Title: Overall performance and taxonomic bias of antimicrobial peptide prediction tools in invertebrates

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Abstract

Invertebrate antimicrobial peptides (AMPs) are at the forefront in the search for agents of therapeutic utility against multi-resistant microbial pathogens, and in recent years substantial advances took place in the *in silico* prediction of antimicrobial function of amino acid sequences. A yet neglected aspect is taxonomic bias in the performance of these tools. Owing to differences in the prediction algorithms and used training data sets between tools, and phylogenetic differences in sequence diversity, physicochemical properties and evolved biological functions of AMPs between taxa, notable discrepancies may exist in performance between the currently available prediction tools. Here, we tested if there is a taxonomic bias in prediction power in 8 tools with a total of 15 prediction algorithms, in 19 invertebrate taxa. We found that most of the tools exhibited considerable variation in performance between tested invertebrate groups, based on which we provide guidance in choosing the adequate prediction tool for specific taxa.

Keywords: antimicrobial peptides, AMP prediction, taxonomic bias, amino acids, prediction performance

Introduction

Antimicrobial peptides (AMPs) are low molecular weight components of the innate immunity, present virtually in all living organisms. Their importance has long been recognized, especially in the search for efficient therapeutic agents against multi-resistant bacteria (Toke, 2005). In the last few decades, invertebrates (mainly arthropods) have become central model organisms to identify and classify novel AMPs with potential utility in medicine (Mylonakis et al., 2016; Vizioli & Salzet, 2002). With the rapid accumulation of available genomic and proteomic data from a wide range of taxa, and novel methods for the prediction of structural, physicochemical, and biological properties of peptides, the search for therapeutic-potential AMPs is one of the most exciting and promising areas in the life sciences (Lee et al., 2018; Porto et al., 2017). In fact, by today a considerable number of AMP activity prediction tools has been built, mainly utilizing modern machine-learning algorithms to identify amino acid sequences with potential antimicrobial capacities, generally on the basis of their physicochemical parameters (Lee et al., 2018; Wu et al., 2018).

Beside to other challenges of AMP prediction using peptides from databases (see Porto et al., 2020 and references therein), neglected taxonomic (and, indeed, phylogenetic) information may also contribute to biases in the training of prediction algorithms. Although AMP prediction tools are quite available, and some articles made attempts of benchmarking how efficiently these tools can identify AMPs in general (e.g. Gabere & Noble, 2017), it still remains overlooked, whether or not these prediction tools may provide the same performance across the taxa from which putative AMPs are extracted. This could be rather problematic, owing to that even among phylogenetically related AMPs substantial differences may exist in the evolutionary trajectories of the amino acids between species. This might be expected to lead to differences in, for example, taxon-specific gene clustering (i.e. sequence diversity, affecting scalar physicochemical properties), or in the evolved functionality of peptides belonging to the given AMP family (Patil et al., 2004; Patil et al., 2005). Indeed, the importance of evolutionary origin of sequence diversity in these molecules was emphasized recently in invertebrates (Tassanakajon et al., 2015). While there is little knowledge

about how such diversity may be associated with physicochemical properties and structural features of AMPs, it is not unreasonable to assume that unaccounted taxon-specific variation in them might affect the performance of prediction tools in recognizing antimicrobial function (e.g. due to taxon-based differences in the evolved physicochemical properties). Also, alternative tools using different methodological approaches and training sets further complicate the picture, and may lead to discrepant performances, i.e. to differences between the prediction tools in how efficiently they recognize AMPs and non-AMPs in different taxa, which might depend on the taxonomic composition of the training datasets. Consequently, neglecting this aspect may lead to an increased incidence of both type I and type II errors when using inadequate tools, by falsely labelling non-antimicrobial peptides as (putative) AMPs, or not recognizing true AMPs to have antimicrobial activity, respectively.

This issue is anything but trivial, as a wide range of researchers rely on these AMP prediction tools and their outputs, from the search for invertebrate AMPs with therapeutical potential (Mylonakis et al., 2016), to ecophysiological and ecoimmunological studies (Shelomi et al., 2020), and to the studies on the evolutionary history of invertebrate AMPs (Tassanakajon et al., 2015). If “taxonomic bias” exists in prediction performance of available tools (i.e. if there are differences between prediction tools in how reliably they can distinguish between AMPs and non-AMPs in given taxa), a practical resolution could be identifying which tool performs best for specific taxa, and consistently using these tools in the given groups. As a first approach to address this problem, taxon-specific assessment of the performance of currently available AMP prediction tools may help us to shed light on whether or not a taxon-bias is present at all. Accordingly, in the present study our aim is not to provide a comprehensive list of AMP prediction tools, or to introduce their algorithms and/or modern methods for AMP prediction, but merely to provide guidance in choosing the adequate prediction tool for specific taxa. To do so, we acquired 1525 unique AMP and 3050 non-AMP sequences sequences from 19 invertebrate taxa, and used 8 freely available AMP prediction tools

(with a total of 15 prediction algorithms), not only to assess the prediction tools' overall performance, but their performance per taxa.

Methods

All data handling and analyses were performed in the R software for statistical computing (ver. 4.0.1, Team R Core, 2020). AMP sequences were acquired from the protein database of the National Center for Biotechnology Information (*NCBI Protein Database*, 2020; NCBI Resource Coordinators, 2018) and the Antimicrobial Peptide Database APD3 (Wang et al., 2016). Taxa for which we acquired AMP sequences were Acari (n = 76), Araneae (n = 45), Blattodea (n = 162), Coleoptera (n = 62), Decapoda (n = 64), Diptera (n = 447), Hemiptera (n = 63), Hymenoptera (n = 183), Lepidoptera (n = 136), Mytiloida (n = 31), Neogastropoda (n = 5), Orthoptera (n = 8), Ostreoida (n = 62), Pectinoida (n = 4), Scolopendromorpha (n = 10), Scorpiones (n = 103), Unionida (n = 4), Veneroida (n = 48), and Xiphosura (n = 12). Non-AMP sequences were downloaded from UniProt (The UniProt Consortium, 2019): for each taxon, a large batch of non-antimicrobial proteins were acquired, between the length of 10 and 200 amino acids, from which 2n for each taxon (where "n" refers to the number of AMP sequences in the given taxa) were randomly drawn, excluding any duplicate sequences. After the acquisition and manual check of sequences, both AMPs and non-AMPs were used with 8 AMP prediction tools; as some of the tools included multiple algorithms, in total there were 15 algorithms for the prediction of AMP properties for each acquired sequence (for the detailed list of the used prediction tools see the Table 1).

Table 1. List of the used prediction tools (and algorithms within some tools).

Prediction tool	Algorithm	URL	Reference
ADAM	Hidden Markov models	http://bioinformatics.cs.ntou.edu.tw/ADAM/tool.html	(Lee et al., 2015)
	Support vector machine		
AMP scan 2	Deep neural network	https://www.dveltri.com/ascan/v2/ascan.html	(Veltri et al., 2018)
$CAMP_{R3}$	Artificial neural network	http://www.camp.bicnirrh.res.in/predict/	(Waghu et al., 2016)
	Discriminant analysis		
	Random forest		
	Support vector machine		
Class AMP	Random forest	http://www.bicnirrh.res.in/classamp/predict.php	(Joseph et al., 2012)
	Support vector machine		
CS-AMPpred	Support vector machine: linear kernel	https://sourceforge.net/projects/csamppred/	(Porto et al., 2012)
	Support vector machine: polynomial kernel		
	Support vector machine: radial kernel		
iAMP	Support vector machine	http://cabgrid.res.in:8080/amppred/server.php	(Meher et al., 2017)
iAMP-2L	Multi-label fuzzy K-nearest neighbour	http://www.jci-bioinfo.cn/bioinfo/iAMP-2L	(Xiao et al., 2013)
StM	Hydrophobic momentum threshold	http://www.portoreports.com/stm	(Porto et al., 2020)

Results from the prediction tools were evaluated using the typical binary classification performance measures, namely accuracy ((TP+TN)/(TP+FP+TN+FN)), precision (TP/(TP+FP)), sensitivity (TP/(TP+FN)), and specificity (TN/(TN+FP)); TP, TN, FP, and FN stand for True Positive, True Negative, False Positive, and False Negative results, respectively. We also used the harmonic mean of the four aforementioned performance measures as a singular quantity reflecting overall performance (henceforth referred to as HMP). Similarly to the four classical performance measures, HMP ranges between zero and one, and values closer to one reflect good performance (high accuracy and precision). Tool performances were also assessed separately for taxa. Additionally, taxonomic bias in prediction power of tools was calculated as the median distance between HMP values within taxa, separately for each prediction tool. Hence, this variable had low values for prediction tools yielding similar HMP values for the different taxa, whereas values were high when HMP varied substantially across taxa within the same prediction tool.

Results and Discussion

Overall, ADAM showed the best performance with both of its algorithms (hidden Markov model, and support vector machine). Also, the artificial neural network classifier of $CAMP_{R3}$ had relatively good performance, similarly to CS-AMPpred (all kernels, although the linear kernel did not perform as good as the polynomial and radial), and to iAMP-L2 (Fig. 1). The weakest overall performance was observed in the case of Class AMP.

AMP prediction tools showed substantial variation in how well they performed in different taxa (Fig. 1; also see Supplementary Fig. S1). Class AMP classifiers showed almost no between-taxa variation, which was due to their very low performance, as they unanimously showed weak prediction efficiency in all taxa. The largest taxonomic bias (i.e. largest median difference in tool performance between taxa) was observed in iAMP-L2, in the linear kernel CS-AMPpred, and in StM; in these tools the discrepancy between the minimum and maximum HMP was 0.94, 0.86, and 0.78, respectively. Even those prediction tools showing best performances based on their HMP had marked taxonomic biases: for example in the hidden Markov model ADAM the difference between minimum and maximum HMP was 0.32. Still, based on the HMP values calculated per taxa, this classifier yielded the highest performance for most of the taxa (Table 2). Notably, taxonomic biases were tool specific, meaning that there weren’t any taxa for which most of the prediction tools yielded consistently low or high performance.

Table 2. Best preforming prediction tools for given taxa, based on the harmonic mean of performance measures (HMP).

Taxon	**Best performing prediction tool**	**HMP**
Acari	ADAM (support vector machine)	0.90
Araneae	iAMP-L2	0.94
Blattodea	ADAM (support vector machine)	0.88
Coleoptera	ADAM (hidden Markov model)	0.92
Decapoda	iAMP-L2	0.88
Diptera	ADAM (hidden Markov model)	0.96
Hemiptera	ADAM (hidden Markov model)	0.90
Hymenoptera	ADAM (hidden Markov model)	0.92
Lepidoptera	ADAM (hidden Markov model)	0.93
Mytiloida	IAMP-L2	0.87
Neogastropoda	ADAM (hidden Markov model)	0.76
Orthoptera	$CAMP_{R3}$ (artificial neural network)	0.89
Ostreoida	ADAM (hidden Markov model)	0.85
Pectinoida	ADAM (hidden Markov model)	0.80
Scolopendromorpha	CS-AMPpred (linear kernel)	0.84
Scorpiones	iAMP-L2	0.81
Unionida	ADAM (support vector machine)	0.90
Veneroida	ADAM (support vector machine)	0.77
Xiphosura	ADAM (support vector machine)	0.90

Based on these results the prediction tool ADAM, using hidden Markov model, provides the best performance in most assessed taxa. While it is outside of the scope of this study to give in-detail insights on the algorithms behind the prediction tools, it should be noted that this tool's strength likely comes from its set of homologues: it uses a probabilistic model based on which the already

known AMPs are used to find the most likely homologue for the query peptide. This is a strength that few currently available prediction tools possess, namely that, instead of only using scalar physicochemical properties based on the amino acid sequence (which approach has its weaknesses, see Porto et al., 2020), sequences are classified based on their resemblance to known AMPs. Notably, such an approach will yield null-findings when presented a previously unknown AMP, but might potentially enable for accounting for evolutionary information. Although the hidden Markov model ADAM tool technically does not consider phylogeny for the taxa from which the query sequences originate, in future prediction tools homologue (and, indeed orthologue) finding may be a quite useful approach in the reliable *in silico* identification of AMPs. Surprisingly, tools accounting for (either species, or gene) evolution are still lacking, and studies addressing the question of functional typization of AMPs generally use amino acid composition (Porto et al., 2020), and/or quantitative structure–activity relationships (Juretić et al., 2011). While, arguably, understanding the association between sequence, structure, and biological function is the forefront in the study of AMPs with therapeutical potential (Ahmed & Hammami, 2019; Torres et al., 2018), evolutionary information may also help us in the identification (and, indeed, *de novo* design and synthesis) of candidate AMPs.

Substantial variation exists in how reliably given tools can predict AMP function of amino acid sequences belonging to different taxa. From a practical perspective this gives reason for caution when selecting the prediction tool for identifying putative AMPs from amino acid sequences of a given invertebrate taxon *in silico*. The substantial taxonomic bias in the different prediction tools' performance hints at important differences in the properties of amino acid sequences across taxa, but interpretation of this result should be done with care, as this bias can have multiple origins. For example, various AMP families (e.g. defensins, cecropins) may have quite different physicochemical properties (e.g. Cytryńska et al., 2007), and there may be substantial between-taxa variation in amino acid sequences of same AMP family (Tassanakajon et al., 2015). Although these

aspects should be taken into account when working with AMPs, it is outside of the scope of our present study to go into details of the background and their possible resolutions.

Arguably, a taxonomically, or even phylogenetically informed perspective would be quite useful not only in the more robust and reliable identification of AMPs in invertebrates, but also in designing and engineering peptides with desirable properties (e.g. efficacy against antibiotic resistant bacteria, but low citotoxicity to eukaryotic cells), through a deeper understanding of the evolution of the wide variety of AMPs evolved in invertebrates. Hence, comparative phylogenetic analyses of AMPs would be an important aspect helping to understand the evolution of these molecules.

It should be noted that our current approach is not without drawbacks. As was mentioned, the different tools use different methodological approaches (e.g. CS-AMPpred uses support vector machine trained on 310 cysteine-stabilized peptides, utilizing scalar physicochemical properties, whereas ADAM hidden Markov model is based on profile models of more than 7000 AMPs and 759 identified structures). Differences in the focus of AMP types arguably restricts the potential range of peptides that can be recognised as to have antimicrobial function by a given prediction tool. Also, taxonomic composition of the AMP and non-AMP sequence datasets used to train the different models likely shape the models' performance, in terms of whether or not the tested putative AMP sequence comes from the taxa included in the model training. Additionally, due to the variable availability of AMP sequences for the different taxa, AMP family could not have been included when testing performance, even though there might be tool-specific differences in it for the different AMP families (i.e. a given tool may differ in its capacity to recognize defensins *versus* cecropins as AMPs). Notably, we know about no studies explicitly assessing the effect of AMP family on the efficiency of AMP prediction, so this aspect might be a quite relevant and important one to address in future studies. Furthermore, owing to practical reasons, non-AMPs (used both for training prediction tools, or for the assessment of their performance) are not standardized, i.e. these peptides are generally drawn randomly from those peptides that are available for the given taxa, with no known functions in antimicrobial immunity. Non-overlapping biological functions between

peptides from different taxa might introduce additional bias in the performance of prediction tools. For example, some membrane-active peptides might be expected to be falsely recognized as AMPs more often than others, due to their structural and physicochemical properties similar to that of AMPs (Lee et al., 2018). Hence, if the randomly drawn non-AMPs in a given taxon contain higher proportion of such membrane peptides than in other taxa, this might confound the per-taxa-performance of the used prediction tool. Using standardized sets of non-AMP protein families (preferably of several, distinct biological functions) that have available sequence data for a wide range of taxa might help improve the performance of new prediction tools.

Overall, the results presented here highlight the necessity of taking taxonomic information into account when predicting antimicrobial function of putative AMPs. We hope that our results will serve as a guide in choosing the appropriate AMP prediction tool for testing candidate peptides in invertebrates. We argue that this could be useful for the big-data / bioinformatic data style search for candidates in open databases and repositories, and for the identification of AMPs in invertebrates for ecophysiological studies. In the search for putative AMPs based on genomic and/or proteomic data, and immune profiling based on transcriptomics (i.e. screening produced peptides using transcriptome) in invertebrates may also benefit from our proposed approach. In addition, in future studies to implement refined AMP prediction techniques, inclusion of taxonomic and/or phylogenetic information, utilization of explicit categorization of AMPs, and standardized sets of non-AMP sequences will arguably help us to achieve more robust prediction of antimicrobial function in invertebrate models on our endeavour to identify peptides of potential therapeutical utility.

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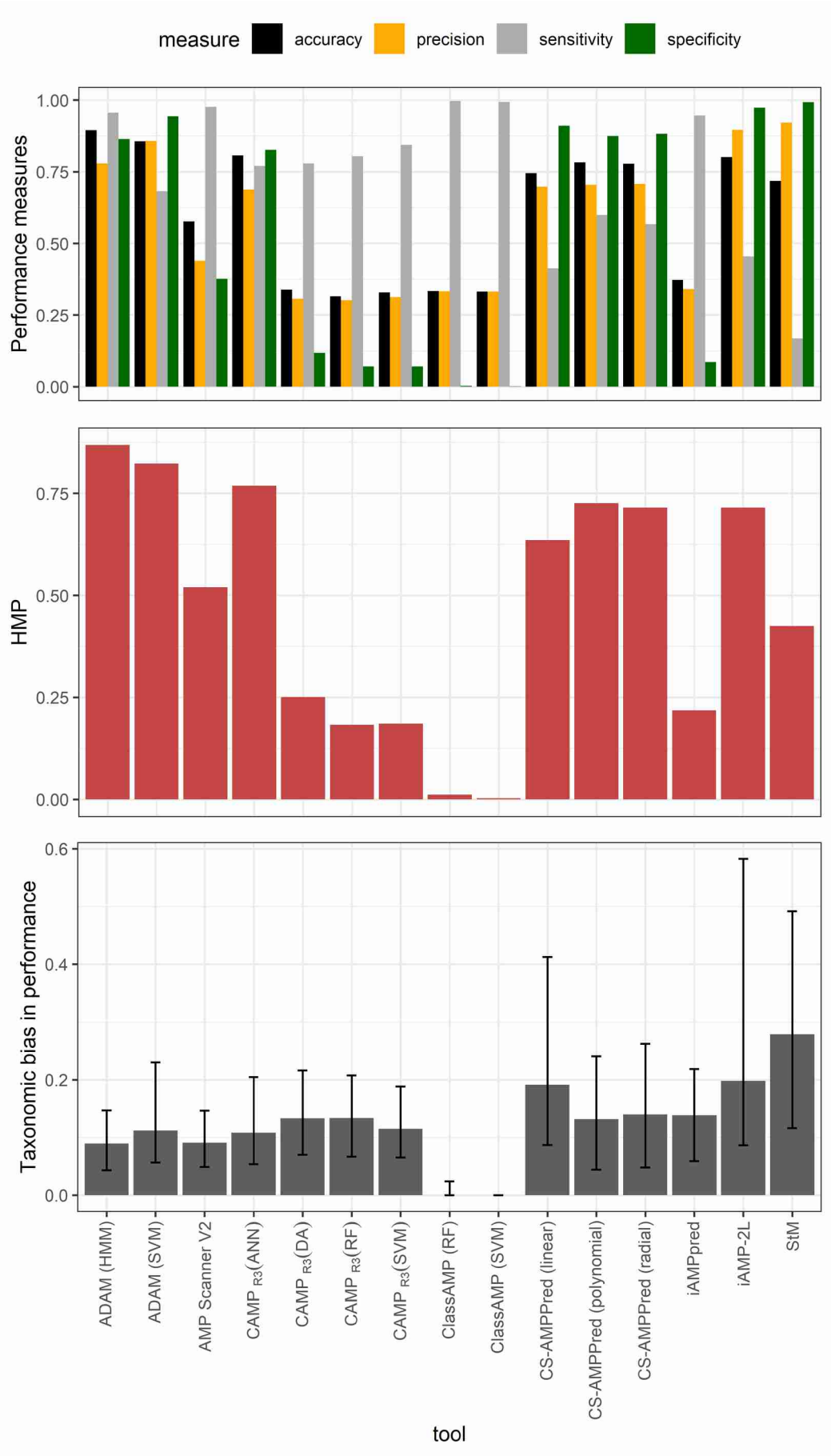
Figures


measure
accuracy
precision
sensitivity
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Performance measures
1.00
0.75
0.50
0.25
0.00
HMP
0.75
0.50
0.25
0.00
Taxonomic bias in performance
0.6
0.4
0.2
0.0
ADAM (HMM)
ADAM (SVM)
AMP Scanner V2
CAMP R3(ANN)
CAMP R3(DA)
CAMP R3(RF)
CAMP R3(SVM)
ClassAMP (RF)
ClassAMP (SVM)
CS-AMPPred (linear)
CS-AMPPred (polynomial)
CS-AMPPred (radial)
iAMPpred
iAMP-2L
StM
tool

Figure 1. Performance measures (top panel), harmonic mean of performance measures (referred to as HMP; middle panel), and taxonomic bias in performance assessed as median of the differences in HMP between taxa, within a given prediction tool (see Methods for details; bottom panel); black segments on the bottom panel represent the value range between the first and third quartiles.