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RESEARCH NOTE

REVISED RNA-seq assembler artifacts can bias expression counts and differential expression analysis - case study on the chickpea transcriptome emphasizes importance of freely accessible data for reproducibility [version 2; peer review: 2 not approved]

Previously titled: RNA-seq assembler artifacts can bias expression counts and differential expression analysis - application of YeATS on the chickpea transcriptome

Sandeep Chakraborty

Celia Engineers, T. T. C Industrial Area, Rabale, Navi Mumbai, India

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Abstract

The unprecedented volume of genomic and transcriptomic data analyzed by software pipelines makes verification of inferences based on such data, albeit theoretically possible, a challenging proposition. The availability of intermediate data can immensely aid re-validation efforts. One such example is the transcriptome, assembled from raw RNA-seq reads, which is frequently used for annotation and quantification of genes transcribed. The quality of the assembled transcripts influences the accuracy of inferences based on them. Here the publicly available transcriptome from *Cicer arietinum* (ICC4958; Desi chickpea, <http://www.nipgr.res.in/ctdb.html>)¹ was analyzed using YeATS². This revealed that a majority of the highly expressed transcripts (HET) encoded multiple genes, strongly indicating that the counts may have been biased by the merging of different transcripts. TC00004 is ranked in the top five HET for all five tissues analyzed here, and encodes both a retinoblastoma-binding-like protein (E-value=0) and a senescence-associated protein (E-value= 5e-108). Fragmented transcripts are another source of error. The ribulose biphosphate carboxylase small chain (RBCSC) protein is split into two transcripts with an overlapping amino acid sequence "ASNGGRVHC", TC13991 and TC23009, with length 201 and 332 nucleotides and expression counts 17.90 and 1403.8, respectively. The huge difference in counts indicates an erroneous normalization algorithm in determining counts. It is well known that RBCSC is highly expressed and expectedly TC23009 ranks fifth among HETs in the shoot. Furthermore, some

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1. **Björn Voß** , University of Stuttgart, Stuttgart, Germany
2. **Lilah Toker**, University of British Columbia, Vancouver, USA

Any reports and responses or comments on the article can be found at the end of the article.

transcripts are split into open reading frames that map to the same protein, although this should not have any significant bearing on the counts. It is proposed that studies analyzing differential expression based on the transcriptome should consider these artifacts, and providing intermediate assembled transcriptomes should be mandatory, possibly with a link to the raw sequence data (Bioproject).

Keywords

RNA-seq , transcriptome , Computational genomics , chickpea , Cicer arietinum , re-validation , Intermediate assembly data , Big Data , Bioproject

Corresponding author: Sandeep Chakraborty (sanchak@gmail.com)

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REVISED Amendments from Version 1

The revised manuscript has the following changes based on the referee suggestions:

1. Changed title.
2. Analyzed the ribulose biphosphate carboxylase small chain genes to ascertain the nucleotide homology with corresponding transcripts.
3. Major re-writing of the Methods section.
4. Minor corrections based on referee inputs.

See referee reports

Introduction

The lack of reproducibility of results in biology is a contentious subject^{3,4}. In computational studies, the exact replication of the output of most computer programs is difficult as most non-trivial algorithms are nondeterministic. The problem is compounded by recent technological advances generating “Big Data” involving multiple programs and pipelines^{5,6}. However, inferences based on these results should not be subject to the same, or ideally any, unpredictability. The availability of software used at each stage and the intermediate data generated is key in enabling debugging and tracking the veracity of results by subsequent researchers⁷.

Chickpea (*Cicer arietinum* L.) is an important pulse crop having numerous nutritional and health benefits⁸. Several online resources exist for chickpea genomes and transcriptomes (<http://www.cicer.info/databases.php>, <http://www.nipgr.res.in/ctdb.html>, <http://gigadb.org/dataset/100076>, <http://nipgr.res.in/CGAP/>). Interestingly, the 68th United Nations General Assembly has declared 2016 as the International Year of Pulses (IYP).

The transcriptome of chickpea has been sequenced¹ using RNA-seq^{10,11}. In contrast to other traditional methods like RNA: DNA hybridization¹² and short sequence-based approaches¹³, RNA-seq can detect transcripts with very low expression levels by increasing sequencing depth. YeATS is a work-flow for analyzing RNA-seq data², and was used to detect a second homolog of a polyphenol oxidase gene and ~130 genes in the large gallate 1- β -glucosyltransferase in walnut¹⁴. YeATS analysis of RNA-seq data from 20 different tissues of walnut in California unravelled detailed, tissue-specific information of ~400 transcripts encoded by a large family of resistance (R) genes and elucidated the biodiversity and possible plant-microbe interactions¹⁵.

In the current work, errors arising from the assembly step as identified by YeATS are shown to have a bearing on the transcript quantification. The chickpea transcriptome (<http://www.nipgr.res.in/ctdb.html>) and its quantification in different tissues provided by an online interface is analyzed. This demonstrates that transcripts which are tagged with high counts predominantly encode multiple genes. While some studies provide the assembled sequences^{16,17}, the author could not find the relevant data, even after personal communication¹⁸.

It is also proposed that the availability of intermediate assembly sequences be made mandatory, in line with the recent initiative Global Open Data (GODAN:<http://www.godan.info/>).

Materials and methods

The transcriptome of the *Cicer arietinum* (transHybrid.fasta, ICC4958; Desi chickpea) “represents optimized de novo hybrid assembly of 454 and short-read sequence data. About 2 million 454 reads were assembled using Newbler v2.3 followed by hybrid assembly with 53 409 transcripts generated by optimized shortread data assembly reported previously¹ using TGICL program” (<http://www.nipgr.res.in/ctdb.html>). Quantification of transcripts in different tissues is provided by an online interface. The chickpea genome (*Cicer arietinum*_GA_v1.0.fa) is obtained from <http://gigadb.org/dataset/100076>¹⁹.

There are three classes of errors. Type I error occurs when a single transcript has multiple ORFs with significant matches to different genes. In a Type II error a single gene is broken into two separate transcripts. For a type III error, a single transcript has multiple ORFs, but they all map to the same gene. Multiple sequence alignment was done using MAFFT (v7.123b)²⁰, and sequence alignment figures were generated using the ENDscript server²¹. ORFs were obtained using the ‘getorf’ utility in the EMBOSS suite²².

The focus of this study are highly expressed transcripts, which result from have Type I errors. The top five transcripts from different tissues are extracted from the online interface, and the three longest ORF from each transcript BLAST’ed to the ‘nr’ BLAST database. These ORFs can overlap on different strands, with the assumption that the homology results will select a unique ORF. The transcripts having more than one unique ORF with a significant match (E-value=1E-8, BLAST bitscore>=75) are noted.

In order to analyze Type II errors, all transcripts need to be assigned to ORFs. YeATS first excluded transcripts that did not align to the chickpea genome (using BLAST), since transcriptomes can have contamination’s^{15,23}. These are not removed in some studies before making inferences¹⁶, which is worrying since there are mis-annotation of certain genes (a transcript, CSTC018609, encoding a heat shock protein from *Cladosporium cladosporioides* has been annotated as a saffron gene)²³. While using the full ‘nr’ BLAST database is the best option, a smaller BLAST database of protein peptides (plantpep.fasta:1M sequences) using ~30 organisms (list.plants) from the Ensembl genome was created to reduce computational times²⁴.

Results and discussion

The chickpea transcriptome (transHybrid.fasta:n=34760¹) was first mapped to the chickpea genome (*Cicer arietinum*_GA_v1.0.fa) obtained from <http://gigadb.org/dataset/100076> using BLAST (E-value=1E-8, BLAST bitscore>=75). There were 60 unmapped transcripts, some of which are mitochondrial transcripts (list.mito in Dataset1), some are contamination (list.meta in Dataset1), and the rest have no match in the complete BLAST ‘nt’ database (list.nomatchinNT in Dataset 1). The metagenomic transcripts are removed from further processing.

Subsequently, the three longest ORFs from each transcript (list.3ORFS:n=104K in Dataset 1) was BLAST²⁵ed to a subset of plant proteins created from the Ensembl²⁴ database (E-value=1E-8, BLAST bitscore>=75).

Type I error: multiple ORFs mapping to different proteins

There are ~1300 transcripts encoding more than one significant peptide (see list.duplicate in Dataset 1). The top five highly expressed transcripts (HET) from five tissues flower bud (FB), mature leaf (ML), root (RT), shoot (SH), young plant (YP) were obtained from <http://www.nipgr.res.in/ctdb.html> (tissues.txt in Dataset1). The number of transcripts encoding multiple genes, as found from 'list.duplicate', were as follows FB:4, ML:5, RT:3, SH:4 YP:5, indicating an over-representation of merged transcripts in HET.

The top five HET in the root, and the genes encoded by them are listed in Table 1. The ORFs of TC00004 encoded in the reverse direction map to a partial retinoblastoma-binding-like protein (~500 out of 549 amino acids) and the complete senescence-associated protein (157 amino acids) (Figure 1). TC00004 is computed to be highly transcribed (ranked in the top five) in all five tissues studied here

(tissues.txt in Dataset 1). The top ranking transcript, TC00002, has two ORFs - ORF.11 and ORF.38 (TC00002.orf in Dataset 1) - which align to an ATP synthase subunit beta (327 aa) and senescence-associated protein (615 aa), respectively. This transcript is highly fragmented and encodes on both strands in an overlapping manner.

When two genes are adjacent to each other in the genome, and are both transcribed it is possible that an assembler merges these into one transcript. Also, it is possible that these loci are under the same transcriptional control and the expression counts are correct. Thus, high levels of expression of one gene should correlate with high expression level of the other, assuming a proper normalization. However, the over-representation of such merged transcripts in HET suggests that there might be some errors in counting.

Type II error: fragmented ORFs of the same protein encoded by different transcripts

There are transcripts that encode fragmented ORFs which map to the same protein. YeATS has a merging algorithm that identifies overlapping amino acid sequences in transcripts. For example, TC13991 (47 aa) and TC23009 (110 aa) have an overlapping amino

Table 1. Five highly expressed transcripts in the root of Chickpea: These are obtained from the online interface <http://www.nipgr.res.in/ctdb.html>. TrLen: length of transcript, PLen: length of the full protein, OLen: length of ORF, RPKM: Reads Per Kilobase of transcript per Million mapped reads. Three out of the five transcripts have Type I errors, where two different transcripts are merged.

TrID	TrLen	PLen	OLen	Accession	Description	E-value	RPKM
TC00002	894	615 327	79 70	XP_013443004.1 CEG35068.1	senescence-associated [<i>M. truncatula</i>] ATP synthase subunit beta [<i>P. halstedii</i>]	1e-28 3e-36	25543
TC00004	3040	549 157	353 193	XP_003628041.1 XP_013443005.1	retinoblastoma-binding-like [<i>M. truncatula</i>] senescence-associated [<i>M. truncatula</i>]	0 5e-108	10290
TC22821	1303	303	323	XP_004488666.1	chitinase 2-like [<i>C. arietinum</i>]	0	6162
TC07055	652	115	121	AlI99866.1	seed storage/ltf family [<i>C. arietinum</i>]	5e-74	4998
TC00120	3859	378 138	163 108	CDY45505.1 YP_173374.1	BnaCnng12640D [<i>B. napus</i>] hypothetical NitaMp027 [<i>N. tabacum</i>]	1e-109 1e-20	4681

Figure 1. Type I error: different ORFs from the same transcript map to the different protein: TC00004 (3040 nt long) has ORFs encoded in the reverse direction (3040 ->1) that map to different proteins: retinoblastoma-binding-like protein (RBLP: 549 aa) and the complete senescence-associated protein (SAP: 157 aa). The RBLP ORFs are fragmented, and combine to ~500 aa. TC00004 is computed to be highly transcribed (ranked in the top five) in all five tissues studied here.

There are ~3000 transcripts which encode more than one ORFs mapping to the same peptide (list.splitORF in Dataset 1). TC01688 is one such transcript having ORF.70 and ORF.89 (see TC01688.orf in Dataset 1) mapping to an aspartyl protease family protein (TAIRid:AT1G05840.1) with BLAST bitscores 250 and 285, respectively. Merging ORF.70 and ORF.89 (inserting 'ZZZ') results in an increased BLAST bitscore of 507 (Figure 3). This should have minimal effects on the counts, unless Type I or Type II also occur simultaneously.

Figure 2. Type II error: different transcripts that encode fragmented ORFs which map to the same protein: TC13991 and TC23009 have an overlapping amino acid sequence “ASNGGRVHC”, and should have been ideally merged. Also, their counts are significantly different - TC13991 has a count of 17.90, while TC23009 has a count of 1403.8, indicating an erroneous normalization algorithm. The ORF of TC13991 does not have other significant matches among other transcripts. Furthermore, the C-terminal peptide “IGFDNVRQVQCISFIAHTPKEF” has no matches in the transcripts.

Table 2. Nucleotide mapping of ribulose biphosphate carboxylase small chain (RBCSC) transcripts to the chickpea genome: RBCSC has three genes in the genome. Two (RBCSC-A and RBCSC-B) of them map to chromosome 2, and the third (RBCSC-C) to SCAFFOLD2575. The transcripts mapping to RBCSC-A and RBCSC-B are TC13991, TC23009 and TC01487. TC13991 and TC23009 can be merged into one transcript based on an overlapping amino acid sequence "ASNGGRVHC". There is no other transcript homologous to TC13991 - either through nucleotide or peptide BLAST. TRS: transcript, FR: Fragment.

TRS	TRS FR	Genomic FR	Length	%	Gene
TC13991 (nt len=201)	1::44	248692::248735	44	100	RBCSC-A
	37::201	248695::248531	166	99	RBCSC-A
	1::44	??	??	??	??
	37::201	258193::258029	166	92	RBCSC-B
TC23009 (nt len=332)	1::35	248559::248525	35	100	RBCSC-A
	34::168	247928::247794	135	100	RBCSC-A
	163::332	247519::247350	170	98	RBCSC-A
	1::35	258057::258023	35	100	RBCSC-B
	34::168	257583::257449	135	100	RBCSC-B
	163::332	257168::256999	170	99	RBCSC-B
TC01487 (nt len=617)	1::569	247928::247366	574	97	RBCSC-A
	569::617	??	??	??	??
	1::569	257583::257015	569	99	RBCSC-B
	569::617	256986::257034	49	100	RBCSC-B

Table 3. ORFs encoding ribulose biphosphate carboxylase small chain (RBCSC) mapping to two genomic region on chromosome 2 : The two RBCSC genes under consideration are highly homologous, making it difficult to assign transcripts TC23009 and TC01487 exactly to the two genomic regions uniquely. For example, TC23009 has the exact homologies to both genomic regions, and has an intron (of length 131 nt in RBCSC-A and 137 nt in RBCSC-B). TC01487 does not have an intron. However, as mentioned previously, TC13991 and TC23009 can be merged based on a overlapping sequence. GS: start in genomic sequence of ORF in chromosome 2, GE: end in genomic sequence of ORF in chromosome 2, BBS = BLAST bitscore.

Transcript	GS	GE	BBS	E-value	Gene
TC23009 (ORF len=110)	247636	247286	120	1e-35	RBCSC-A
	247928	247767	91.3	6e-25	RBCSC-A
	257285	256935	120	1e-35	RBCSC-B
	257583	257422	91.3	6e-25	RBCSC-B
TC01487 (ORF len=106)	247636	247286	191	2e-63	RBCSC-A
	257285	256935	183	3e-60	RBCSC-B
TC13991 (ORF len=47)	248724	248515	80.9	1e-21	RBCSC-A
	258216	257986	79.3	5e-21	RBCSC-B

Table 4. Expression profiles of different ribulose biphosphate carboxylase small chain (RBCSC) proteins in different tissues: Counts are in RPKM (Reads Per Kilobase of transcript per Million mapped reads). Although TC13991 and TC23009 map to the same gene, there is a huge discrepancy in their counts.

Gene	Transcript	Length (nt)	Shoot	Root	Mature leaf	Flower bud	Young pod
RBCSC-A	TC13991	201	17.9	0	0	0	0
	TC23009	332	1403.8	0	441.5	99.5	153.8
RBCSC-B	TC01487	617	49.2	0	23.2	0	3.7
RBCSC-C	TC10770	538	17.9	0	0	0	11

Dataset 1. Raw data for YeATS on chickpea transcriptome

A description of each file is provided in the text file 'Dataset_Description'.

transcriptomes be linked to the Bioproject. Such initiatives have been adopted in the Global Open Data (<http://www.godan.info/pages/statement-purpose>) to “to make agricultural and nutritionally relevant data available, accessible, and usable for unrestricted use worldwide”.

No competing interests were disclosed.

Grant information

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Björn Voß

University of Stuttgart, Stuttgart, Germany

Although the manuscript has been revised, it was not substantially improved, and therefore many of my previous concerns were not properly addressed.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Version 1

Reviewer Report 05 December 2016

<https://doi.org/10.5256/f1000research.10416.r17521>

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Lilah Toker

University of British Columbia, Vancouver, BC, USA

The article "RNA-seq assembler artifacts can bias expression counts and differential expression analysis application of YeATS on the chickpea transcriptome" by Sandeep Chakraborty describes the use of a workflow developed by the author for identification of assembly artifacts in the chickpea transcriptome. The author suggests a possible impact of these artifacts on the

expression counts and concludes that when de-novo transcriptome assembly is implemented as a part of RNAseq study, the intermediate assembly transcripts should be provided by the authors.

While the concluding statement is true regardless of the content of the manuscript, there are several issues that need to be addressed for the readers to benefit from this study:

1. The mention of YeATS in the title and the abstract without providing explanation of what it is, unnecessarily complicates the understanding of the text.
2. The title and the abstract of the manuscript are quite misleading. It is true that assembly artifacts can bias expression counts and differential expression analysis, in fact, it is a quite trivial statement. Nevertheless, nothing in the manuscript addresses this claim. In the best case, the author might have detected several artifacts in the assembly of chickpea transcriptome speculating about their impact on the expression counts, but he has not evaluated the impact of the proposed artifacts on expression counts or differential expression analysis. In addition, the author analysed a single dataset and thus should avoid generalizing his statements.
3. The manuscript is entirely based on a workflow developed by the author. The workflow was never validated and was not previously used by other researchers, questioning the reliability of the results. The main step of the workflow is based on ORFs identified by the getorf tool, which by default define ORFs as regions between two STOP codons. This is quite puzzling, since traditionally ORFs are defined as sequences beginning with a START codon and might or might not contain a STOP codon. The author should validate his results using a tool based on the conventional definition of ORF such as NCBI's ORFfinder.
4. More generally, the author should evaluate the performance of his workflow and verify the proposed artifacts by analysing of well annotated organism such as *Arabidopsis*. Do the identified "errors" indeed represent artifacts or do they represent biological truth? For example, one intuitive explanation for a single ORF aligned to two proteins would be that the two proteins are truly transcribed from a single transcript, as often observed in other organisms. Similarly, the "Type II error" can represent cases where multiple isoforms exist for a single protein (e.g. due to alternative splicing events).
5. p.4 – "However, the overrepresentation of such merged transcripts in HET suggests that there might be some errors in counting". This is entirely based on the author's opinion. Is there any evidence that this is the case?
 - The manuscript needs to be proofread in order to improve its readability. For example, the interchangeable use of past and present tenses while describing the work make it difficult to understand the workflow of the study (e.g. p.3, 4th paragraph).
 - p.3, 3rd paragraph: "In contrast to other traditional methods like RNA:DNA hybridization and short sequence-based approaches". Why does the author mention the other methods? This sentence doesn't really make any sense, especially in light of the fact that RNAseq methodology is also a short sequence based approach.
 - p.3, 3rd paragraph: "RNA-seq detects transcripts with very low expression levels". This

sentence is irrelevant to the manuscript. Moreover, it is not entirely valid since the ability of the method to detect low abundant transcripts is related to the depth of the sequencing.

- p.3, 3rd paragraph: "YeATS is a work-flow for analyzing RNA-seq data". The author should clearly indicate that YeATS workflow was developed and implemented by the author himself instead of presenting it as well established approach.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Reviewer Report 11 November 2016

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Björn Voß

University of Stuttgart, Stuttgart, Germany

The article "RNA-seq assembler artifacts can bias expression counts and differential expression analysis - application of YeATS on the chickpea transcriptome" reports putative assembly artifacts in the chickpea transcriptome and discusses possible impacts on the expression counts. The main message is that it should become mandatory to make intermediate data, such as assembled transcripts, accessible. With respect to this the title is not appropriate. Additionally the title should not explicitly mention YeATS, and the fact the artifacts can bias expression counts is not surprising. The title needs to be changed.

There are some wrong claims in the introduction, but overall the intro is acceptable. In contrast, the Methods section is insufficient in its current form. It lacks information to reproduce the results. Interestingly, this is a point that the author stresses in the introduction. Furthermore, some of the used tools and datasets seem inappropriate (e.g. getorf to translate transcripts to peptides. Further concerns are provided in the detailed comments below).

In the Results, the author presents some examples of putative assembly artifacts found in highly expressed transcripts. Unfortunately, no information about the actual errors that result from the artifacts is provided. Furthermore, finding such artifacts in a single dataset does not satisfy to claim these as a widespread problem.

Overall, the manuscript is hard to read. One reason is the quality of the language that makes it hard to follow the authors reasoning. Another reason, is that there seems to be no clear story, that is to be presented. What is the main point the author wants to make? Is it reproducibility, the

problem of assembly artifacts or that more data needs to be made freely accessible?

p.3: 'In computational studies, the exact replication of the output of most computer programs is difficult as most non-trivial algorithms use heuristics'

Comment: Heuristics do not contradict reproducibility.

p.3: 'The three longest ORFs were obtained using the 'getorf' utility in the EMBOSS suite 2'

Comment: Is assured that these ORFs do not overlap on either strand?

p.3: 'BLAST'ed 2'

Comment: Provide details, like E-value cutoff, and so on.

p.3: 'Type I error occurs when a single transcript has multiple ORFs with significant matches to different genes'

Comment: How is significance assessed? How are multiple significant matches of the same ORF treated? Is the best selected?

p.3: 'the 'plantpep.fasta''

Comment: It is not clear to me why the author uses a database of plant proteins rather than looking at the mapping to the genome, to identify assembly errors. Another option would be to use chickpea protein sequences from the genome.

p.3: 'In a Type II error a single gene is broken into two separate transcripts'

Comment: How is this detected from the BLAST results?

p.3: 'For a type III error, a single transcript has multiple ORFs, but they all map to the same gene'

Comment: Are the ORFs perhaps overlapping?

p.3: 'was first mapped to the chickpea genome'

Comment: How?

p.3: 'The RNA-seq [8,9] derived transcriptome of chickpea has also been sequenced [10]'

Comment: This sentence makes not much sense.

p.3: 'short sequence-based approaches [12], RNA-seq detects transcripts with very low expression levels.'

Comment: RNA-seq is also based on short sequence reads, which depends on the used sequencing technology. The detection of low expressed transcripts heavily depends on the used sequencing depth and is not an inherent feature of it.

p.3: 'metagenomic contamination'

Comment: This term does not exist. Simply say contamination.

p.3: 'split '

Comment: Split is misleading.

p.3: 'significant'

Comment: How is significance measured?

p.3: 'FB:4, ML:5, RT:3, SH:4 YP:5'

Comment: Some transcripts occur several times in different tissues, TC00004 for example. What would be the non-redundant numbers? Does it at all make sense to differentiate between tissues for this study?

p.3: 'are'

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Comment: Does this annotation make sense for a plant protein?

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p.3: 'This transcript is highly fragmented and encodes on both strands in an overlapping manner'

Comment: This sound like this is a transcript from a pseudogene and the predicted ORFs are wrong.

p.4: 'TC13991 has a count of 17.90, while TC23009 has a count of 1403.8. The large difference indicates an erroneous normalization algorithm, since there is only one expressed transcript of this gene (the ORF of TC13991 has no other significant match among other transcripts), although there are other genomic variants.'

Comment: First, the author argues that these two transcripts should be merged, but then he is concerned about the fact that, although different genomic variants of the corresponding gene exist, there is only one transcript. It looks like the transcripts resemble the genomic situation, although very poorly. It would be interesting to see the alignment on the nucleotide level.

p.4t: 'Furthermore, there is a missing 35 aa stretch in the C-terminal peptide "IGFDNVRQVCISFIAHTPKEF", which has no match in the transcripts'

Comment: What is meant with the missing 35aa stretch? The missing C-terminal part has 22aa.

p.4: 'merged transcripts are proximally located in the genome'

Comment: Please clarify what this is intended to mean. Which merged transcripts?

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 24 Nov 2016

Sandeep Chakraborty, Celia Engineers, T. T. C Industrial Area, Rabale, Navi Mumbai, India

Dear Dr Voß,

I would like to thank you for taking the time to review this paper in detail, and providing constructive criticism on the overall manuscript. I also appreciate the opportunity to make

suitable changes where appropriate, and defend some of the critiques that are not correct in my opinion. Reference numbering below is based on the main manuscript.

It is apparent to me that the concept of identifying assembly errors by breaking a transcript into ORFs is not lucid. Mapping a transcript to the genome will not identify such errors, since there is no way to differentiate introns and inter-gene sequences. However, I have attempted to the best of my ability to clarify your doubts.

Getting access to the assembled transcriptome is not the general case, even in cases when inferences are made based on quantification (maybe not in this particular chickpea study). In another case, I have been unable to obtain the transcriptome through personal communication. (<http://bmcbgenomics.biomedcentral.com/articles/10.1186/s12864-015-1894-5>).

Another paper (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4725360/>) makes inferences on differential gene expression under drought and salinity stress, but I could not find intermediate data. Although, I have not communicated with the authors of this paper, the point here is that one should not have to make personal contact, as not getting responses to valid queries is not a particularly good feeling. So, I think studies that make inferences on transcriptomes should always provide them.

In general, I am focused on studies that make inferences based on transcriptomic data since I believe they have a degree of impenetrability due to complex pipelines. For example, certain studies (which have provided intermediate data) have made inferences on the saffron transcripts, without removing extraneous transcripts from other genomes [16]. In a different study (pre-print) it has been shown that there is a fungal transcript annotated as a saffron gene (and possibly others) [23].

Below I outline a point-by-point response to your comments.

The article "RNA-seq assembler artifacts can bias expression counts and differential expression analysis - application of YeATS on the chickpea transcriptome" reports putative assembly artifacts in the chickpea transcriptome and discusses possible impacts on the expression counts. The main message is that it should become mandatory to make intermediate data, such as assembled transcripts, accessible. With respect to this the title is not appropriate. Additionally the title should not explicitly mention YeATS, and the fact the artifacts can bias expression counts is not surprising. The title needs to be changed.

The title has been changed as suggested. However, I would not take "the fact the artifacts can bias expression counts is not surprising" since understanding the basis for these biases can possibly lead to algorithms that fix it (maybe an assembler that does a quick analysis and does not merge transcripts that map to different genes).

There are some wrong claims in the introduction, but overall the intro is acceptable. Please point them out so that they can be fixed, even if it takes multiple revisions.

In contrast, the Methods section is insufficient in its current form. It lacks information to reproduce the results. Interestingly, this is a point that the author stresses in the introduction.

I agree that, if true, this is a major oversight my part. I assumed, wrongly, that these would be really trivial to reproduce.

(1) Find the top five transcripts. (2) Find the ORFs - choose the three longest. (3) Find whether they are annotated using BLAST (possibly on a smaller database as I have done, but best done the 'nr' database). (4) See if the most highly transcribed transcripts encode more than one gene.

The complete analysis of all transcripts need to be done for identifying Type II errors, which highlights another problem of discrepancies in counts of split transcripts from the same gene. While using the full 'nr' BLAST database is the best option, a BLAST database of protein peptides (plantpep.fasta:1M sequences) using 30 organisms (list.plants) from the Ensembl genome was created to reduce computational times. I have re-written the Methods - hopefully it is more lucid now.

Furthermore, some of the used tools and datasets seem inappropriate (e.g. getorf to translate transcripts to peptides. Further concerns are provided in the detailed comments below).

I disagree with this point. 'getorf' from the EMBOSS suite is a well-known tool for getting ORFs. While it is simple, and I have my own version written before I found this tool, I use this standardized version.

In the Results, the author presents some examples of putative assembly artifacts found in highly expressed transcripts. Unfortunately, no information about the actual errors that result from the artifacts is provided. Furthermore, finding such artifacts in a single dataset does not satisfy to claim these as a widespread problem.

All that is shown here that RNA-assembly merges transcripts (already shown before for a different plant and assembler [2]), which casts suspicion on counts - and the fact that most highly expressed genes in the chickpea database analyzed are merged further strengthens this doubt. Admittedly, the particular chickpea study made no inferences based on the counts - but that still does not refute the point that counts might be wrong, even in this single study. I use the word 'might', since I have not analyzed the downstream raw data to establish. And if there are insinuations in the manuscript that this is widespread (which I suspect is true, but will take time to establish), kindly point it out so that I may correct that.

Overall, the manuscript is hard to read. One reason is the quality of the language that makes it hard to follow the authors reasoning.

Please let me know how to improve this.

Another reason, is that there seems to be no clear story, that is to be presented. What is the main point the author wants to make? Is it reproducibility, the problem of assembly artifacts or that more data needs to be made freely accessible?

The 'problem of assembly artifacts (Point 1)' (Trinity) encountered during the Walnut

genome project [14] has led to the development of methods to detect these artifacts [2], which the current work could reproduce (Point 2) for another transcriptome (and a different assembler, Newbler) as it was possible to find 'freely accessible data' (Point 3). Point 2 and 3 are well-known issues, the little contribution in the current paper is to emphasize Point 1.

p.3: 'In computational studies, the exact replication of the output of most computer programs is difficult as most non-trivial algorithms use heuristics' Comment: Heuristics do not contradict reproducibility.

Agreed, I have changed the statement to 'as most non-trivial algorithms are non-deterministic.'

p.3: 'The three longest ORFs were obtained using the 'getorf' utility in the EMBOSS suite 2' Comment: Is assured that these ORFs do not overlap on either strand?

No, there is no such assumption. However, this is deliberate since the E-value of matches of these ORFs will determine their relevance.

p.3: 'BLAST'ed 2' Comment: Provide details, like E-value cutoff, and so on.

Done.

p.3: 'Type I error occurs when a single transcript has multiple ORFs with significant matches to different genes' Comment: How is significance assessed? How are multiple significant matches of the same ORF treated? Is the best selected?

Significance is assessed using: E-value=1E-8, BLAST bitscore=75. Yes, the best match is selected for multiple significant matches of the same ORF.

p.3: 'For a type III error, a single transcript has multiple ORFs, but they all map to the same gene' Comment: Are the ORFs perhaps overlapping?

No, these ORFs would never be overlapping. They have been created by sequencing or assembly errors, resulting in the false insertion of stop codons. Note, that the 'getorf' program always ends at a stop codon, but does not (need to) start from a start codon.

p.3: 'was first mapped to the chickpea genome' Comment: How?

Using BLAST through the YEATS pipeline. Mentioned in the text now.

p.3: 'The RNA-seq [8,9] derived transcriptome of chickpea has also been sequenced [10]' Comment: This sentence makes not much sense.

Changed to 'The transcriptome of chickpea has been sequenced [1] using RNA-seq [10, 11].'

p.3: 'short sequence-based approaches [12], RNA-seq detects transcripts with very low expression levels.' Comment: RNA-seq is also based on short sequence reads, which depends on the used sequencing technology. The detection of low expressed transcripts heavily depends on the used sequencing depth and is not an inherent feature of it.

I concur, changed the line to '... RNA-seq can detect transcripts with very low expression levels by increasing sequencing depth.'

p.3: 'metagenomic contamination' Comment: This term does not exist. Simply say contamination.

Corrected.

p.3: 'split' Comment: Split is misleading.

Corrected.

p.3: 'significant' Comment: How is significance measured?

E-value=1E-8, BLAST bitscore=75. Mentioned in the text.

p.3: 'FB:4, ML:5, RT:3, SH:4 YP:5' Comment: Some transcripts occur several times in different tissues, TC00004 for example. What would be the non-redundant numbers? Does it at all make sense to differentiate between tissues for this study?

Having multiple tissues strengthens the main point highlighted in this paper, that expression counts might be biased due to merged transcript. The claim would be weaker if there were just one tissue in which this were true. There are common (TC00004) transcripts, but there are specific ones too (TC00462 in mature leaf). Also, the very high RPKM of these merged transcripts compared to other transcripts indicates the possibility of some errors in counting.

p.3: 'are' Comment: as

Corrected.

p.3: 'retinoblastoma-binding-like protein' Comment: Does this annotation make sense for a plant protein?

Yes - <http://nar.oxfordjournals.org/content/27/17/3527.full>. Even though this is correct, annotation of genes is not critical to the narrative here.

p.3: 'have' Comment: be

Corrected.

p.3: 'This transcript is highly fragmented and encodes on both strands in an overlapping manner' Comment: This sound like this is a transcript from a pseudogene and the predicted ORFs are wrong.

This is not a pseudogene, by virtue of being transcribed. It seems there is an assembly error, for example Uniprot:A0A072THK0 Senescence-associated protein has three homologous segments in TC00002 - fwd:249-485, reverse:211-35 and fwd:730-894 with E-values 2e-34, 2e-14 and 9e-14, respectively.

p.4: 'TC13991 has a count of 17.90, while TC23009 has a count of 1403.8. The large difference indicates an erroneous normalization algorithm, since there is only one expressed transcript of this gene (the ORF of TC13991 has no other significant match among other transcripts), although there are other genomic variants.' Comment: First, the author argues that these two transcripts should be merged, but then he is concerned about the fact that, although different genomic variants of the corresponding gene exist, there is only one transcript. It looks like the transcripts resemble the genomic situation, although very poorly. It would be interesting to see

the alignment on the nucleotide level.

This is an excellent suggestion, I have looked at the nucleotide mapping of these transcripts to the genome more closely. There is one new paragraph addressing this (three new tables).

p.4t: 'Furthermore, there is a missing 35 aa stretch in the C-terminal peptide "IGFDNVRQVCISFIAHTPKEF", which has no match in the transcripts' Comment: What is meant with the missing 35aa stretch? The missing C-terminal part has 22aa.
Corrected.

p.4: 'merged transcripts are proximally located in the genome' Comment: Please clarify what this is intended to mean. Which merged transcripts?

I have rephrased the statement. 'When two genes are adjacent to each other in the genome, and are both transcribed it is possible that an assembler merges these into one transcript. Also, it is possible that these loci are under the same transcriptional control and the expression counts are correct. Thus, high levels of expression of one gene should correlate with high expression level of the other, assuming a proper normalization. However, the over-representation of such merged transcripts in HET suggests that there might be some errors in counting.'

I hope that your concerns have been addressed suitably.
Best regards,

Sandeep

Competing Interests: No competing interests were disclosed.

Comments on this article

Version 2

Reader Comment 05 Feb 2017

Scott Edmunds, GigaScience, BGI-Hong Kong Co Ltd, Hong Kong

Where you link to the chickpea data in GigaDB, can you please use the DOI (<http://dx.doi.org/10.5524/100076>) rather than the unstable URL (likely to change when the database is updated). Or even better, you could follow the data citation principles and cite the datasets discussed in the references (see: <https://www.force11.org/group/joint-declaration-data-citation-principles-final>).

Competing Interests: Work on GigaDB database.

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