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Proteomics in non-model organisms: a new analytical frontier

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Abstract

For the last century we have relied on model organisms to help understand fundamental biological processes. Now, with advancements in genome sequencing, assembly and annotation, non-model organisms may be studied with the same advanced bioanalytical toolkit as model organisms. Proteomics is one such technique, which classically relies on predicted protein sequences to catalog and measure complex proteomes across tissues and biofluids. Applying proteomics to non-model organisms can advance and accelerate biomimicry studies, biomedical advancements, veterinary medicine, agricultural research, behavioral ecology, and food safety. In this post-model organism era we can study almost any species, meaning that many non-model organisms are in fact important emerging model organisms. Herein we focus specifically on eukaryotic organisms and discuss steps to generating sequence databases, analyzing proteomic data with or without a database, interpreting results, and future research opportunities. Proteomics is more accessible than ever before, and will continue to rapidly advance in the coming years, enabling critical research and discoveries in non-model organisms that were hitherto impossible.

Graphical Abstract

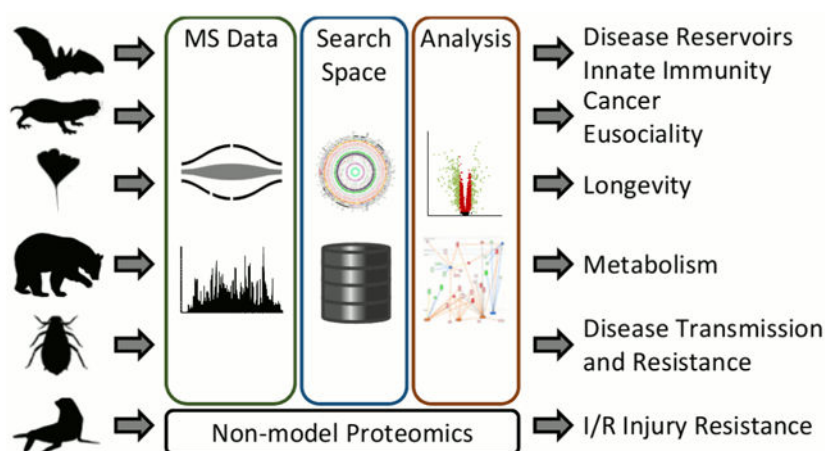
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Introduction

Beginning with fruit flies at the turn of the 20th century, model organisms have enabled biological discoveries by bringing testable yet complex biological systems to the lab. These organisms, including mice, rats, frogs, zebra fish, roundworms, yeast, maize, *Arabidopsis*, and *Escherichia coli*, are easily maintained in the lab, have well established methods and molecular tools, can be manipulated at the molecular level, and have a vast body of associated literature¹⁻². Over the last 100 years, these model organisms have been crucial to revealing fundamental biological truths and developing the theoretical framework for molecular biology. But as cutting-edge biomolecular analysis in non-model organisms is becoming more routine, we are on the cusp of a new frontier³⁻⁴. Over billions of years, natural selection has favored countless adaptations to allow organisms to inhabit and thrive in all corners of the earth. Through the study of non-model organisms, scientists are revealing insights into the molecular basis of a vast array of human conditions. Naturally occurring adaptations and susceptibilities inform the evolutionary and genetic underpinning of chronic and genetic disease in humans, from cancer to neurodegeneration (expertly reviewed by Stenvinkel *et al.*⁵⁻⁶). For instance, elephants and naked mole rats illuminate molecular mechanisms at play in cancer resistance⁷⁻⁹, deer antlers hold clues to organ regeneration¹⁰⁻¹¹, diving mammals inform ischemia reperfusion injury resistance¹²⁻¹⁶, hibernating arctic squirrels and grizzly bears teach lessons about neuroprotection and metabolism¹⁷⁻¹⁹, and naked mole rats and bats possess secrets to longevity²⁰⁻²³. Plants are also treasure troves of information including how compartmentalization in plants avoids senescence²⁴⁻²⁵. This field of study, biomimetics, seeks to understand these adaptations and provide insight into chronic disease in humans, and is made possible by non-model organism research.

In addition to biomimetics, non-model organisms are important in other key areas, including agricultural research, veterinary medicine, behavioral ecology, and food safety. Emerging

plant diseases in high value agricultural crops, such as citrus greening disease^{26–28} and cotton blue disease^{29–33}, are forcing rapid development of bioanalytical capabilities to study non-model plant species, plant pathogens and insect vectors of plant pathogens. Knowledge of these virus-interacting host proteins paves the way for genome editing approaches to develop durable resistance. Non-model organism research is also critical in veterinary medicine for industries such as zoos and aquariums or pets (\$3 billion and \$18 billion annual, respectively^{34–35}), which rely on bioanalytical measurements for development and validation of new techniques translated from human medicine. In cases such as bats, genomic and proteomic analysis is being harnessed to better understand longevity, as well as their innate immunity and ability to serve as disease reservoirs^{36–38}. Maybe less apparent is the applicability of proteomics to behavioral ecology (expertly reviewed by Valcu and Kempenaers³⁹), such as eusociality in the naked mole-rat⁴⁰. Lastly, with growing concerns of food authenticity, proteomic analysis in non-model organisms offers promising avenues of tracking and validation in aquaculture and agriculture^{41–45}. These topics are economically critical and rely on our ability to rapidly develop cutting edge analytical capabilities in non-model organisms. Classical model organisms are vitally important, but there is an enormous promise of untold discoveries and applications by studying non-model organisms (Figure 1).

Given the current SARS-CoV-2 pandemic, it is important to specifically highlight the importance and potential of non-model organism research when studying zoonotic viruses. Although predicting zoonosis includes many non-molecular factors^{46–47}, it has been shown that sequence homology between host receptors (*e.g.*, ACE2 in SARS and SARS-CoV-2 infection) can predict zoonotic potential and species tropism^{48–50}. For instance, SARS-CoV-2 entry potential correlates with ACE2 receptor homology between reservoirs and hosts⁵⁰. Current studies of SARS-CoV-2 utilizing structural information such as receptor binding domain⁵¹ and glycosylation of the viral spike protein⁵² will continue to improve predictive power even further^{53–54}. Undoubtedly proteomic analysis of these non-model reservoirs can provide empirical evidence of structural and glycosylation predictions on vital molecular targets, which is made possible only by directly studying the non-model organism. Furthermore, determining host-virus interactions by protein-protein interaction (PPI) proteomic analysis is critical to understanding infection, such as with Ebola virus⁵⁵, Zika virus⁵⁶ and SARS-CoV-2⁵⁷, but similar PPI studies in viral reservoirs themselves could be equally enlightening for innate and adaptive immunity as well as zoonotic spillover potential. Broad proteomic studies across hundreds of reservoir and host species will improve our understanding of viral infection, zoonotic prediction, and highlight possible treatments.

Proteomics in non-model systems is driven by genomics

Currently, the most common method of proteomic analysis is shotgun proteomics (also referred to as bottom-up proteomics). By digesting proteins to peptides, proteomics researchers use mass spectrometers and database search algorithms to identify and quantify near complete proteomes (such as recent studies in human^{58–59} and yeast⁶⁰). Traditional data-dependent acquisition (DDA) or newer techniques such as data-independent acquisition (DIA) or BoxCar⁶¹ may be used if the search space can be defined using protein sequences and appropriate post-translational modifications. When using shotgun proteomics to study a

non-model organism that may not have an annotated genome available, the search space is the major hurdle^{62–63}. If an annotated genome is available, any limitation that applies to proteomics in model organisms will also apply to a non-model (*e.g.*, studying glycosylation with DIA data is difficult regardless). Knowledge of current solutions and best practices in non-model organisms is limiting widespread adoption. For starters, in the past, genome sequencing and annotation could prove an insurmountable or very expensive problem to overcome, but this is no longer the case.

In recent years, post-2015, there has been an explosion of genome sequencing as the cost to generate accurate highly-contiguous genome assemblies has continued to drop⁶⁴. There are ongoing large sequencing efforts (*e.g.*, Earth BioGenome Project, Vertebrate Genomes Project, DNA Zoo, Zoonomia Project, Bat1K Project, Bird 10 000 Genomes Project) that are beginning to hit their stride and soon will be releasing hundreds of genomes per year to achieve many thousands of genomes by completion. Of the roughly 5400 mammal species, currently 430 have genomes, while of the roughly 400 000 plant species, there are 630 plant genomes, and of the nearly 1 million named species of insects less than 500 genomes are complete⁶⁵ (Figure 2). When a genome is not available, especially in the case of mammals the most affordable path to *de novo* sequencing is to use short-read sequencing with proximity ligation techniques (such as Hi-C or Chicago). The resulting genome assemblies are accurate for transcriptomic and proteomic studies (*e.g.*, proteomic analysis using a Chicago-based mammalian assembly⁶⁶). In cases of highly heterogeneous and repetitive genomes (*e.g.*, Mollusca), additional and more expensive techniques may be required such as long-read sequencing based on Oxford Nanopore Technologies or PacBio, as well as optical mapping. But in the case of many species, a *de novo* assembly can be generated in the matter of months for minimal cost and expertise. Once completed, quality measures including contiguity (*i.e.*, scaffold and contig N50) and completeness (*i.e.*, BUSCO⁶⁷) can be evaluated, but since there are no universal quality thresholds, we recommend comparing quality metrics of phylogenetically similar published assemblies. Alternatively, proteogenomics represents experimental validation of a genome assembly using proteomics^{66, 68–69}. Peptide identification can help validate polymorphisms and predicted alleles while top-down proteomic methods can confirm predicted isoforms. Future development of genome annotation pipelines may include ways to integrate mass spectrometry-based proteomic data.

Genome sequencing and availability does not seem to be the main roadblock to proteomics in non-model species: the current bottleneck is genome annotation. Complete and accurate genome annotation is key to proteomic workflows. Broadly there are two paths to annotation: in-house annotation or making the data publicly available for annotation by NCBI or Ensembl. In the case of in-house annotation, publicly available and often free, high-performance computing (HPC) resources such as XSEDE (Extreme Science and Engineering Discovery Environment; including JetStream), ACI-REF (Advanced Cyberinfrastructure Research and Education Facilitators) Network, NCI (National Computational Infrastructure) Australia, PRACE (Partnership for Advanced Computing in Europe) and the ELIXIR (the European life-sciences Infrastructure for biological Information) network, have enabled novice bioinformaticians without a local HPC to annotate genome assemblies using pipelines like MAKER (see tutorials^{70–71}). Although still

an iterative task, this is made possible by having thousands of processing hours on a virtual cluster. The second path to genome annotation is using free public resources like NCBI RefSeq or Ensembl. Specifically, the RefSeq project maintains and curates new and updated genome annotations^{72–73}. RefSeq has published guidelines on how genomes are selected for annotation⁷⁴, related to assembly contiguity, secondary RNA-seq data, and need by researchers. Once a high-quality genome assembly has been made public on DDBJ (DNA Data Bank of Japan), ENA (European Nucleotide Archive) or GenBank, it may be annotated and publicly released by RefSeq in a matter of weeks. But of the hundreds of genomes on GenBank, only a fraction have been annotated (Figures 2 and 3). What may not be evident is that a researcher not associated with the original genome project but with access to RNA-seq data may upload new sequence read archives (SRA), which can help begin or improve annotations of already existing high-quality genome assemblies. This is also an excellent way to demonstrate interest and improve annotation as more secondary gene evidence and transcript diversity typically results in more complete and accurate annotations. For instance, multiple tissue single-stranded RNA sequencing data were used by RefSeq to annotate the California sea lion genome⁷⁵. The current annotation system is ripe for democratization and team science approaches, especially for pre-existing publicly available genome assemblies.

Following in-house or RefSeq genome annotation as described above, there will be a sequence database (a collection of protein sequences, typically as a FASTA, XML or GFF file) that can be used to search proteomic data. There are secondary resources such as UniProtKB that may import and evaluate annotations from RefSeq and Ensembl, but it is important to note that UniProtKB does not import all available high-quality annotations. Moreover, whereas RefSeq groups genome annotations by release or by user submission, the provenance of protein assignments from other resources may be difficult to determine. For these reasons, retrieving species-specific proteomes from UniProtKB, RefSeq and Ensembl will yield different protein databases, often based on different genome assemblies, annotation pipelines, and curation. Since interpretations of proteomic data in non-model organisms are only as useful as the functional annotation of the proteome, a careful manual inspection of automated annotations may be required before proceeding with biological and functional experiments, as even RefSeq annotations may not be perfect due to issues with homology-based assignments, likely because orthologs in well-annotated species may not always exist⁷⁷. When proteins of interest are unknown or hypothetical, manual single protein curation is required using tools like UniRule⁷⁸. In insects, community and student-led annotation efforts have helped identify families of well-conserved genes and proteins^{79–81}, but species-specific genes and genes under positive selection are more difficult to annotate and require detailed, tissue-specific proteomic investigation^{82–85}. As more annotations come online, and with continued curation by the community, pipeline generated genome annotations will continue to improve.

There is a wealth of public genome assemblies, even multiple genomes from the same species, challenging the concept of a reference genome. In this pan-genome era, researchers are realizing that one individual cannot capture the genomic (and therefore proteomic) diversity of a population or species. In other words, more than a single human, aphid or African elephant reference genome may be required to describe the genome or proteome. Moreover, even with reference genomes there will be numerous annotation versions as gene

predictions improve, more evidence is acquired, assemblies are improved, or more genomes are completed. The reference human annotation is on its tenth release, and less obvious species like the cow are on their seventh release. For protein databases to evolve and effectively capitalize on pan-genomic data, a new framework will need to be developed.

Analyzing proteomic data from non-model organisms

Given the global resources devoted to genome annotation and how these data products are maintained and propagated throughout data hubs, when possible the greatest community benefits will come from applying resources to genome annotation (described above), as opposed to one-off transcriptomes. Yet there are still occasions where it is not possible due to resources, time, or simply availability of quality samples for genomic sequencing. If samples can be acquired with RNA stability in mind, it should be possible to generate predicted protein sequences using RNA sequencing (such as⁸⁶; Figure 4A). In cases such as biofluids, choosing a relevant tissue to sequence may be difficult since protein provenance is unknown. For instance, due to its proximity to all organs the complete blood proteome is translated in many tissues, not the blood itself⁸⁷. Another consideration is that when using short-read RNA sequencing, *de novo* assembly is required, which can be computationally costly and may not provide sequence coverage of protein isoforms or very large proteins (*e.g.*, human titin isoform N2BA mRNA is over 100 000 residues in length). To overcome these issues, full-length RNA sequencing can be employed⁸⁸, negating the need for transcriptome assembly since the direct transcript evidence can define the search space. As the capability and accessibility of full-length RNA sequencing improves, this could become a viable parallel approach to support mass spectrometry-based proteomics.

Proteomic analysis of a non-model organism in the absence of any additional nucleic acid sequencing is also possible (Figure 4B). Homology searching is when the database of a closely related species is used for conventional database searching. In this case, only tryptic peptides with complete homology will be identified. Depending on the clade this may work better than others (*e.g.*, using the Weddell seal and Pacific walrus annotations to search California sea lion proteomic data provided valid conclusions⁸⁹). Recently an approach was developed that can assist in selecting species by evaluating how well a homologous sequence database can explain an unknown proteome⁶³. Other search approaches can involve using wide mass tolerances such that single amino acid variants (SAAVs) can be tolerated between the known and unknown species. This is accomplished by employing an open search with a wide mass tolerance, and searching using a closely related species with a tool like MSFragger⁹⁰ or ANN-SoLo⁹¹. Similarly, utilizing a hybrid spectral library approach may work with closely related species⁹². Also, TagGraph⁹³ should likewise be applicable with an unknown proteome by combining database and *de novo* searching to identify unknown post-translational modifications (PTMs). Another approach is to take the remaining unmatched spectra from any of these approaches and perform *de novo* searching with tools such as Kaiko⁹⁴, PEAKS⁹⁵, or those packaged within DeNovoGUI⁹⁶. Using *de novo* proteomic techniques in conjunction with database searching, or as a stand-alone search, is a viable alternative when database searching of shotgun proteomic data isn't possible. Although the accuracy of *de novo* is still improving, recent and ongoing advancements continue to make *de novo* a valid approach. Finally, it is important to note that employing these search

approaches may be useful even in well studied organisms where we have complete confidence in the validity of sequence databases. Until we appreciate and account for the genetic diversity within a species, there will always remain the possibility that the reference sequence database is incomplete or inaccurate.

In the given suggestions, it is assumed that the source of the proteome is known. In some cases there may be a primary or secondary unidentified species. In the case of unknown disease agents, we can use proteomics where other methods fail due to antibody reactivity, RNA degradation or unknown PCR probes. This can present an enormous search space (such as the nearly 12 million proteins listed on UniProtKB for Fungi), but one that is not insurmountable given the numerous databases available, though high computational costs and significant false-discovery concerns exist. Conversely, we may be tasked with identifying non-model organisms within model systems. For instance, when studying the gut proteome there is the microbiome and host, but there are also remnants of ingested food. In an effort to correctly define search space and accurately identify mass spectra, we must account for numerous unknowns. In these cases, multiple search steps, greater reliance on known spectral libraries, or *de novo* is required.

Technical capabilities, hurdles, and future advancements

The discussion and solutions presented so far have focused on current approaches to define and evaluate the search space for shotgun proteomic analysis in non-model organisms, but there are other proteomic approaches worth mentioning. In contrast to digesting proteins prior to analysis, intact proteins can be analyzed using mass spectrometry by top-down proteomics^{97–99}. Although top-down is not yet routinely used in non-model organisms, it would face similar hurdles as those in humans, but given accurate protein sequences top-down non-model proteomics is possible. Protein arrays, composed of antibodies, lectins, or aptamers, are another possible approach to proteomics in non-model organisms. Though these arrays are typically geared towards human proteins, such as the SOMAscan, PETAL¹⁰⁰, or Olink platforms, they may be used in other species with varying success when there is enough cross-reactivity between conserved epitopes^{101–102}. It seems likely that arrays will be developed for animal-models if the market exists. There is a paucity of commercial antibodies against proteins from non-model organisms, therefore targeted mass spectrometry-based proteomics is a convenient alternative and a tremendously valuable tool to aid researchers interested in quantification of specific proteins in non-model organisms^{31, 103–104}. Overall, it seems only a matter of time before other proteomic techniques become routine in non-model organism studies.

Once protein identifications have been made, data interpretation in non-model organisms presents its own unique challenges. A typical workflow will result in a list of differentially abundant proteins, or proteins that are drivers of some difference, and this list is used for downstream analysis. Depending on the source of the non-model organism sequence database, the protein identification may be meaningful (*e.g.*, alpha-2 microglobulin) or it may simply be a generic locus or transcript ID which is unsuitable for biological interpretation. One possible first step is to convert the identifications to a known model-species by using BLAST to assign protein sequences to their model-species equivalent (a

notable implementation is the PAW BLASter¹⁰⁵). When determining orthology, the conversion may still require manual inspection due to the high degree of homology between certain proteins (*e.g.*, human alpha-2 microglobulin and pregnancy zone protein are 72% identical). Once the protein identifications have been converted to a model-species, a typical downstream analysis is gene set enrichment or pathways analysis. The underpinning biology and literature inform these lists, which means results from a non-model organism may be irrelevant or at least filled with caveats. Currently, the Molecular Signatures Database (MSigDB v7.0) includes five species (the vast majority being human and mouse), the Reactome pathway database and WebGestalt have 15 and 12 species, respectively, while the Plant Reactome has 97 plant species¹⁰⁶ and the OmicsDB::Pathogens has 11 species¹⁰⁷. But the further away, phylogenetically speaking, you move from the few well-studied model organisms, the less applicable the results may be. One alternative is to use co-expression or stabilized regression analysis¹⁰⁸, which can utilize abundance patterns to identify novel pathways or gene sets. Although these approaches are far from perfect, they do allow conditional conclusions to be reached and generation of testable hypotheses.

Proteomics provides unique insights into biology

Given the hurdles described, an apparent question is why use proteomics to study non-model organisms. Studying biology at the molecular level is not mutually exclusive to nucleic acid sequencing techniques, but there are cases where proteomics provides more relevant and actionable insights as compared to gene or transcript studies. Since the abundance of the majority of proteins does not correlate with transcript or gene abundance^{109–115}, proteomic analysis can provide a clearer understanding of the plasticity of phenotypes. Moreover, there are specific cases where proteomics can provide more relevant and actionable insights, such as biofluids (blood, urine, cerebrospinal fluid, insect hemolymph, *etc.*) where the mRNA in the fluid is not related to the proteome of the biofluid. Instead, the proteome of a biofluid is reflected by organs proximal to the fluid and any pathology causing flux between the fluid and organ(s). Also, it is important to note that genes do not change with age, but protein abundance, turnover, localization, modifications and interactions do change^{116–117}. In other words, phenotypes may arise from unique combinations of protein interactions and protein abundance, which will not be captured from genomic information alone. In addition to examining protein abundance differences, proteomic analysis can capture measurements of functionally important endogenous peptides^{118–119}, PTMs¹²⁰ and proteoforms¹²¹. In the case of proteoforms especially, abundance is highly dynamic, such as histone H4 with over 40 proteoforms that may vary over two orders of magnitude¹²². It is not to say that other techniques cannot answer these questions, but it is important to note where proteomics is uniquely capable.

The complexity of the proteome is further magnified when one considers that different proteoforms can interact to form functional protein complexes with unknown roles in cellular homeostasis and pathogenesis (*e.g.*, tumor suppressor protein PTEN¹²³). Only proteomic methods can provide quantitative measurements of protein complex formation in cells at proteome-wide scales^{29, 124–132}, improving our understanding of the evolutionary origins of protein interactions, protein co-localization, and functional protein interaction networks¹³³. Genetic and proteomic diversity also extends to an organism's microbiota¹³⁴.

This proteomic diversity is critical to understanding biological phenotypes, including cancer¹³⁵ and transmission of viruses^{134, 136}. Metaproteomic techniques can also provide insights into host-symbiont functional compartmentalization of proteomes, predictions on resident microbiota in eukaryotic host tissues and knowledge on co-evolutionary mechanisms regulating symbiosis in non-model organisms is crucial to our understanding of eukaryotic biology¹³⁷⁻¹³⁹. It is evident that proteomics can provide an additional modality to improve nucleic acid based analyses, while also providing unique insight into the molecular landscape responsible for a given phenotype or pathology.

Future Outlook

There is great potential in applying proteomics in non-model organisms and the technical hurdles are not formidable. This is best exemplified by a recent large scale proteomic study that cataloged 340 000 proteins from 100 species¹⁴⁰. In the future, proteomic studies of non-model organisms will undoubtedly become more commonplace. As it becomes possible to define the molecular landscape in species across the tree of life, the next step is finding the non-model organism experts. But this doesn't need to be for an exotic species in a distant land. In the shallow waters along the eastern seaboard is a euryhaline stingray that can achieve and maintain an extraordinarily high renal transtubular osmotic gradient¹⁴¹ and regenerate glomeruli¹⁴². Understanding the elasmobranch kidney could provide insight into chronic disease in humans. Exciting topics like this are all around us if we just look, explore and experiment. Proteomics researchers must work with biologists in these adjacent fields who can direct experimental design of naturally occurring phenotypes to empower comparative studies, and may be unaware of the power of proteomics or its accessibility via cores and collaborations. Empowering these scientists with advanced bioanalytical capabilities is a major goal of proteomics in non-model organisms. There is great potential to address pressing issues such as food supply security, help facilitate commerce, protect human and animal health, and use these techniques in biomimetics to help accelerate biomedical breakthroughs. The post-model organism era opens the door to new applications of maturing technology, hopefully leading to new fundamental biological truths unattainable using classic model organisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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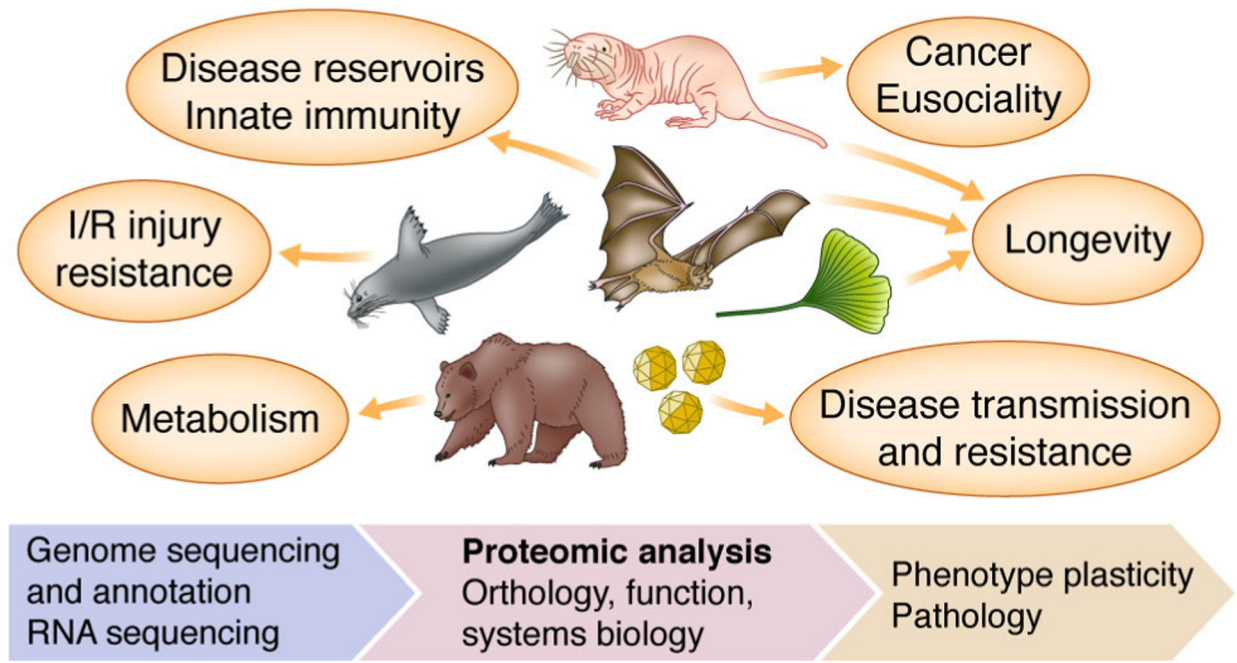


Figure 1. General overview of proteomics in non-model organisms. Some notable examples of non-model proteomics are shown (clockwise from top): naked mole-rat, bat, ginkgo, viruses, hibernating mammals, and diving mammals. I/R refers to ischemia reperfusion.

all	P	eukaryotes	P	G	A	animals	P	G	A	chordates	P	G	A	mammals	P	G	A
eukaryote	1308	animals	601	1958	487	chordates	298	1060	323	mammals	151	430	138	even-toed ungulates	32	120	26
bacteria	876	green plants	343	630	108	arthropods	165	593	140	bony fish	55	280	80	rodents	28	93	25
virus	106	fungi	209	3210	0	molluscs	55	44	8	lizards	53	33	10	carnivores	25	60	23
archaea	54	apicomplexans	40	143	0	nematodes	22	134	0	birds	19	240	76	primates	20	63	28
		kinetoplastids	29	82	0	cnidarians	20	35	10	amphibians	8	17	5	odd-toed ungulates	11	12	4
		diatoms	11	13	0	flatworms	17	39	0	carilaginous fish	3	9	2	rabbits & hares	5	3	2
		ciliates	8	32	0	echinoderms	11	17	2	turtles	2	24	5	anteaters and sloths	5	5	0
		oomycetes	8	89	0	segmented worms	7	4	0	alligator	2	2	2	bats	4	37	12
		haptophytes	5	4	0	sponges	2	2	1	dinosaurs	2	0	0	elephants	4	1	1
		diplomonads	5	8	0	placozoans	1	2	0	lungfish	1	1	0	herbivorous marsupials	3	3	2
		red algae	5	13	0	rotifers	1	10	0					elephant shrews	2	1	1
		trichomonads	4	4	0	brachiopods	1	1	1					aardvarks	1	2	1
		dinoflagellates	4	12	0	priapulids	0	1	1					manatees and dugongs	1	1	1
		cellular slime molds	3	20	0	hemichordates	0	2	1					carnivorous marsupials	1	2	1
		brown algae	3	5	0									possums	1	1	1
		forams	2	2	0	green plants	P	G	A					armadillos	1	3	1
		euglenoids	1	1	0	flowering plants	303	517	106					egg-laying mammals	0	1	1
		cryptomonads	1	2	0	green algae	15	87	0					insectivores	0	8	3
		other eukaryotes	25	490	0	fems	2	1	0					golden moles	0	1	1
						club-mosses	1	3	0					tenrecs	0	2	1
						other green plants	18	16	0					flying lemurs	0	1	1
														tree shrews	0	3	1
														pangolins	0	3	1

Figure 2. Distribution of species with proteomic data sets (P), genome assemblies (G), and genome annotations (A) across phylogenetic clades.

This series of charts was created by cross-referencing taxon IDs of genome assemblies on Genbank and genome annotations on RefSeq (valid as of 12 March 2020) with published proteomic data sets on the Proteomics Identifications Database (PRIDE; as of 17 January 2020) in order to emphasize clades lacking proteomic analysis, or clades that should be focused on for genomic sequencing and annotation. Specific to Eukaryotes, there are approximately 1308 species with published proteomic results, 6718 with available genomes and 595 species with complete genomic annotations. Note that these charts do not show the total number of species per clade (e.g., 950 000 named insect species⁶⁵), or the number of proteomic data sets per species (e.g., 68 % of the 12 660 PRIDE data sets are human and mouse). For this cataloging, we focused only on genome and annotation resources at NCBI and acknowledge that for non-animal non-flowering plants, there are other more appropriate repositories such as the Joint Genome Institute. Terms in tables are accepted common or scientific NCBI designations, except in a few cases under ‘mammals’ (the full mammal table with taxon IDs is available in Supplemental Table S1), and that these numbers may not be exact due to self-reporting on PRIDE and matching taxon identifiers between sources (note that the two dinosaur proteomic data sets are from *Brachylophosaurus canadensis* and *Tyrannosaurus rex*).

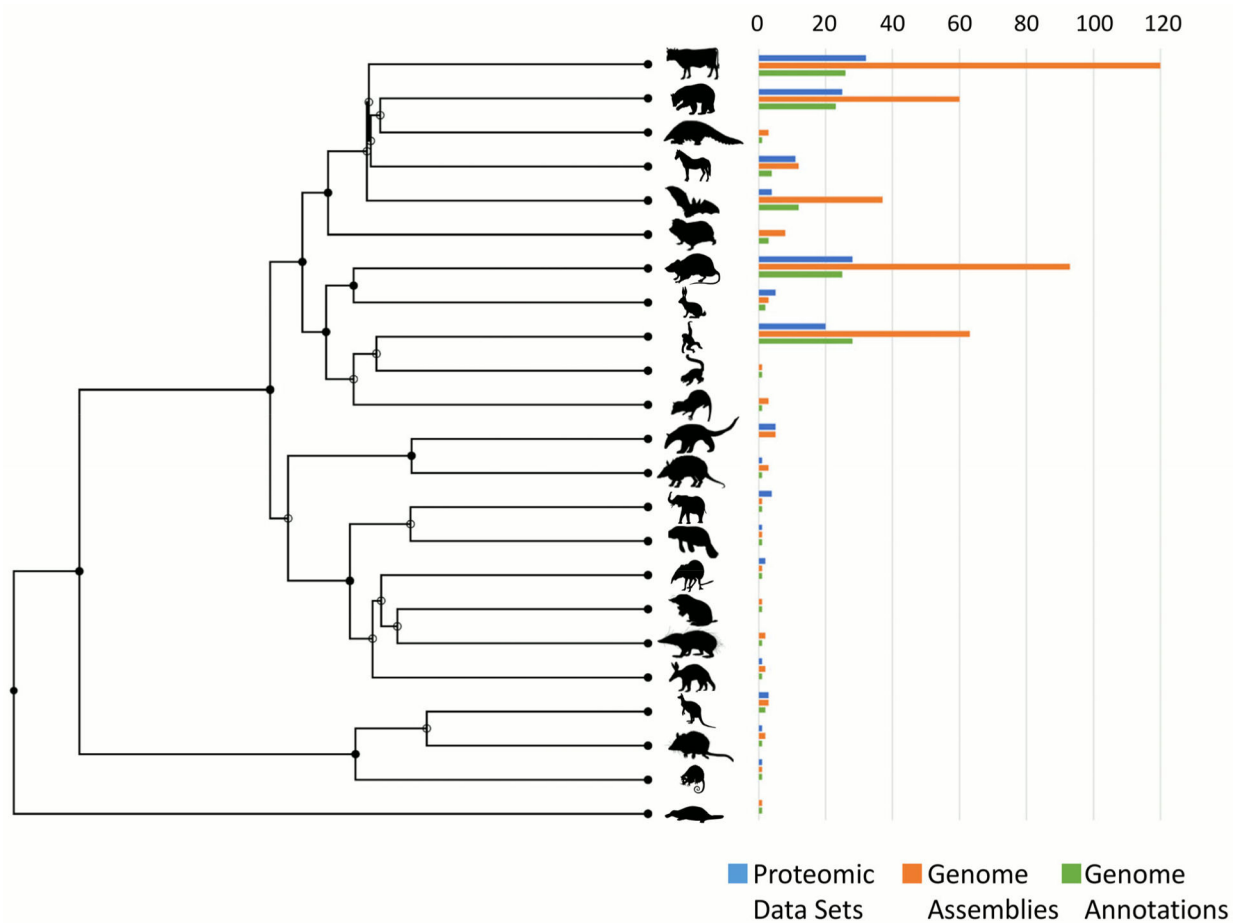


Figure 3. Distribution of mammals with proteomic data sets or genome annotations. Using a time tree (<http://www.timetree.org>), which incorporates fossil evidence⁷⁶, information regarding the 23 groups of mammals with proteomic data sets on the Proteomics Identifications Database (PRIDE; as of 17 January 2020), genome assemblies on Genbank and genome annotations on RefSeq (valid as of 12 March 2020) was plotted. This is per taxa and does not include the number of proteomic data sets, genome assemblies or genome annotations within taxa. The order, from top to bottom is: even-toed ungulates, carnivores, pangolins, odd-toed ungulates, bats, insectivores, rodents, rabbits and hares, primates, flying lemurs, tree shrews, anteaters and sloths, armadillos, elephants, manatees and dugongs, elephant shrews, golden moles, tenrecs, armadillos, herbivorous marsupials, carnivorous marsupials, possums, egg-laying mammals.

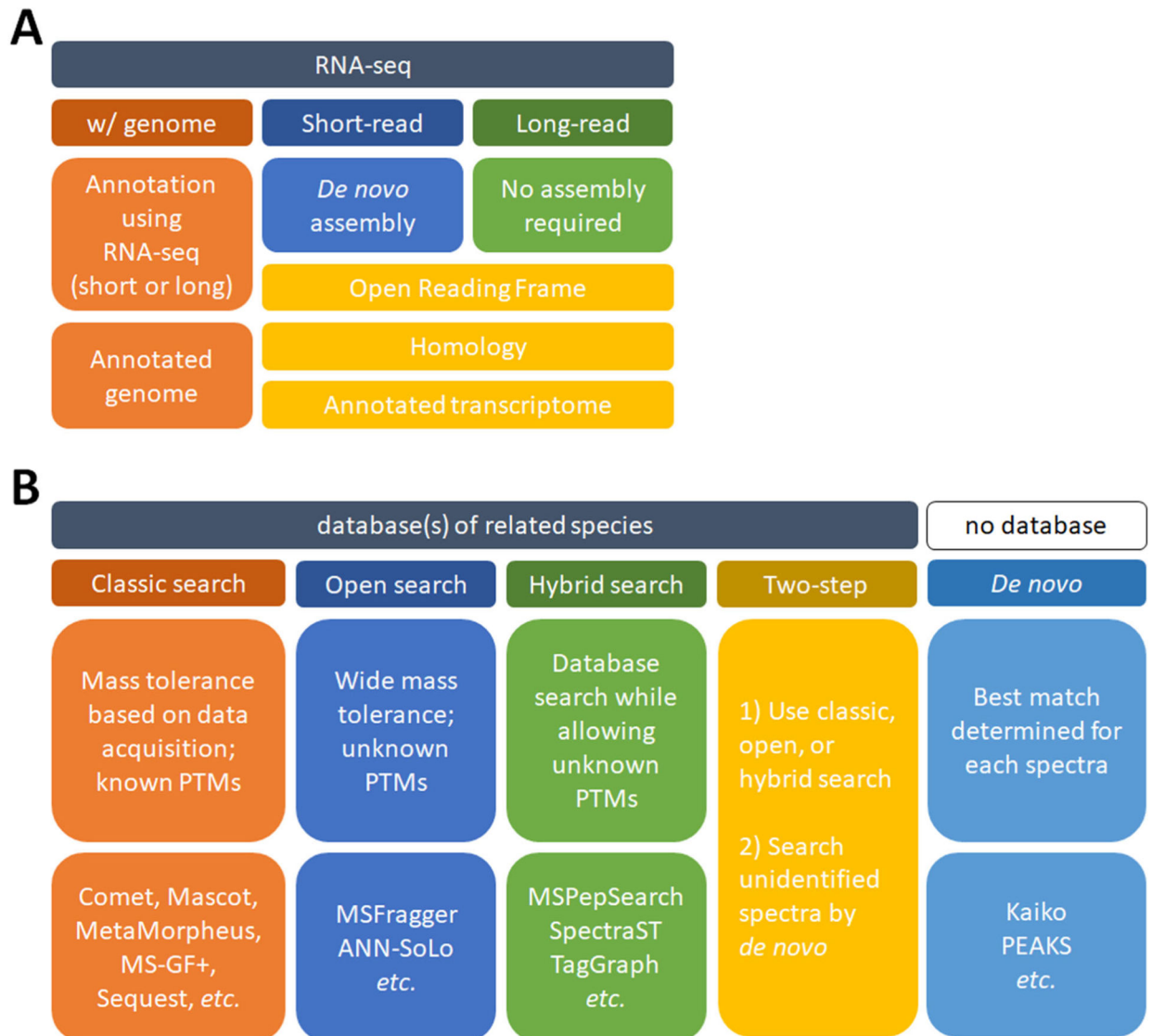


Figure 4. Different approaches to analyzing proteomic data without an annotated genome.

A. Using nucleic acid sequencing to generate an annotated genome or transcriptome. These can be used to analyze shotgun proteomic data. **B.** Searching shotgun proteomic data without prior/relevant nucleic acid sequencing. The software listed is not exhaustive and there are many suitable alternatives.