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Omics-based strategies to discover novel classes of RiPP natural products

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Ribosomally synthesized and post-translationally modified peptides (RiPPs) form a highly diverse class of natural products, with various biotechnologically and clinically relevant activities. A recent increase in discoveries of novel RiPP classes suggests that currently known RiPPs constitute just the tip of the iceberg. Genome mining has been a driving force behind these discoveries, but remains challenging due to a lack of universal genetic markers for RiPP detection. In this review, we discuss how various genome mining methodologies contribute towards the discovery of novel RiPP classes. Some methods prioritize novel biosynthetic gene clusters (BGCs) based on shared modifications between RiPP classes. Other methods identify RiPP precursors using machine-learning classifiers. The integration of such methods as well as integration with other types of omics data in more comprehensive pipelines could help these tools reach their potential, and keep pushing the boundaries of the chemical diversity of this important class of molecules.

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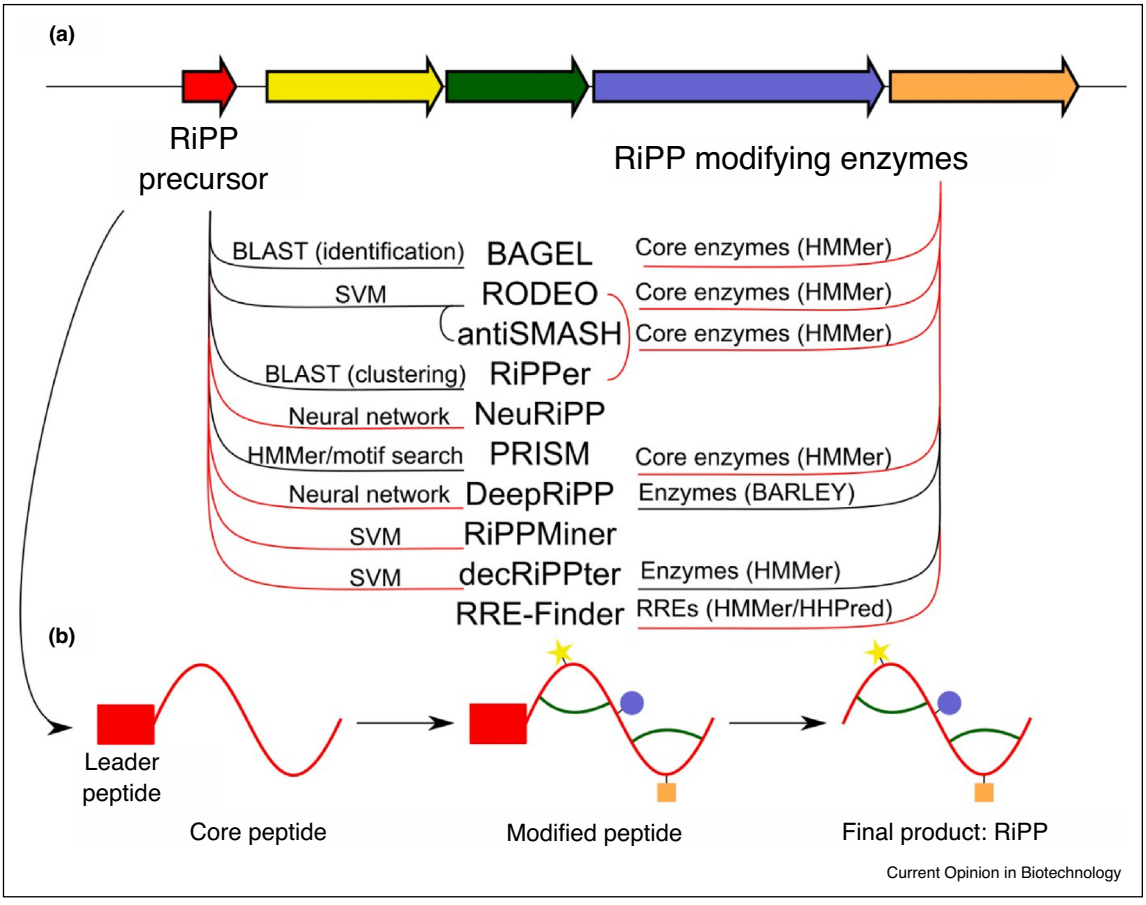
Introduction

Small organic molecules from a biological origin, collectively called natural products, comprise a dazzling array of diverse chemical structures [1]. These molecules play many different roles in nature, including interspecies signaling, resource competition and host defense. As such, bioactive natural products find their way into the clinic, as a result of their antimicrobial, antifungal, anticancer, antiviral or immunosuppressant activities. The discovery of antibiotics has had a huge impact on human life span, and in the mid-20th century bacterial infections seemed like a call from the past. However, multi-drug resistance and newly emerging infectious diseases once more impose serious health threats, and new drugs are now needed more than ever [2,3]. Meanwhile, traditional high-throughput screening (HTS) efforts lost their power, primarily due to the problem of replication, the rediscovery of known compounds [4,5]. Next-generation sequencing efforts surprisingly revealed that the capacity of bacteria to produce natural products had been grossly underestimated. This has led to a revolution in drug discovery based on the efficient mining of the rapidly growing genome sequence data [6]. Numerous tools and databases have been developed to explore, compare and catalogue biosynthetic gene clusters (BGCs) and their chemical products [7–11]. The discrepancy between the low return on investment of HTS and the apparent massive reservoir of biosynthetic potential is likely explained by the fact that the compounds are not produced under laboratory conditions. In other words, the BGCs that specify these natural products are only activated in response to specific environmental signals [12,13], and are therefore referred to as cryptic or silent BGCs.

The biosynthesis of natural products typically requires a distinct set of conserved enzymes that is responsible for their biosynthesis. Their encoding genes can be used as a basis for the detection of BGCs of that class [14,15]. Completely new biosynthetic pathways and natural product classes are much harder to discover with bioinformatics alone, however. A few methods that do not rely on the detection of known marker domains have been developed for that purpose, leading to the discovery of new chemical scaffolds [16–19].

One class of natural products which likely still comprises a large amount of hidden chemical diversity, is that of the ribosomally synthesized and post-translationally modified peptides (RiPPs) [20,21••]. RiPP biosynthesis always follows the same logic—a precursor peptide is ribosomally synthesized, biochemically modified and finally cleaved, resulting in the finished product (Figure 1)—but numerous different precursors and sets of modifying enzymes acting on them leads to large structural diversity (Figure 2). Their functions are equally diverse, and

Figure 1



Common biosynthetic logic as foundation for genome mining.
(a) RiPP BGCs contain a gene for a precursor peptide and genes encoding modifying enzymes, either of which can be used as a target. The red lines indicate a method's main way of identifying a BGC of interest. **(b)** Template for RiPP biosynthesis. A gene is translated into a precursor peptide, which has both a leader and a core region. The core is modified by modifying enzymes, and the leader is cleaved off, resulting in the final product.

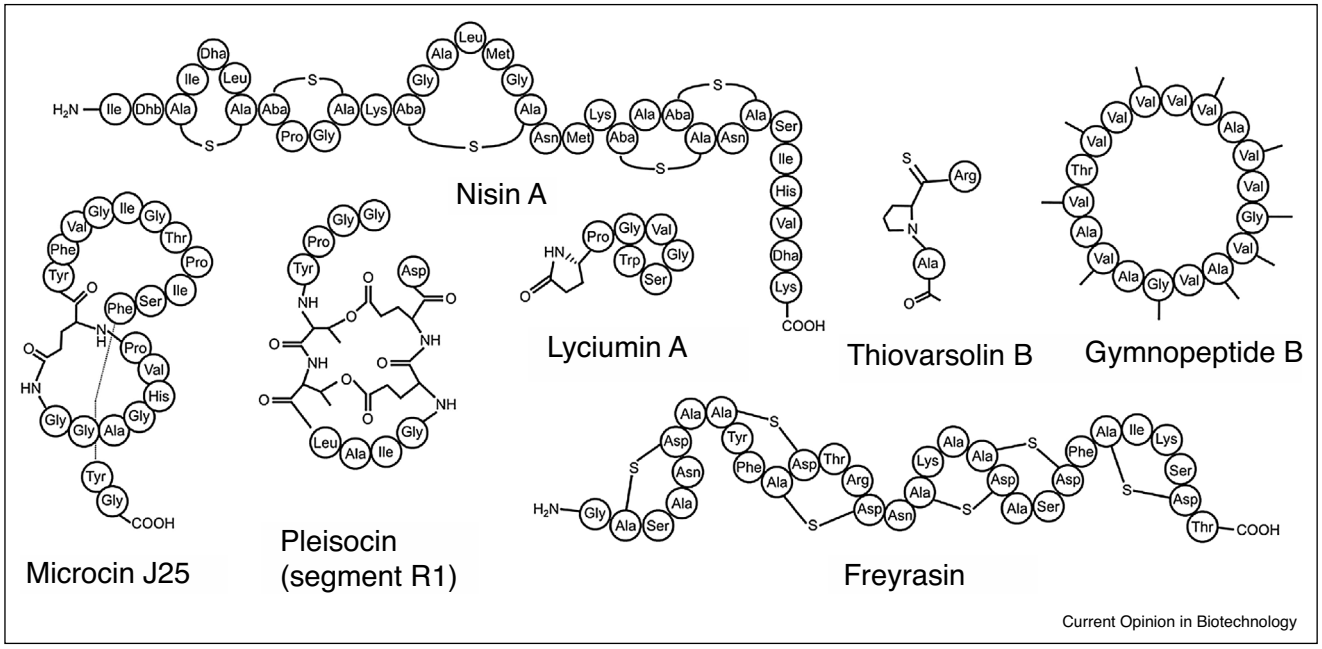
include quorum sensing, acting as enzyme co-factors, roles in cellular development, mediating host–microbe interactions, but also the much sought-after antibacterial and antifungal properties that would make them interesting for clinical applications [22].

RiPPs are classified into subclasses or families, each of which is defined by one or more characteristic modifications. For example, all lasso peptides have at least a single crosslink, forming a small loop through which the amino acid chain is threaded. Given the simple architecture of a RiPP BGC and the expected ease with which they might have evolved, it is likely that many more RiPP classes exist that do not contain the marker genes of known classes. This is exemplified by the rapid increase of known RiPP classes, which have nearly doubled between 2013 and 2020 [20,21••]. Discovery of novel RiPPs presents itself as a promising avenue to identify novel chemical scaffolds and drug leads. However, the lack of a single genetic marker among all RiPP classes requires novel strategies to leverage genomic data to this end. In this review, we discuss approaches and strategies for explorative RiPP genome mining aimed at the discovery of novel RiPP families (Table 1).

Taking the bait: how to prioritize regions of interest

Marker-based genome mining has been the main strategy for the high-confidence detection of BGCs from known classes of RiPPs. For many RiPP classes, modifying enzymes are split into core and accessory types, depending on the type of modification installed. RiPP BGCs are then detected by targeting the core modifying enzymes of one class, and many tools like antiSMASH [14], BAGEL [23], PRISM [24] and RODEO [25•,26–30] have implemented this strategy. The rules for each RiPP class result in the high-confidence detection of BGCs from known RiPP classes. For the detection of novel RiPP classes,

Figure 2



Examples of the rich chemical diversity of RiPPs.
RiPP precursors are highly diverse in sequence and can be modified in many ways. Several old and new examples are shown here: nisin A (lanthipeptide) [66], lyciumin A (lyciumin) [58], thiovarsolin B (thioamitide) [31••], gymnopeptide B (borosin) [58], microcin J25 (lasso peptide) [67], plesiocin segment R1 (omega-ester containing peptide/graspetide) [68] and freyrasin (ranthipeptide) [27]. Abu-S-Ala: beta-methyllanthionine. Dha: dehydroalanine. Dhb: dehydrobutyirine.

however, these rules need to be broken, even if this will result in more false positives.

A method exploring this principle, RiPPer, allows a user to find novel BGC architectures based on a single enzyme query [31••]. Candidate precursors are prioritized based on their conservation among the candidate BGCs of interest. The authors themselves use an enzyme involved in thioviridamide maturation to find the thiovarsolins, expanding the thioamitide class. Arguably, any enzyme can be used in such a strategy, as long as it is frequently associated with RiPP biosynthesis. For enzymes like YcaO and LanD, this has long been known to be the case [32,33]. The discovery of lanthinidins as intermediates between lanthipeptides and linaridins suggests that the exchange of domains between different RiPP classes may be more common than previously thought [34,35]. Another versatile enzyme is the radical *S*-adenosyl methionine (RaS) enzyme, involved in the maturation of RiPPs [36]. Exploration of RaS genes in novel contexts has recently led to the identification of new RiPP classes, including the spliceotides [37•], ryptides [38], rotapeptides [39] and WGK peptides [40•]. In rare cases, overlap in domains can even lead to the discovery of novel non-RiPP BGCs using RiPP modifying enzymes. LanB enzymes, vital for the maturation of type I lanthipeptides, have also been found in new genetic contexts, which led to the identification of the pearlins [41].

A key identifier for many different RiPP families is the RiPP Recognition Element (RRE). RREs can be encoded in small peptides or fused to modifying enzymes, and are required for the recognition of the precursor peptide. Although their secondary structure is conserved, RREs have highly diverse sequences, and could until recently only be effectively identified using HHPred [42,43], a highly sensitive but compute-intensive algorithm that compares families of sequences and their secondary structures to each other. RRE detection can be sped up using HMMer instead of HHPred, although novel RiPP classes will then only be detected if their RREs are similar on the sequence level. To detect an RRE-like secondary structure, HHPred can be used with a smaller database aimed specifically at RREs, rather than the large uniclust database which is used by default [44]. Both methods have been implemented in RRE-Finder [45•]. With this tool, several RREs were detected fused to enzymes not known to be involved in RiPP biosynthesis. RREs are not a high-confidence marker, as they are not contained in all RiPPs [46], and sometimes the domain is vestigial

Table 1

Tools currently available for RiPP genome mining

Name	BGC identification target	Method description	Possibilities of identifying novel classes	Reference
antiSMASH	Core enzymes	Identifies RiPP BGCs with core enzymes per class. Identifies precursor peptides with RODEO's SVMs.	Focuses on known classes. Can identify novel classes if they share core enzymes.	Blin *et al.* [14]
BAGEL	Core enzymes	Identifies RiPP BGCs with core enzymes per class. Identifies precursor peptides with BLAST and a known precursor database.	Focuses on known classes. Can identify novel classes if they share core enzymes.	Van Heel *et al.* [23]
RiPP-PRISM	Core enzymes	Identifies RiPP BGCs with core enzymes per class. Identifies precursor peptides with HMMer and a motif search.	Focuses on known classes. Can identify novel classes if they share core enzymes.	Skinnider *et al.* [15,24]
RODEO	Core enzymes	Identifies RiPP BGCs with core enzymes per class. Identification of precursor peptides with SVMs.	Focuses on known classes. Can identify novel classes if they share core enzymes (e.g. ranthipeptides). Custom queries possible.	Tietz *et al.* [29], Schwalen *et al.* [28], Hudson *et al.* [27], DiCaprio *et al.* [30], Walker *et al.* [25•], Georgiou *et al.* [26]
RiPPer	Any enzyme	Identifies RiPP BGCs with any query enzyme. Prioritizes candidate precursor peptides with prodigal-short and BLAST-based clustering.	Can identify novel classes if a query is selected that is associated with RiPP biosynthesis in a class-independent manner, with a higher chance of false positives depending on the query used.	Santos-Aberturas *et al.* [31••]
RRE-Finder	RiPP Recognition Elements (RREs)	Identifies RREs with HMMer or HHPred-like pipeline. Can identify novel RRE-fusions, depending on the cutoffs used.	Can identify novel RRE-fusions with exploratory mode or with precision mode and lower cutoffs, at the cost of more false positives.	Kloosterman *et al.* [45•]
RiPPMiner	Precursor peptides	Identifies and classifies precursors with a single SVM.	Can detect precursors of novel classes if they are similar from the perspective of the classifier.	Agrawal *et al.* [53]
NeuRiPP	Precursor peptides	Identifies precursors with a neural network.	Can detect precursors of novel classes if they are similar from the perspective of the classifier.	De Los Santos [55]
DeepRiPP	Precursor peptides	Identifies and classifies precursors and BGCs with a neural network (NLPPrecursor). Predicts products and estimates novelty based on genetic context and known modifications (BARLEY). Compares metabolomics and matches MS/MS spectra to predicted products (CLAMS).	Focuses on known classes. Can detect precursors of novel classes if they are similar from the perspective of the precursor classifier.	Merwin *et al.* [56••]
decRiPPter	Precursor peptides	Identifies and classifies precursors with a single SVM. Uses genetic context to prioritize novel RiPP BGCs. Forms candidate RiPP families based on precursor and BGC similarities.	Can detect precursors of novel classes if they are similar from the perspective of the classifier. Comparative genomics is used to prioritize hits.	Kloosterman *et al.* [54••]
DEREPLICATOR	NA	Clusters peptide natural products based on MS/MS spectra.	Focuses on finding spectra of known peptidic natural products. Can find novel RiPPs if part of the RiPP can be related to previously identified products.	Mohimani *et al.* [62]
VarQuest	NA	Matches peptide natural products to their variants with unknown modifications based on MS/MS spectra.	Focuses on finding spectra related to known peptidic natural products. Can find novel RiPPs if part of the RiPP can be related to previously identified products. More flexible with regards to unknown modifications than DEREPLICATOR.	Gurevich *et al.* [64]
MetaMiner	Core enzymes	Identifies RiPP BGCs with antiSMASH. Predicts products based on genetic context and known modifications. Matches predicted products to MS/MS spectra.	Can identify RiPP subclasses with novel modifications using a blind modification search.	Cao *et al.* [65•]

[47]. Still, these results show that RREs can serve as excellent beacons leading to the discovery of novel RiPP classes.

Prioritizing BGCs of interest without the use of a query domain is much more challenging, but has the potential to identify completely novel machinery. These approaches are particularly important for eukaryotic BGCs, for which few BGC markers are known [48,49]. Bacteria, fungi, plants and other eukaryotes each produce their own unique RiPP subclasses. The discovery of RiPPs across these branches of life is therefore mostly independent from one another, from a bioinformatic point of view. Most of the subclasses have been found in bacteria, although increasingly more subclasses are being uncovered for fungal and plant RiPPs, and likely many more exist with still unknown modifying enzymes [49]. The bioinformatics discovery of these could rely on query-independent strategies. The conserved genomic location and co-regulation of BGCs, for example, has been exploited to prioritize regions of interest in fungi, resulting in the discovery of a novel RiPP [19,50,51]. Similar methods can be exploited for other eukaryotes as well, when sequencing of many of their large genomes becomes viable. ClusterFinder [17], and more recently, DeepBGC [16], both detect BGCs without being confined to specific domains. Such methods have not been developed and trained with a specific focus on RiPP BGCs, but could be highly valuable when combined with precursor detection (see below).

Finding the needle: new methods for the detection of precursor peptides

The first step in RiPP biosynthesis is the translation of the precursor gene. Since these genes are small, they are notoriously hard to detect by gene-finding algorithms. Often, the precursor genes are found nearby their modifying enzymes, which limits the search space. BAGEL4, for example, identifies precursor peptides by BLASTing all small ORFs against a large database of previously characterized precursors peptides [23]. Precursor gene detection is even more important when they are not encoded near the genes encoding their modifiying enzymes, such as for animal RiPPs and cyanobactins [49,52].

Machine-learning based classification of precursor peptides is quickly gaining traction as a viable way for their detection with high accuracies and low false discovery rates (FDR). Support Vector Machines (SVMs) are especially popular. Rather than the peptide sequence itself, SVMs use features calculated from the sequence, such as charge, hydrophobicity, or abundance of amino acids or amino acid pairs.

Depending on the features selected, a wealth of information can be extracted from the peptide sequence, to precisely separate precursors and non-precursors. This method is used by RODEO [25•,26–30], which uses a different SVM per RiPP class to detect precursors in detected BGCs. RiPP-MINER [53] and decRiPPter [54••] are available as standalone tools and use a single SVM to identify precursor peptides regardless of class.

Neural networks bypass the need for feature selection by taking in the raw sequence of the peptides as a vector. Two tools have been developed that use neural networks to identify and classify precursors: NeuRiPP [55] and NLPPrecursor [56••]. The tools use different network architectures: NeuRiPP's most successful architecture is the parallel convoluted neural network (CNN), while NLPPrecursors uses a Universal Language Model Fine-Tuning (ULMFiT) neural network to detect encoded precursor peptides. The latter is a neural network architecture used for language processing that has shown to be highly effective in building models from training sets with low amount of data.

Both neural networks, as well as the SVMs from RiPP-MINER and decRiPPter, can detect precursors of many different classes using only a single model. Interestingly, both NeuRiPP and decRiPPter can identify RiPP precursors that are not included in the training data, suggesting that they should allow the detection of precursors of currently undiscovered families of RiPPs. Apparently, some properties are common to RiPP precursors regardless of their class. These are not directly obvious from their sequence, but are still picked up by the classifiers. How suitable precursor classifiers are for detection of novel RiPP classes likely depends on the selected features and model architecture. A standardized test with curated databases could be a valuable addition to benchmark and compare classifiers. By leaving out RiPP classes during the training process and testing how well they are still detected, the approximate 'explorativeness' of a classifier can be measured. This process could also give valuable insights into how RiPP precursors from different classes relate to one another, from the perspective of the classifier and the features it is trained on. Some classes of RiPP precursors may be more similar than others, which would mean that some classes are more easily discovered with our current training data than others.

Another interesting feature that has been reported in increasingly more studies is the presence of multiple core regions in a single precursor peptide. The encoding of multiple copies of the same core region allows for the efficient production of several RiPP variants, while only needing a single leader peptide. These repeats are found often in eukaryotic RiPPs [49,57,58], and could provide a handhold for their identification without prior knowledge of their primary sequence.

Assembling the whole: the integration of –omics

Exploiting the capabilities of a precursor classifier to detect novel RiPP classes is a promising route that has only been partially explored. However, any precursor-based approach faces a difficult challenge with regard to the ratio of false positives to true positives, as the number of small ORFs far exceeds the number of expected RiPP precursors. Integration of these tools into larger pipelines and combination of –omics datasets could help these tools reach their potential.

Purely genomic approaches can help prioritize regions of interest around predicted precursors. Any of the methods mentioned above could be combined, such as the requirement of a novel RRE-enzyme fusion and a predicted precursor. decRiPPter builds on its precursor predictions to prioritize regions of interest with a marker-independent strategy [54••]. Candidate RiPP BGCs are prioritized by filters, such as requiring the presence of a transporter gene or the lack of household genes that are common to the taxonomic group of organisms studied. Candidate RiPP families are formed by clustering identified BGCs based on their precursor sequences and encoded enzymatic domains, which resulted in the identification of 42 new candidate RiPP BGC families across 1295 *Streptomyces* genomes.

Transcriptomic and proteomic data can be used to identify co-regulated genes and link them to their cognate bioactivity [59,60]. This principle was previously used to identify ustiloxin B [51]. More recently, a pipeline that integrates the use of RNASeq data was used to identify novel RiPP BGCs in the fungus *Trichoderma* spp. [61]. The authors used ClusterFinder [17] to identify candidate BGCs, in which candidate BGCs were identified with RiPPMINER [53]. The results were further filtered by removing gene islands which were not activated, and clustering the predicted precursors, resulting in the prediction of several candidates.

The integration of metabolomics data could accelerate up the identification of novel classes. There is a large potential for the identification of RiPP-like compounds by automated detection from spectral data. In contrast to normal proteins, however, RiPPs contain modified amino acids and are rarely linear. For known RiPP classes, the modifications can be predicted based on genomic information. Predicted peptide fragments containing these modifications can be matched to the spectra with tools like DEREPLICATOR [62] (recently updated with NPS [63]) and CLAMS (available within the DeepRiPP pipeline [56••]). DeepRiPP is perhaps the most integrative pipeline for RiPP discovery. Besides structure prediction based on the identification of known modifications, it also combines comparative genomics with comparative metabolomics, to prioritize peaks whose presence/absence matches that of the BGCs of interest. While DeepRiPP mostly prioritizes RiPPs of known classes, a similar pipeline could be conceptualized aimed at the discovery of novel classes. All of this will depend on whether compounds are expressed at sufficiently high levels to facilitate their detection. Elicitors should therefore be added to activate the expression of cryptic BGCs, whereby comparative metabolomics combined with transcriptomics or proteomics will allow linkage of BGC expression profiles to changes in metabolites. This will allow scientists not only to observe more metabolites than under one specific growth condition, but also to predict which metabolites are produced by which BGCs.

A major challenge for automated MS/MS analysis that remains is dealing with new modifications. VarQuest [64], an extension of DEREPLICATOR, can identify peptide variants based on known peptides, even if these variants contain unknown modifications. MetaMiner [65•] combines genomics and metabolomics to predict precursor modifications and find associated spectra, which can contain unknown modifications. Completely *de novo* identification of novel RiPPs with only unknown modification has yet to be explored by tools like these, but represents a sizable computational challenge. Even so, just matching a small sequence of unmodified amino acids to part of a candidate novel RiPP precursor is a valuable addition to more explorative RiPP searches. Identified, novel precursors could then be fed back to the training data of the precursor classifiers, creating an iterative process in which the classifiers will become increasingly specific and tuned toward a larger variety of RiPP classes.

Conclusions and final perspectives

RiPPs represent a diverse class of natural products within which new subclasses are being quickly discovered. The lack of universal genetic markers makes genome-based mining for novel RiPP BGCs challenging, but the amount of sequence data and the level of computational power available allows for many highly interesting strategies. As more RiPP classes are being discovered, more modifications are found to be shared between different classes, which can lead the way to novel variants. Precursor classification is a powerful addition to the list of available tools, both for known and novel RiPP identification. Finally, integrative approaches combining comparative genomics, eliciting strategies, transcriptomics, proteomics and metabolomics will help us explore the vast and diverse chemical space of this promising class of natural products.

Conflict of interest statement

M.H.M. is on the scientific advisory board of Hexagon Bio and co-founder of Design Pharmaceuticals.

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