple of DG (*FG* and *BG*) of size l_{FG} and l_{BG}, as formalized in [2]. The probability to obtain a CAAS from random species is calculated as the probability of extracting concomitantly k_{FG} and k_{BG} objects from a population of size N over a number of extractions *n*, provided the conditions in [4], i.e. $k_{FG} \cap k_{BG} = \emptyset$ and $wk_{FG} == 1 \; or \; wk_{BG} == 1$ where *wk* is the number of symbols in the resampling *k*. This probability can be calculated through the probability mass function from the hypergeometric distribution [5].

[5] $$P(k) = \frac{\binom{K}{k}\binom{N-k}{n-k}}{\binom{N}{n}} = Hyp(N, K, k, n)$$

$$P(CAAS) = P(FG) * P(BG)$$

$$\begin{cases} P(FG) = Hyp(N_{FG}, K_{FG}, k_{FG}, n_{FG}) \\ N_{FG} = q\,; \\ k_{FG} = l_{FG} - null_{FG} \\ n_{FG} = k_{FG} \end{cases}$$

$$\begin{cases} P(BG) = Hyp(N_{BG}, K_{BG}, k_{BG}, n_{NG}) \\ N_{BG} = q - l_{FG} \\ k_{BG} = l_{BG} - null_{BG} \\ n_{BG} = k_{BG} \end{cases}$$

Note that the size of the population N in *P(FG)* differs from the one considered in *P(BG)*. In the first case, the probability of obtaining a convergence in the *FG* is calculated on the total number of sequences in the alignment. In *BG*, the size considered is the difference between the total number of sequences in the alignment *q* and the size of the other group ($q - l_{BG}$), ($q - l_{FG}$), as the two events are concomitant but not independent. The number of extractions k_{FG} and k_{BG} are equal to the number of the difference between the size of the DGs and the number of indels and missing species allowed by the user (*null*). The terms K_{FG} and K_{BG} represent the number of successes in the population. In [6], [7]

and [8], we see how this value can be calculated considering all the possible combinations of amino acid symbols that meet the requirements for CAAS detection [4].

[6] $$C_{P1,2} = \{K_{FG} = [f_j];\ K_{BG} = [q - f_j]\ \forall\ a_j \in pos_i\}$$
$$C_{P1,2} = [(K_{FG_1};\ K_{BG_1}), (K_{FG_2};\ K_{BG_2}) \dots (K_{FG_z};\ K_{BG_z})]$$

[7] $$C_{P1,3} = \{K_{FG} = [q - f_j];\ K_{BG} = [f_j]\ \forall\ a_j \in pos_i\}$$
$$C_{P1,3} = [(K_{FG_1};\ K_{BG_1}), (K_{FG_2};\ K_{BG_2}) \dots (K_{FG_z};\ K_{BG_z})]$$

[8] $$C_{P1} = \{K_{FG} = [f_j];\ K_{BG} = [f_h]\ \forall\ a_j, a_k \in pos_i\}$$
$$C_{P1} = [(K_{FG_1};\ K_{BG_1}), (K_{FG_2};\ K_{BG_2}) \dots (K_{FG_z};\ K_{BG_z})]$$

These combinations are based on patterns (P). Note that $C_{P1,2}$ and $C_{P1,3}$ overlap, and that the intersection coincides with C_{P1}. We can now calculate the CAAS probability separately for each pattern [9].

[9] $$P(CAAS_{P1,3}) = \sum_{x=1}^{z} Hyp(N_{FG}, K_{FG_x}, k_{FG}, n_{FG}) * Hyp(N_{BG}, K_{BG_x}, k_{BG}, n_{NG})$$
$$P(CAAS_{P1,2}) = \sum_{x=1}^{z} Hyp(N_{FG}, K_{FG_x}, k_{FG}, n_{FG}) * Hyp(N_{BG}, K_{BG_x}, k_{BG}, n_{NG})$$
$$P(CAAS_{P1}) = \sum_{x=1}^{z} Hyp(N_{FG}, K_{FG_x}, k_{FG}, n_{FG}) * Hyp(N_{BG}, K_{BG_x}, k_{BG}, n_{NG})$$

The probability to obtain a CAAS in position pos_i is hence calculated as it follows:

[10] $$pvalue_{pos_i} = P(CAAS_{pos_i}) = P(CAAS_{P1,3}) + P(CAAS_{P1,2}) - P(CAAS_{P1})$$

2.1 Correction for discovery groups of equal size.

If the species found in the alignment are the same for FG and BG sizes ($l_{FG} = l_{BG}$), the probability of retrieving pattern 2 and pattern 3 are equal. In this case, the p-value is equal to the $P(CAAS_{P1,2})$.

[11] $$\begin{cases} pvalue_{pos_i} = P(CAAS_{pos_i}) = P(CAAS_{P1,2}) \\ \qquad\qquad if\ l_{FG} = l_{BG} \end{cases}$$

Supplementary 3. CAAS discovery from Farré et al., 2021.

As a test run for CAAStools, we repeated the CAAS discovery from the results published by Farré et al., in 2021 and entitled "*Comparative Analysis of Mammal Genomes Unveils Key Genomic Variability for Human Life Span*" (DOI: 10.1093/molbev/msab219). In this work, 13,035 MSA from UCSC public database (https://genome.ucsc.edu/, accessed August, 2019) were scanned to find CAAS between two groups of species with divergent maximum lifespan. The "long lived" group is formed by *Homo sapiens* (hg38), *Nomascus leucogenys* (nomLeu3), *Heterocephalus glaber* (hetGla2), *Myotis davidii* (myoDav1), *Myotis lucifugus* (myoLuc2), *Eptesicus fuscus* (eptFus1). The "short lived" group is

formed by *Mesocricetus auratus* (mesAur1), *Rattus norvegicus* (rn6), *Pantholops hodgsonii* (panHod1), *Sorex araneus* (sorAra2), *Condylura cristata* (conCri1), *Monodelphis domestica* (monDom5). Farré et al., filtered the results from CAAS discovery to those CAAS having no gaps or missing species, and focused their analysis on the CAAS of scenarios 1 and 2, which correspond to patter 1 and 2 in CAAStools terminology.

We have repeated this analysis under the same conditions, filtering for pattern 1 and 2 and for no gaps in foreground (*short-lived* group) and background (*long-lived* group). The results (*Supplementary dataset 1*) and the phenotype configuration (*Supplementary dataset 2*) are available in the supplementary.material.xls spreadsheet. Our analysis confirmed the identification of 2737 mutations in 2004 MSA.

Supplementary 4. An example of p-value calculation from *Farré et al.* dataset.

The gene AKAP13 (RefSeq code: NM_006738) is part of the dataset published by Farré et al. in 2021. In that analysis, authors selected only those positions that were associated with no gaps in both Foreground and Background, obtaining 3 CAAS from this gene. Here, we repeated the CAAS detection without any filtering for gaps or missing species. The result is shown in *Supplementary Table 2*. The extended CAAStools discovery output can be found in *Supplementary Dataset 3*.

Supplementary Table 2*: Resume of the CAAStools discovery analysis for AKAP13 with phenotype information from Farré et al., 2021.*

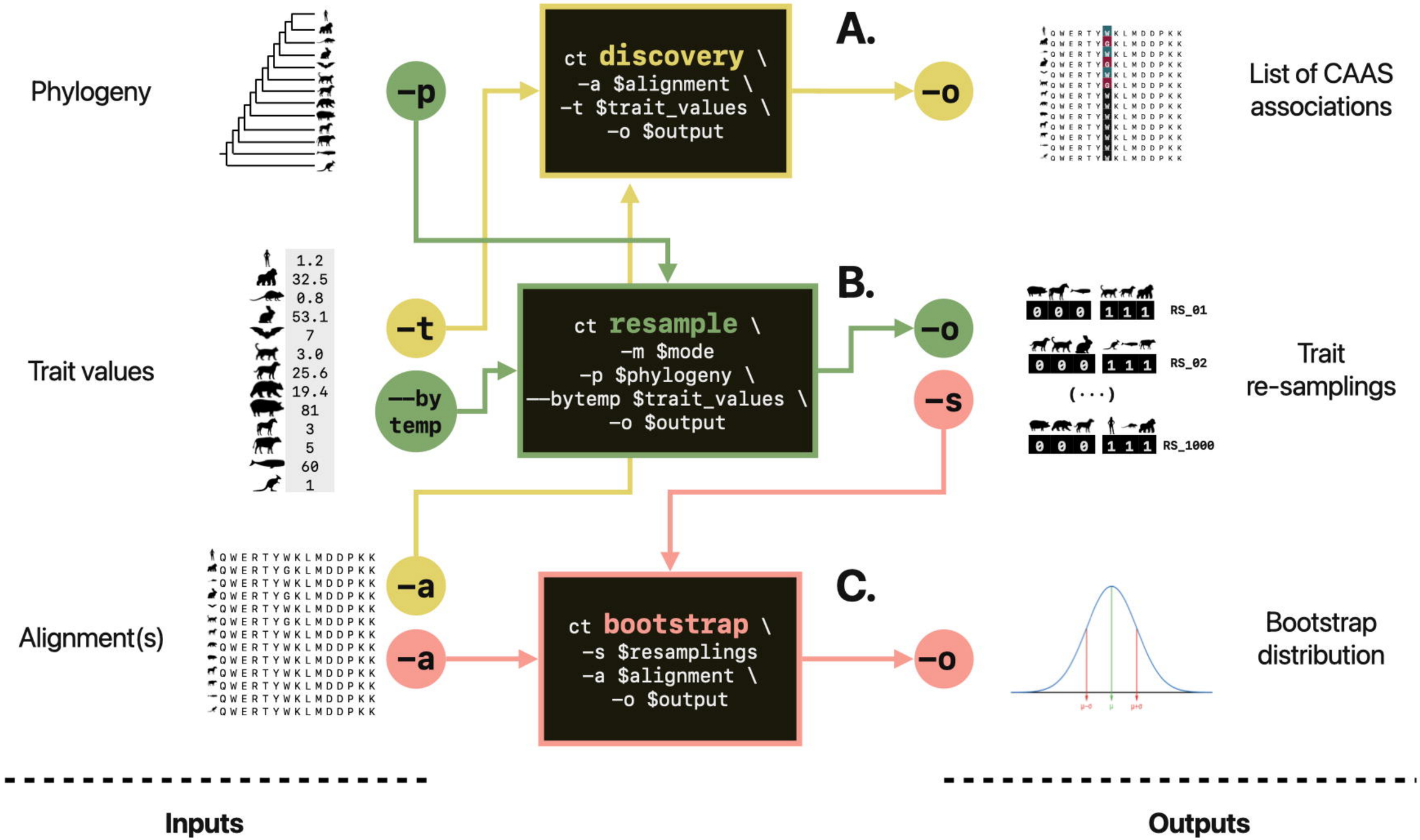
Gene	Position	Substitution	P-value	Pattern	Gaps FG	Gaps BG
AKAP13	392	T/AIS	0.0009037398709	2	0	1
AKAP13	451	M/GLST	0.001177073702	2	0	1
AKAP13	577	A/INST	0.0005992501486	2	0	1
AKAP13	657	D/AEGSV	0.0002559580225	2	0	0
AKAP13	**931**	**V/AMRT**	**0.0001223275131**	**2**	**0**	**0**
AKAP13	1180	D/EPRS	0.007729389679	2	3	0
AKAP13	1185	V/ADP	0.0152264739	2	2	3
AKAP13	1204	D/AENP	0.001044565793	2	1	1
AKAP13	1210	G/CDV	0.0121208014	2	3	3
AKAP13	1212	R/DK	0.02023676039	2	3	3
AKAP13	1213	E/HKQ	0.01388995848	2	3	3
AKAP13	**1219**	**GP/V**	**0.1087036943**	**3**	**3**	**4**
AKAP13	1298	K/EHQST	0.0001593229323	2	0	0
AKAP13	1315	E/GIKR	0.0008881559737	2	1	1
AKAP13	1827	S/GN	0.00135352871	2	0	1
AKAP13	2685	R/CHQ	0.004566143925	2	1	2
AKAP13	2692	H/NRY	0.0007280567925	2	0	1

In order to show the details of the p-value calculation through the hypergeometric function, we decided to focus on the position 931 and 1219. Both positions present a

CAAS, but their p-values are largely different. In position 931, the p-value is 0.0001223275131, while the p-value associated with position 1219 is 0.1087036943. The details of the p-value calculations are presented in *Supplementary Table 3*. For each amino acid combination, the algorithm calculates the probability to obtain a convergence in the *FG* (*P(FG)*) and the probability to obtain a *BG* with different amino-acids (*P(BG)*).

Supplementary Table 3. *P-value calculation for positions 931 and 1219 in AKAP13 gene (NM_006738).*

P 931	**FG**	**BG**	**P(FG)**	**P(BG)**	**P(CAAS)**	**Patterns**
	V	L,G,T,A,R,M	0.0731423047	0.00167128469	0.000122241614	1,2
	A	L,V,G,T,R,M	1.14E-07	0.8928571429	1.02E-07	1,2
				P-value	**1.22E-04**	
P 1219	**FG**	**BG**	**P(FG)**	**P(BG)**	**P(CAAS)**	**Patterns**
	A	I,P,S,G,V,T	0.03024193548	0.5310344828	0.01605951057	1,2
	I,P,S,G,V,T	A	0.2822580645	0.1264367816	0.03568780126	1,3
	P	I,A,S,G,V,T	0.06671554252	0.3931034483	0.02622610982	1,2
	I,A,S,G,V,T	P	0.1776026393	0.2091954023	0.03715365558	1,3
	V	I,A,P,S,G,T	0.0007331378299	0.9333333333	0.0006842619746	1,2
	I,A,P,S,G,T	V	0.6697214076	0.01379310345	0.009237536657	1,3
	A	P	0.03024193548	0.2091954023	0.00632647386	1
	A	V	0.03024193548	0.01379310345	0.0004171301446	1
	P	V	0.06671554252	0.01379310345	0.0009202143796	1
	P	A	0.06671554252	0.1264367816	0.00843529848	1
	V	A	0.0007331378299	0.1264367816	9.27E-05	1
	V	P	0.0007331378299	0.2091954023	0.0001533690633	1
				P-value	**0,1087**	


Phylogeny
Trait values
Alignment(s)
-p
-t
--by temp
-a
-a
A.
ct discovery \
-a $alignment \
-t $trait_values \
-o $output
B.
ct resample \
-m $mode
-p $phylogeny \
--bytemp $trait_values \
-o $output
C.
ct bootstrap \
-s $resamplings
-a $alignment \
-o $output
-o
-o
-s
-o
List of CAAS associations
Trait re-samplings
RS_01
RS_02
(...)
RS_1000
Bootstrap distribution
Inputs
Outputs