Origins and evolution of trans-splicing of bursicon in mosquitos

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Broad transcriptomic sequencing of eukaryotes has revealed the ubiquity of splicing of nuclear genes. While the vast majority of splicing events join segments of the same RNA transcript, various studies have found a few intriguing cases of trans-splicing of introns, in which splicing events within protein coding regions join segments of different RNA transcripts. The most structurally intricate case known involves the bursicon gene in mosquitos, in which an internal exon is encoded at a distinct locus, requiring multiple trans-splicing events form the mature mRNA. This arrangement is known to be ancestral to mosquitos, however the exact timing of the origin of trans-splicing and the history of the bursicon gene within mosquitos is unknown. Taking advantage of the recent availability of genomes from various *Anopheles* mosquitos and from relatives of mosquitos, I determined *trans* versus *cis* encoding of bursicon across Culicomorpha. I conclude that trans-splicing emerged in the last common ancestor of mosquitos, and that trans-splicing has been retained in all 19 studied *Anopheles* species. The retention of trans-splicing could indicate functional importance of this arrangement, or could alternatively reflect the rarity of mutations giving rise to viable allelic alternatives.

In eukaryotic nuclear genes, the protein-coding regions of mRNA transcripts do not represent a single contiguous genomic region, but rather are spliced together from RNA segments representing different DNA regions (Roy and Irimia 2014). Almost always, these segments represent subsegments of a single RNA transcript which are joined by a molecular machine called the spliceosome. Portions that are retained in the mature mRNA are termed exons while the internal removed sections are termed introns. Much more rarely, the protein-coding region of an mRNA can be formed by splicing together portions of RNA transcripts transcribed from different genomic loci, that is, trans-splicing of introns (Li et al. 1999; Takahara et al. 2000; Dorn et al. 2001; Robertson et al. 2007; Nageshan et al. 2011; Kamikawa et al. 2011; Roy et al. 2012). (This project should be distinguished from so-called spliced leader trans-splicing, in which a short RNA 'tag' (the spliced leader) is added to the 5' end of various mRNA species, typically donating a (partial) UTR (Lasda and Blumenthal 2011)). Trans-splicing of introns within coding regions is thought to be very uncommon, with only a handful of cases known across eukaryotes.

The most structural intricate known case of nuclear trans-splicing involves bursicon, an important developmental hormone in insects (Robertson et al. 2007). In characterized mosquitos, the mature bursicon mRNA consists of four exons, derived from two pre-mRNA transcripts transcribed from different genomic loci. One transcript encodes exons 1, 2 and 4, and the second encodes exon 3. Two spliceosomal reactions occur between the two RNA species, one joining the 3' end of exon 2 to the 5' end of exon 3 and the other joining the 3' end of exon 3 to the 5' end of exon 4 (Figure 1a). This arrangement is known to be ancestral to mosquitos as it is found in the divergent species *Anopheles gambiae*, *Aedes aegypti* and *Culex pipiens* (Robertson et al. 2007). However, the timing of the origin of this arrangement, and its evolutionary history within other lineages of mosquitos, remain obscure.

To trace the history of the bursicon gene, I downloaded recently available genomes from various mosquitos and related species (Vicoso and Bachtrog 2015; Neafsey et al. 2015). First, I downloaded species within the very ancient genus *Anopheles* (dating back ~100My; see Neafsey et al. 2015) and performed TBLASTN searches of the *A. gambiae* bursicon protein sequence against each species' genome. For all 15/19 searched species, sequences with very high levels of protein-level similarity to exons 3 and 4 of *A.gambiae* bursicon were found on different DNA molecules, consistent with a pattern of trans-splicing similar to that found in previously characterized mosquitos. For the other 4 species, a single hit was found to exon 3, but no sequence (either proximal or distal to the exon 3 hit) was found for exon 4. One possible explanation for this pattern is that the exon 4 sequence is not represented in the current genome assembly. For no species did I find a sequence homologous to exon 2, possibly because exon 2's function in encoding a signaling peptide gives it a higher degree of protein sequence flexibility (and thus rate of evolution). All these results are consistent with retention of the *A.gambiae* trans-splicing pattern across *Anopheles* species.

To trace the origins of the trans-splicing arrangement in mosquitos, I downloaded the genomes of five related species within the larger infraorder Culicomorpha. TBLASTN searches of the *A.*gambiae bursicon protein gave clear hits for four of the five genomes. Figure 1d shows the phylogeny of the studied species. For all cases, sequences homologous to exon 3 and exon 4 appeared on the same genomic DNA molecule in the order expected for co-linear transcription within a single molecule. For all cases, intervening sequences between the exon 3 and exon 4 regions contained stop codons or frameshifts, strongly suggesting the presence of a *cis*-spliced intron at or near the position of the trans-spliced intron in mosquitos. This suggests that trans-splicing evolved in the ancestor of mosquitos.

Concluding remarks

It is striking that the peculiar arrangement of the trans-spliced bursicon gene has been independently retained in a wide variety of mosquitos. Conservation is often taken as evidence of function, and the possibility that the trans-spliced arrangement imparts a function that could not be accomplished by a cis-spliced form is enticing. However, an important alternative is that production of viable cis-spliced alternatives by random mutation is an extremely rare occurrence. Reinsertion of exon 3 into the genomic region between exons 2 and 4 would require a very precise non-homologous double recombination, and even then might lead to mis-splicing due to disruption of splicing signals or to disrupted functioning of splicing signals in cis. Another possibility is retroposition of a spliced mRNA at a third genomic locus, however such a gene would likely lack various intronic and upstream regulatory motifs, and thus might not be able to replace the ancestral function.

References

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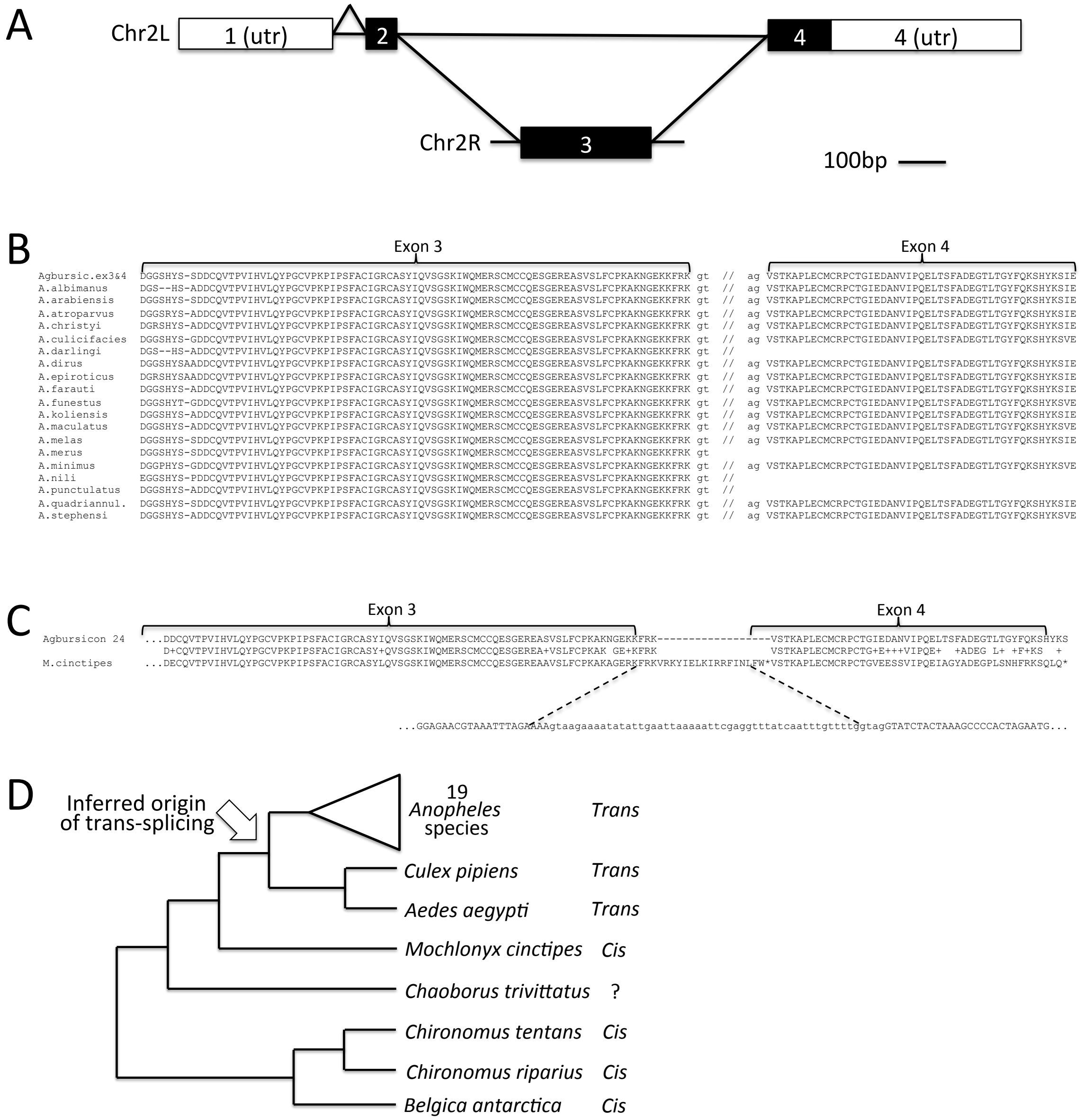

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Agbursic.ex3&4  DGGSHYS-SDDCQVTPVIHVLQYPGCVPKPIPSFACIGRCASYIQVSGSKIWQMERSCMCCQESGEREASVSLFCPKAKNGEKKFRK gt  //  ag VSTKAPLECMCRPCTGIEDANVIPQELTSFADEGTLTGYFQKSHYKSIE
A.albimanus     DGS--HS-ADDCQVTPVIHVLQYPGCVPKPIPSFACIGRCASYIQVSGSKIWQMERSCMCCQESGEREASVSLFCPKAKNGEKKFRK gt  //  ag VSTKAPLECMCRPCTGIEDANVIPQELTSFADEGTLTGYFQKSHYKSIE
A.arabiensis    DGGSHYS-SDDCQVTPVIHVLQYPGCVPKPIPSFACIGRCASYIQVSGSKIWQMERSCMCCQESGEREASVSLFCPKAKNGEKKFRK gt  //  ag VSTKAPLECMCRPCTGIEDANVIPQELTSFADEGTLTGYFQKSHYKSIE
A.atroparvus    DGGSRYS-ADDCQVTPVIHVLQYPGCVPKPIPSFACIGRCASYIQVSGSKIWQMERSCMCCQESGEREASVSLFCPKAKNGEKKFRK gt  //  ag VSTKAPLECMCRPCTGIEDANVIPQELTSFADEGTLTGYFQKSHYKSIE
A.christyi      DGRSHYS-ADDCQVTPVIHVLQYPGCVPKPIPSFACIGRCASYIQVSGSKIWQMERSCMCCQESGEREASVSLFCPKAKNGEKKFRK gt  //  ag VSTKAPLECMCRPCTGIEDANVIPQELTSFADEGTLTGYFQKSHYKSIE
A.culicifacies  DGGSHYS-GDDCQVTPVIHVLQYPGCVPKPIPSFACIGRCASYIQVSGSKIWQMERSCMCCQESGEREASVSLFCPKAKNGEKKFRK gt  //  ag VSTKAPLECMCRPCTGIEDANVIPQELTSFADEGTLTGYFQKSHYKSVE
A.darlingi      DGS--HS-ADDCQVTPVIHVLQYPGCVPKPIPSFACIGRCASYIQVSGSKIWQMERSCMCCQESGEREASVSLFCPKAKNGEKKFRK gt  //
A.dirus         DGGSHYSAADDCQVTPVIHVLQYPGCVPKPIPSFACIGRCASYIQVSGSKIWQMERSCMCCQESGEREASVSLFCPKAKNGEKKFRK gt  //  ag VSTKAPLECMCRPCTGIEDANVIPQELTSFADEGTLTGYFQKSHYKSIE
A.epiroticus    DGRSHYSAADDCQVTPVIHVLQYPGCVPKPIPSFACIGRCASYIQVSGSKIWQMERSCMCCQESGEREASVSLFCPKAKNGEKKFRK gt  //  ag VSTKAPLECMCRPCTGIEDANVIPQELTSFADEGTLTGYFQKSHYKSIE
A.farauti       DGGSHYS-ADDCQVTPVIHVLQYPGCVPKPIPSFACIGRCASYIQVSGSKIWQMERSCMCCQESGEREASVSLFCPKAKNGEKKFRK gt  //  ag VSTKAPLECMCRPCTGIEDANVIPQELTSFADEGTLTGYFQKSHYKSIE
A.funestus      DGGSHYT-GDDCQVTPVIHVLQYPGCVPKPIPSFACIGRCASYIQVSGSKIWQMERSCMCCQESGEREASVSLFCPKAKNGEKKFRK gt  //  ag VSTKAPLECMCRPCTGIEDANVIPQELTSFADEGTLTGYFQKSHYKSVE
A.koliensis     DGGSHYS-ADDCQVTPVIHVLQYPGCVPKPIPSFACIGRCASYIQVSGSKIWQMERSCMCCQESGEREASVSLFCPKAKNGEKKFRK gt  //  ag VSTKAPLECMCRPCTGIEDANVIPQELTSFADEGTLTGYFQKSHYKSIE
A.maculatus     DGGSHYS-ADDCQVTPVIHVLQYPGCVPKPIPSFACIGRCASYIQVSGSKIWQMERSCMCCQESGEREASVSLFCPKAKNGEKKFRK gt  //  ag VSTKAPLECMCRPCTGIEDANVIPQELTSFADEGTLTGYFQKSHYKSVE
A.melas         DGGSHYS-SDDCQVTPVIHVLQYPGCVPKPIPSFACIGRCASYIQVSGSKIWQMERSCMCCQESGEREASVSLFCPKAKNGEKKFRK gt  //  ag VSTKAPLECMCRPCTGIEDANVIPQELTSFADEGTLTGYFQKSHYKSIE
A.merus         DGGSHYS-SDDCQVTPVIHVLQYPGCVPKPIPSFACIGRCASYIQVSGSKIWQMERSCMCCQESGEREASVSLFCPKAKNGEKKFRK gt
A.minimus       DGGPHYS-GDDCQVTPVIHVLQYPGCVPKPIPSFACIGRCASYIQVSGSKIWQMERSCMCCQESGEREASVSLFCPKAKNGEKKFRK gt  //  ag VSTKAPLECMCRPCTGIEDANVIPQELTSFADEGTLTGYFQKSHYKSVE
A.nili          EGGSHYS-PDDCQVTPVIHVLQYPGCVPKPIPSFACIGRCASYIQVSGSKIWQMERSCMCCQESGEREASVSLFCPKAKNGEKKFRK gt  //
A.punctulatus   DGGSHYS-ADDCQVTPVIHVLQYPGCVPKPIPSFACIGRCASYIQVSGSKIWQMERSCMCCQESGEREASVSLFCPKAKNGEKKFRK gt  //
A.quadriannul.  DGGSHYS-SDDCQVTPVIHVLQYPGCVPKPIPSFACIGRCASYIQVSGSKIWQMERSCMCCQESGEREASVSLFCPKAKNGEKKFRK gt  //  ag VSTKAPLECMCRPCTGIEDANVIPQELTSFADEGTLTGYFQKSHYKSIE
A.stephensi     DGGSHYS-ADDCQVTPVIHVLQYPGCVPKPIPSFACIGRCASYIQVSGSKIWQMERSCMCCQESGEREASVSLFCPKAKNGEKKFRK gt  //  ag VSTKAPLECMCRPCTGIEDANVIPQELTSFADEGTLTGYFQKSHYKSVE
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Agbursicon 24   ...DDCQVTPVIHVLQYPGCVPKPIPSFACIGRCASYIQVSGSKIWQMERSCMCCQESGEREASVSLFCPKAKNGEKKFRK------------------VSTKAPLECMCRPCTGIEDANVIPQELTSFADEGTLTGYFQKSHYKS
                   D+CQVTPVIHVLQYPGCVPKPIPSFACIGRCASY+QVSGSKIWQMERSCMCCQESGEREA+VSLFCPKAK GE+KFRK                  VSTKAPLECMCRPCTG+E+++VIPQE+  +ADEG L+ +F+KS  +
M.cinctipes     ...DECQVTPVIHVLQYPGCVPKPIPSFACIGRCASYLQVSGSKIWQMERSCMCCQESGEREAAVSLFCPKAKAGERKFRKVRKYIELKIRRFINLFW*VSTKAPLECMCRPCTGVEESSVIPQEIAGYADEGPLSNHFRKSQLQ*

...GGAGAACGTAAATTTAGAAAAgtaagaaaatatattgaattaaaaattcgaggtttatcaatttgttttggtagGTATCTACTAAAGCCCCACTAGAATG...
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Figure 1. Evolutionary history of the trans-spliced bursicon gene in mosquitos. A. Schematic of expression of the bursicon gene in *A. gambiae*, adapted from Robertson (2007). Two pre-mRNA transcripts are produced, one from chromosome 2L and one from chromosome 2R. The former contains three exons (1,2 and 4, with exon 1 encoding the 5' UTR and exon 4 encoding the C-terminus of the protein and the 3' UTR), and the latter contains one exon (exon 3). The mature message is created by three splicing reactions including two joining portions of the two pre-mRNA transcripts. B. Alignment of the *A. gambiae* bursicon protein with homologous sequences for the 19 species of *Anopheles* mosquito characterized here. For each species for which sequence homologous to both exon 3 and exon 4 were found, they were found on different DNA molecules, suggesting trans-splicing. C. Alignment of the *A. gambiae* bursicon protein with homologous sequence on contig JXPH01093764.1 in the *M. cinctipes* genome, showing *cis* orientation of exons 3 and 4 and an intervening putative intron with gt...ag splice boundaries and in-frame stop codons. D. Phylogeny of studied species within the infraorder Culicomorpha, showing species with evidence for *trans* or *cis* encoding of bursicon.

Additional data. Results from TBLASTN search of *A. gambiae* bursicon protein against various *Anopheles* species genomes. For each line, the format is subject_scaffold:genomic_coordinates, then the amino acid positions of the *A. gambiae* bursicon protein that fall within the BLAST hit.

A.albimanus	
KB672320.1:524518-524784	Agbursicon:25-115
KB672342.1:143410-143261	Agbursicon:114-163
::::::::::::::	
A.arabiensis	
KB704451.1:4928193-4928459	Agbursicon:27-115
KB704396.1:2542421-2542570	Agbursicon:114-163
::::::::::::::	
A.atroparvus	
AXCP01008157.1:7448-7170	Agbursicon:23-115
KI421891.1:6686038-6685889	Agbursicon:114-163
::::::::::::::	
A.christyi	
KB697805.1:644-922	Agbursicon:23-115
KB678907.1:3115-3285	Agbursicon:107-163
::::::::::::::	
A.culicifacies	
AXCM01005500.1:1165-893	Agbursicon:27-117
AXCM01017223.1:760-611	Agbursicon:114-163
::::::::::::::	
A.darlingi	
ADMH02002125.1:942117-941851	Agbursicon:27-117
::::::::::::::	
A.dirus	
KB672979.1:6926299-6926015	Agbursicon:28-121
KB672602.1:5830665-5830513	Agbursicon:113-163
::::::::::::::	
A.epiroticus	
KB671576.1:75830-75540	Agbursicon:20-115
KB671690.1:102779-102928	Agbursicon:114-163
::::::::::::::	
A.farauti	
KI915040.1:593704-593438	Agbursicon:27-115
KI915044.1:11246457-11246606	Agbursicon:114-163
::::::::::::::	
A.funestus	
KB669425.1:142513-142241	Agbursicon:27-117
KB669203.1:467130-466981	Agbursicon:114-163
::::::::::::::	
A.koliensis	

JXXB01041064.1:3306-3025	Agbursicon:22-115
JXXB01016478.1:5058-4909	Agbursicon:114-163
:::::::::::::	
A.maculatus	
AXCL01025102.1:272-6	Agbursicon:27-115
AXCL01011182.1:483-319	Agbursicon:109-163
:::::::::::::	
A.melas	
AXCO02002142.1:785-1087	Agbursicon:27-127
KI919430.1:57035-56874	Agbursicon:110-163
:::::::::::::	
A.merus	
KI915301.1:220765-220499	Agbursicon:27-115
KI915256.1:770693-770532	Agbursicon:110-163
:::::::::::::	
A.minimus	
KB663610.1:4819334-4819062	Agbursicon:27-117
KB663655.1:917356-917207	Agbursicon:114-163
:::::::::::::	
A.nili	
ATLZ01013352.1:519-253	Agbursicon:27-115
:::::::::::::	
A.punctulatus	
JXXA01016707.1:1714-1980	Agbursicon:27-115
:::::::::::::	
A.quadriannulatus	
KB667777.1:4536606-4536878	Agbursicon:27-117
KB668088.1:1031074-1030925	Agbursicon:114-163
:::::::::::::	
A.sinensis	
KE524456.1:61412-61708	Agbursicon:17-115
KE525275.1:769221-769370	Agbursicon:114-163
:::::::::::::	
A.stephensi	
KE388949.1:917632-917354	Agbursicon:23-115
ALPR02015437.1:909-760	Agbursicon:114-163