Discovery and Characterization of Terpene Synthases Powered by Machine Learning

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Abstract

Terpene synthases (TPSs) generate the scaffolds of the largest class of natural products, including several first-line medicines. The amount of available protein sequences is increasing exponentially, but computational characterization of their function remains an unsolved challenge. We assembled a curated dataset of one thousand characterized TPS reactions and developed a method to devise highly accurate machine-learning models for functional annotation in a low-data regime. Our models significantly outperform existing methods for TPS detection and substrate prediction. By applying the models to large protein sequence databases, we discovered seven TPS enzymes previously undetected by state-of-the-art computational tools and experimentally confirmed their activity. Furthermore, we discovered a new TPS structural domain and distinct subtypes of previously known domains. This work demonstrates the potential of machine learning to speed up the discovery and characterization of novel TPSs.

Introduction

Terpene synthases (TPSs) are ubiquitous enzymes that produce the hydrocarbon scaffolds for the largest and the most diverse class of natural products called terpenoids, which include widely used flavors, fragrances, and first-line medicine. The most scents a human has ever experienced are terpenoids[1]. Some sesquiterpenes treat malaria (e.g., a Nobel-prize winning artemisinin[2] with a market size projected to reach USD 697.9 million by 2025[3]), bacterial infections, and migraines[4]. Some diterpenes possess anticancer or anti-inflammatory properties and treat cardiovascular diseases[5,6], e.g., taxol is the first-line anticancer medicine with billion-dollar pick annual sales[7]. Triterpenes can improve wound healing and blood circulation[8]. Several other complex terpenes exhibit anticancer properties by acting on different stages of tumor development[8,9]. Furthermore, terpenes are being used as biofuels[10,11].

Yet, these fantastic small molecules, terpenes and terpenoids, are too complex to be efficiently synthesized industrially[12], and are typically extracted from plants, which is resource-intensive. For instance, each patient requires 3-10 Pacific yew trees for the mentioned anticancer treatment taxol[13]. A more sustainable and efficient production of terpenoids can be achieved via synthetic biology [14].

However, the discovery of TPSs responsible for the biosynthesis of these invaluable natural products is not trivial. The amount of available protein sequences is increasing exponentially. Preprocessing the results of high-throughput DNA sequencing by detecting the most likely candidates for TPS function can significantly accelerate the progress in bioprospecting, natural product biosynthesis, and synthetic biology.

Recent machine learning (ML) advances enable high-quality automatic data annotation employing models trained on large datasets. Breakthroughs in biological applications of machine learning are best exemplified by protein language models and protein structure prediction models like AlphaFold2[15–17]. Protein language models (PLM) enabled state-of-the-art prediction of enzymatic activity when trained on all available annotated data from Uniprot[18], and the analysis of AlphaFold2 predictions at scale led to new biological insights about protein structure space[19,20]. However, when studying a specific enzymatic family, researchers do not have the luxury of working with labeled data at scale but rather deal with small datasets of characterized enzymes[21]. A low-data regime makes the application of the recent deep-learning techniques challenging. As a result, established computational approaches for *in-silico* TPS characterization are based on techniques such as PSI-BLAST[22] and Profile Hidden Markov Models[23].

In the case of TPSs, existing datasets of characterized sequences are sparse[21]. Mere dozens of characterized proteins cover TPS classes such as sesterTPSs or tetraTPSs. As a result, there are no tools to predict these rare classes. The state-of-the-art TPS detection and characterization method[24] predicts only the most abundant substrate classes.

Here, we present a curated dataset of over a thousand characterized TPS sequences (Fig 1a-b) and use it to train a machine-learning pipeline for automated TPS detection and substrate prediction. A protein sequence is the only required input to the pipeline. Our method leverages

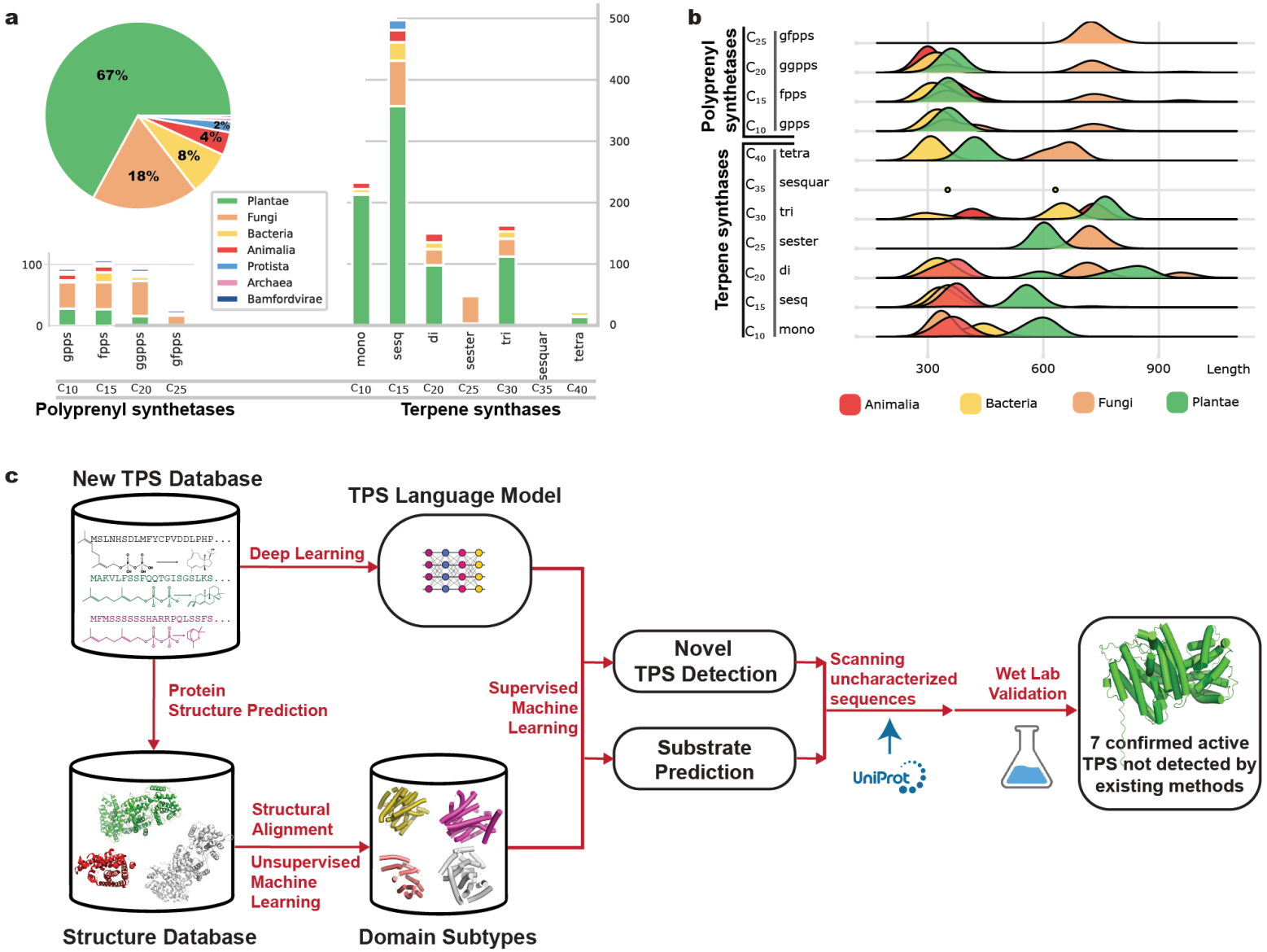

Fig. 1 | Curated dataset and workflow overview. a) *The dataset comprises more than 1,000 TPSs, three times more than the state-of-the-art TPS dataset[25]. Our dataset covers a larger variety of TPS classes, notably a significant amount of triterpene synthases. In addition, less than 70% of the TPSs come from plants, while related work [25] was based solely on plant TPSs.* ***b)*** *The novel comprehensive dataset enables population-wide insights, such as revealing differences and similarities of protein length distributions per TPS classes and kingdoms.* ***c)*** *An overview of our solution workflow: from curating a TPS database of characterized reactions to the wet-lab characterization of seven novel TPS enzymes, which were missed by all computational tools integrated into InterProScan for protein function, domains, and families annotation[26].*

and builds on recent PLM[27] and AlphaFold2[17] models as well as established methods for structural alignment, unsupervised, and supervised ML. Fig.1c overviews our proposed workflow. Final models are highly accurate, even for rare classes like sesterTPSs or tetraTPSs. By increasing the mean average precision of TPS substrate classification from 0.69 to 0.89, our solution greatly outperforms existing methods based on PSI-BLAST[22], Profile Hidden Markov Models[23], and recent state-of-the-art deep-learning models Foldseek[20] and CLEAN[18]. We applied our method to the UniRef50 database and selected challenging detections for wet

lab validation. The selected TPS hits were completely undetected by traditional bioinformatic approaches like Pfam[28], SUPFAM[29], or InterProScan[26], some came from organisms not typical for TPS activity, like archaea or viruses. We conducted experimental validation by expressing newly detected TPSs in budding yeast. Using this approach, we confirmed the predicted activity for seven TPSs. Furthermore, our systematic computational analysis revealed subtypes of structural TPS domains, with one subtype possessing distinct properties to be considered a separate structural domain. We derived a discriminative sequence motif for the discovered domain by leveraging ML explainability methods.

Results

Computational characterization of terpene synthases

The goal is to develop an in-silico method for accurate TPS detection in large datasets of protein sequences available thanks to high-throughput DNA sequencing. Moreover, we aim to estimate substrate(s) for each detected TPS, including predicting underrepresented substrate types. For this task, the main challenge is the sparsity of the TPS sequence space due to the low-data regime. Here, we describe a three-step strategy to overcome this challenge.

Our first step to tackle the sparsity of the TPS sequence space is to gather more characterized examples. To that end, we mined over 700 TPS sequences from Swiss-Prot, the expertly curated UniProtKB component produced by the UniProt consortium[30]. In addition, we gathered characterized TPS sequences from academic literature. Manual curation of the related academic literature resulted in more than 417 proteins not included in Swiss-Prot. We enriched the dataset with over 100 polyprenyl synthetases that produce TPS substrates to enable a more comprehensive view of TPS-related activity. Details about the curated TPS dataset are in Fig.1a-b and in Methods.

Next, to overcome the sparsity of small data, we leverage recent Protein language models (PLMs) pre-trained on all available uncharacterized protein sequences[16,27,31]. For small enzymatic datasets, a pre-trained PLM can serve as a starting point for model training or as a feature extractor. Recently, PLMs enabled a performance boost in the sequence-based prediction of enzymatic activity, best exemplified by a state-of-the-art EC number predictor CLEAN[18]. Nevertheless, we found that the prediction of TPS substrates remains challenging for the CLEAN model, see Fig. 4c. We build our models on top of pre-trained PLMs, tailored for highly accurate characterization of TPSs.

Our third step to overcome the sparsity of TPS sequence space is the inclusion of structural information. TPS structure evolves slower than sequence[21], and TPSs with low sequence similarity can share common 3D folds[24,32]. In related work, it was demonstrated that handcrafted features derived from homology-based structure modeling enable binary classification of sesquiTPSs into two broader groups of products[21]. Since then, AlphaFold2 has enabled highly accurate structure prediction from sequence[17]. A recent deep-learning model Foldseek enables

the related protein search in a structure space[20], analogously to BLAST-based search in a sequence space. However, similar global structural TPS folds might differ in catalytic activity[24]. Extracting information about fine local differences in structural folds holds promise to significantly improve the modeling of enzymatic function[33]. Yet, for small datasets, the curse of dimensionality challenges data-driven learning of subtle differences in the 3D representation of proteins. We leverage the domain knowledge about TPS structural composition to include structural information in modeling while escaping the curse of dimensionality. Different terpene synthases share similar local structural modules called α, β, γ domains[24,34,35], and we developed algorithms for their detection.

Discovery of structural domain subtypes

Fig. 2a gives a high-level overview of the TPS domain detection pipeline; more details can be found in the Methods section. The devised computational method for segmenting a protein structure into TPS-specific domains enabled us to analyze the domains at the scale of all characterized TPSs.
The main contribution of domain detection is the possibility of analyzing TPSs at the resolution of individual structural modules. At the same time, the devised domain-based workflow leads to a better quality of whole-protein clustering compared to state-of-the-art sequence-based and structure-based methods (Fig. 2b).

We compared the detected domains among each other via structural alignment[36]. On top of structural similarity, we used a state-of-the-art unsupervised machine learning approach[37] and clustered the detected domains. Each cluster defines a group with a distinct fold. We consider the discovered groups to be subtypes of the established α, β, γ structural domains in TPSs.

Analysis of the domain subtypes led to several insights.
First, there is a subtype related to γ TPS domain, which has a distinct structure, in Fig. 2c it is denoted as δ. One can see in Fig. 2c/ii that structures grouped in the δ cluster align on top of each other almost perfectly. Fig. 2c/iii-v show that the δ cluster structure differs from any other subtypes within classical β and γ TPS domains. Folds aligned in Fig. 2c/v are obviously different but used to be considered the same γ TPS domain.
The next insight highlights the distinct biochemistry of the δ cluster. Fig. 2d enlists all substrates at least one TPS with the δ-cluster domain can accept. Basically, no TPS without a δ-cluster domain can accept any of these substrates.
Further insight enabled by the developed domain-level analysis sheds light on TPSs with αα architecture. We discovered that the second α domain is different from all other α TPS domains and is closer to the domain found in polyprenyl synthetases (Fig. 2e).

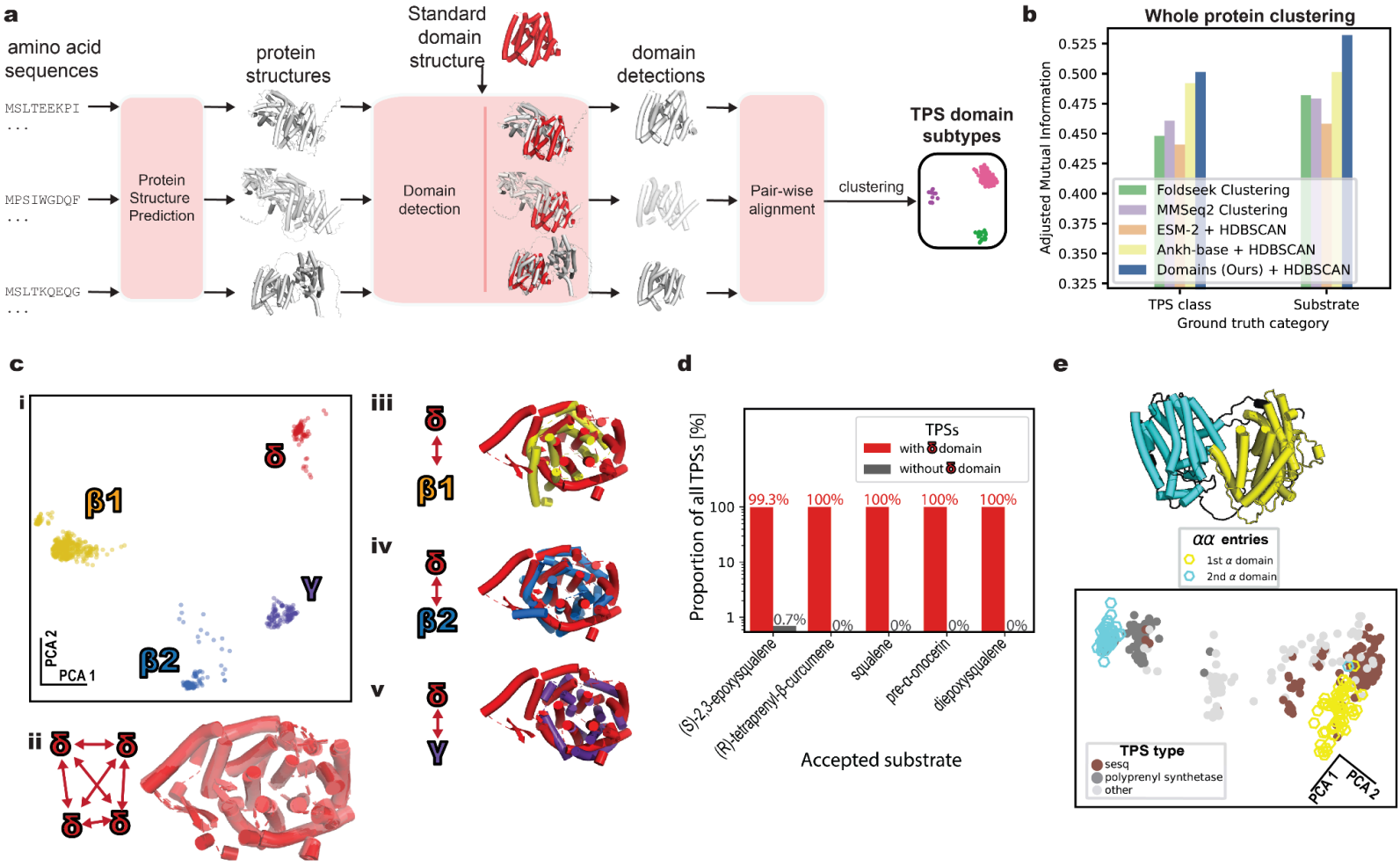

Fig. 2 | Structural domains analysis. a) *Overview of our structure-based workflow.* ***b)*** *Assessment of the quality of whole-protein clustering.* ***c) i)*** *Pairwise comparisons of detected β and γ domains reveal four clusters with distinct folds. We used Principal Components Analysis (PCA), which preserves the global structure of the space of structural similarities.* ***c) ii)*** *Domains grouped in the δ cluster align almost perfectly on top of each other.* ***c) iii-v)*** *There are substantial differences in folds when aligning representants of different clusters.* ***d)*** *Analysis of substrates accepted by all TPSs from the δ cluster in the panel c). All possible substrates the δ cluster representatives accept are enumerated on the x-axis. Each substrate is exclusively accepted by δ-cluster TPSs. This finding highlights that TPSs from the δ structural domain cluster have a distinct catalytic function.* ***e)*** *Domain-level resolution of our analysis shed light on TPSs with αα architecture. Their α domains differ from each other substantially. The first domain is structurally similar to the domain found in sesquiTPSs (sesq in legend), while the fold of another α domain resembles polyprenyl synthetases.*

Furthermore, we described 18 different domain configurations of natural TPS enzymes that can be clearly distinguished based on the developed structural analysis (Fig. 3). The order of domains in the TPS sequence confirms that β1 and β2 clusters from Fig. 2c correspond to subtypes of the established β domain, while γ and δ clusters used to be considered the same γ domain. Please note in Fig. 3 that the δ cluster is essential for triTPSs activity, being present in all triTPSs with βγ architecture and only there. TriTPSs with βγ architecture constitute approximately 80% of all triTPSs in the curated dataset. Moreover, we have shown that the δ cluster has a unique biochemical profile (Fig. 2d) and is distinct structurally (Fig. 2c).

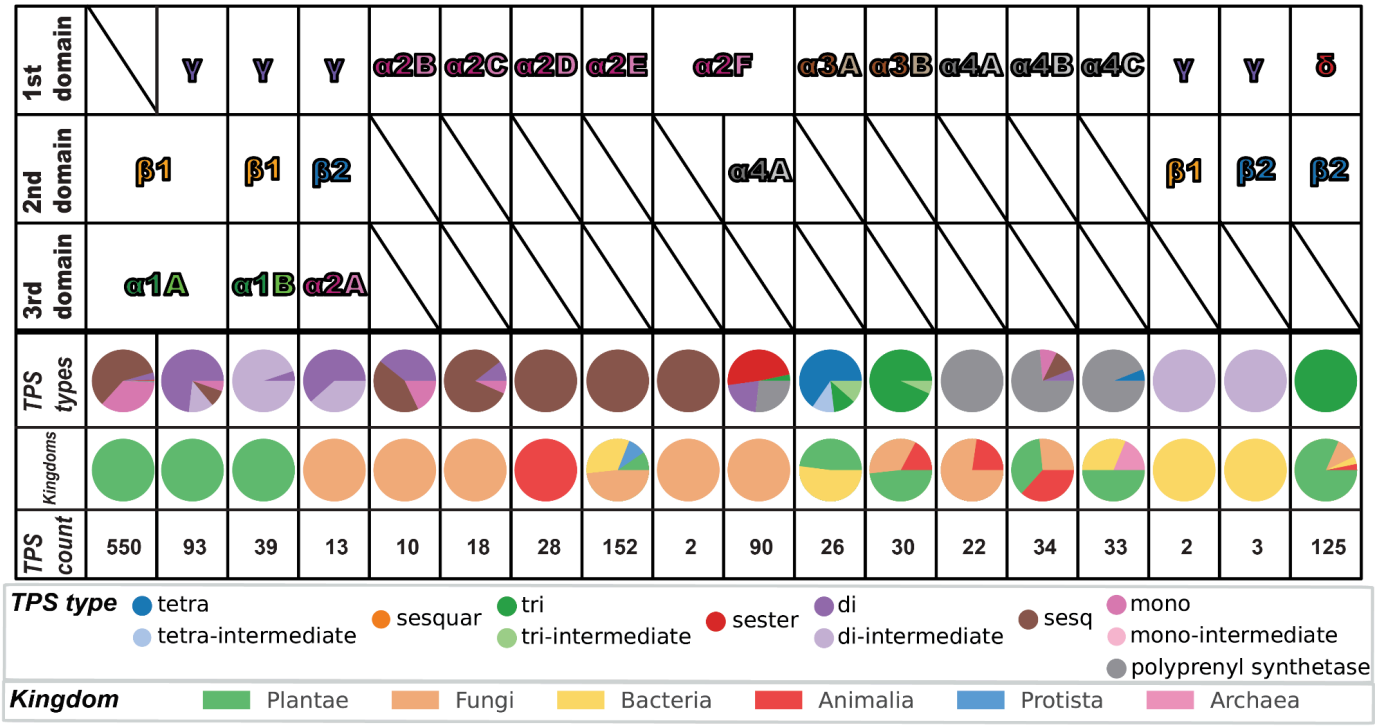

Fig. 3 | Domain configurations of natural TPS enzymes. *Sequences of domain subtypes in a TPS sequence and corresponding TPS types and kingdoms profiles.*

Considering all these, we propose to consider the δ cluster a separate structural domain and denote it as a *δ domain*.

When analyzing the clusters, it is apparent that there are four larger groups of the α TPS domain (Extended data Fig. 2a). Nevertheless, we uncovered that each larger group has its subclusters. In addition to the hierarchical organization of the α domain subtypes, it is apparent that the space of possible α subtypes represents a spectrum of gradual structural variations (Extended data Fig. 4b). The space of β/γ domain subtypes, on the other hand, has very distinct four subtypes (Extended data Fig. 2b, Extended data Fig. 4a).

Furthermore, we performed a phylogenetic analysis of TPS sequences corresponding to different domain configurations from Fig. 3. The phylogenetic tree is in Extended data Fig. 3. One can see the evolutionary relations between the discovered domain configurations. The identified configurations of domain subtypes tend to cluster together in subtrees of the phylogenetic tree, which is another indicator of the biological relevance of the discovered subtypes. In addition, it is fascinating to attempt reading the course of evolution from the constructed tree in Extended data Fig. 3. For instance, one can observe that some of TPSs with configuration γ+β+α1A likely lost their γ domain throughout evolution, while others got their α domain mutated from the α1A subtype to α1B.

Analysis of TPS-type profiles per domain configurations in Fig. 3 shows a correlation with catalytic activity. Motivated by this insight, we leverage information about structural domains for TPS detection and substrate classification.

Predictive models for TPS detection and substrate prediction

The goal is, given a protein sequence, to detect TPS activity and predict the exact substrate for each TPS detection.
The main challenge is underrepresented TPS classes that have less than a dozen characterized sequences. Training a detector with a protein structure as an input is very challenging for such small data because of the curse of dimensionality. To address this issue, we design an approach that leverages our segmented structural domains and compares each domain to the most similar ones from characterized TPSs. In detail, we store the segmented AlphaFold2-predicted structures corresponding to the underrepresented classes and, at inference time, compare a segmented structure of an unknown protein to the stored domain structures. Leveraging our procedure for segmenting a TPS structure into separate domains, it is possible to compare domains of the same type instead of whole structures all at once. Then, for a detected domain in the unknown protein, we store its alignment-based similarity to training domains of the same type and use the vector of similarities as input features for a predictive model. We use a Random Forest[38] as our model for its resilience to overfitting and ability to automatically perform feature selection[39]. We take sequence embeddings from a TPS language model as additional input features to the Random Forest model. We started with a protein language model (PLM)[27] pre-trained on the whole UniProt[40]. To tailor the PLM to our task, we mined sequence databases using TPS-specific Pfam[28] and SUPFAM[29] domains and retrieved sequences resembling TPSs. Using this mined dataset of putative TPS sequences, we fine-tuned the pre-trained PLM using a masked language modeling objective.

An overview of our predictive pipeline is provided in Fig. 4a, while in Fig. 4b, we report an evaluation of TPS detection performance. We benchmark our method against well-established Pfam[28], SUPFAM[29] domains, PSI-BLAST[22], Profile Hidden Markov Models[23], the recent deep-learning model Foldseek[20] for protein search in the structure space of AlphaFold2 predictions[20], and a state-of-the-art EC number predictor CLEAN[18]. We found classical methods to have strong performance in the task of TPS detection. Still, our pipeline further improved the detection quality by a large margin. Additionally, Fig. 4c illustrates that the existing approaches struggle to distinguish different TPS types, while our pipeline improves the performance of substrate prediction from the best baseline mean average precision of 0.69 to 0.89. Notably, our TPS classification across all TPS types with the strongest performance improvement for underrepresented classes of sesterTPSs and tetraTPSs, see Fig. 4d.

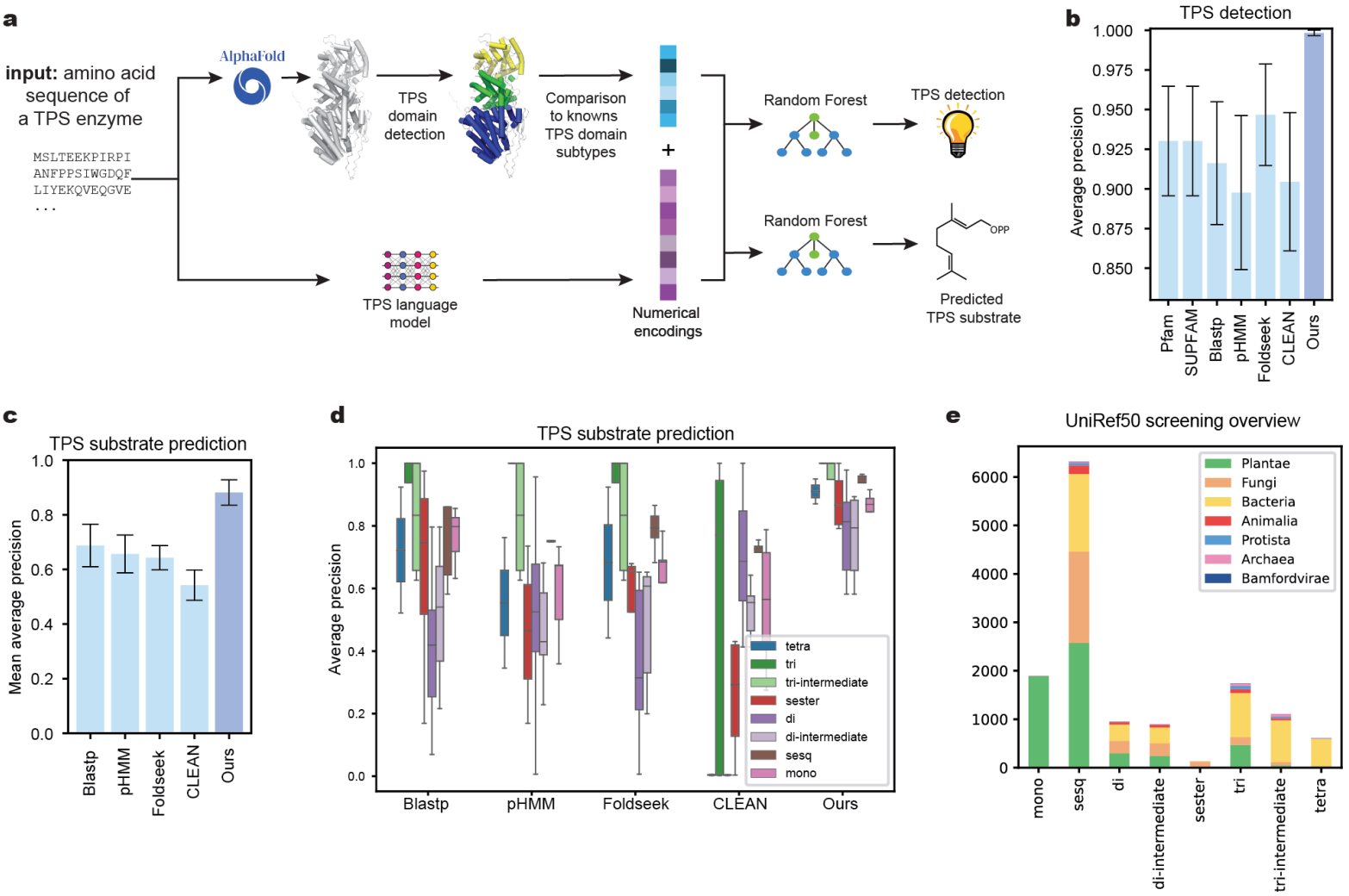

Fig. 4 | Our predictive pipeline outperforms existing methods on TPS detection and substrate classification. a) *Overview of our predictive pipeline.* ***b)*** *Evaluation of TPS detection performance.* ***c)*** *Evaluation of TPS substrate classification performance.* ***d)*** *TPS substrate classification performance for different TPS classes.* ***e)*** *Summary of TPS types detected by screening UniRef50*[41].method improves

Finally, we used the developed predictive pipeline to screen UniRef50[41] to get an estimate of the abundance of different TPS types across kingdoms (Fig. 4e). We compared distributions of TPS type proportions and kingdom proportions between the curated dataset and the results of the screening, see Extended data Fig. 7. As a result of repeated Pearson's chi-square tests with Holm correction, for each comparison the null hypothesis was rejected in favor of the alternative that there is a discrepancy between the curated dataset and the annotated UniRef50. The differences in TPS-type proportions are statistically significant, but the difference is not as dramatic as the discrepancies in kingdom proportions. The most notable difference in kingdom proportions suggests that bacterial TPSs are significantly understudied and, as a result, underrepresented in our database. This observation is aligned with recent remarkable findings about TPSs in bacteria[42,43]. Technical details of our in-silico evaluation protocol and modeling are described in Methods.

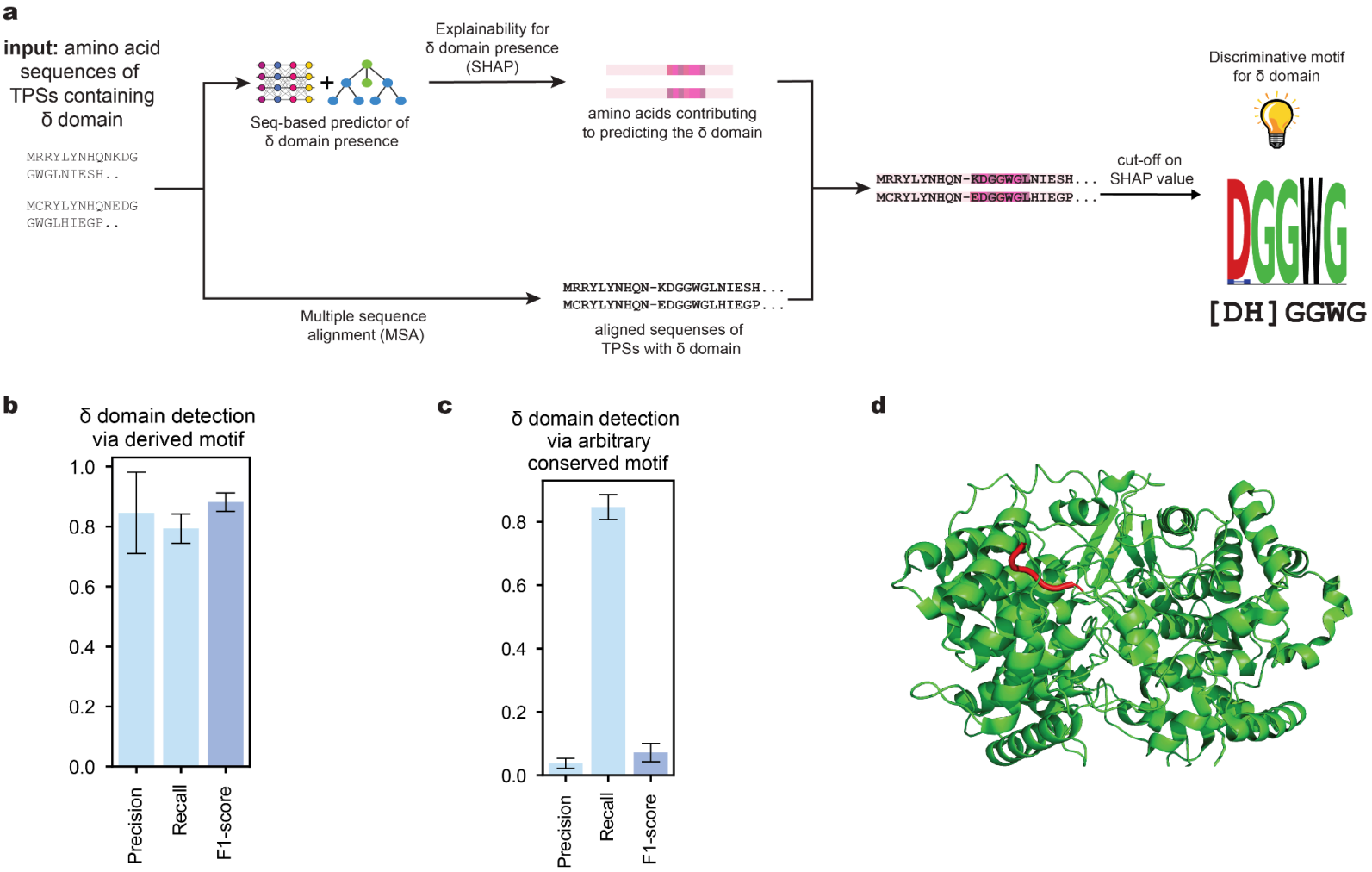

Fig. 5 | Discovery of the key δ-domain motif using predictive model explainability. a) *An approach based on multiple sequence alignment (MSA) and explainable AI for the derivation of δ domain motif,* ***b)*** *performance of δ domain retrieval with the automatically derived motif,* ***c)*** *performance of δ domain retrieval with the arbitrarily selected conserved region of the MSA,* ***d)*** *localization of the derived motif in Human lanosterol synthase, depicted in red.*

Analysis of the new structural δ domain

The proposed δ domain has a distinct structural fold and corresponds to a unique biochemical profile. The domain is a major determinant of triTPS activity. TriTPSs are responsible for the biosynthesis of steroids and have important medical applications, e.g., in treating diabetes or cancer[44–46]. Motivated by the uniqueness of the δ domain and its biochemical importance, we conducted additional analysis of amino acid sequences of TPSs containing the δ domain.

The goal of our analysis was to identify the δ-domain sequence motifs.
The challenge was to derive a discriminative motif that is not only contained in all δ-domain sequences (high recall of δ-domain detection) but also does not appear in other sequences (high precision).

For this, we defined the following procedure that leverages and builds on explainable-AI (XAI) techniques and multiple sequence alignment (MSA). The overview of our XAI-based procedure for sequence motif derivation is depicted in Fig. 5a. Inputs to the procedure are TPS sequences labeled by the presence or absence of the δ domain in the corresponding TPS structures. The

procedure output is a discriminative sequence motif reliably detecting the presence of the δ domain in a TPS.

Our intuition to apply XAI for the δ-domain motif identification arises from the observation that our TPS classifier has almost perfect performance on triTPSs. At the same time, roughly 80% of triTPSs are defined by the distinct δ domain. We hypothesized that triTPSs might be easy to detect and reliably distinguish from other TPS types thanks to a discriminative δ-domain-specific sequence motif. If that was the case, then the ML-based TPS classifier must have learned to pay attention to that discriminative motif. XAI techniques can shed light on regions in input data that an ML model used for predictions[47], and, therefore, can help identify a δ domain discriminative motif if there is one.

We first trained a separate sequence-based detector of proteins containing a structural δ domain. Then we applied XAI to the trained model. This way, for each δ-domain-containing TPS, we obtain discriminative sequence regions the model leveraged to predict the presence of the δ-domain. Next, all sequences of the δ-domain-containing TPSs were aligned into a δ-domain-specific MSA, and the XAI-based discriminative regions were mapped onto the MSA. Next, we leave only the most discriminative regions for each sequence in the MSA by applying a cut-off on the XAI scores. Finally, from these discriminatory parts of the MSA, we created a sequence logo with a corresponding regular expression.

After obtaining the discriminative sequence logo, we used a corresponding motif to retrieve δ-domain-containing sequences in the test set, which was hidden during model training. The quality of the corresponding hold-out retrieval is reported in Fig. 5b, with a mean F1-score of 0.88. We also compared the performance of the derived motif against randomly picked motifs of conserved regions from the MSA. We selected regions with sequence coverage similar to the coverage of the derived motif. The conserved regions had a similar or better recall as our motif but had poor precision. It means that even though most δ-domain-containing TPSs have those conserved motifs from their MSA, other TPS classes also have it.

There is only a single δ-domain-containing triTPS with a corresponding characterized structure in the Protein Data Bank[48]. The triTPS is a Human lanosterol synthase (LSS), which plays a crucial role in cholesterol biothynthesis[49]. We checked that our sequence motif is in a loop system of LSS, see Fig. 5d. Interestingly, in the related medical literature, the motif region was reported to be crucial for the lanosterol synthase structural stability[50], and mutations in the motif region are associated with cataract and a rare genetic condition hypotrichosis simplex, when scalp hair is either absent or sparse[51]. This provides a biological indication of our motif importance for the correct function of a δ-domain-containing triTPSs.

Experimental validation

The goal of experimental wet laboratory validation is to assess the practical value of the proposed approach. We show the utility of our predictive models by discovering novel active TPS, which would be impossible to spot in sequence databases without our method. From the

results of our UniRef50 screening, we selected 17 hits without any known function, domain, or family predicted by any computational tool integrated into InterProScan[26]. Moreover, we selected hits from organisms not typical for TPS activity, like an archaeon and a virus.

We cloned the 17 selected enzymes downstream of tdh3 promoter and upstream of ssa1 terminators into the genome *S. cerevisiae* JW501 and used the pESC plasmid (restoring Ura3 prototrophy) as a control. (Table S1, S2). Then, we cultivated the strains bearing the terpene synthase candidate in selective media with 10 % glucose and 90 % galactose to induce overexpression of GGPP. We proceeded to the analysis of ethyl acetate extract of the 48h culture by GC-EI-MS and LC-ESI-MS.

For GC-EI-MS data, we generated the extracted ion chromatogram for *m/z* 204.2 for sesquiterpenes and *m/z* 272.2 for diterpene. Then, we compared the extracted ion chromatogram of each *S. cerevisiae* JW501 strain expressing a terpene synthase candidate against the extracted ion chromatogram of *S. cerevisiae* JW501 with the empty ESC plasmid.

We detected a new signal at *m/z* 272.2 for the strain A0A5S9IQ85 (FigSI_WetLab1_a). For four enzymes, A0A2POVN22, A0A5E4I9B1, A0A2H0W7N1, A0A450SFA8 we detected new unique signals at *m/z* 204.2 (FigSI_WetLab1_b).

For LC-ESI-MS data, we generated the extracted ion chromatogram for m/z 205.1951 (with 5 ppm error). Within the 5 ppm accuracy, [C15H24 +H]+ is the only possible molecular formula for *m/z* 205.1951. We also generated the extracted ion chromatogram for *m/z* 273.2577 (with a 5 ppm error). Within the 5 ppm accuracy, [C20H32 +H]+ is the only possible molecular formula for *m/z* 273.2577. Then, we compared the extracted ion chromatogram of each *S. cerevisiae* JW501 strain expressing a terpene synthase candidate against the extracted ion chromatogram of *S. cerevisiae* JW501 with the empty ESC plasmid. We detected a new signal at *m/z* 273.2577 for enzyme A0A537EJD0 (FigSI_WetLab2_a). Then for *m/z* 205.1951 we detected new signals for four enzymes A0A2H0W7N1, A0A2P0VN22, A0A0E3NXY0, A0A5E4I9B1 (FigSI_WetLab2_b).

Overall, out of 17 tested putative protein sequences, we detected sesquiterpenes or diterpenes for 7 of them. Two signals were only detected using GC-EI-MS (diterpene from A0A5S9IQ85 , sesquiterpene from A0A450SFA8), two signals were only detected by LC-ESI-HRMS (diterpene from A0A537EJD0, sesquiterpene from A0A0E3NXY0) and 3 signals were detected in both GC-EI-MS and LC-ESI-HRMS.

Those wet lab results on the most difficult UniProt entries without any predicted function, domain, or family by computational tools integrated into InterProScan indicate the ability of our model to discover new terpene synthases currently not captured by existing databases or bioinformatical tools.

Discussion

Characterization of terpene synthases is of high practical importance, as TPSs are responsible for the largest class of natural products, including first-line medicines or prevalent flavoring

agents[4–6,8,9]. While existing computational tools[25,28,29] assist in detecting the most abundant TPS classes (monoTPSs, sesquiTPSs, and diTPSs), in-silico characterization of underrepresented TPS types has remained an unsolved challenge. Yet, rare TPS classes like sesterTPSs have biomedically important activities, including cancer cell growth suppression, modulation of receptor signaling, antimicrobial effects, or analgesic properties[52,53].

Here, we presented a method for accurately detecting substrates corresponding to six TPS classes: monoTPSs, sesquiTPSs, diTPSs, sesterTPSs, triTPSs, and tetraTPSs. This result is based on several pillars of our work. Firstly, we curated a rich dataset of characterized TPSs. The dataset comprehensively covers different TPS types, including underrepresented sesterTPSs and tetraTPSs. While the detection of triTPSs was impossible with state-of-the-art methods, triTPSs are so well represented in our dataset that established approaches re-trained on the new data have high accuracy of triTPS detection out of the box. Next, we further improved the classification of TPSs by developing computational techniques to efficiently leverage the latest machine learning advancements, AlphaFold2[17] and protein language models (PLMs)[27]. Our carefully designed procedure for the segmentation of a protein structure into TPS-specific domains enabled the comparison of corresponding structural modules between each other instead of working with the whole enzyme at once. That particularly boosted the performance of sesterTPSs and tetraTPSs detection. SesterTPSs and tetraTPSs are represented with too few sequences in the available databases, which makes modeling difficult for sequence-based methods.

We used our accurate predictive models to screen a large protein sequence space given by the UniRef50[41] set produced by the UniProt consortium[30]. A comparison of the screening results with our comprehensive dataset of characterized TPS sequences suggests that bacterial TPSs are significantly understudied. This supports recent findings about TPSs in bacteria, signifying the importance of their deeper exploration[42,43].
Next, we focused on our predictions for challenging UniRef50 entries having no predicted function, domain, or family by any computational tool integrated into InterProScan[26]. In the wet lab, we confirmed the predicted activity for 7 out of 17 of these challenging TPS hits by heterologous expression in budding yeast.

Furthermore, the developed procedure for automatic and reliable detection of TPS domains enabled novel biological insights about TPSs. We discovered subtypes of known structural domains in TPSs using unsupervised machine learning. Rigorous analysis of the discovered subtypes revealed the uniqueness of one domain subgroup. As the subgroup is a major determinant of triTPS activity, as it has a distinct structural fold and a unique biochemical profile, we propose to differentiate it from other domain types by naming it the δ domain. By leveraging techniques of explainable AI, we derived a sequence motif uniquely identifying the new δ domain. The discovered motif was reported to be responsible for the stability of a Human lanosterol synthase[50], and mutations in the motif regions are associated with genetic disorders such as hypotrichosis simplex or cataract[51]. This finding provides a verification of the motif's biological importance for the function of triTPSs containing the δ domain.

Our analysis of the TPS domain configurations enables novel insights into TPS biochemistry in a broader context. We observe that all sesterTPSs characterized to date have αα architecture, and the first α domain is analogous to sesquiTPS α architecture, while the second sesterTPS α domain is very distinct from all TPS α domains, resembling the polyprenyl synthetases domain.

The curated dataset, representative structures of discovered TPS domain subtypes, and our models are freely available via a web server, and we anticipate it will speed up the discovery of TPSs with novel properties. Likewise, we make the source code of the developed methods publicly available with the potential to be further expanded to other enzyme families and thus accelerate biological discoveries. Furthermore, we expect the development of ML pipelines on top of the published TPS dataset and the proposed method to enable full computational characterization of the whole TPS enzyme family.

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Methods

Dataset

We mined Swiss-Prot, the curated UniProtKB component produced by the UniProt consortium[30] for protein sequences that match the TPS-specific Pfam domains PF01397, PF03936, PF19086, PF13249, PF13243, PF01397 and SUPFAM domain combinations for Terpenoid synthases sf48239 and sf48576. Furthermore, we mined the Rhea[54] knowledgebase of biochemical reactions to retrieve TPSs and polyprenyl synthetases from Swiss-Prot. It resulted in 669 TPS sequences and 109 polyprenyl synthase sequences. Finally, we conducted a manual literature search to retrieve 416 characterized TPSs not included in Swiss-Prot. We have also included 6 hard negatives, which are single-point mutants of TPSs without TPS activity. The final dataset contains 1194 sequences and is available online at 10.5281/zenodo.10567437.

Computational evaluation of predictive models

We use the novel TPS dataset as a source of positive TPS examples and the Swiss-Prot knowledgebase[30] as a source of negative samples (excluding TPSs, we randomly sampled 10000 proteins from Swiss-Prot). For TPSs catalyzing multiple reactions, we used only reactions corresponding to major products. In the dataset, we still included reactions for which we did not find reliable information about the product being major, but we did not use those for model training or evaluation, as this information can introduce noise into our models and evaluation. For each evaluated model, we perform multiple experiments when part of the data is hidden as a test set. We use 5-fold cross-validation, i.e., during a train/test experiment, a single fold serves as a hold-out dataset untouched in the process of training the predictive model, while the remaining four folds serve for training the predictive model.

Motivated by the pursuit of discoveries, our goal for the in-silico evaluation is to assess the ability of models to generalize learned patterns to proteins far from the training set. To that end, we split proteins into folds, minimizing the overlap of sequences between folds while preserving the proportions of different classes across folds. We used a stratified group k-fold with groups defined based on clades from a phylogenetic tree. We used IQ-Tree software[55] to create the phylogenetic tree of TPS sequences on top of MSA computed with the MAFFT program[56]. We require at most 70% of TPSs that accept a particular substrate to belong to a single clade. Otherwise, the substrate is excluded from evaluation, as the absolute majority of representatives of the TPS substrate class belong to a single fold. We still include such entries in the model creation, but they are flagged in the column “ignore_in_evaluation” in the published dataset. The folds are in the “validation_split” column in the dataset. Entries, for which we were not able to reliably identify the major product, have the value “-1” in the column “validation_split”.

We assess the performance of the models using mean average precision (mAP). The output of our models is confidence in detection/classification. In other words, we create score-based classifiers. Average precision (AP), closely related to the area under the precision-recall curve,

summarizes the performance of score-based classifiers over different detection thresholds. For score-based models and imbalanced problems, it is a recommended evaluation metric[57].

Additionally, we evaluate our models using the area under the ROC curve (ROC-AUC) and recently proposed MCC-F1 score based on the MCC-F1 curve[58]. Our pipeline also has dominant performance when using these metrics; see Extended data Fig. 5. The error bars in the evaluation plots represent standard deviation over five folds.

Discovery of structural domain subtypes

The goal is to develop a reliable method for the segmentation of a TPS structure into domains. Furthermore, once all TPS structures are segmented into domains, we aim to detect groups of similar folds within domains. The input to our computation procedure is a dataset of TPS structures. The results of the devised procedure are subtypes of established TPS domains and computation tools for analysis of novel TPSs at resolution of individual domains.

We use structural domains of characterized TPSs from related work[34] as our standards for TPS-specific structural domains. Namely, the fold of pentalenene synthase[59] represents a standard for the α domain. Next, we aligned the α domain standard to the characterized structure of 5-*epi*-aristolochene synthase[60] and used the remaining, unaligned part of the 5-*epi*-aristolochene synthase fold as a standard for the β domain. Finally, after aligning both α and β standards to the structure of taxadiene synthase[34], we defined a standard for the γ domain as the remaining unaligned part.

For sequence-independent alignment, we use PyMOL[61] and TM-align[36]. It is known that the modeling of protein loops is challenging[62]. Therefore, to align a new structure to the defined standards of the structural modules, we use only α helixes and β sheets. The TM-align algorithm does not map all residues of the target standard to the novel TPS structure. In order to overcome this issue, we post-process the mapping of residues between a structure-module standard and an unknown TPS structure by pairing all residues in the standard to corresponding residues in the novel structure. To do so, for each residue in the TPS domain standard, we find the closest domain residues with mappings reported by the TM-align algorithm and derive the local shift between the domain sequence and the novel-structure sequence based on the shift of the mapped neighboring residues. In order to have reliable domain detections, we require at least 60% of the structure-module standard to be mapped. Furthermore, we only consider residues predicted by AlphaFold2 with high confidence (pLLDT higher than 90). As a domain detection threshold, we used 0.4, i.e. structures with TM-score above 0.4 are deemed matched. The threshold selection was given by the fact that with a TM-score of pairwise similarity under 0.4, there are almost no pairs of structures with the same fold according to the consensus definition of SCOP and CATH[63].

After detecting all TPS-specific domains, we process the unassigned ***α*** helixes. For each such helix, we find the closest helix from any detected domain and assign the unmapped α helix to the corresponding domain if the helix is not too far from the domain in terms of physical

proximity. The threshold on the maximum allowed distance from the detected domain is computed based on the domain statistics. For each helix in a particular domain, we check how far it is from the closest helix in the same domain and use the maximum distance among all domain helices as a threshold for inclusion into this domain.

After all TPS structures were segmented into domains by the described procedure, we used the same methods for pairwise alignment of the domain detections. The TM-score of pairwise alignment was then used as a precomputed metric for K-medoids[64] and HDBSCAN[37] clustering algorithms with default parameters. To determine an optimal number of clusters for the K-medoids method, we performed Silhouette analysis[65]. Considering how structurally different β/γ domains are from α domains, for computational efficiency, we analyzed the space of α domains separately from β/γ domains. Based on Silhouette analysis, the space of β/γ domains has four apparent clusters.

On the other hand, the space of α domains does not have as straightforward clustering patterns as β/γ domains. For α domains, the optimal value of the silhouette score corresponded to two clusters. This fact is due to the uniqueness of domains found in polyprenyl synthetases. The score for four clusters is comparably high, suggesting three major groups in non-polyprenyl-synthetase α domains. Therefore, we use K-medoids with k=4 to define four global types of α domain. We notice an increase in the silhouette score as the number of α-domain medoids goes up to fourteen, see Extended Fig. 2. It suggests the presence of smaller local subgroups of α domains. The HDBSCAN automatically determines an optimal number of clusters, excluding noise. For the space of α domains, HDBSCAN found thirteen clusters and a set of noisy points. As both methods independently suggested a similar number of α domain subclusters, we defined thirteen subtypes of α domains based on clusters automatically found by HDBSCAN.

Predictive modeling

We mined the BFD,[66] UniParc,[67] Mgnify,[68] 1KP,[69] Phytozome,[70] and NCBI Transcriptome Shotgun Assembly (TSA) databases for protein sequences that match the TPS-specific Pfam domains PF01397, PF03936, PF19086, PF13249, PF13243, PF01397 and SUPFAM domain combinations for Terpenoid synthases sf48239 and sf48576. This mining procedure resulted in roughly two hundred thousand TPS-like sequences.
Using these mined sequences, we finetuned the complete encoder-decoder Ankh base model[27]. As a masking strategy, we employ unigram T5 span masking (Experiment 4 in the Ankh paper[27]) and dynamically sample new masking tokens every epoch. We set the maximum sequence length to 512 and randomly select 20% of tokens for masking, following the approach described in the Ankh paper. For optimization, we use the AdamW optimizer, a batch size of 256, and a learning rate of 5e-5. The finetuning is performed with early stopping until the training loss convergence, corresponding to approximately seven training epochs.

TPS-specific domains are detected using the procedures described in the Methods section "Discovering domain subtypes."

For the Random Forest model[38], we use Scikit-learn implementation with default parameters[71].
For Foldseek[20] and CLEAN[18] models, we use implementations from the official GitHub repositories as per instructions, with recommended or default parameters. For profile Hidden Markov models, we have followed the procedure from the Terzyme paper[25] and trained models on top of our currated dataset using the HMMER library[72]. The PSI-BLAST[22] was used with default parameters.

We conducted an ablation study of our approach, see Extended data Fig. 6. It turns out that the finetuning of the protein language model (PLM) has almost no effect on the performance of the pipeline both for TPS detection and TPS substrate prediction. Nevertheless, both PLM and structural-domain features contribute to the superior performance of our pipeline. Interestingly, PLM features are more important for TPS detection, while structural domains are more critical for TPS substrate prediction. Structural domains have a notable effect on the pipeline performance for minor TPS types like tetraTPSs or sesterTPSs, see Extended data Fig. 6c. PLM features shine for major classes like sesqTPSs and contribute to the dominant performance of the pipeline for minor classes.

UniRef50 screening was performed using PLM features only for the sake of computational efficiency for large-scale analysis. Based on the above ablation, this simplification does not corrupt the performance of our approach to the TPS detection task. However, it likely decreases the quality of TPS substrate prediction for underrepresented classes like sesterTPSs or tetraTPSs.
For the screening, we used a detection threshold on TM-score of 0.3. This threshold was selected by cross-validation, as described in the Methods section "Computational evaluation of models." The threshold corresponded to both a precision and a recall higher than 0.9.

For checking the consistency between class and kingdom proportions in the curated TPS dataset vs. in Uniref50 screening results, we performed Pearson's chi-square tests with a significance level of α=5%. We omitted all categories with less than five samples. We corrected p-values with Holm correction as we tested multiple hypotheses on our data. For statistical analysis, we used standard Python libraries SciPy[73] and Statsmodels[74].

δ domain sequence motif derivation

We detected the δ domain in the protein structures of our dataset using the developed procedure for TPS domain detection described in the section "Discovery of structural domain subtypes". The presence of the δ domain is a binary label we aim to predict.

As described above, we used the same pipeline with a TPS language model and a Random Forest to build a predictive model for detecting sequences corresponding to structures with the δ domain.

MSA was created with the MAFFT program[56]. As a model explainability technique, we used SHAP[75]. Sequence logos were created with a library Logomaker[76]. Visualization of Human lanosterol synthase and motif position was performed in PyMOL[61]. The cut-off threshold on SHAP values was optimized using the classifier's training data, and the derived motif's retrieval performance was evaluated on the held-out set.

As training data for the classifier, we used our curated TPS dataset combined with randomly sampled 10000 proteins from the Swiss-Prot knowledgebase[30].

Wet laboratory material and methods

Strains, Growth Media.

Chemicals used for media preparation were purchased from either Sigma-Aldrich (St. Louis, Missouri, United States), Duchefa Biochemie (Haarlem, Netherlands), Lach:ner (Neratovice, Czech Republic) or Penta chemicals (Prague, Czech Republic). Solvent for metabolic sample preparation, LC and GC MS were purchased from Fisher Chemical (Waltham, Massachusetts, United States) and were LC-MS grade.
The list of all strains used in the study is available in Table S2
S. cerevisiae JW501 derivative strains were used to express terpene synthases and produce terpenes. Selective medium (SCE) used to grow transformants contained 1.92 g.L-1 (w/v) Yeast Synthetic Drop-out Medium Supplements without Uracil (Sigma-Aldrich, Y1501), 6.7 g.L-1 Yeast nitrogen base without amino acid (Sigma-Aldrich, Y0626), and 20 g.L-1 glucose.
DH10β electrocompetent E. coli cells were used for all cloning experiments. Transformed cells were selected on Lysogeny Broth (LB) with the appropriate antibiotics (ampicillin, chloramphenicol, or spectinomycin). SOC was used for recovery after electroporation.

Growth condition.

For maintenance S. cerevisiae strains were cultivated in solid selective medium at 30 °C.
For general preculture, S. cerevisiae strains were cultivated in a liquid selective medium (SCE) at 30 °C, 200 RPM in an orbital shaker.
For the production run, S. cerevisiae strains were cultivated in selective medium SCE in 24 deep well plates (CR1426, Enzyscreen, Hamburg, Germany, Netherlands) sealed with AeraSeal™ (Excel Scientific, Victorville, California), at 30 °C, 800 RPM on an Eppendorf ThermoMixer® C (Eppendorf, Hamburg, Germany).
For plasmid amplification, E. coli strains were cultivated in LB medium in 24 deep well plates (CR1426, Enzyscreen, Hamburg, Germany, Netherlands) sealed with AeraSeal™ (Excel Scientific, Victorville, California), at 37 °C, 800 RPM on an Eppendorf ThermoMixer® C (Eppendorf, Hamburg, Germany).

Plasmids

The list of all plasmids used in the study is available in Table S1
All pTP plasmids were generated using GoldenGate assembly based on the Lee et al. toolkit and overhangs.[77]

Part plasmids (Table S1) use pYTK001 as a backbone. Terpene synthases DNA parts were synthesized by TwistBioscience (South San Francisco, California, United States) as gene fragments. Cassette plasmids for genomic integration (Table S4) use pYTK096 as a backbone. Plasmid extraction was achieved from 2 mL of LB of E. coli harboring plasmid overnight culture. Plasmid extraction was achieved using the QIACube robot (Quiagen, Hilden, Germany) and QIAprep Spin Miniprep Kit (27104, Quiagen) with the QIAprep miniprep “rapid” protocol. Plasmids were eluted in 50 µL ddH2O.

Polymerase chain reactions (PCR)

For colony PCR and yeast genotyping we used Phire Green Hot Start II PCR Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts, United States) with the following conditions:

In 10 µL final, Phire Green Hot Start II PCR Master Mix 5 µL, 25 µM forward primer 0.2 µL, 25 µM reverse primer 0.2 µL, DNA template 4.6 µL. Reactions were conducted in ProFlex™ 3 × 32-well PCR System thermocycler (Waltham, Massachusetts, United States). Thermocycling conditions used the following template: 98°C for 2 min as initial denaturation, for 30 cycles: 98 °C for 10 s, annealing temperature for 10 s, 72 °C for 10 s.kb-1 and a final extension 72 °C for 2 minutes. PCR products were separated on agarose gel (0.8 % w/v), 130 V, 30 min.

E. coli colonies were selected with a toothpick and spotted 4 times on selective media. The remaining bacteria were thus resuspended in 10 µL ddH2O, and boiled for 10 minutes before being used as a DNA template.

Yeast genotyping was adapted from Lõoke et al.[78], S. cerevisiae colonies were selected with a toothpick and resuspended in 100 µL 200 mM LiOAc, 1 % SDS solution, and boiled for 10 minutes. Then 300 µL of EtOH 96 % were added and the solution was a vortex and centrifuge 15 000 × g for 3 minutes. The supernatant was discarded and the pellet washed with 500 µL EtOH 70% before centrifugation 15 000 × g for 1 min. The supernatant was discarded and the pellet dried for 1 minute at room temperature. The precipitated DNA was dissolved in 100 µL ddH2O and cell debris spined down 15 000 × g for 1 min.

GoldenGate assembly reaction

Part plasmids were generated in 10 µL reaction volume. T4 ligase buffer 1 µL, T4 ligase 0.5 µL (M0202L, New England Biolabs, Ipswich, Massachusetts, United States), BsmBI-v2 0.5 µL (R0739L, New England Biolabs, Ipswich, Massachusetts, United States), pYTK001 0.5 µL, DNA part 0.5 µL (20 fm), ddH2O up to 10 µL. Reactions were conducted in ProFlex™ 3 x 32-well PCR System thermocycler (Waltham, Massachusetts, United States). Thermocycling conditions used the following template: for 25 cycles, 42 °C for 2 min, 16 °C for 2 min, then 60 °C for 30 min and 80 °C for 10 min.

Cassette plasmids were generated in 10 µL reaction volume. T4 ligase buffer 1 µL, T4 ligase 0.5 µL (M0202L, New England Biolabs, Ipswich, Massachusetts, United States), BsaI-HF®v2 0.5 µL (R3733L, New England Biolabs, Ipswich, Massachusetts, United States), DNA parts 0.5 µL (20 fm), ddH2O up to 10 µL. Reactions were conducted in ProFlex™ 3 × 32-well PCR System thermocycler (Waltham, Massachusetts, United States). Thermocycling conditions used the following template: for 25 cycles, 37 °C for 5 min, 16 °C for 5 min, then 60 °C for 30 min and 80 °C for 10 min.

E. coli transformation

Electrocompetent cuvettes were cooled down at 4°C 30 min before the experiment. Electrocompetent 20 µL of E. coli cells were thawed at 4°C 10 min before the experiment. Once thawed, 0.5 µL of plasmid was added to the cell with gentle mixing. The electroporator was set to 1700 V. For chloramphenicol, spectinomycin and kanamycin selective markers, cells recovered in 1 mL of SOC for 1 h at 37 °C, 200 RPM. Thus, cells were concentrated to 100 µL through centrifugation 5000 × g for 3 min and plated to their respective LB + selection marker. In case of ampicillin, cells were resuspended into 100 µL of SOC after electroporation and directly plated in LB + ampicillin. Cells were then grown overnight at 37 °C.

S. cerevisiae transformation

Budding yeast strains were generated by standard lithium acetate transformation protocol[79] and selected using auxotrophy.

For genomic integration, 500-1500 ng of Plasmid were digested using NotI-HF® in 10 µL final volume. For plasmid integration, 500-1500 ng of plasmid were used. Cells were precultured overnight in YPD medium, 30 °C, 200 RPM. Then, cells were diluted to OD 0.1 and cultivated in YPD medium, 30 °C, 200 RPM until they reached OD 0.5. For 5 mL of cells at OD 0.5: the cells were pelleted, 2500 × g, 5 min and washed in 5mL sterile sorbitol wash buffer (600 mM sorbitol, 100 mM K2HPO4). Cells were pelleted again 2500 × g, 5 min and resuspended in 1 mL sterile sorbitol wash buffer, and pelleted again 16 000 × g, 1 min and resuspended in 1 mL TE/LiAc. Then, cells were concentrated in 100 µL TE/LiAc and 108 µL of digested plasmid and 10 µL of Salmon sperm were added, mixed gently and incubated for 10 min at room temperature. Then, 260 µL of TE/LiAc/PEG (40 % w/v) was added and the cells were incubated for 45 min at 42 °C. Cells were then centrifuged 6000 × g for 1 min and washed in water before plating on selective medium. Cells were grown for 3-5 days at 30 °C.

Metabolite extraction

Cells were maintained in solid selective medium and preculture in 1 mL selective medium media in 96 deep well plate (CR1496, Enzyscreen, Hamburg, Germany, Netherlands) sealed with AeraSeal™ (Excel Scientific, Victorville, California), at 28 °C, 1500 RPM on an Eppendorf ThermoMixer® C (Eppendorf, Hamburg, Germany) for 1 day. Cells were seeded at OD 0.05 in 2.2 mL in inducible selective medium SCE (10 % glucose, 90 % galactose) in 24 deep well plates (CR1426, Enzyscreen, Hamburg, Germany, Netherlands) sealed with AeraSeal™ (Excel Scientific, Victorville, California), at 28 °C, 300 RPM for 48 hours.
Then, 1 mL of ethyl acetate (E196-4, Fisher Scientific) was added to the culture medium and mixed for 1 hour at 28 °C, 250 RPM. Then 1 mL of ethyl acetate was added and thoroughly mixed by pipetting. The sample was collected in a 2 mL round bottom tube (Eppendorf, Hamburg, Germany) centrifuged for 5 mins, 14 100 × g and the organic phase was carefully collected and dried under N2 flow.

LC-ESI-MS

For LC-MS analysis, samples were resuspended in 100 µL ethyl acetate.
LC-MS analyses were performed using Vanquish™ Flex UHPLC System interfaced to an Orbitrap ID-X Tribrid mass spectrometer, equipped with heated electrospray ionization (H-ESI). The LC conditions were as follows: column, Waters BEH (Ethylene Bridget Hybrid) C18 50 × 2.1 mm, 1.7 µm; mobile phase, (A) water with 0.1 % formic acid; (B) acetonitrile with 0.1% formic acid; flow rate, 350 µL.min-1; column oven temperature, 40 °C, injection volume, 1 µL, linear gradient of 5 to 100 % B over 5 min and isocratic at 100 % B for 2 min. Electrospray ionization was achieved in positive mode and mass spectrometer parameters were as follows: ion transfer tube temperature, 325 °C, auxiliary gas flow rate 10 L.min-1, vaporizer temperature 350 °C; sheath gas flow rate, 50 L.min-1; capillary voltage, 3000 V, MS resolution 60 000, quadrupole isolation, scan range from m/z 100-1000, RF Lens 45 %, maximum injection time 118 ms.

GC-MS

For GC-MS analysis, samples were resuspended into 100 µL ethyl acetate.
GC-MS analyses were performed using a 7890A gas chromatograph coupled with a 5975C mass spectrometer, equipped with electron ionization (EI) and quadrupole analyzer (Agilent Technologies, Santa Clara, CA, USA). The samples (1 µl) were injected into split/splitless inlet in split mode (split ratio 10:1). The injector temperature was 250 °C. A DB-1ms fused silica capillary column (30 m ✕ 250 µm; a film thickness of 0.25 µm, J&W Scientific) was used for separation. The carrier gas was helium at a constant flow rate of 1.0 ml/min. The temperature program was: 40 °C (1 min), then 5 °C.min-1 to 100 °C, followed by 15 °C.min-1 to 230 °C . The temperatures of the transfer line, ion source and quadrupole were 320 °C, 230 °C and 150 °C, respectively. EI spectra (70 eV) were recorded from 25 to 500 m/z.

Sesquiterpene Electronic impact spectra database

To constitute a database of sesquiterpene, data from NIST-EI (https://chemdata.nist.gov/), NIST Chemistry Book (https://webbook.nist.gov/chemistry/), MS-Finder (http://prime.psc.riken.jp/compms/msfinder/main.html), WILEY GC database (https://sciencesolutions.wiley.com/solutions/technique/gc-ms/), MassBank RIKEN and MassBank NIST (https://github.com/MassBank/MassBank-data/releases/tag/2022.12.1) were extracted for all molecular formula starting by “C15H22”, “C15H24”, C15H26 or “C15H28”. All sesquiterpene spectra were concatenated using Python into a .mgf file to generate a Molecular Network based on MS spectra similarity.

Data analysis

LC-MS .raw data files were directly imported into MZmine 3.4.16. Extracted ion chromatograms for compounds of interest were generated using the raw data overview feature and exported as .pdf.
GC-MS .dx files were analyzed using OpenLab CDS 2.4. Extracted ion chromatograms for compounds of interest were exported as .csv files and built using the ggplot2 package in R.

Figures were generated using Rstudio and the following packages: ggplot2, gridExtra, patchwork, dplyr, forcats, ggthemes, ggprism, DescTools, tidyverse, scales and Adobe Illustrator CS6.

Acknowledgments

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Author contributions

T.P. conceptualized the project. R.S. developed structural domain segmentation and clustering; derived biological insights from domain analysis; built, trained, and evaluated predictive models; screened UniRef50; developed XAI-based procedure for sequence motif derivation; automatically mined characterized TPSs; wrote the manuscript with inputs from all authors. T.H. performed metabolic engineering, LC-ESI-MS, and GC-MS analysis; processed wet lab data and wrote corresponding parts of the manuscript. R.B., A.B., and J.K. performed fine-tuning of protein language models. R.Ch. performed error analysis. R.Ch., T.Č., R.S., R.B., and A.B. performed exploratory data analysis. T.Č. curated the TPS database and mined putative TPSs. M.P., M.E., and A.T. curated the TPS database. J.S. and T.P. supervised the project.

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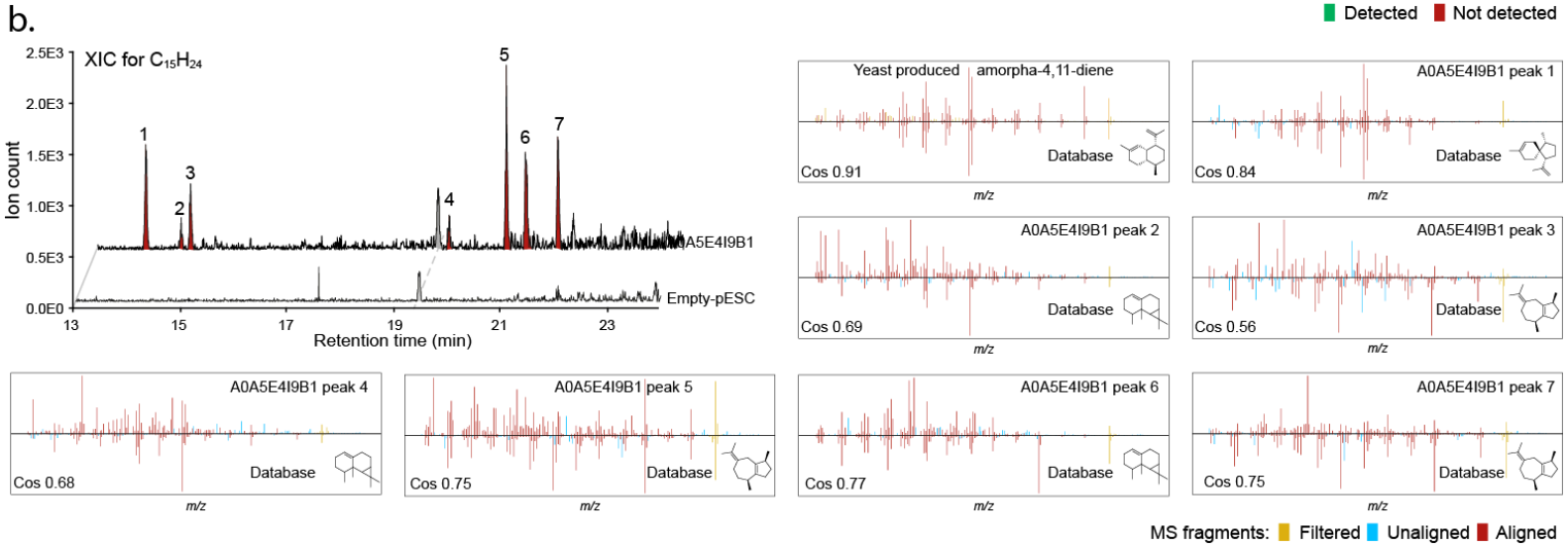
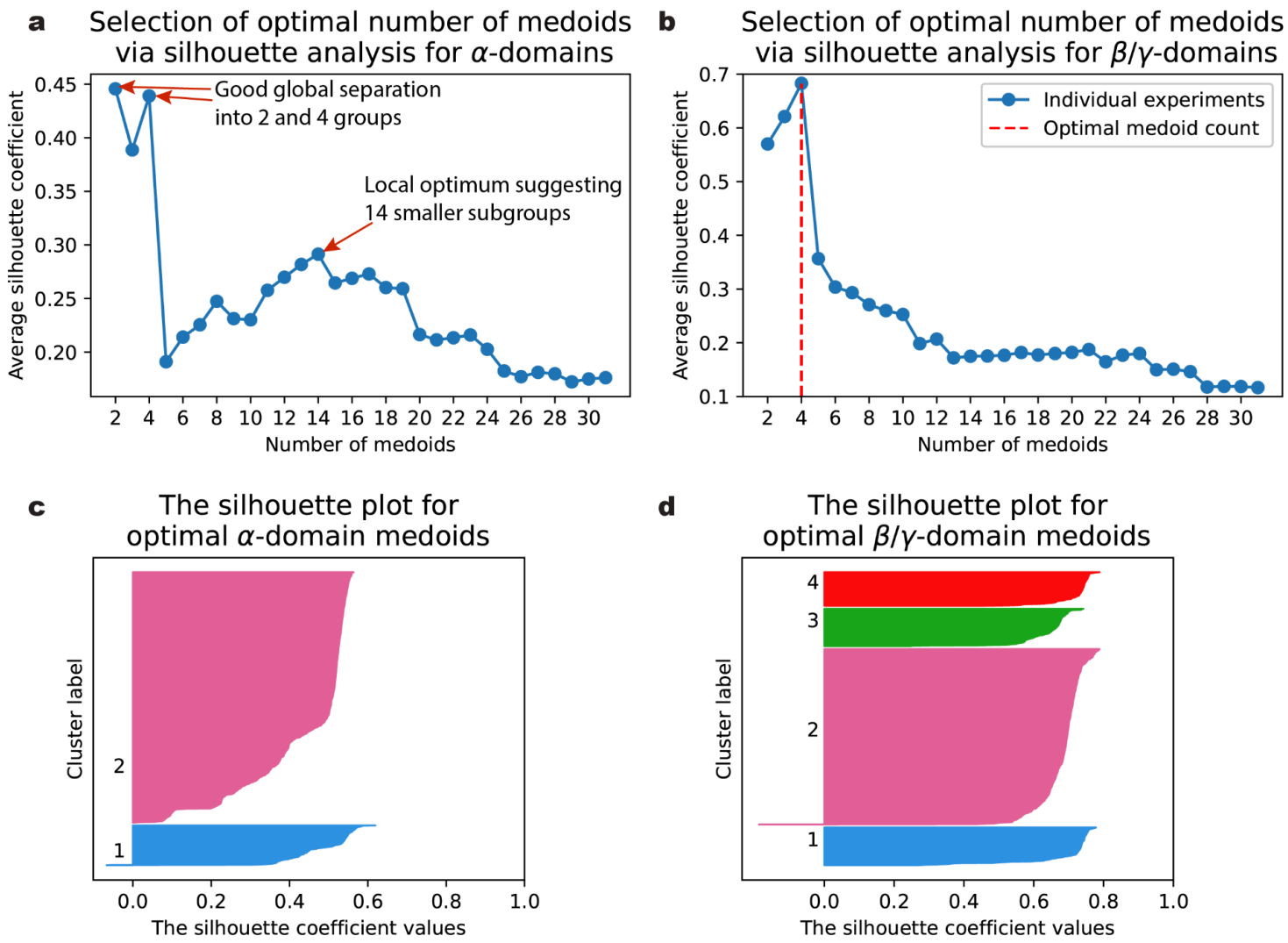
a.

	A0A0E3NXY0	A0A5S9IQ85	A0A2H0W7N1	A0A2P0VN22	A0A450SFA8	A0A5E4I9B1	A0A537EJD0
GC-EI-MS							
LC-ESI-HRMS							

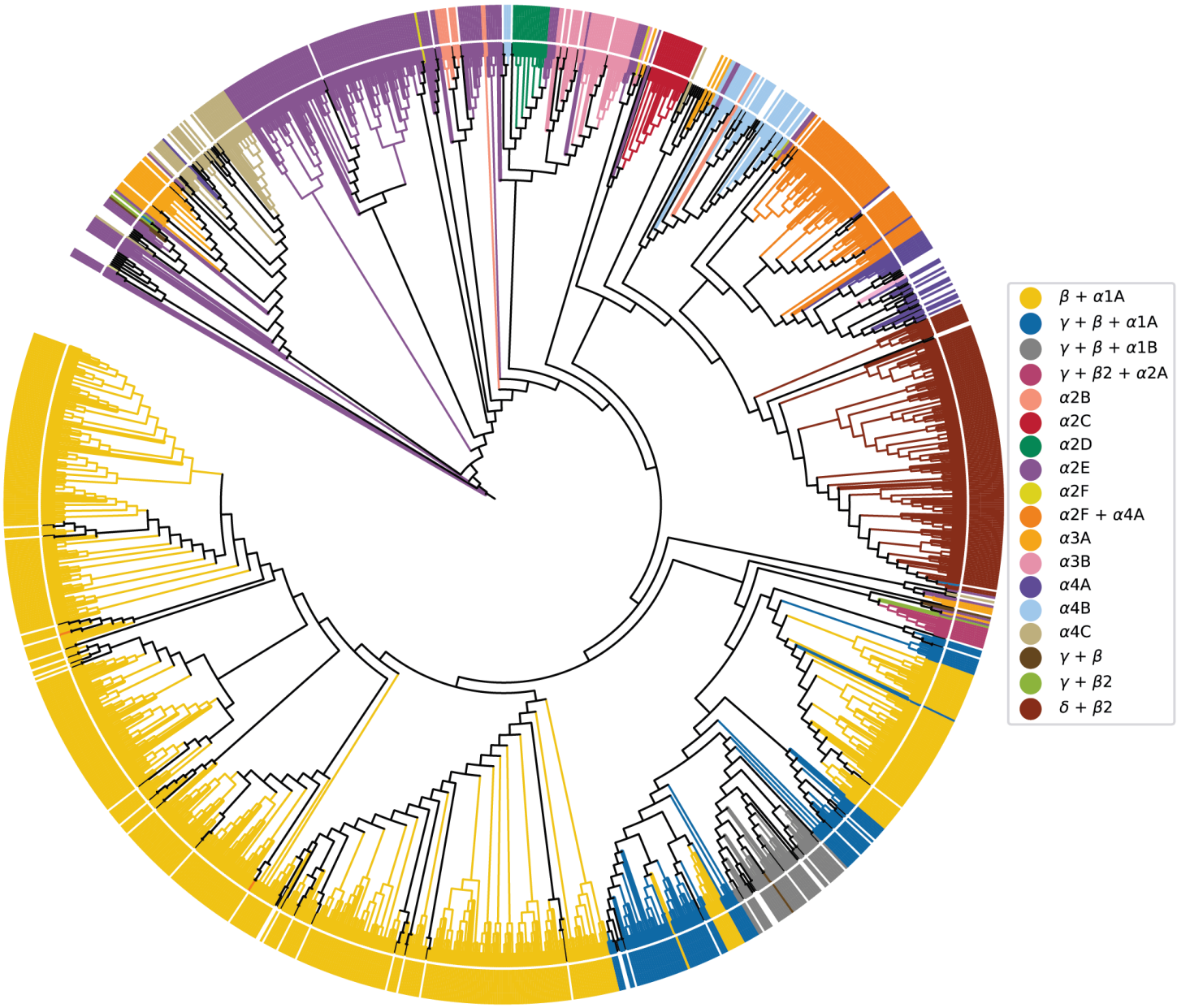
b.



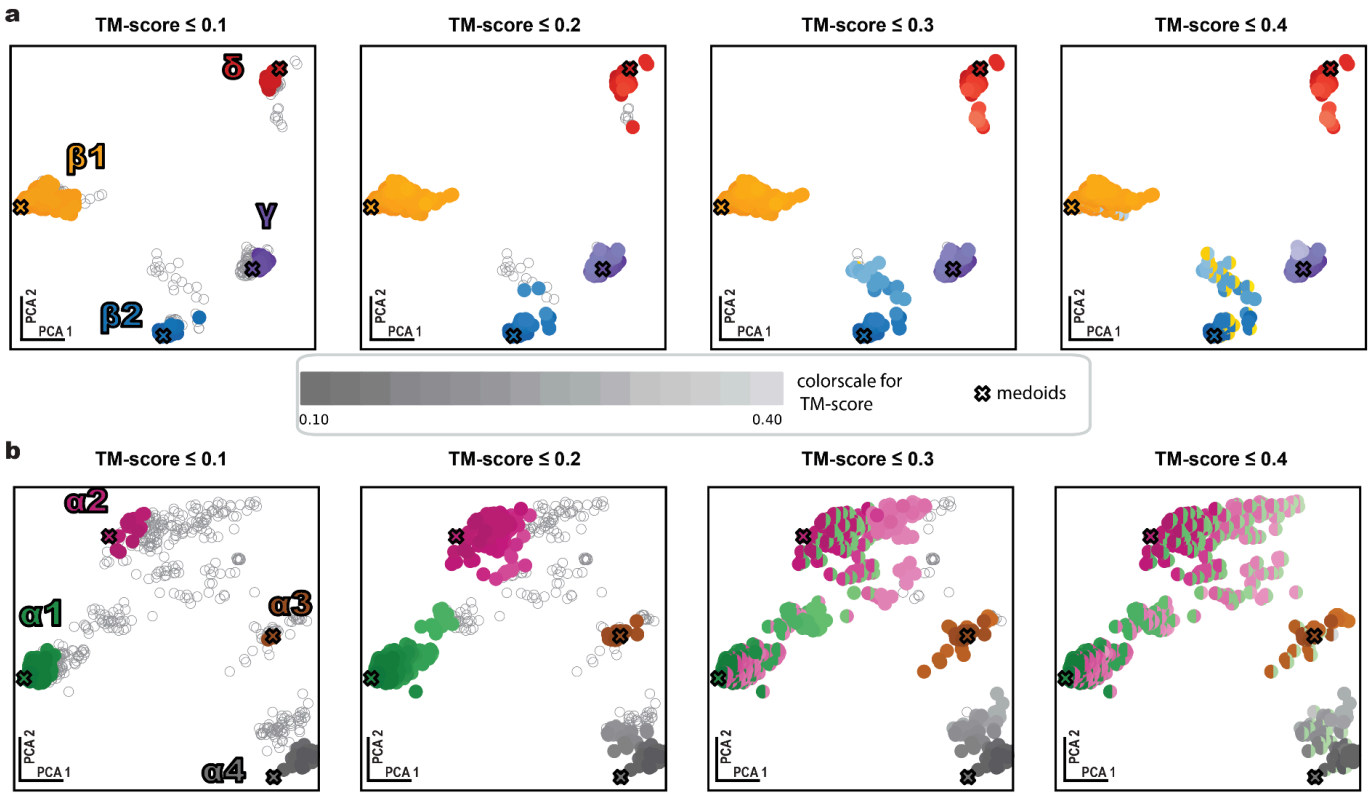
Extended data Fig. 1 | Wet lab results. a) *7 out of 17 predicted TPSs with a detected sesquiterpene or diterpene (see* FigSI_WetLab1 and FigSI_WetLab2*).* ***b)*** *GC-EI-MS chromatogram for A5E4I9B1 enzyme with closest matches in NIST-EI database for each signal corresponding to a sesquiterpene*



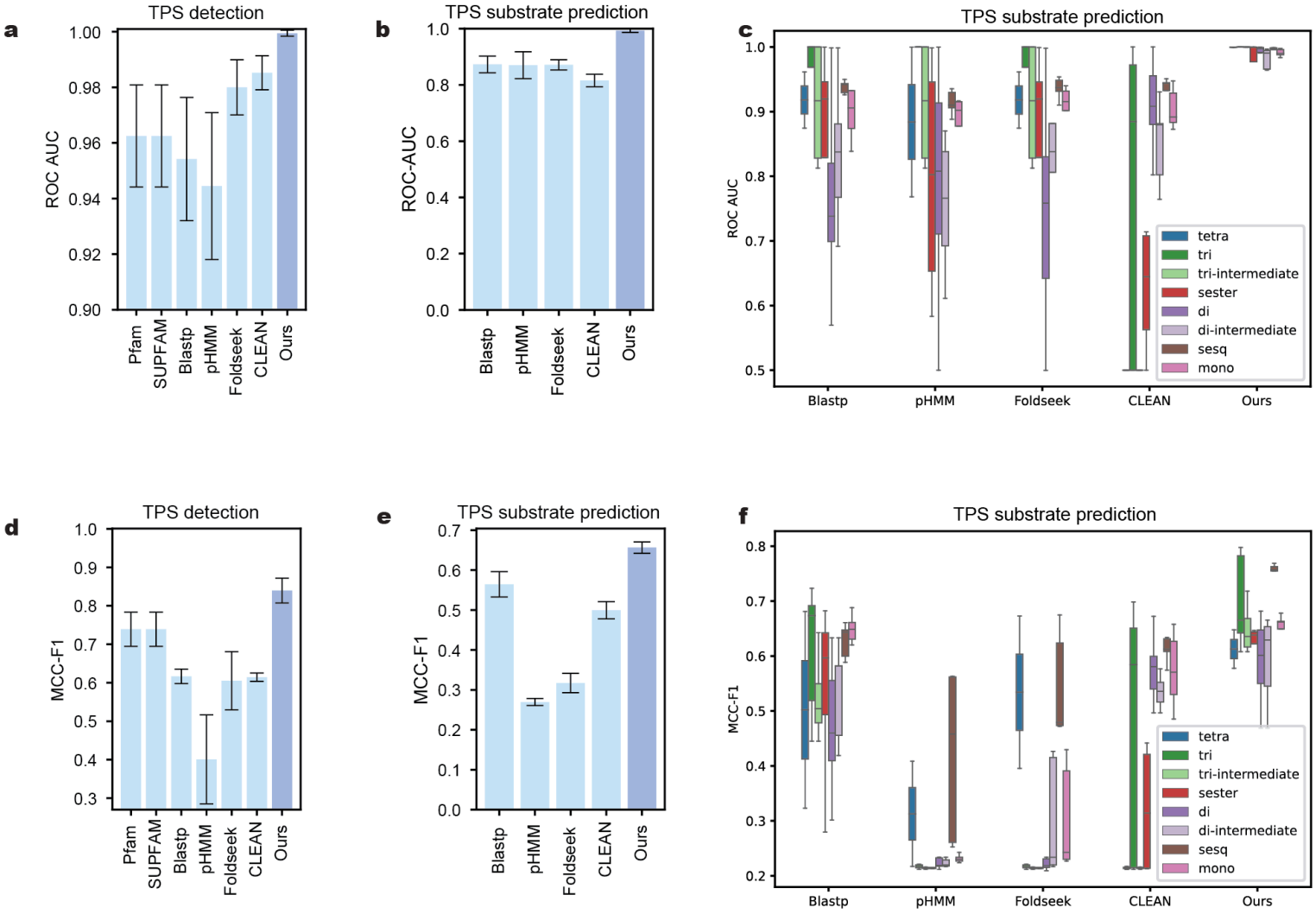
Extended data Fig. 2 | Determining domain clusters. a-b) *Silhouette analysis for α domains and β/γ domains.* ***c-d)*** *Silhouette plots for optimal numbers of K-medoid clusters. Unlike α domains, the space of β/γ domains has apparent four clusters.*



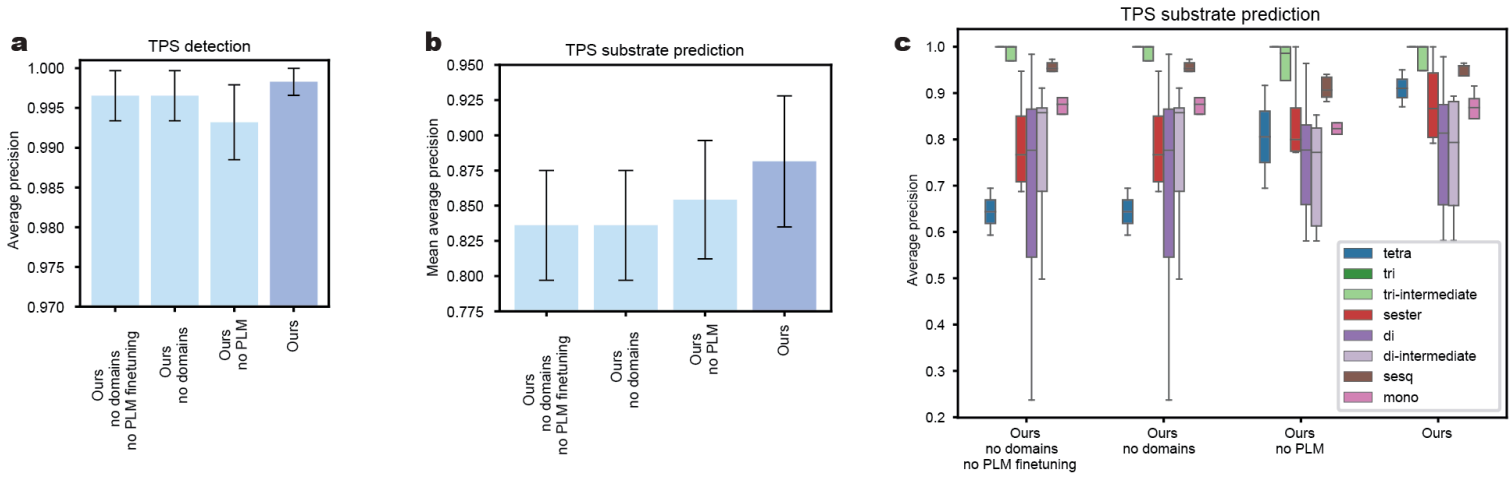
Extended data Fig. 3 | Domain subtypes order on top of a phylogenetic tree of characterized TPSs. *Please see the consistent clustering of the detected domain configurations and evolutionary relationships between the discovered domain subtypes.*



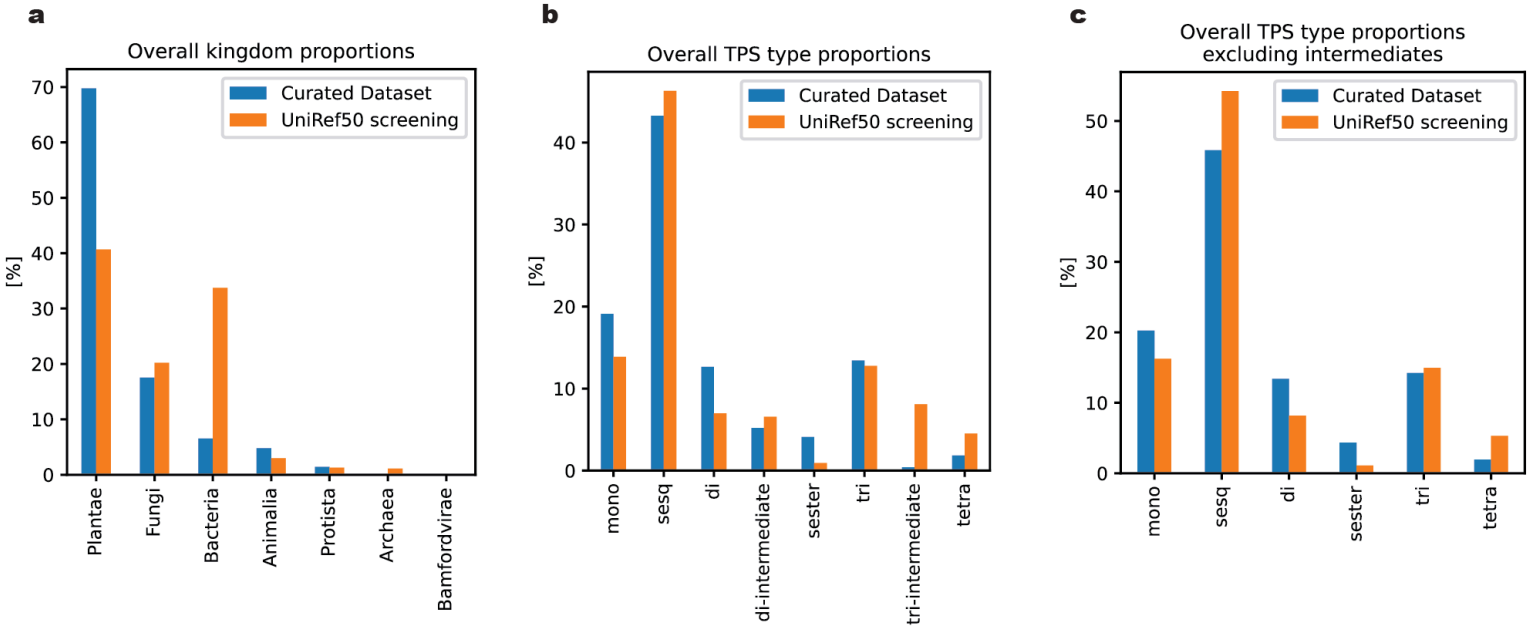
Extended data Fig. 4 | Clustering TPSs by structural domains. *Cluster membership under different thresholds on the maximum allowed TM-score distance from the cluster medoid. If a point can be associated with two clusters, then it has two colors, with the left one corresponding to a closer cluster.* the *The space of possible α subtypes represents a spectrum of gradual structural variations. The space of β/γ domain subtypes, on the other hand, has very distinct four subtypes*



Extended data Fig. 5 | Benchmarking performance of our TPS detection and substrate prediction methods against existing approaches using alternative metrics. a) *area under the ROC curve (ROC-AUC) for TPS detection,* ***b)*** *ROC-AUC for substrate prediction,* ***c)*** *ROC-AUC for substrate prediction computed separately per different TPS types,* ***d-f)*** *analogous experiments reported using the MCC-F1 score*[58]

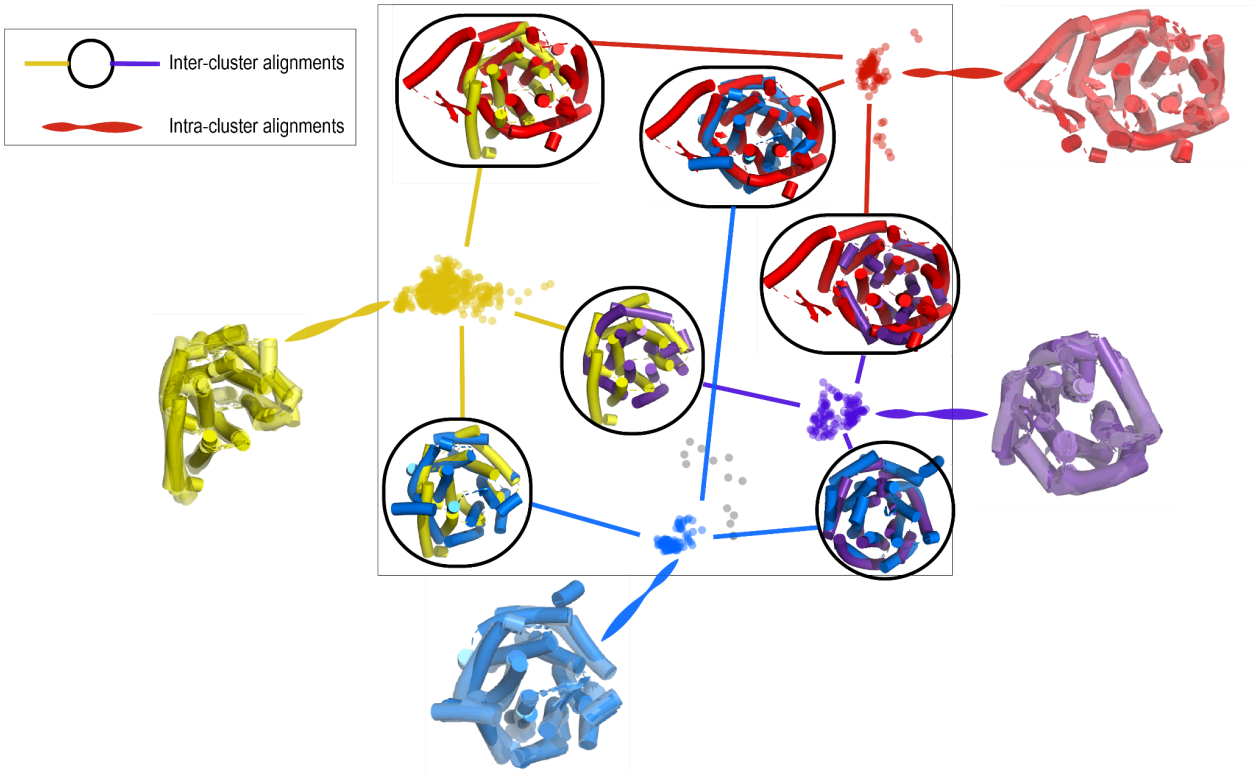


Extended data Fig. 6 | Ablation study. a) *TPS detection task,* ***b)*** *overall performance in the TPS substrate prediction task,* ***c)*** *performance in TPS substrate prediction task per TPS type. See methods for the discussion.*

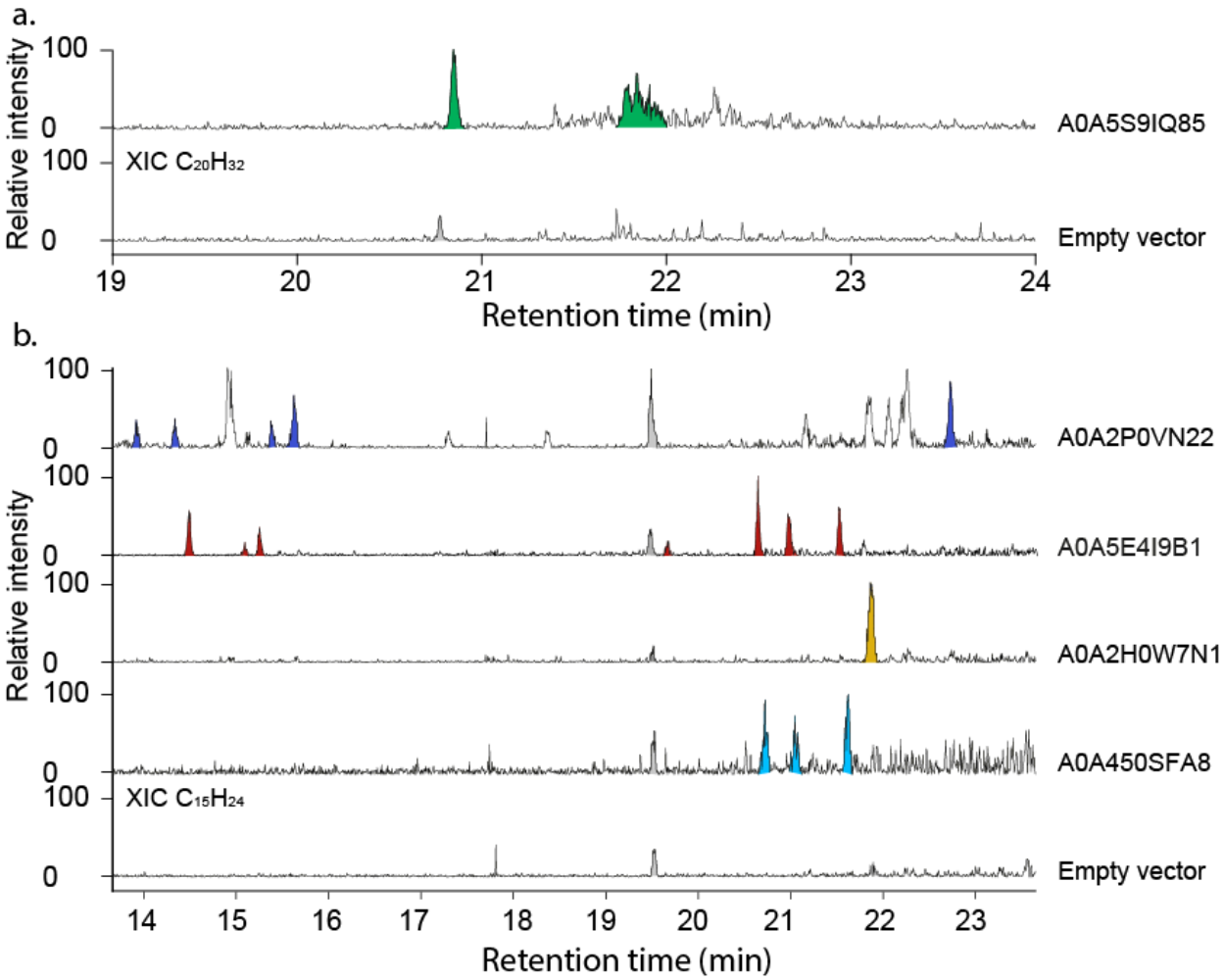


Extended data Fig. 7 | Differences in categorical distributions between the curated TPS dataset and results of UniRef50 screening. a) *Kingdom proportions comparison,* ***b)*** *TPS type proportion comparison,* ***c)*** *comparison of TPS type proportions excluding intermediates.*

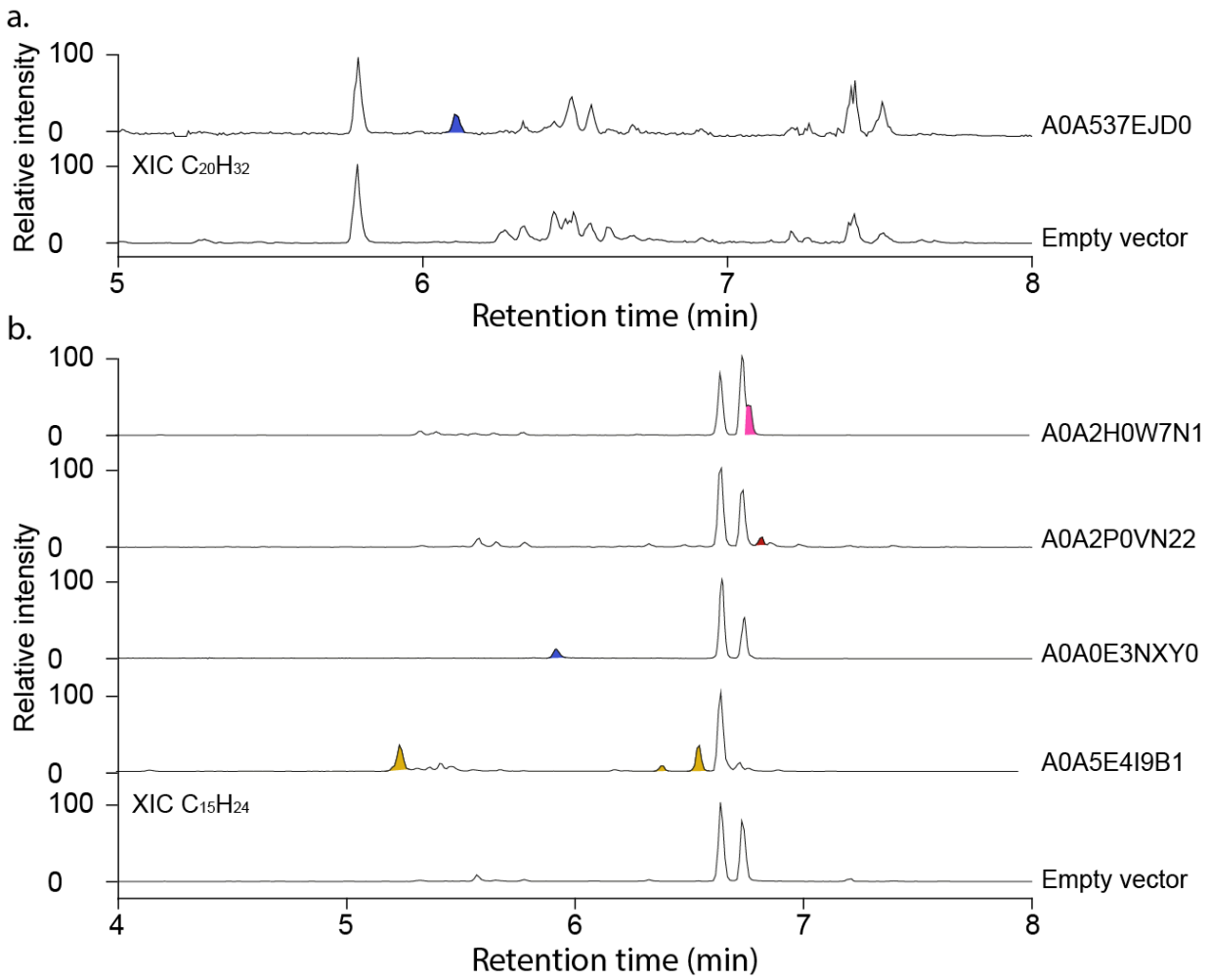
Supplementary



FigSI_Domain_subtypes_comparison. All-to-all pairwise comparisons for β/γ domain subtypes.



FigSI_WetLab1. Analysis of Yeast extract by GC-EI-MS. a. Extracted ion chromatogram for diterpene signal, *m/z* 272.2. b. Extracted ion chromatogram for sesquiterpene signal at *m/z* 204.2.



FigSI_WetLab2. Analysis of Yeast extract by LC-ESI-MS. a. Extracted ion chromatogram for diterpene signal at *m/z* 273.2577 (+/- 5 ppm). b. Extracted ion chromatogram for sesquiterpene signal at *m/z* 205.1951 (+/- 5 ppm).

SI Wet Lab

Table S1: List of plasmids

Plasmid name	Plasmid type	Backbone&Selection marker	Description	Reference

pYTK001	Backbone	pYTK001, Cm	Storage plasmid	Lee et al.[77]
pYTK009	Part type 2	pYTK001, Cm	P*tdh3*	Lee et al.[77]
pYTK052	Part type 4	pYTK001, Cm	T*ssa1*	Lee et al.[77]
pYTK096	Backbone	pYTK096, Amp	Ura3 targeting integration vector	Lee et al.[77]
pESC-Ura	Backbone	Backbone, Amp	Ura3 resistance marker	Agilent
pTP0209	Part type 3	pYTK001, Cm	A0A0E3NXY0	This study
pTP0210	Part type 3	pYTK001, Cm	A0A5S9IQ85	This study
pTP0214	Part type 3	pYTK001, Cm	A0A8I0Q8N9	This study
pTP0215	Part type 3	pYTK001, Cm	C4ZEM0	This study
pTP0217	Part type 3	pYTK001, Cm	A0A8J2KXK4	This study
pTP0219	Part type 3	pYTK001, Cm	A0A1G0GBF6	This study
pTP0220	Part type 3	pYTK001, Cm	A0A8J2P3A2	This study
pTP0221	Part type 3	pYTK001, Cm	F0ZD71	This study
pTP0222	Part type 3	pYTK001, Cm	A0A1I7GSV9	This study
pTP0225	Part type 3	pYTK001, Cm	A0A2H0W7N1	This study
pTP0226	Part type 3	pYTK001, Cm	A0A417ATN1	This study
pTP0228	Part type 3	pYTK001, Cm	A0A2P0VN22	This study
pTP0229	Part type 3	pYTK001, Cm	A0A450SFA8	This study
pTP0230	Part type 3	pYTK001, Cm	A0A5E4I9B1	This study
pTP0231	Part type 3	pYTK001, Cm	A0A537EJD0	This study
pTP0234	pYTK009, pTP0209, pYTK0052	pYTK096, amp	P*tdh3*, A0A0E3NXY0, T*ssa1*	This study
pTP0235	pYTK009, pTP0210, pYTK0052	pYTK096, amp	P*tdh3*, A0A5S9IQ85, T*ssa1*	This study
pTP0239	pYTK009, pTP0214, pYTK0052	pYTK096, amp	P*tdh3*, A0A8I0Q8N9, T*ssa1*	This study
pTP0240	pYTK009, pTP0215, pYTK0052	pYTK096, amp	P*tdh3*, C4ZEM0, T*ssa1*	This study
pTP0242	pYTK009, pTP0217, pYTK0052	pYTK096, amp	P*tdh3*, A0A8J2KXK4, T*ssa1*	This study
pTP0244	pYTK009, pTP0219, pYTK0052	pYTK096, amp	P*tdh3*, A0A1G0GBF6, T*ssa1*	This study
pTP0245	pYTK009, pTP0220, pYTK0052	pYTK096, amp	P*tdh3*, A0A8J2P3A2, T*ssa1*	This study
pTP0246	pYTK009, pTP0221, pYTK0052	pYTK096, amp	P*tdh3*, F0ZD71, T*ssa1*	This study
pTP0247	pYTK009, pTP0222, pYTK0052	pYTK096, amp	P*tdh3*, A0A1I7GSV9, T*ssa1*	This study
pTP0250	pYTK009, pTP0225, pYTK0052	pYTK096, amp	P*tdh3*, A0A2H0W7N1, T*ssa1*	This study
pTP0251	pYTK009, pTP0226, pYTK0052	pYTK096, amp	P*tdh3*, A0A417ATN1, T*ssa1*	This study
pTP0253	pYTK009, pTP0228, pYTK0052	pYTK096, amp	P*tdh3*, A0A2P0VN22, T*ssa1*	This study
pTP0254	pYTK009, pTP0229, pYTK0052	pYTK096, amp	P*tdh3*, A0A450SFA8, T*ssa1*	This study
pTP0255	pYTK009, pTP0230, pYTK0052	pYTK096, amp	P*tdh3*, A0A5E4I9B1, T*ssa1*	This study
pTP0256	pYTK009, pTP0231, pYTK0052	pYTK096, amp	P*tdh3*, A0A537EJD0, T*ssa1*	This study

Table S2: List of strains

Name	Genotype	Ref
JW501	MATa leu2–3112::His3MX6_PGAL1-ERG19/PGAL10-ERG8 ura3–52::URA3_PGAL1-mvaS(A110G)/PGAL10-	Wong *et al.*[80]

	mvaE(CO) his3Δ1::hphMX4_PGAL1-ERG12/PGAL10-IDI1 trp1–289::TRP1_PGAL1-crtE(X.den)/PGAL10-ERG20 yprcδ15::natMX_PGAL1-crtE(opt)/PGAL10-crtE (ura3–52 prototrophy removed for use of Cas9 system)	
JW501_TP0027	JW501 with pTP0027	This study
JW501_TP0234	JW501 ura3-52::ura3::P*tdh3*::A0A0E3NXY0::T*ssa1*	This study
JW501_TP0235	JW501 ura3-52::ura3::P*tdh3*::A0A5S9IQ85::T*ssa1*	This study
JW501_TP0239	JW501 ura3-52::ura3::P*tdh3*::A0A8I0Q8N9::T*ssa1*	This study
JW501_TP0240	JW501 ura3-52::ura3::P*tdh3*::C4ZEM0::T*ssa1*	This study
JW501_TP0242	JW501 ura3-52::ura3::P*tdh3*::A0A8J2KXK4::T*ssa1*	This study
JW501_TP0244	JW501 ura3-52::ura3::P*tdh3*::A0A1G0GBF6::T*ssa1*	This study
JW501_TP0245	JW501 ura3-52::ura3::P*tdh3*::A0A8J2P3A2::T*ssa1*	This study
JW501_TP0246	JW501 ura3-52::ura3::P*tdh3*::F0ZD71::T*ssa1*	This study
JW501_TP0247	JW501 ura3-52::ura3::P*tdh3*::A0A1I7GSV9::T*ssa1*	This study
JW501_TP0250	JW501 ura3-52::ura3::P*tdh3*::A0A2H0W7N1::T*ssa1*	This study
JW501_TP0251	JW501 ura3-52::ura3::P*tdh3*::A0A417ATN1::T*ssa1*	This study
JW501_TP0253	JW501 ura3-52::ura3::P*tdh3*::A0A2P0VN22::T*ssa1*	This study
JW501_TP0254	JW501 ura3-52::ura3::P*tdh3*::A0A450SFA8::T*ssa1*	This study
JW501_TP0255	JW501 ura3-52::ura3::P*tdh3*::A0A5E4I9B1::T*ssa1*	This study
JW501_TP0256	JW501 ura3-52::ura3::P*tdh3*::A0A537EJD0::T*ssa1*	This study