MSProGene: integrative proteogenomics beyond six-frames and single nucleotide polymorphisms

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Abstract

Summary: Ongoing advances in high-throughput technologies have facilitated accurate proteomic measurements and provide a wealth of information on genomic and transcript level. In proteogenomics, this multi-omics data is combined to analyze unannotated organisms and to allow more accurate sample-specific predictions. Existing analysis methods still mainly depend on six-frame translations or reference protein databases that are extended by transcriptomic information or known single nucleotide polymorphisms (SNPs). However, six-frames introduce an artificial sixfold increase of the target database and SNP integration requires a suitable database summarizing results from previous experiments. We overcome these limitations by introducing MSProGene, a new method for integrative proteogenomic analysis based on customized RNA-Seq driven transcript databases. MSProGene is independent from existing reference databases or annotated SNPs and avoids large six-frame translated databases by constructing sample-specific transcripts. In addition, it creates a network combining RNA-Seq and peptide information that is optimized by a maximum-flow algorithm. It thereby also allows resolving the ambiguity of shared peptides for protein inference. We applied MSProGene on three datasets and show that it facilitates a database-independent reliable yet accurate prediction on gene and protein level and additionally identifies novel genes.

Availability and implementation: MSProGene is written in Java and Python. It is open source and available at http://sourceforge.net/projects/msprogene/.

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1 Introduction

High-throughput technologies in both genomics and proteomics have driven the development of a variety of methods to analyze the large amounts of data generated. RNA-Seq techniques measure the transcriptome (Wang et al., 2009), while mass spectrometry allows identification and quantification of proteins that were expressed (Nilsson et al., 2010). The field of proteogenomics combines this multi-omics data for more accurate and sample-specific analyses (Castellana and Bafna, 2010; Nesvizhskii, 2014).

In recent years, proteogenomic studies have become more and more popular, focusing on deeper understanding of model organisms or exploring currently unannotated genomes (Ahn et al., 2013; Castellana et al., 2008; Fanayan et al., 2013; Kelkar et al., 2014). Despite this popularity, methods that are jointly focusing on genomics, transcriptomics and proteomics so far mainly rely on six-frame translations (Kelkar et al., 2011; Krug et al., 2013) or extensions of existing reference protein databases (Ahn et al., 2013; Li et al., 2010). Six-frame translation has the advantage of being independent from any a priori annotation of the nucleotide sequence. However, it introduces an artificial sixfold increase of the (unknown) target database, which can result in a bias in peptide identification (Blakeley et al., 2012; Branca et al., 2014; Jeong et al., 2012; Reiter et al., 2009).

In contrast, reference protein databases, for instance extended by known single nucleotide polymorphisms (SNPs) from databases such as dbSNP (Sherry et al., 2001), are not as prone to this bias as six-frame translations. But these approaches depend on existing annotations and thus cannot be applied to unannotated organisms without reference proteomes. Further, they might not contain all information necessary to identify mutated or novel genes, and even error-tolerant search approaches (Renard et al., 2012) may not be sufficient to recover these unannotated genes.

Thus, recent studies also rely on transcriptome information to provide better suited databases (Krug et al., 2014; Ning and
Nevizhskii, 2010; Safavi-Hemami et al., 2014; Wang and Zhang, 2014). They focus on a more specific choice of six-frame translated open reading frames and on enhancing databases in a data-driven fashion, for instance by only including spliced parts or variations to the database (Wang and Zhang, 2013; Wang et al., 2011; Woo et al., 2013).

These approaches are either only suitable for eukaryotes (having splicing events) or are still only seen as an addition to or refinement of the standard approach using protein databases to identify peptides. Other approaches rely on the de novo assembly of transcript sequences, which are then six-frame translated to provide a sample-specific database (Evans et al., 2012; Mohien et al., 2013).

Further, all of these efforts are targeted on improving peptide identification, but rely on standard approaches to perform protein inference. Because of shared peptides that are present in more than one protein, often parsimonious approaches are employed that group proteins instead of selecting one specific match per peptide (Claassen, 2012; Huang et al., 2012; Serang et al., 2010). However, a possibility to select the most likely protein per peptide is desirable. Here, RNA-Seq is a valuable source to assist protein inference, as it provides an additional layer of confidence for a specific protein.

In this article, we present MSProGene (Mass Spectrometry and RNA-Seq based Protein and Gene Identification) as an integrative proteogenomic method that goes beyond the extension of existing reference databases by constructing customized transcript databases based on RNA-Seq. This sample-specific database avoids unnecessary enlargement by six-frame translations and increases the confidence in identified proteins. Further, RNA-Seq information is used to approach shared peptide protein inference without the need for protein grouping. To do so, MSProGene represents transcriptomic and peptide evidence in a network and performs a maximum-flow optimization formulated as an integer linear program.

We applied MSProGene on a Bartonella henselae and a Litomosoides sigmodontis dataset where it shows reliable and accurate identifications. Further, in a simulation based on Escherichia coli we demonstrate the suitability of the network optimization and RNA-Seq integration to resolve shared peptides for protein inference.

2 Methods

Figure 1 shows the overall workflow of MSProGene: First, an RNA-Seq read mapping is analyzed to infer transcript sequences, which are updated by including variations present in the RNA-Seq reads (Fig. 1.1.). These sequences are translated to amino acid sequences to serve as a database for a peptide search of tandem mass spectra (Fig. 1.2.). The resulting set of peptide spectrum matches (PSMs) is represented by a network. MSProGene then performs protein inference by re-assigning shared peptides using a linear program approach based on RNA-Seq information (Fig. 1.3.). Finally, peptide identifications are controlled with regard to their false discovery rate (FDR) and transcripts with a sufficient number of peptide hits are reported (Fig. 1.4.).

2.1 Transcript database

MSProGene uses evidence from RNA-Seq reads to derive a customized transcript database for the spectra search. This database reflects sample-specific mutations present in the reads and is independent from any a priori knowledge, in particular it is independent from known annotations or protein sequences. Per default, the gene finder GIIRA (Zickmann et al., 2014) is used to extract transcripts based on a mapping of the RNA-Seq reads. However, also other methods for gene and transcript prediction can be used, for instance Cufflinks (Trapnell et al., 2010).

MSProGene analyzes the read mapping and refines the transcript sequence according to mutations present in the RNA-Seq reads. A variation (SNP or insertion or deletion) is integrated if (i) it is present in more than one read (this ensures that regions with low coverage are not biased towards more mutations) and (ii) it is supported by the majority of the reads. Note that the first condition is only a default threshold specified to reduce bias introduced by low coverage. This threshold can be changed by the user. Further, also a vcf file with previously called mutations by external tools can be provided. These mutations are directly integrated to the reference sequence and are thus respected in the transcript reconstruction.

In case a specific database is intended for the peptide spectrum search, MSProGene can also be provided with custom sequences in fasta format, without the need for RNA-Seq evidence. Also gene models based on evidence different from RNA-Seq or the combined results of varying prediction methods [for instance combinations by IPred (Zickmann and Renard, 2015), or EVidenceModeler (Haas et al., 2008)] are accepted as input for MSProGene. Note that in this case mutations already need to be included in the sequences, and the sequence header must contain information on the strand and start position of the gene (an example file is provided with the MSProGene installation).

To be suitable for spectra search, nucleotide sequences need to be translated into amino acid sequences. Initially, we rely on a three-frame translation since in RNA-Seq experiments the ends of genes are often not recovered with high precision. Hence, the predicted start codon might not be the correct one and translating only one frame would potentially lead to a loss in peptide identifications. However, (i) increasing the transcript database with a six-frame translation is only necessary if no strand information is available (as it for instance is the case for unspliced Cufflinks predictions). Thus, bias resulting from unnecessary extension of the database can be avoided. Further, (ii) in order to create a tailored transcript database without artificial increase we perform a second MSProGene iteration based on the analysis of the first spectra search.
Note that only one out of the initial three frames is correct; hence, the translated protein sequence of the incorrect frames might contain stop codons. Since an early stop codon can also be due to an incorrectly inserted mutation, MSProGene does not stop the entire translation in case of a stop codon but can extract several amino acid subsequences per transcript frame. Since the user can specify a minimum peptide length for spectra search (per default 5 amino acids), subsequences with smaller length are removed.

Finally, each transcript \( t \) with sequence length \( l' \) is initially scored based on the original GIIRA gene score (or score from other prediction methods) \( s' \) and its read coverage \( c' \). The coverage is calculated by taking the number of reads \( n' \) mapping to the transcript and their corresponding length \( l' \) into account:

\[
    c' = \frac{n' \cdot l'}{l}
\]

The initial transcript score \( s' \) is normalized over minimum \( (m') \) and maximum \( (m'') \) score of all original gene scores to indicate the relative evidence for a transcript in comparison to other transcripts:

\[
    s' = s' \cdot \frac{c'}{m'' - m' + 1}
\]

2.2 Peptide spectrum search

Once the transcript database has been created, the input tandem mass spectra are searched against the resulting set of amino acid sequences. Per default, MSProGene uses MSGF+ (Kim and Pevzner, 2014) as the search engine, but can easily be adapted to also work with other search methods. After the search, the resulting PSMs are extracted by MSProGene, independent of whether they are unique peptides or shared peptides (i.e. one peptide mapping to more than one transcript sequence). Further, the PSM score provided by the search engine is extracted, and normalized to the [0,1] interval.

2.3 Proteogenomic network

After the spectra search, each identified spectrum is assigned to one peptide sequence that can be found in one or more transcript sequences. Since each spectrum can only arise from one peptide and one transcript, we (i) need to assign shared peptides to their most likely origin. An additional challenge is the presence of potentially multiple supported reading frames per transcript. Since we initially provide at least three frames (sister frames) per transcript, a peptide can independently be mapped to each of the frames, although only one of the frames can be correct. Hence, (ii) we also have to identify the correct frame for each transcript and erase all incorrectly mapped peptides. Furthermore, not necessarily all PSMs are correct. Thus, (iii) we have to detect and remove incorrect identifications.

To meet these three objectives we first represent the inference problem as a network, which is then optimized in order to solve the inference. The network \( G = \{N, E\} \) (depicted in Fig. 2) with edge set \( E \) and node set \( N = P \cap F \cap T \cap so \cap st \cap d \) has nodes \( p_i \in P \) representing the individual peptides and nodes \( f_j \in F \) representing the sister frames of each transcript. Further, also the transcript itself is included as a node \( t_k \in T \). For technical purposes, also a source node \( so \) and a sink node \( st \) are integrated to the network, as well as a dummy node \( d \).

For each match between peptide \( p_i \) and frame \( f_j \), a directed edge \( e_{p_i, f_j} \in E \) is integrated to \( G \) connecting the two nodes. Further, all sister frames are connected to their corresponding transcript. Note that each peptide node is not only connected to its mapped frames but also to the dummy node. This ensures that whenever no target frame remains possible for a peptide, this peptide can be assigned to the dummy without creating inconsistency. The set of connections of a peptide \( p_i \) can become infeasible in case \( p_i \) only maps to frames that were marked as incorrect because their competing sister frames have more support. In this case, \( p_i \) is likely to be an incorrect identification, which is indicated by assigning \( p_i \) to \( d \). For an example refer to Figure 2: here \( p_2 \) and \( p_3 \) match to different frames of the same transcript; hence, only one match can be correct, and the other peptide is assigned to \( d \).

Since we aim at choosing connections between nodes that reflect the most likely correct identification, each edge is assigned a capacity representing the reliability of the associated match. Edges starting from the source are connected to peptide nodes and have an unlimited capacity, whereas edges \( e_{p_i, f_j} \) connecting peptides to frames have a capacity \( q_{p_i, f_j} \) that is initially determined by the score calculated by the peptide search engine. Further, it is restricted by a binary variable \( y_{p_i, f_j} \in \{0,1\} \) indicating whether this connection is chosen as the most likely connection \((y_{p_i, f_j} = 1)\) or not \((y_{p_i, f_j} = 0)\):

\[
    0 \leq y_{p_i, f_j} \leq q_{p_i, f_j} \forall e_{p_i, f_j} \in E.
\]

Further, edges \( e_{so, u} \in E \) connecting transcript nodes \( t_k \in T \) to \( si \) have a capacity \( o_k \) that is determined by the initial transcript score calculated in step 1 (Equation 2) of the overall workflow. The capacity \( \theta_{j, a} \) of connections of sister frames to their transcript is initially set to this transcript score, weighted by the number of peptides originally associated to the frame.

Since only one of the sister frames can be correct, \( \theta_{j, a} \) is also restricted by a binary variable \( m_{j, a} \in \{0,1\} \) that indicates whether a frame is chosen or not:

\[
    0 \leq \theta_{j, a} \leq m_{j, a} \forall e_{so, u} \in E.
\]

Two additional constraints ensure that only one match per peptide (Equation 5) and only one frame per transcript (Equation 6) is selected, respectively:

\[
    \sum_{f_j} y_{p_i, f_j} = 1 \forall i \mid p_i \in P.
\]

\[
    \sum_{t_k} m_{j, a} = 1 \forall k \mid t_k \in T.
\]
this case the reliability of connections—that is passed from source
towards sink node:

$$\max \sum_{e_{p,d} \in E} \varphi_{p,d} + \sum_{e_{p,u} \in E} \theta_{p,u} + \sum_{e_{a} \in E} \omega_{a} + \sum_{e_{p,d} \in E} \lambda_{p,d} y_{p,d}.$$  \hspace{1cm} (7)

where $\lambda_{p,d}$ corresponds to a penalty term similar to a Lagrange multiplier for connections to the dummy node: In the maximum-
flow description above, all capacities of chosen edges add to the
overall maximal flow. However, an important difference holds for
the dummy node $d$: since assignments to $d$ are required for peptides
that are likely incorrect identifications, a chosen connection to the
dummy results in a penalty on the overall flow. This is realized by a
form of Lagrangian relaxation on constraints describing edges to the
dummy node. Whenever such a connection is chosen (i.e. $y_{p,d} = 1$),
a penalty $\lambda$ (i.e. the Lagrange multiplier) that equals the confidence
score of the PSM is applied to the overall objective.

Although nodes have an unlimited throughput, a requirement of the
maximum-flow is that for each node the input has to equal the
output flow. Hence, the number of peptides that can be assigned to
each frame and transcript is restricted by the overall evidence for
this transcript because the higher $\omega_{a}$, the more flow can be assigned
to the transcript. Given the capacities $\theta_{p,u} \leq \omega_{a}$ of the connections of sister frames to their corresponding transcript, we derive the following
constraint:

$$\sum_{i \in X_{i} \in E} \varphi_{p,i} \leq \theta_{p,u} \forall e_{p,u} \in E.$$ \hspace{1cm} (8)

Note that the dummy node has an unlimited outgoing capacity, such
that in theory an unlimited number of peptides can be assigned.
However, due to the penalty this connection is only chosen if the
penalty is balanced by the benefit of supporting the competing
frames.

Finally, the described maximum-flow problem is formulated as an
integer linear program, which can be solved for instance using
the CPLEX Optimizing studio (CPLEX, 2011). As a result, each
peptide is either indicated as an incorrect match or associated to the
most likely transcript frame. Note that thereby the graphical model
ensures that the reassignment is performed in a non-greedy fashion
that for instance distributes peptides between multiple observed
alternative isoforms, rather than selecting only one isoform.

### 2.4 Postprocessing

After all PSMs have been reassigned to their most likely frame or are
indicated as likely incorrect predictions, the confidence in each tran-
script sequence and corresponding frame has to be recalculated.

MSProGene proceeds through the original transcripts and as-
signs the frame chosen in the linear program. Note that at this point
MSProGene uses the sequences supported by the spectra search for a
second iteration: The supported frames are used to create a second
and more specific amino acid database for a second run with a pep-
tide spectrum search engine. The initial database was artificially
increased by the three-frame translation, whereas the updated data-
based is tailored to the (unknown) true database. Also the second
PSM results are represented in a network to resolve shared peptides
and identify incorrectly mapped peptides (refer to former section). Afterwards, the transcripts are finally analyzed for their peptide sup-
port and FDR controlled.

Since decoy protein sequences which are classically used for
FDR computation in proteomics are artificial sequences without
RNA-Seq evidence, the network representation and maximum-flow
optimization is not applicable to decoy identifications. Hence, only
target peptide hits are reassigned in the maximum-flow and can thus
be used for FDR calculation. Therefore, the FDR cannot be calcu-
lated by a standard target-decoy approach, but is determined in a
decoy-free approach based on the expectation-maximization (EM)
algorithm (Dempster et al., 1977). The aim is to fit two distributions
on the frequencies of overall scores, one that explains the correct
(i.e. target) and one the incorrect (i.e. decoy) identifications, similar
to the approach in (Renard et al., 2010). The observed frequencies
of scores should be a mixture of these two distributions, where we
assume an underlying normal distribution for both target and decoy
identifications (assumption confirmed in independent experiments,
data not shown).

Note that since the EM is not guaranteed to find the global max-
imum, the search is performed several times with differing initial
values to identify the model best fitting the data. With the resulting
target $N_{T}$ and decoy $N_{D}$ distribution we can compute a false discov-
ery rate $FDR$, at each PSM $p_{i}$ with score $s'_{i}$, using the cumulative
density functions $F_T(s'_{i})$ for $N_{T}$ and $F_D(s'_{i})$ for $N_{D}$:

$$FDR_{i} = \frac{w_{T} \cdot (1 - F_D(s'_{i}))}{w_{T} \cdot (1 - F_T(s'_{i})) + w_{D} \cdot (1 - F_D(s'_{i}))}.$$ \hspace{1cm} (9)

where $w_{T}$ and $w_{D}$ are the weights of the target and decoy distribu-
tion, respectively.

### 2.5 Output

After the reassigned peptides are FDR controlled and hits below the
threshold are removed, the set of transcripts with spectra support is
reported. For postprocessing and visualization, the coordinates as
well as confidence score and number of spectrum matches are pre-
sented in the well-established GTF format, accompanied by the ac-
tual sequences in fasta format.

The final confidence score $s'$ combines the original transcript
score $s^{'}$ with its coverage and quality of PSMs (set denoted as $P'$):

$$s' = \frac{1}{l'} \sum_{i:j_{i} \in P'} s'_{i} \cdot \hat{p}_{i},$$ \hspace{1cm} (10)

where $l'$ is the length of the transcript sequence and $\hat{p}_{i}$ is the length
of a peptide $p_{i} \in P'$ with score $s'_{i}$.

Since the combination of RNA-Seq read support and tandem
mass spectra support does not only increase the confidence in pro-
tein identifications, but can also be used to verify variation observed
in the read mapping, MSProGene additionally outputs a VCF file.
This file contains all mutations present in the transcripts compared
to the given reference sequence. Further, we indicate whether muta-
tions are also supported by spectra (as an additional layer of
confidence).

### 3 Experimental setup

#### 3.1 Algorithm evaluation

As a proof-of-principle evaluation of the algorithm for peptide re-
assignment we conducted a simulation experiment. We used the
NCBI reference annotation of Escherichia coli (NCBI accession:
NC_000913.3) and integrated SNPs simulated with a mutation-rate
of 1% to the gene sequences (to simulate deviations from the refer-
ence sequence as occurring in real datasets). Based on the mutated
sequences, we simulated Illumina RNA-Seq reads with the read
simulator Mason (Holtgrew, 2010) in varying expression levels.
Tandem mass spectra were generated with the spectra simulator MS Simulator (Bielow et al., 2011; OpenMS Release 1.11) with a gradient of 3000s, an instrument resolution of 200,000, 10 tandem mass spectra per retention time bin, and default settings otherwise. Each of the resulting spectra is linked to its original peptide and protein, such that we can compare the peptide assignments of the network optimization integrated in MSProGene against the ground truth peptides.

### 3.2 Bartonella henseae

MSProGene was tested on data of *B. henselae*, a pathogenic bacterium that causes infections such as the cat scratch disease (Omasits et al., 2013). Tandem mass spectra and RNA-Seq reads originate from a study by Omasits et al. (2013) (GEO Series accession number: GSE44564). We pooled data from the two conditions (induced and uninduced) of replicate 1 resulting in 1.16 million tandem mass spectra and 211 million AB-Solid RNA-Seq reads. Reads were mapped to the *B. henselae* reference genome (strain Houston-1, NCBI accession: NC_005956) using BFAST (Homer et al., 2009; version: 0.7.0.a). For settings we followed the mapping pipeline and parameters recommended in the BFAST manual. As in the original study, the resulting mapping was filtered using samtools (Li et al., 2009) to remove contamination with rRNA. Further, all raw spectra were converted to MGF format using the Trans-Proteomic Pipeline (Deutsch et al., 2010). MSProGene was applied with default settings, using GIIRA in prokaryote mode for construction of the transcript database, also with default settings.

To analyze the performance of reference-independent methods, we compared MSProGene to the approach by (Evans et al., 2012; in the following called Assembly) based on de novo assembly with Trinity (Grabherr et al., 2011), as well as a standard six-frame translation of the *B. henselae* genome (in the following denoted as six-frame). Assembly was applied with default settings in its genome-guided mode (using the BFAST mapping as a guide). The resulting assembly contained 1907 transcripts, which were six-frame translated to identify open reading frames. These frames served as the database for MSGF+ search. Six-frame translation was performed using the program getorf from the EMBOSS package (Rice et al., 2000; version EMBOSS 6.4.0.0), requiring a minimum length of 200bp. These three reference-independent methods were analyzed regarding the overall number of identified proteins and the spectra coverage of identifications.

For a general analysis of the robustness of our method we also randomly divided the original set of 1.16 million spectra into two smaller sets, each including half of the spectra. The compared methods were applied using the smaller samples of spectra separately and the resulting predicted protein sequences were compared between runs. The higher the overlap between two runs on differing input samples, the more robust the method. As a measure of overlap we counted the number of proteins coinciding in both runs and divided it by the highest number of proteins predicted in one run.

Further, we compared our method to a standard database search (in the following denoted as Standard) on the 1488 annotated *B. henselae* proteins available at NCBI (http://www.ncbi.nlm.nih.gov/). In addition, we performed a standard search on a database including SNPs indicated by a samtools (Li et al., 2009) mpileup variant call on the RNA-Seq mapping (in the following denoted as Mutated).

For all evaluations we chose the set of annotated *B. henselae* proteins as a ground truth reference protein set (note that not necessarily all of these proteins are actually expressed simultaneously).

The output of the Standard and Mutated approach was directly compared to the reference. In contrast, for the reference-free methods we first compared the coordinates of predicted proteins to the reference coordinates in order to map predictions to reference proteins.

For evaluation of method quality we employed the metrics of recall and precision. Recall is calculated as the number of identified annotated proteins, divided by the total number of annotations (1488). Precision is calculated as the number of predicted proteins matching the annotation, divided by the total number of proteins predicted by the method. Note that by nature of the analysis, the Standard and Mutated method always have a precision of 100% because they are exclusively searched against the reference annotation.

We also calculated an annotation-based FDR on the protein identifications of reference-free methods, sorted by identification score. We regard an identified protein as incorrect in case it did not match the reference annotation. We note that since not necessarily all unmatched predictions are false positives, this is a conservative estimate that likely overestimates the actual rate of incorrect identification.

### 3.3 Litomosoides sigmodontis

We also compared MSProGene to a six-frame based analysis on a *L. sigmodontis* dataset (assembly nLs.2.1 from www.nematodes.org). *Litomosoides sigmodontis* is a popular model organism for filarial nematodes, that amongst other diseases cause lymphatic filariasis (‘elephantiasis’) and are the human-parasitic species with the highest overall impact on public health (Armstrong et al., 2014).

Tandem mass spectra originate from a study by Armstrong et al. (2014) (PRIDE Project PXD000756, in total 856,380 spectra).

For this organism only very few proteins are already annotated (a search at NCBI on January 9, 2015 resulted in 75 protein sequences). Hence, here we only compare methods in regard to their overall identification confidence, the number of predicted proteins and their spectra coverage.

Transcript prediction methods such as Cufflinks (Trapnell et al., 2010) and GIIRA work best on high coverage RNA-Seq datasets. Hence, since at the time of this study only low coverage 454 transcriptome data was available for *L. sigmodontis*, we chose RNA-Seq data from *Brugia malayi*, a close relative of *L. sigmodontis*. We pooled 14 samples from different life cycle stages of *B. malayi* (BioProject-accession: PRJEB2709) and mapped the reads to the *L. sigmodontis* draft genome using TopHat2 (Kim et al., 2013; version 2.0.11) with error tolerant parameter setting (N 5, read-gap-length 5, read-edit-dist 5). Transcript coordinates were obtained using Cufflinks (version 2.2.0) on the resulting mapping. The resulting GTF file was converted in-house scripts to generate a fasta file with transcript sequences for MSProGene analysis. For the six-frame analysis the *L. sigmodontis* draft genome was translated using the program getorf from the EMBOSS package, requiring a minimum length of 200 bp.

In addition to the transcripts predicted by either Cufflinks or getorf, we included protein sequences from the Wobbachia symbiont of *L. sigmodontis*, obtained from www.nematodes.org (release wLs 2.0, 1042 sequences) for spectra search.

For further evaluation, we used BLAST (Altschul et al., 1997) to compare the identified sequences to *B. malayi* proteins. Similar to (Armstrong et al., 2014), we specified a bit score cutoff of 50. We did not use the BLAST E value for threshold definition to allow a fair comparison since an E value threshold may have favored the
evaluation towards MSProGene because it has a smaller query database size than the six-frame translation.

3.4 Peptide search parameters

All spectra searches were conducted using MSGF+ (Kim and Pevzner, 2014; version v9881) with a precursor mass tolerance of 5 ppm, a minimum peptide length of five amino acids, specifying a high-resolution LTQ, and using default settings otherwise. All analyses were performed in regard to a 1% FDR cutoff and excluding proteins with fewer than two spectra hits.

4 Results and discussion

4.1 Algorithm evaluation

We analyzed the PSMs before and after the network optimization of MSProGene. Details are shown in Figure 3. Of 21,715 spectra that MSGF+ matched to the original protein (sometimes among multiple proteins), 21,617 were assigned correctly (99.5%) by MSProGene. Overall, the algorithm correctly reassigned over 90% of the spectra that had multiple protein hits (933 of 1031).

This demonstrates that the network representation with integrated RNA-Seq information and its optimization is suitable to successfully resolve shared peptide protein inference, without the need for protein grouping.

4.2 Bartonella henselae data

4.2.1 Verification of transcripts with spectra support

First, we investigated the effect of integrating RNA-Seq evidence and spectra on the actual identification accuracy. As shown in Table 1, the transcript database constructed for spectra search contains 1568 sequences. This number is reduced to 1397 when taking spectra support into account. This leads to a decrease in recall from 78.2 to 76.5%. This shows that although generally transcriptome and proteome correlate well, we have to be aware of potential losses in protein identifications. For comprehensive studies on mRNA and protein, a suitable source and verification technique for protein level analysis and evidence on the transcriptome level is a good indication for protein measurements. However, if possible, combinations with other methods are a good indication for protein measurements.

In contrast to sensitivity, the precision strongly increases from 79.0% to 85.1% when spectra support is taken into account. This shows that the combination of RNA-Seq data and tandem mass spectra is a suitable verification method for accurate protein identification.

4.2.2 Comparison to reference-free methods

For the three methods compared we counted the number of annotations that were identified and the number of predictions that actually match the annotation. Both numbers can differ since a single annotated protein might be covered by several smaller predictions.

The results of the analysis are summarized in Table 2. The transcript database constructed for spectra search by MSProGene contains 1568 sequences. This is significantly smaller than the number of sequences searched in the six-frame analysis and Assembly and shows the suitability of RNA-Seq data to provide smaller and more tailored search databases.

Table 1. Prediction results of MSProGene, exclusively based on RNA-Seq, verified by spectra support, and in addition excluding proteins with only one spectrum hit

|                      | Without spectra | With spectra | With spectra single hits |
|----------------------|-----------------|--------------|-------------------------|
| Predicted            | 1164            | 1109         | 1276                    |
| # matches to annotation | 1143           | 1189         | 1143                    |
| # identified annotations | 1164       | 1139         | 1109                    |
| Recall (%)           | 78.2            | 76.5         | 74.5                    |
| Precision (%)        | 79.0            | 85.1         | 88.9                    |

Evaluation on the B.henselae dataset, compared to the reference annotation comprising 1488 genes. Best values for each category are marked in bold.

Table 2. Prediction of reference-free methods on the B.henselae dataset, compared to the reference annotation comprising 1488 genes

|                      | MSProGene | Six-frame | Assembly |
|----------------------|-----------|-----------|----------|
| Database size        | 1568      | 6091      | 5894     |
| Predicted            | 1286      | 1502      | 1276     |
| # matches to annotation | 1143     | 1207      | 447      |
| # identified annotations | 1164     | 1163      | 372      |
| Recall (%)           | 74.5      | 78.2      | 25.0     |
| Precision (%)        | 88.9      | 80.4      | 35.0     |
| Recall 1%-AnnotationFDR (%) | 51.5  | 1.1       | 0.0      |
| Median # spectra per protein | 90   | 77        | 50       |

The row indicated as ‘1%-AnnotationFDR’ shows results for an additional 1% annotation-based FDR on the protein level. The best value for each category is marked in bold.

Overall the six-frame approach predicts the highest number of spectra-supported genes and also achieves the highest recall given the peptide level FDR. At first this is surprising given the supposedly high number of spurious sequences in six-frame translated databases (which should lead to reduced sensitivity). We suspect that the overall high coverage of this dataset (1.16 million spectra) prevents the originally expected loss in protein identifications. A loss in sensitivity is rather reflected in the spectra coverage of protein identifications, where six-frame shows 77 median spectra hits per protein, compared to 90 for MSProGene. The drawback of six-frame
translations is also reflected on the precision level, where the high recall comes at the cost of specificity: six-frame has 3.7% higher recall but 8.5% less precision than MSProGene (also refer to Fig. 4). Hence, although MSProGene identifies slightly fewer proteins, it provides higher confidence in the resulting predictions. Further, if in addition to the peptide level FDR also an annotation-based FDR is applied on the protein level, the recall of six-frame decreases to 1% because of early false positive identifications. In contrast, MSProGene still achieves a recall of 51%.

The Assembly approach shows low agreement between predicted transcripts and the annotation, resulting in reduced precision and recall. This indicates that the two-step integration of RNA-Seq data (first de novo assembly followed by six-frame translation and later the independent spectra search) is not as suited for proteogenomic analysis as the integrative approach employed by MSProGene.

As illustrated in Figure 5, MSProGene and the six-frame approach coincide in 941 of the 1488 annotations. In contrast, Assembly only shared 304 and 317 annotations with MSProGene and six-frame, respectively.

Taken together, the three methods identified 1340 of the 1488 annotated B. henselae proteins. However, all methods identified proteins that were not predicted by the other methods, such that no approach shows a complete prediction by itself. Six-frame is sensitive, but lacks confidence and precision. MSProGene is specific but dependent on the quality of predicted transcript sequences. Here, gene identifications exclusively based on RNA-Seq (as performed by GIIIRA for this dataset) might not identify all possible transcripts and a more comprehensive RNA-Seq based prediction might be more sensitive. Some of the missing transcripts can be recovered by the de novo assembly used in Assembly; however, this approach overall has the least accuracy. Hence, in regard to precision, customized transcript databases as employed by MSProGene should be preferred.

All three methods performed well in the robustness analysis. The overlap of six-frame (97.5%) is slightly higher than for MSProGene (96.0%) and Assembly (95.5%). However, all three approaches only vary little, indicating that they are robust to differing input data.

4.2.3 Comparison to reference-based methods

To generate the mutated database 2592 variants were called with samtools on the RNA-Seq read mapping and included in the reference protein sequences. Both Standard and Mutated method identified 1274 of the annotated proteins (recall: 85.6%). Interestingly, including mutations observed in the RNA-Seq mapping did not improve the overall recall, but instead even decreases the median spectra support for identified proteins from 106 (Standard) to 95 (Mutated) spectra. This indicates that some of the included SNPs are incorrect. Since thresholds for the filtering of incorrect mutations are hard to define (Giese et al., 2014), this is a likely bias when including sample-specific mutations to reference proteins.

With 1109 identified proteins, MSProGene has a lower recall than both Standard and Mutated method. However, as shown in Figure 6, it identifies 84 proteins not detected by the standard searches.

When comparing MSProGene and the Mutated approach, 92 proteins are unique to MSProGene, and 257 proteins are unique to Mutated. The latter are not identified due to missing or incorrectly constructed transcript sequences. MSProGene not only needs to correctly identify the correct PSMs for a protein sequence, but also the correct coordinates of a transcript. Hence, the sensitivity of MSProGene strongly depends on the quality of constructed transcript sequences. Since RNA-Seq is challenging as the exclusive source for gene prediction (performed by GIIIRA for this dataset), integrating additional evidence or other methods for prediction might lead to a more comprehensive set of transcripts and hence improved recall. We believe that the extensive studies dedicated to RNA-Seq analysis (a search of the term ‘RNA-Seq’ on google scholar resulted in more than 17 300 entries in year 2014) will also benefit MSProGene. Since our method is independent of the method used for transcript construction (except scores and mutations that need to be provided), better methods for RNA-Seq based gene and transcript prediction will lead to improved recall by MSProGene.

The proteins exclusively detected by MSProGene often have shared peptide support and in addition are supported by peptides that have scores below the FDR threshold in the Mutated approach. For instance, 51 of the missing 92 proteins of Mutated can be identified with an FDR threshold of 5%. This indicates the precision of MSProGene peptide assignments since it identifies these proteins under a more conservative FDR.

In general, the comparison against the complete reference can only be regarded as a relative rather than an absolute comparison between methods (since not all genes are necessarily expressed at the same time). Further, transcripts that do not match the reference are
not necessarily false positives but might be unannotated genes. However, for the evaluation of sensitivity and specificity all transcripts not matching the annotation are regardless counted as false positives. Hence, the evaluation is slightly biased against MSProGene. Reference-dependent approaches fail to detect novel genes (examples detailed below) and in addition, even databases adapted or extended with SNPs are not always suited to identify mutated proteins. Hence, even for annotated organisms or fast evolving organisms such as viruses it is worth to employ alternative search strategies.

When compared to the annotated reference database comprising 1488 genes, MSProGene predicted 76 genes with RNA-Seq and spectra support that do not match the annotation. Two of these genes located at position 1 357 979 to 1 358 722 and 1 180 052 to 1180672, respectively were chosen for further verification with BLAST (Altschul et al., 1997). The first protein with length 248 was supported by 94 spectra, the second one of length 207 received 36 spectra.

A protein BLAST search of the two sequences (predicted by MSProGene on the Houston-1 reference strain) revealed that both proteins are annotated in other *B. henselae* strains. The first sequence shows high similarity to a peptide ABC transporter substrate-binding protein (BLAST E value: 1 e−178, identity: 99%), for instance present in strain BM1374165. The second one shows high similarity to a hemin binding protein E, for instance present in strain BM1374163 (BLAST E value: 5 e−145, identity: 100%).

Thus, both genes are likely candidates for novel genes in the *B. henselae* taxonomy. This highlights the relevance of reference database independent approaches because standard database searches cannot identify genes that are not already annotated.

### 4.3 *Litomosoides sigmodontis* data

The results of the evaluation on the *L. sigmodontis* dataset are shown in Table 3. Also for this dataset the RNA-Seq based transcript database used by MSProGene is significantly smaller than the six-frame translation.

Although the overall number of predicted sequences is higher for the six-frame approach, MSProGene sequences receive higher spectra support. The greater confidence of MSProGene transcripts is also significantly shown in the BLAST search: As illustrated in Figure 7, the confidence of BLAST hits is considerably improved for MSProGene sequences. Further, only 42% of the six-frame sequences receive a BLAST hit at all, while in contrast 68% of MSProGene predictions can be mapped. With 1804 hits, the overall number of hits for the six-frame approach is still higher, but only in case no score cutoff for confidence control for the BLAST search is applied. When using a bit score cutoff of 50 as in (Armstrong et al., 2014), the number of remaining BLAST hits of MSProGene is an order of magnitude higher than for the six-frame analysis. Hence, MSProGene identifies fewer transcripts with more confidence.

We are aware that the comparison against a *B. malayi* database can only identify proteins that are *L. sigmodontis* orthologs and does not determine proteins specific to *L. sigmodontis*. However, *L. sigmodontis* and *B. malayi* are close relatives. Hence, the BLAST search against *B. malayi* is a good indicator of the quality of *L. sigmodontis* protein identifications.

### 4.4 System requirements

The computational performance of MSProGene is evaluated using the transcripts predicted by GIIRA (for *B. henselae*) or Cufflinks (for *L. sigmodontis*). The main contributors to run time are the two spectra searches performed by MSGF+: The search of 1.16 million spectra on the *B. henselae* dataset required 35.7h. The search of 856 380 spectra on the *L. sigmodontis* dataset required 40.8h. Overall,
MSProGene used 30 GB RAM and 36.5 h to analyze the *B. henselae* dataset, and 30 GB RAM and 41.6 h to analyze the *L. sigmodontis* dataset.

5 Conclusion and outlook

We present MSProGene as a novel proteogenomic method for integration of proteomic, genomic and transcriptomic data beyond six-frame translation and the dependency on reference databases. We demonstrate the benefits of the new method in a comparison on three datasets and show that MSProGene provides an automated integrative framework for robust and precise proteogenomic analysis. We show that MSProGene performs peptide and protein identification with higher specificity than existing methods and constructs smaller customized spectra search databases. It is independent of *a priori* annotations and allows the identification of mutated and novel genes. Further, the network optimization employed by MSProGene successfully resolves shared peptides for protein inference without the need for protein grouping. This way, MSProGene distinguishes alternative isoforms and genes sharing homologous regions. Since the algorithm for peptide reassignment is independent of the constructed gene model, MSProGene can be combined with any prediction method or previously defined gene sequences of choice. Thus, given a suitable gene prediction, our method is also applicable to higher eukaryotes and polyploid organisms and can respect polyploid SNPs. Future applications of the software include a more thorough analysis of the simultaneous verification of SNPs on the transcriptome and proteome level and the analysis of variant peptides.

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