Curation and analysis of tRNA and aminoacyl-tRNA synthetase genes in the nuclear genome of *Myceliophthora thermophila*

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ABSTRACT

Curation and analysis of tRNA and aminoacyl-tRNA synthetase genes in the nuclear genome of *Myceliophthora thermophila*

Thi Truc Minh Nguyen

Transfer ribonucleic acids (tRNAs) and aminoacyl-tRNA synthetases (AARSs) have long been known for their indispensable roles in the translational process to synthesize proteins in living cells. Therefore, detecting and analyzing the comprehensive sets of tRNA and AARS genes would enhance understanding of the translation system and the results could potentially be used to optimize the translation efficiency for the protein production in the organisms of interest. This research aims to detect the complete sets of cytoplasmic tRNA genes and AARS genes from the nuclear genome of *Myceliophthora thermophila*, a filamentous fungus used in the enzyme production industry and the first thermophilic eukaryote with a finished genome sequence. In this study, 194 cytoplasmic tRNA genes and 35 aminoacyl-tRNA synthetase genes in *M. thermophila* were determined by comparing the gene models predicted from the genome with the sequenced RNAs and the experimentally characterized tRNA and AARS genes. The experimentally verified tRNA genes in *M. thermophila* can encode all of the 20 universal amino acids. The 35 AARS genes code for cytoplasmic and mitochondrial AARS enzymes of all of the 20 amino acid specificities except for the mitochondrial glutaminyl-tRNA synthetase. Four commonly used tools – tRNAscan-SE, SPLITSX, ARAGORN and tRNAfinder – were deployed for the tRNA gene prediction, and we showed that tRNAscan-SE and SPLITSX give more accurate results than ARAGORN and tRNAfinder. Analysis of the tRNA genes showed that there are significant correlations between tRNA gene number in the genome and tRNA abundance in the cell, as well as between tRNA gene number and codon usage bias of protein-encoding genes in *M. thermophila*. Based on the complete tRNA gene set and the codon frequencies in the protein-encoding gene set, a set of preferred codons in *M. thermophila* was determined. The manually curated tRNA and AARS genes of *M. thermophila*, as well as the experimentally characterized ones collected from tRNA and protein databases along with the supporting literature provided in this study, can be used as reliable datasets for tRNA and AARS gene annotation processes.

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LIST OF ABBREVIATIONS

AARS	Aminoacyl-tRNA synthetase
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BHB	Bulge - Helix – Bulge
BLAT	BLAST-like alignment tool
BW	Burrows-Wheeler
BWT	Burrows-Wheeler Transform
CAI	Codon Adaptation Index
CBI	Codon Bias Index
FPKM	Fragments Per Kilobase of exon per Million fragments mapped
GBrowse	Generic Genome Browser
GDP	Guanosine 5′-diphosphate
GO	Gene Ontology
GTP	Guanosine-5'-triphosphate
GtRNAdb	Genomic tRNA database
HMM	Hidden Markov Model
JGI	Joint Genome Institute
kb	Kilobase
mRNA	messenger ribonucleic acid
MSA	Multiple Sequence Alignment
mTP	mitochondrial targeting peptide
N_c	Number of effective codons

ORF	:	Open reading frame
Pyl	:	Pyrrolysine
RSCU	:	Relative Synonymous Codon Usage
SeC	:	Selenocysteine
SECIS	:	SeC insertion sequence
SF	:	synonymous family
tAI	:	tRNA Adaptation Index
TE	:	Transposable Element
tRNA	:	transfer ribonucleic acid
tRNAdb	:	Transfer RNA database

CHAPTER 1. INTRODUCTION

1.1. Overview of tRNAs and tRNA genes

1.1.1. Roles of tRNAs in the cell

Transfer ribonucleic acid, or tRNA, has been known for its crucial role in protein synthesis since 1962 when it was identified as an adaptor molecule to convert the genetic code in a messenger RNA (mRNA) into a polypeptide chain (Chapeville *et al.* 1962). Each tRNA attaches to its conjugate amino acid by an aminoacylation reaction catalyzed by an appropriate aminoacyl-tRNA synthetase enzyme. Each tRNA has three consecutive nucleotides called an anticodon, which can form base pairings with the corresponding codon consisting of three successive nucleotides in the mRNA (**Figure 1**). By having these special features, tRNA molecules can specifically recognize codons in an mRNA and decode them into amino acids.

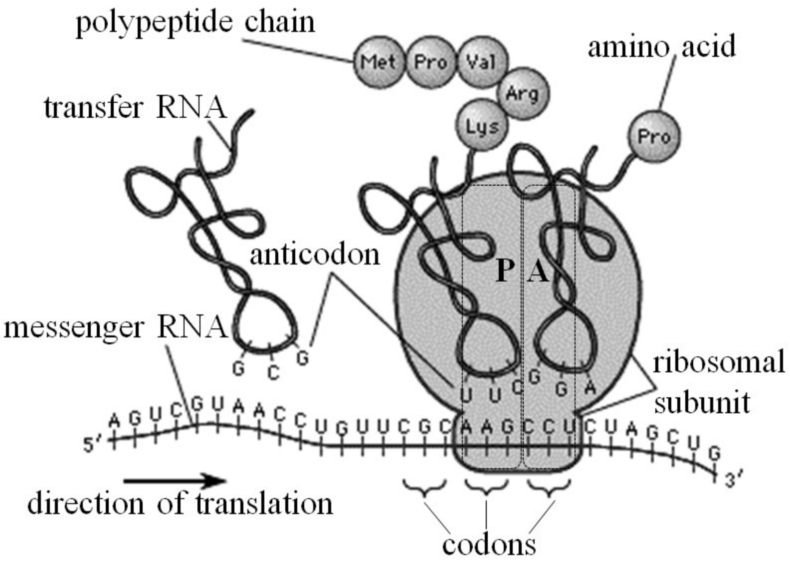


Figure 1. Adaptor function of tRNAs in the translation process

(reproduced from Britannica Encyclopædia 2012)

There are two classes of tRNAs in eukaryotic cells: organelle tRNAs and cytoplasmic tRNAs. Transfer RNAs in mitochondria and chloroplasts are encoded by their own genomes and they function as adaptors of the translational system of their corresponding organelles (Parks *et al.* 1984; Marechal-Drouard, Weil and Dietrich 1993). The cytoplasmic tRNAs are encoded by the nuclear genome and function in the synthesis of a majority of proteins in the cell; therefore, they play a crucial role in the cell's growth (Schmidt and Söll 1981). This study focuses on cytoplasmic tRNAs, and the tRNAs mentioned hereafter refer to cytoplasmic tRNAs unless otherwise specified.

There are two types of tRNAs, the initiators and the elongators, functioning in different stages of the translation process. The initiator tRNA ($tRNA_i^{Met}$) plays an important role in the initiation step. After being charged with Met, $tRNA_i^{Met}$ binds to the guanosine-5'-triphosphate **(**GTP) molecule, initiation factors and the small ribosomal subunit to form a complex which subsequently scans through the mRNA to search for the start codon (AUG). Additionally, unlike in bacteria, a formyl group is not added to the aminoacylated initiator tRNA in eukaryotes. However, it can be formylated by bacterial methionyl-tRNA transformylase while the elongator methionyl-tRNA cannot, and this special feature has been used to distinguish between initiator and elongator Met-tRNAs during the purification procedure (RajBhandary and Ghosh 1969; Gillum *et al.* 1977; Yamashiro-Matsumura and Takemura 1979). Elongator tRNAs are responsible for decoding the codons located downstream of the start codon. They code for all amino acids and can be classified into isoacceptor families according to their amino acid specificity. Within each family, tRNAs can also be divided into subgroups according to their anticodons. During the elongation step, each elongator tRNA is specifically charged with an appropriate amino acid. Subsequently, the aminoacyl-tRNA is delivered to the “A” (aminoacyl) site of the ribosome by a complex consisting of an elongation factor and a GTP molecule. When correct base pairings are established between nucleotides of the anticodon and the codon, the GTP is hydrolysed to a guanosine diphosphate (GDP) molecule, and the complex of GDP and elongation factor is released from the ribosome. Next, the polypeptide chain attached to the tRNA at the “P” (peptidyl) site, which is directly upstream of the “A” site, is transferred to the amino acid attached the tRNA at the “A” site by a peptide linkage (**Figure 1**). The elongation procedure is repeated while the ribosome keeps moving forward to the 3’ end of the mRNA. The translation process is finished when a stop codon (UAA, UAG or UGA) is located at the A site. Finally, a complete polypeptide chain is synthesized.

As reviewed by Goldman (2008), in addition to its primary function as an adaptor in protein synthesis, tRNA is involved in other processes of the cell including transcription, gene regulation and synthesis of the bacterial cell membrane and cell wall. For example, tRNAs play a role in the transcriptional regulation of bacterial amino acid biosynthesis operons and several genes coding for aminoacyl-tRNA synthetases, and tRNA^{Leu} functions as a transcription factor of the RNA polymerase III in silkworm.

1.1.2. Features of tRNA and tRNA gene structures

Lengths of most known tRNAs range from 74 to 98 nucleotides (Westhof and Auffinger 2001). Before becoming a mature tRNA, the precursor tRNA is subjected to modification steps in which its 5’ leader, 3’ trailer and intron are removed (Phizicky and Hopper 2010). Hence, lengths of tRNA genes are much longer than their functional products. However, in most tRNA genes that have been reported so far, the regions encoding the extra sequences at both ends of the precursor tRNAs are excluded. In this thesis, tRNA genes do not include sequences coding for the 5' leader and 3' trailer.

The last three nucleotides at the 3' end of all mature tRNAs are always "CCA". This 3' end CCA is encoded by tRNA genes in prokaryotes while it is added by the enzyme tRNA nucleotidyltransferase after transcription in eukaryotes (Schmidt and Söll 1981; Mohan *et al.* 1999). This CCA-terminus functions in the binding between a tRNA molecule and an amino acid. It has been reported that substitutions of these nucleotides affect the aminoacylation reaction (Zhou *et al.* 2011).

In a tRNA gene, there are two signature sequences to recognize the internal promoters. They are called box A and box B, and located within the coding region. Moreover, like protein-coding genes, tRNA genes also contain intervening sequences, that is, introns. In *S. cerevisiae*, approximately 20% of the tRNA genes contain introns (Goldman 2008). All characterized, intron-bearing tRNA genes of eukaryotes have a single intron which is frequently located one nucleotide downstream of the anticodon (Schmidt and Söll 1981; Sugahara *et al.* 2007; Goldman 2008). In archaeal species, it has been reported that tRNA genes can have multiple introns which are located at various locations in the tRNA (Sugahara *et al.* 2007; Randau and Soll 2008). The lengths as well as the nucleotide composition of tRNA introns are diverse (Schmidt and Söll 1981).

In mature tRNAs, there are a number of unusual nucleotides derived from the modification of the normal ones. There have been 92 types of modified nucleotides found in tRNA sequences from different organisms (Cantara *et al.* 2011). The average number of these nucleotides in each tRNA sequence is 12.6 in *S. cerevisiae* (Phizicky and Hopper 2010). Although they are not crucial for the viability of the cells, they have an important role in maintaining the stability of tRNA structure as well as in the decoding property of tRNAs (Bjork *et al.* 1987; Toh *et al.* 2001).

In the flanking regions of tRNA genes, several repeated sequences have been found and may have some relationship with these genes. In *S. cerevisiae*, the three transposable elements, *sigma, delta* and *tau*, are often found to be located adjacent to the tRNA genes in their upstream sequences (del Rey, Donahue and Fink 1982; Eigel and Feldmann 1982; Genbauffe, Chisholm and Cooper 1984). Among them, the *sigma* element frequently shows a constant distance from 16 to 18 base pairs to the tRNA gene (del Rey *et al.* 1982). Moreover, in *Candida albicans*, another transposable element called *beta* is found to be positioned close to the tRNA genes (Perreau, Santos and Tuite 1997). In the downstream region, a stretch of thymidines is usually found. This poly-T is proposed to be the termination site of the tRNA transcription process (Schmidt and Söll 1981; Pavesi *et al.* 1994; Hamada *et al.* 2000).

The most typical feature of tRNAs is their ability to form a cloverleaf model including four stems and four loops formed from the base pairings of residues in the tRNA sequence. Nucleotides in this secondary structure are numbered from 0 to 76 with the letter suffixes added for the additional nucleotides as shown in **Figure 2**. The residue 0 is only found in tRNAs specific for histidine (Westhof and Auffinger 2001).

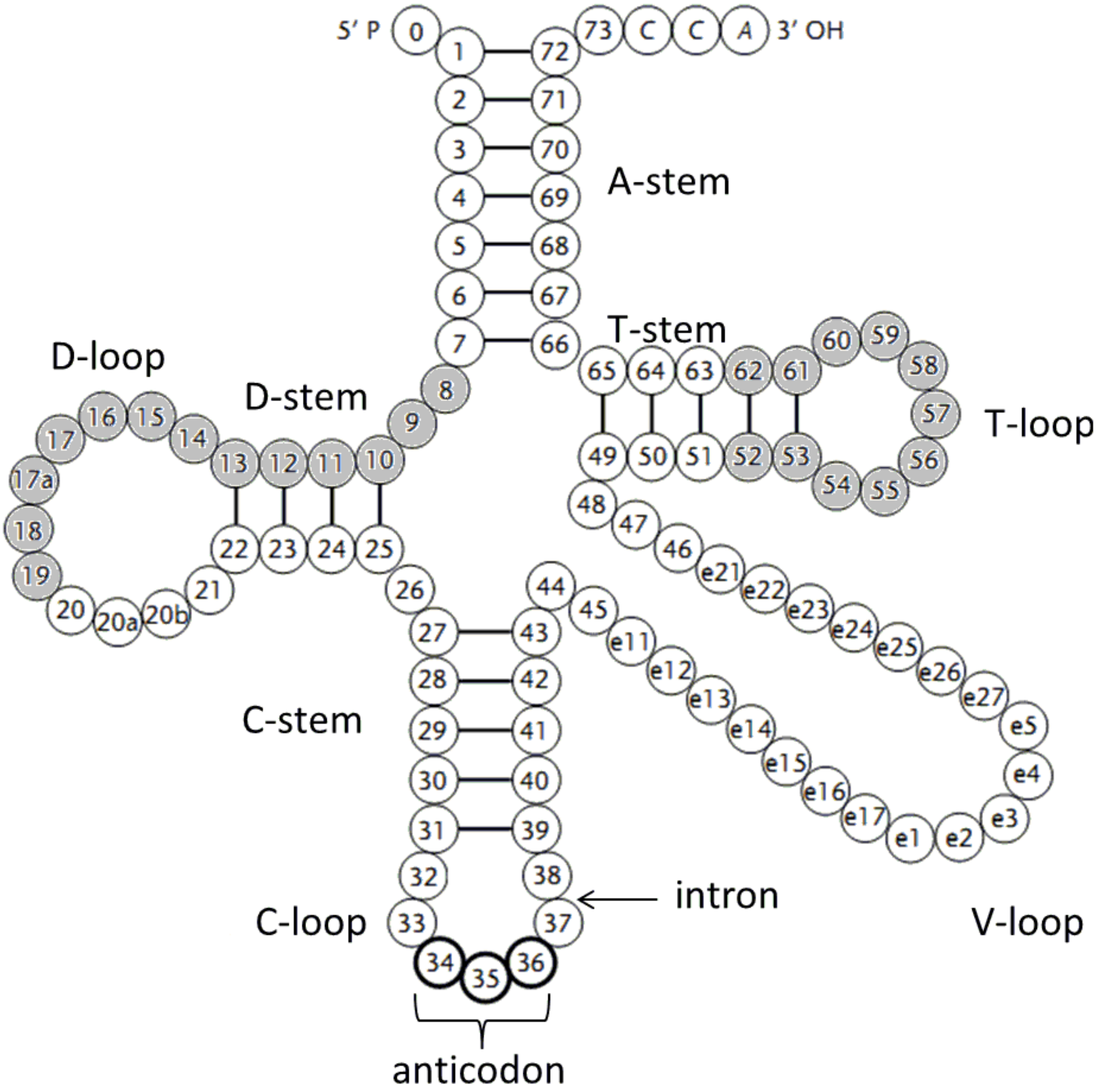


Figure 2. Cloverleaf structure of tRNAs

(reproduced from Westhof and Auffinger 2001)

(The positions highlighted in grey colour within the D stem and loop region as well as within the T stem and loop region represent the box A and box B, respectively)

The following details of the stems and loops of the cloverleaf structure are reviewed by Goldman (2008). The acceptor stem, or the A-stem, consists of seven base pairs. The two strands of this hairpin structure are from nucleotides at both ends of the tRNA sequence except for the 3' CCA terminus and the residue 0. The A-stem is involved in attaching the amino acid to the tRNA; hence, it is called acceptor stem. The hairpin shown on the left hand side of the cloverleaf comprising three to four base pairs is called the D-stem since it usually contains the modified nucleotide dihydrouridine (D). The loop next to this stem is the D-loop, which often comprises 8-11 nucleotides. The anticodon hairpin, also called the AC-stem or C-stem, consists of five base pairs and is followed by a loop of seven nucleotides harbouring the anticodon in the middle. Downstream of the anticodon stem and loop is the variable loop (V-loop). The variable loops of different tRNAs are highly diverse in their lengths which classify tRNAs into two classes. The class I tRNAs have a short V-loop, which spans four to five nucleotides in length, while the class II tRNAs have a much longer V-loop, from 10 to 24 nucleotides in length. In eukaryotic organisms, the class II tRNAs consist of those coding for leucine and serine (Söll and RajBhandary 1995). The T-stem and T-loop are at the right hand side of the cloverleaf model. They are also called the TΨC- stem and loop due to the presence of the unusual nucleotides including ribothymidine (T) and pseudouridine (Ψ). These stem and loop structures contain five base pairs and seven nucleotides, respectively. The two internal promoter signal sequences, box A and box B, are located within the D- and T- regions, at positions 8-19 and 52-62, respectively.

The locations and structures of tRNA introns were reported by Randau and Soll (2008) as well as Sugahara *et al.* (2007). Before the splicing process, the intervening sequence is frequently located in the C-loop of the precursor tRNA, between position 37 and 38. In addition to this canonical location, tRNA introns can be found in other non-canonical positions such as D-, T- loops and C-stem. Both of the canonical and non-canonical introns have been found in archaeal organisms. Most archaeal tRNA introns have a structurally conserved motif at the junctions between exon and intron regions. It consists of a central helix (H) comprising four base pairs and two bulges (B), each of which contains three nucleotides; thus, it is widely known as the BHB motif. Moreover, for introns not located between positions 37 and 38, this motif is often simplified into HBh' as shown in **Figure 3**. In either the BHB motif or the HBh' motif, the splicing sites are positioned right after two nucleotides downstream of the two strands of the central helix. Based on these motifs, the archaeal splicing endonuclease enzymes can identify and remove the intron sequences.

There has been no mention of the BHB motif in eukaryotic tRNA genes containing introns; however, it was reported that the splicing endonuclease enzyme in eukaryotes could recognize this motif and excise the intervening sequence *in vitro* as well as *in vivo* (Randau and Soll 2008). Therefore, it is worth considering these motifs in the detection of tRNA introns in eukaryotic species, especially the ones not located at the canonical intron position.

As described by Goldman (2008), all tRNAs have similar tertiary structures, which have a typical L-shape pattern (**Figure 4**). The base pairings in the cloverleaf model are maintained in this three-dimensional structure. The CCA-terminus and the anticodon, which respectively bind to the corresponding amino acid and codon, are at the two ends of the L-shape model. This arrangement helps tRNAs fit into the ribosomes and function as adaptors in the translation process.

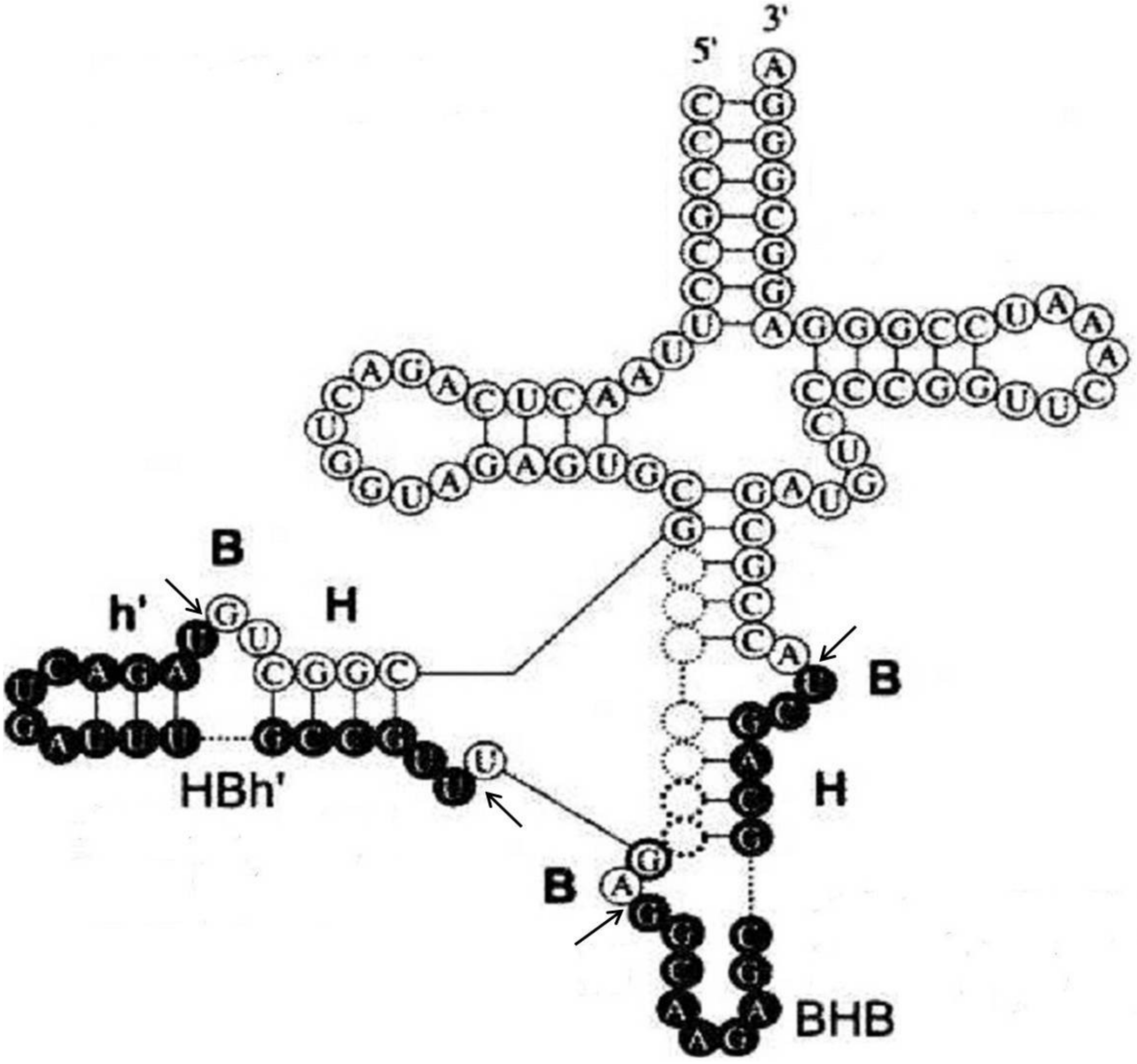


Figure 3. Structures of BHB and HBh' motifs

(reproduced from Sugahara *et al.* 2007)

(*The solid and clear circles illustrate nucleotides in the intron and exon regions, respectively. The arrows show the splicing sites.*)

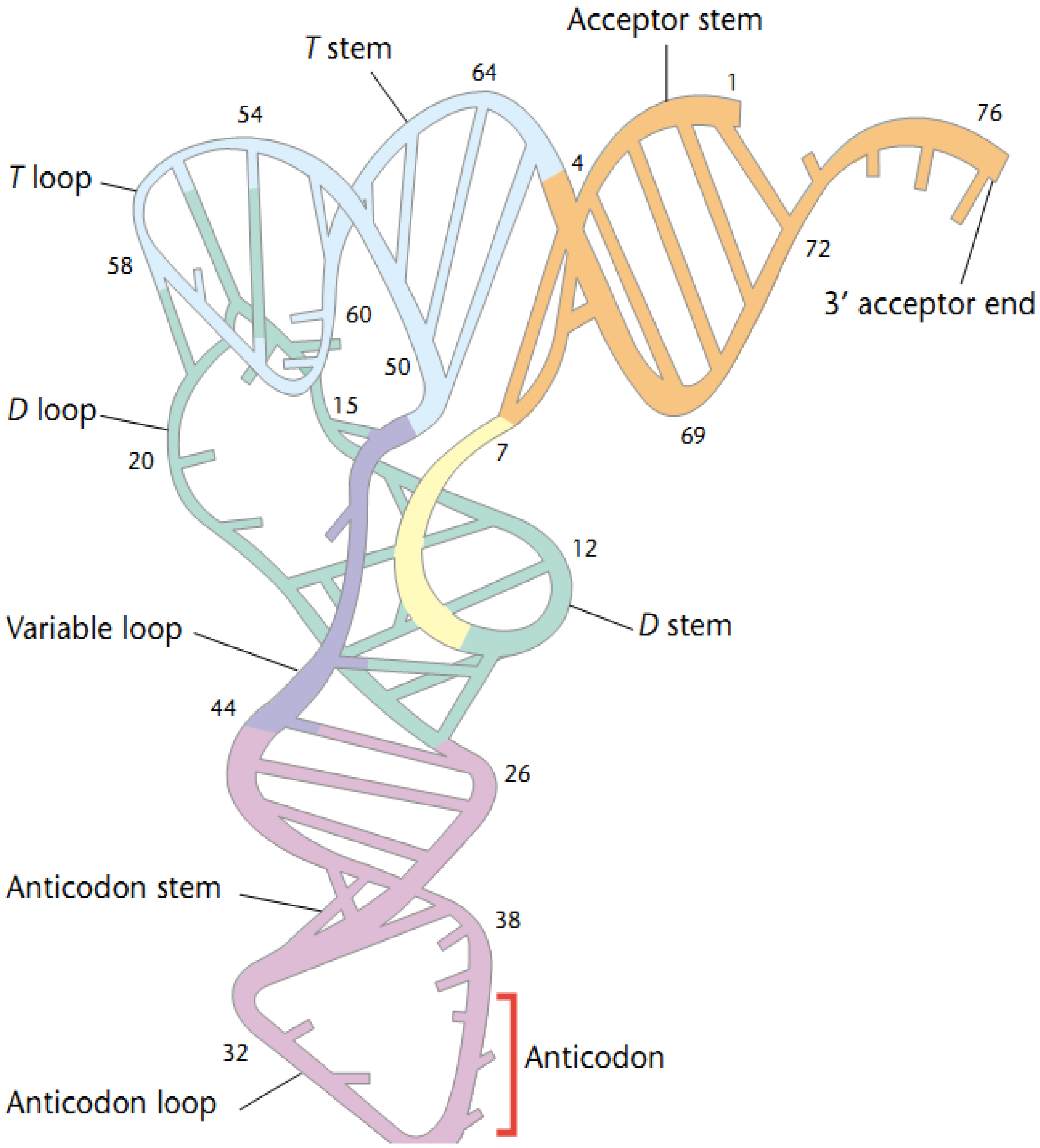


Figure 4. Tertiary structure of tRNAs

(reproduced from Goldman 2008)

1.1.3. Synthesis of tRNA in the cell

The synthesis of a functional tRNA in eukaryotic cells is illustrated in **Figure 5**. First, a tRNA gene is transcribed in the nucleolus by the enzyme RNA polymerase III. In this step, two transcriptional factors, TFIIIB and TFIIIC, recognize and bind to the internal promoter sequences (box A and box B) of the tRNA gene to initiate the transcription process (Toh *et al.* 2001). The product from the transcription harbours extended sequences at both ends, which are removed in the subsequent steps as described by Phizicky and Hopper (2010). The removal of the 5' leader takes place before the elimination of the 3' trailer and is carried out by endonuclease RNase P. The 3' end processing is catalyzed by multiple endonucleases and exonucleases. Next, CCA is added to the 3' end of the precursor tRNA by the enzyme tRNA nucleotidyltransferase in the nucleus. Also, several nucleotides in this pre-tRNA sequence are modified. This modification process is carried out at the inner nuclear membrane. Subsequently, the pre-tRNA is exported to the cytoplasm by a protein called Los1. If the pre-tRNA contains an intervening sequence, this intron is removed during the splicing process that takes place at the cytoplasmic surface of the mitochondria. In the cytoplasm, more nucleotides are modified. The modification process requires various enzymes since there are a large number of different tRNA modifications. Finally, the mature tRNA is available in the cytoplasm and can be charged with its corresponding amino acid. The aminoacyl-tRNA formed by the aminoacylation reaction can then be used in the translation process.

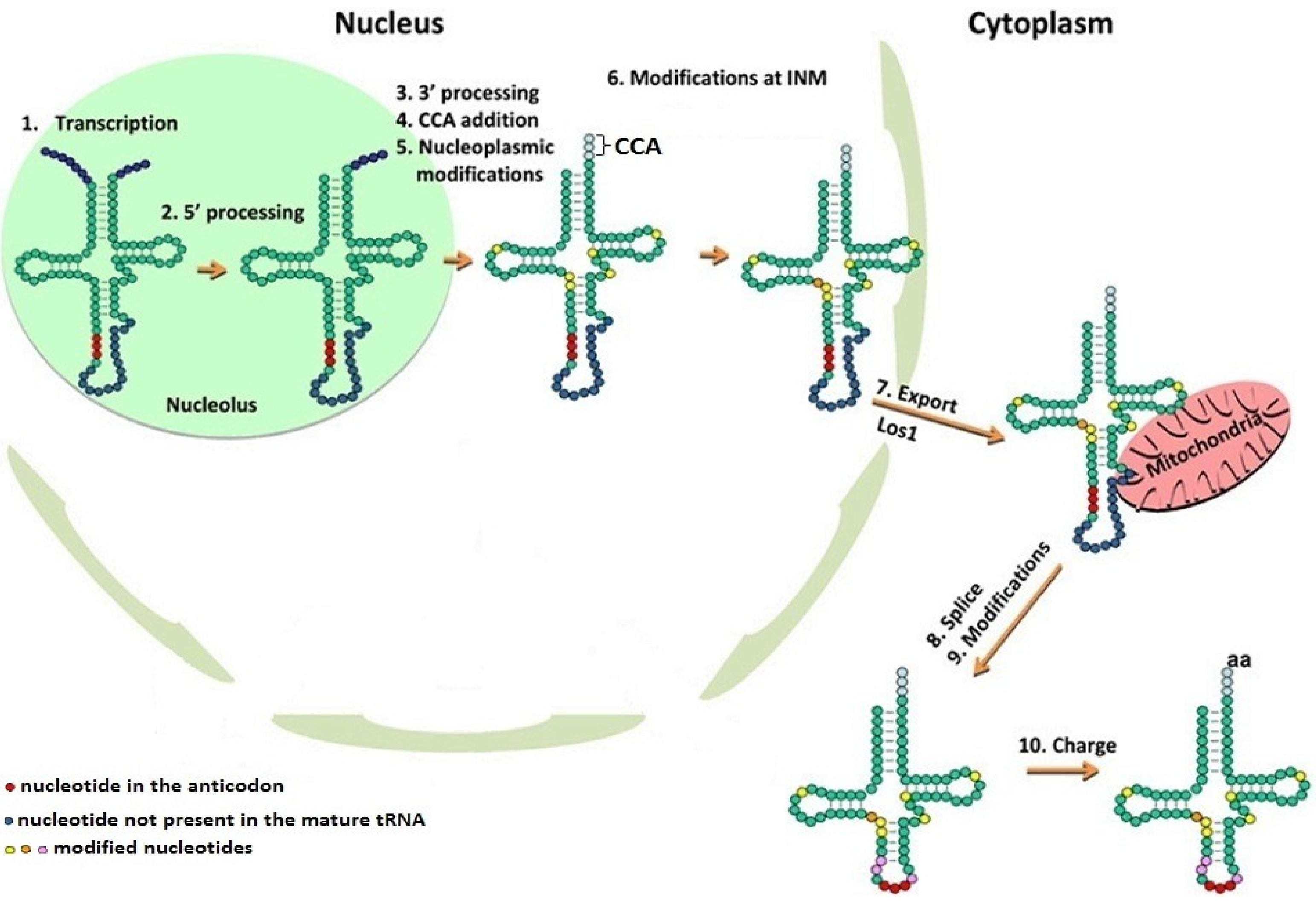


Figure 5. Synthesis of tRNA in a yeast cell (reproduced from Phizicky and Hopper 2010)

(Abbreviation: INM, inner nuclear membrane)

1.1.4. Two special tRNAs: tRNA^{SeC} and tRNA^{Pyl}

1.1.4.1. Selenocysteine and tRNA^{SeC}

The biosyntheses of selenocysteine (SeC) and SeC-containing enzymes as well as functions of tRNA^{SeC} in these processes were reviewed by Turanov *et al.* (2011) and Böck (2001). Although this 21st amino acid is not commonly present in protein sequences as compared to the 20 universal amino acids, this special residue has been found in various enzymes from a number of species belonging to all three domains of life: bacteria, archaea and eukarya. Many enzymes involved in methanogenesis in *Methanococcus jannaschii* are SeC-containing proteins. Morever, enzymes involved in the synthesis of thyroids hormones in some mammals have been found to contain SeC. In addition, it has been reported that when knocking out the gene encoding tRNA^{SeC} in mouse, this organism cannot survive in embryogenesis stage. The codon coding for SeC is UGA, which is generally a stop codon. To distinguish between the UGA codon coding for SeC and the one terminating the translation process, there is a signal element named SECIS (SeC insertion sequence) in the 3'-untranslated region of the corresponding gene. The distance between SECIS and the SeC-encoding UGA codon is at least 55 bases and can be up to 2.7 kb (Commans and Böck 1999; Böck 2001). The SeC insertion sequence has a stem-loop structure that can be recognized and bind to proteins specific for the integration of SeC into the polypeptide chain. In addition to these proteins, tRNA^{SeC} plays an important role in the synthesis of SeC-containing enzymes since it is an adaptor carrying SeC to the polypeptide chain. Moreover, tRNA^{SeC} is involved in the SeC biosynthesis process which consists of two steps. Initially, serine is attached to tRNA^{SeC} by an aminoacylation reaction catalyzed by seryl-tRNA synthetase. Then, seryl-tRNA^{SeC}

is converted into selenocysteinyl-tRNA^{SeC} by different reactions catalyzed by seryl-tRNA synthetase as well as several other enzymes.

Transfer RNAs specific for SeC have been found in all five kingdoms of life: monera, protista, fungi, plantae and animalia (Hatfield *et al.* 1992). This special tRNA has some different features in its structure as compared to other tRNAs. In the secondary structures of all known eukaryotic tRNAs specific for SeC, the numbers of base pairs in the acceptor, D- and T- stems are different from those of normal tRNAs. There are 9, 6 and 4 base pairs in these stems respectively in tRNA^{SeC} instead of 7, 4 and 5 in the general cloverleaf model. Also, the number of nucleotides in the D-loop is only 4 instead of 8-11 as in other tRNAs (**Figure 6**) (Commans and Böck 1999; Böck 2001).

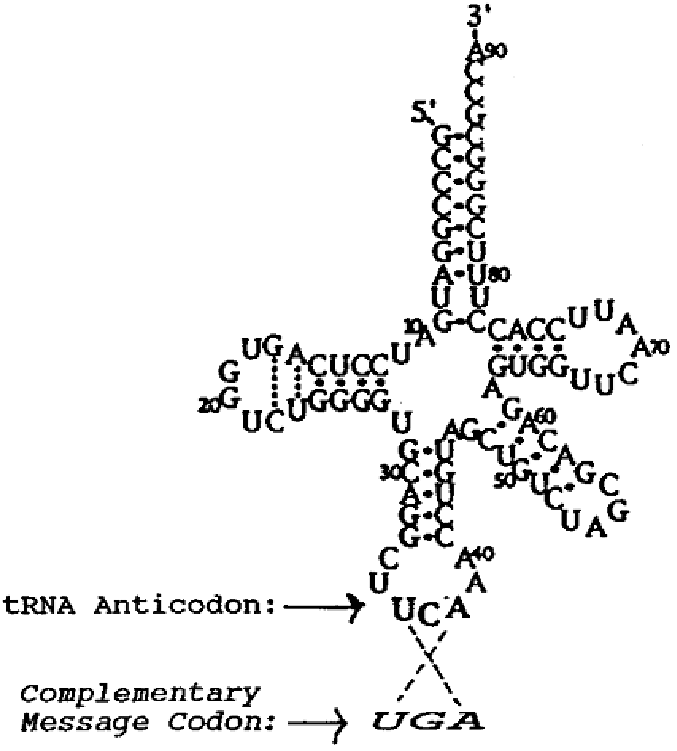


Figure 6. Secondary structure of an eukaryotic tRNA^{SeC} (Stadtman 1996)

1.1.4.2. Pyrrolysine and tRNA^{Pyl}

In addition to SeC, pyrrolysine (Pyl) is another new residue added to the genetically encoded amino acid list. While diverse SeC-containing proteins have been found in various organisms of three domains of life, Pyl has only been found in methylamine methyltransferase and some transposase enzymes in a few archaeal and bacterial species. This 22nd amino acid is encoded by UAG, a stop codon, and Pyl-utilizing species use UAG as a Pyl-encoding codon more effectively than a stop codon (Zhang *et al.* 2005). The biosynthesis of Pyl is dissimilar from SeC since it is independent of tRNA^{Pyl}. In the Pyl biosynthesis process, two lysine residues are converted to one Pyl molecule by using several enzymes including PylB, PylC and PylD. Subsequently, tRNA^{Pyl} is charged with Pyl in an aminoacylation reaction catalyzed by pyrrolysyl-tRNA synthetase. The Pyl-tRNA^{Pyl} complex can be recognized by the normal translational elongator factor and carried to the ribosome for incorporating Pyl into the polypeptide chain. When considering the secondary structures of tRNAs specific for Pyl found in bacterial and archaeal organisms, there are some differences from the general cloverleaf model since these Pyl specific tRNAs have three nucleotides in the V-loop, an anticodon-stem containing six base pairs, a short D-loop and only one nucleotide between D-stem and acceptor-stem (Gaston, Jiang and Krzycki 2011).

1.2. Relationships between tRNA gene number, tRNA abundance and codon usage

According to the universal genetic code, each amino acid, except for Met and Trp, is encoded by multiple codons. However, these synonymous codons are not used equally in most genomes (Plotkin and Kudla 2011). As reported by Ikemura (1981, 1982, 1985), codon usage bias is not a random event. In fact, protein-encoding genes from the same species tend to use similar codon usage systems, and the codon usage patterns from different kinds of organisms do not follow the same manner. For instance, the codon usage patterns of *S. cerevisiae* and *E. coli* are very different. Thus, each genome has its own organism-specific codon-choice pattern. Moreover, the codon selection is highly affected by the tRNA availability. There is a strong correlation between the abundances of tRNAs and the occurrences of the corresponding codons in *S. cerevisiae* as well as in *E. coli*. It is believed that this relationship is found not only in these two species but also in many other organisms. On the other hand, it has been reported that the amount of tRNA in the cell is closely related to the number of tRNA genes. In *S. cerevisiae* and *B. subtilis*, the correlation coefficient values (r) of this relationship are 0.91 and 0.86, respectively (Percudani, Pavesi and Ottonello 1997; Kanaya *et al.* 1999). Thus, the tRNA gene number can be used to estimate the tRNA content in the cell. Furthermore, in *S. cerevisiae*, a strong correlation was found between the tRNA gene number and the synonymous codon occurrence (r = 0.91) (Percudani *et al.* 1997). Also, the number of tRNA genes was shown to have significant relationship with the amino acid frequency in *S. cerevisiae* (r = 0.84) and in *C. elegans* (r = 0.82) (Percudani *et al.* 1997; Duret 2000).

Considering individual genes, the correlation between the tRNA availability and the codon-choice pattern is clearly found in the highly expressed genes as well as the ones encoding abundant proteins. Codons well-matched with the anticodons of the most abundant tRNAs are used more frequently than the other synonymous ones in these genes; therefore, based on the amount of tRNAs and the interaction between codon and anticodon, the most preferred codon, or optimal codon, of each amino acid can be deduced. Since the gene numbers of different tRNAs are correlated with their abundances, tRNA gene numbers in a genome sequence can be used to infer the optimal codons for the corresponding species (Ikemura 1981, 1982; Percudani *et al.* 1997).

1.3. tRNA gene prediction methods

Developed in 1997, tRNAscan-SE has been the most widely used tRNA gene prediction tool for many genome projects due to its high accuracy (Lowe and Eddy 1997). However, this tool cannot deal with genes harbouring non-canonical introns or long introns. Hence, to improve the prediction performance, other predictors have been created. Among them, SPLITSX shows a unique ability to detect tRNA genes containing multiple introns (Sugahara *et al.* 2007). ARAGORN has an advantage in predicting tRNA genes having long introns, which could be thousands of nucleotides (Laslett and Canback 2004). When using tRNAfinder, the intron lengths can be set by users (Kinouchi and Kurokawa 2006). Algorithms of the four most commonly used tRNA gene prediction programs are described below.

1.3.1. The tRNAscan-SE algorithm

tRNAscan-SE predicts tRNA genes by combining three different predictors including tRNAscan (Fichant and Burks 1991), EufindtRNA (Pavesi *et al.* 1994; Lowe and Eddy 1997), and a tRNA gene search tool using probabilistic models (Eddy and Durbin 1994).

The first program, tRNAscan, was developed by Fichan and Burks (1991). It runs the prediction in sequential steps, each of which examines a common feature of either primary or secondary structure of tRNAs. If the scanning fails at any step, the prediction will initiate in the next windowed region of the input sequence. Firstly, tRNAscan begins by searching for T stem and loop regions from position 44 of the examined sequence. The scanning is based on some conserved and semi-conserved nucleotides within the T regions and the ability to form a loop of seven nucleotides and a stem of 4-5 base pairs. Secondly, it scans for D-stem and D-loop in the upstream area of the identified T region. Thirdly, sequences at the 5'end of the D-region and the 3'end of the TΨC-region are considered to see if they can form base pairings in order to examine the aminoacyl stem which consists of six or seven base pairings. Fourthly, the anticodon stem, which includes four or five base pairings, is inferred from the last position at the 3' end of D-stem. The two stems are separated by one nucleotide. Also, the 7-nucleotide anticodon loop is deduced from the 5'-half of the anticodon stem. In case where the four or five nucleotides downstream of the anticodon loop cannot form base pairings with the 5'-half of the anticodon stem, the tRNA is considered to contain an intron, which is located one nucleotide downstream of the anticodon. The initial length of the intron is eight nucleotides. This length is then increased one nucleotide at a time iteratively while the program proceeds to find an appropriate 3'-half of the anticodon stem. While the size of the intron is increased, the length of the variable loop is also considered so that it should not be less than three nucleotides. Once the anticodon stem is found, the nucleotide upstream of the intron, which can only be either A or G, is tested. Finally, the tRNA gene candidate is checked for the presence of T at the 5' end adjacent to the anticodon.

The second program, EufindtRNA, was developed by Pavesi *et al.* (1994). EufindtRNA utilizes an algorithm which is based on the weight matrices and weight vectors established from four features of a tRNA gene. These features include the A and B boxes of the internal promoter, the distance between them and the distance between the B box and the transcriptional termination site. The training data set used in this algorithm comprises 231 tRNA genes obtained from Sprinzl (Gauss and Sprinzl 1983a, 1983b), GenBank (Burks *et al.* 1992), and EMBL (Higgins *et al.* 1992) databases. Each value in the matrices is calculated from the frequency of nucleotides at different positions in the A box and B box regions. On the other hand, distances between the A box and B box and between B box and transcription termination site are calculated for each sequence in the training set to build the two corresponding weight vectors. Based on the weight vectors and weight matrices, the tRNA gene search from a query sequence is carried out in sequential steps (**Figure 7**). In the first step, the algorithm searches for the B box region by calculating a B-box score which is the sum of the values from the corresponding weight matrix of the nucleotides in the B box candidate. If the score is equal or higher than the cut-off value, the process is subjected to the second step in which the A box region is searched in the region of 24-139 nucleotides upstream the previously identified B box. The A-box score is then calculated in a similar way and added to the first score to obtain the intermediate score. If this score is higher than the threshold, the third step is done by searching for the transcriptional termination site which contains at least four thymidines in the region of 133 nucleotides downstream the 3' end of the B box. In the fourth step, the boundaries of the candidate tRNA are identified by considering six nucleotides upstream of the A box region and 11 nucleotides downstream of the B box region. The anticodon is identified by considering the subsequent three nucleotides of the 8-nucleotide region downstream of the A box. When using the selected cut-off values, all of the tRNA genes in the training set are correctly detected except for those of $tRNA^{SeC}$. These special tRNAs are also detected by the algorithm with some modifications in the threshold values and rules which are specific for the $tRNA^{SeC}$ in the training set.

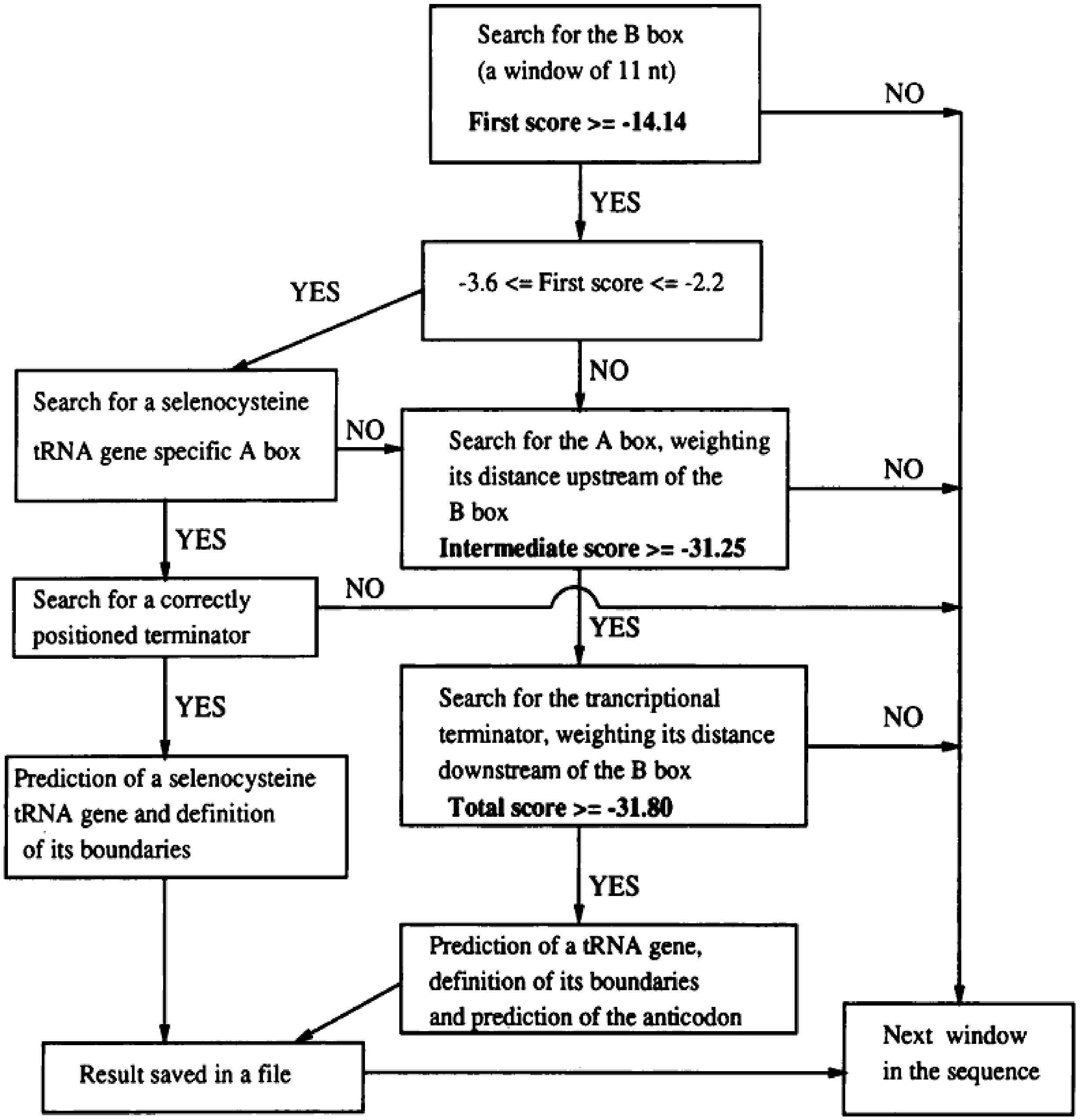

Search for the B box
(a window of 11 nt)
First score >= -14.14
NO
YES
-3.6 <= First score <= -2.2
YES
NO
Search for a selenocysteine tRNA gene specific A box
NO
Search for the A box, weighting its distance upstream of the B box
Intermediate score >= -31.25
NO
YES
Search for a correctly positioned terminator
NO
YES
YES
Search for the trancriptional terminator, weighting its distance downstream of the B box
Total score >= -31.80
NO
Prediction of a selenocysteine tRNA gene and definition of its boundaries
YES
Prediction of a tRNA gene, definition of its boundaries and prediction of the anticodon
Next window in the sequence
Result saved in a file

Figure 7. Schematic description of the algorithm implemented by EufindtRNA

(Pavesi *et al.* 1994)

The third algorithm used in the tRNAscan-SE program was established by Eddy and Durbin (1994). It utilizes a probabilistic model to predict tRNA genes. The probabilistic model is built from the structural sequence alignment profile of 1415 non-redundant tRNA sequences obtained from the Sprinzl database (Steinberg, Misch and Sprinzl 1993). This model can present both primary and secondary structures of the tRNAs; therefore, it is also called a covariance model. The covariance model is based on an ordered tree. To describe an RNA structure in this tree, the base pairs are represented by the pairwise nodes, and the nucleotides in the single strand are represented by the single nodes (**Figure 8**). To describe a RNA multiple sequence alignment, each node in the tree represents a column in the profile. The probabilistic model, or the covariance model, built from the alignment profile is a hidden Markov model represented in the tree structure. Therefore, a covariance model can show not only the primary structure of the sequences in the training set but also their secondary structures. To predict a tRNA gene, an alignment is made between the query sequence and the covariance model to obtain a probabilistic score, which is then used to identify the prediction result. The score is reported in bits. If it is above zero, the matching between the predicted tRNA and the profile is more likely than a random matching. The higher the score is, the more significant the matching is. For the intron prediction, intron sequences were manually inserted into the Sprinzl alignment for the 38 intron-containing tRNA genes in the training set. Therefore, a covariance model can also predict tRNA genes containing intervening sequences.

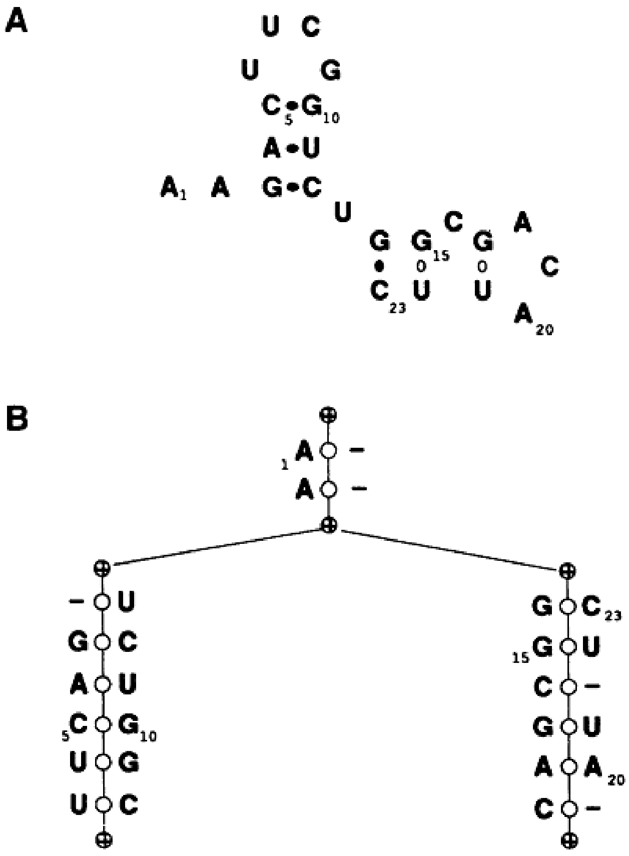


Figure 8. Example of an RNA structure and its presentation in an ordered tree

(reproduced from Eddy and Durbin 1994)

((A) RNA structure; (B) Presentation of the RNA structure as an ordered tree)

Using the three described algorithms, Lowe and Eddy (1997) developed tRNAscan-SE which predicts tRNA genes from the whole genome in different stages (**Figure 9**). Firstly, tRNAscan 1.4 and EufindtRNA are used to initially detect the tRNA genes. There was a little modification in the algorithm of EufindtRNA when it was integrated into tRNAscan-SE. The intermediate score was decreased from -31.25 to -32.10, and the transcriptional termination site was not searched as in the algorithm described above.

Moreover, the introns predicted by tRNAscan 1.4 were discarded since this program did not report reliable introns. The transfer tRNA genes specific for SeC are predicted by EufindtRNA. Secondly, predicted results from the two programs are combined and subjected to the next prediction stage using the previously described tRNA covariance model. There is a covariance model specific for tRNA^{SeC} genes. Each of the tRNA gene candidates from the first stage is aligned to its corresponding covariance model and gets a probabilistic score, or the "*complete tRNA*" score. Those having "*complete tRNA*" scores less than the threshold (20 bits) are eliminated. The others are checked to see if they are true tRNA genes or pseudogenes. In addition, there was a hidden Markov model (HMM) built from the same tRNA alignment profile, which was used to build the covariance model. Unlike the covariance model, this HMM only describes the primary structures of the tRNAs. When aligning a tRNA candidate sequence to this HMM, a "*primary structure-only*" score is obtained, and the "*secondary structure-only*" score is calculated as followed:

"secondary structure-only" score = "complete tRNA" score - "primary structure-only" score

The candidates having *"primary structure-only"* and *"secondary structure-only"* scores less than the cut-off values are considered as the pseudogenes. Lastly, the secondary structure prediction is carried out by the global structure alignments to the tRNA covariance models. If there are more than four consecutive non-consensus nucleotides in the anticodon loop, these are considered as those of the intron.

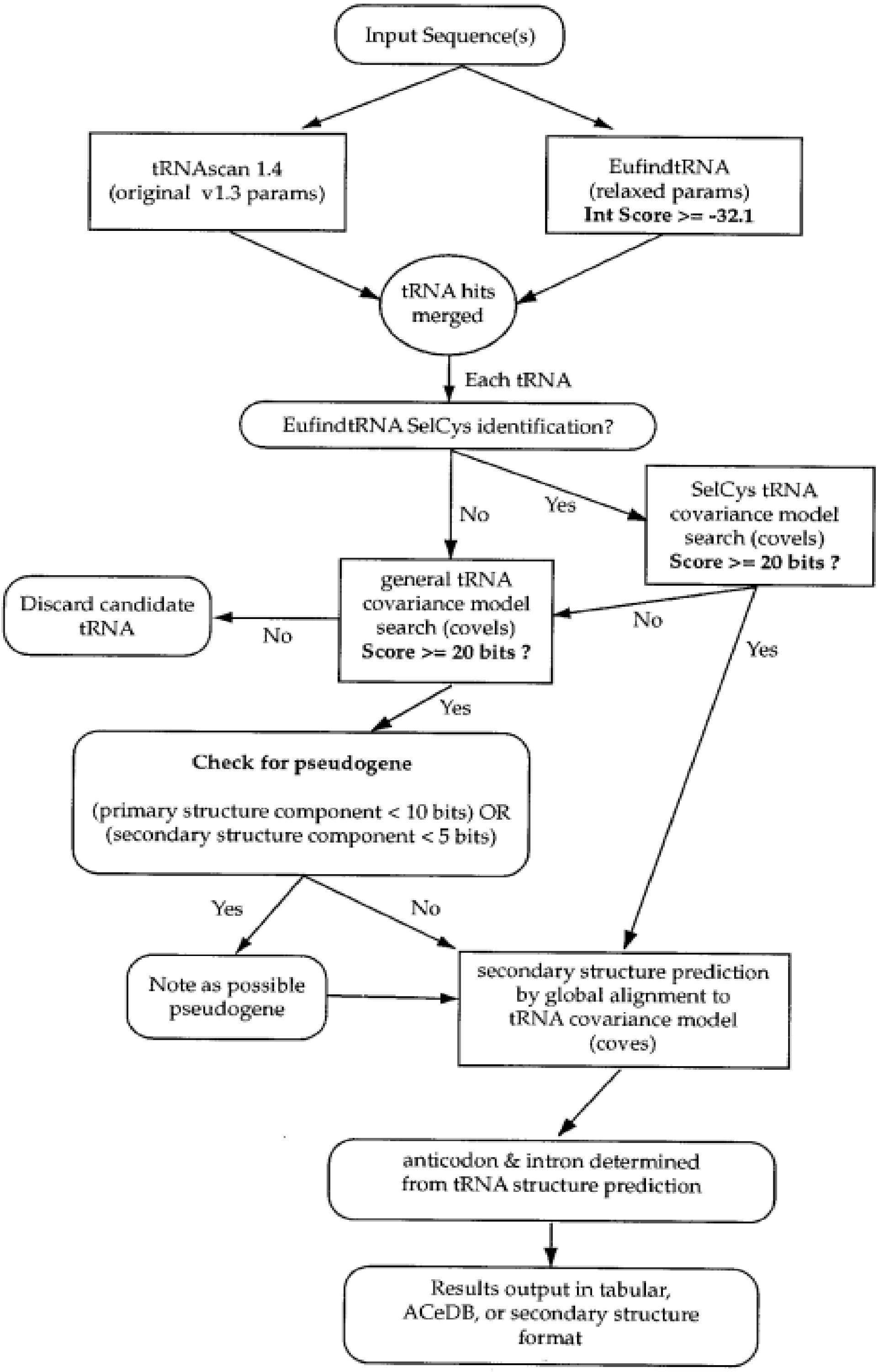

Input Sequence(s)
tRNAscan 1.4
(original v1.3 params)
EufindtRNA
(relaxed params)
Int Score >= -32.1
tRNA hits merged
Each tRNA
EufindtRNA SelCys identification?
No
Yes
SelCys tRNA covariance model search (covels)
Score >= 20 bits ?
Discard candidate tRNA
No
general tRNA covariance model search (covels)
Score >= 20 bits ?
No
Yes
Yes
Check for pseudogene
(primary structure component < 10 bits) OR
(secondary structure component < 5 bits)
Yes
No
Note as possible pseudogene
secondary structure prediction by global alignment to tRNA covariance model (coves)
anticodon & intron determined from tRNA structure prediction
Results output in tabular, ACeDB, or secondary structure format

Figure 9. Description of tRNAscan-SE's algorithm

(Lowe and Eddy 1997)

1.3.2. The SPLITSX algorithm

Unlike other tRNA gene predictors, SPLITSX, which was developed by Sugahara *et al*. (2007), can detect tRNA genes containing multiple introns which can be located in either canonical or non-canonical locations. Firstly, it searches for BHB motifs, which include either the canonical motif (BHB) or the non-canonical one (HBh'), in the genomic sequences (**Figure 3**). The splicing sites are recognized at the two nucleotides downstream of each strand of the central helix (H). Secondly, the intron sequences are removed. Finally, different patterns of genomic sequences created from all possible combinations of intron removals are subjected to the tRNAscan-SE program. The original genomic sequences in which the predicted introns are not eliminated are also used in the input of tRNAscan-SE. For the genes predicted in overlapping regions, the one having the highest score, which is obtained from the covariance model, is selected.

As described by Sugahara *et al.* (2007), the search for BHB motifs is carried out by using sequence homology and structure-based methods. The sequence homology search uses several position weight matrices for sequences of the helices and bulges within the motif structure to scan for the motif regions. These matrices were built from documented BHB motifs. The initial candidates detected by using the position weight matrices are then screened for the minimal BHB secondary structure which should have a four base pair central helix (H) not having more than two mismatches, the 5' bulge (B), and the outer helix (h') having length longer than one base pair. Finally, predicted BHB structures are subjected to the RNAeval program (Schuster *et al.* 1994) to estimate the free energies. Only those having free energies less than the threshold (3 kcal/mol) are selected.

1.3.3. The ARAGORN algorithm

ARAGORN was developed by Laslett and Canback (2004). First, it searches for the T-loop region by looking for the conserved sequence "GTTC" in the B box. When a non-gapped hit is found, the flanking sequences around it are scanned to build the T-loop and T-stem structures which are about 5-9 nucleotides and 4-5 base pairs in length, respectively. Subsequently, the consensus sequence of a part of the A box (TRGYNAA) is searched in the region of 28-85 nucleotides upstream of the T-stem. The region around the A box is then considered to form a D-loop which was 5-11 nucleotides in length and contained the sequence A-----GG-R. The A-stem from seven to nine nucleotides in length is then formed from the regions of 2-3 nucleotides upstream of the D-stem and immediately downstream of the T-stem. Also, the 5'-end sequence of the C-stem is formed in the location one nucleotide downstream of the D-stem. When this 5'-half of anticodon stem, or the C-stem, is found, its complementary strand is searched in the region downstream of the T-stem. The V-loop is constructed from the sequence downstream of the T-stem and upstream of the C-stem. Its length is from 3 to 25 nucleotides. Finally, the C-loop is found from the sequence between the two complementary strands of the C-stem. Introns predicted by ARAGORN are all located in the C-loop and could be up to 3000 nucleotides in length. Like tRNAscan-SE, ARAGORN can also predict pseudogenes.

1.3.4. The tRNAfinder algorithm

tRNAfinder, developed by Kinouchi and Kurokawa (2006), consists of a set of programs written in the C language to detect tRNA genes in sequential stages. First, it searches for all possible regions that could form the secondary structure specific for tRNA genes. This work is carried out in the following steps: (*i*) The A-stem containing seven base pairs is identified, and those having a loop of 56-80 nucleotides between the two 7-nucleotide complementary strands are selected. (*ii*) The sequence upstream of the 3' half of the acceptor-stem is considered to see if it could form the T-loop of seven nucleotides and a T-stem of five base pairings. (*iii*) The region from two nucleotides downstream of the 5' half of the A-stem is checked for its ability to form a D-loop of 7-11 nucleotides, and a D-stem of 3-4 base pairings. (*iv*) The sequence from one nucleotide downstream of the D-stem is checked for the ability to form a C-stem of five base pairings and a C-loop of seven nucleotides. The V-loop is located upstream the T-stem. When scanning for the cloverleaf structure, the base pairing between "G" and "T" is also acceptable in addition to the two normal ones ("A-T" and "G-C"). Secondly, tRNAfinder selects one candidate having the highest mismatch score among the overlapping ones. This score is based on the number of mismatches found in the stems in which the score assigned for "A-T" and "G-C" is 0, those of "G-T" and other base pairings are -1 and -5, respectively. Thirdly, the nucleotides which are found to be conserved in experimentally identified tRNAs from the Sprinzl database (Sprinzl *et al.* 1998) are checked to select more reliable candidates. Lastly, the number of mismatches is checked again, and those less than the threshold value are eliminated. tRNAfinder predicts only tRNA genes containing one intron. The intron length can be set by the user.

1.4. Algorithm of Bowtie, an efficient short-read mapping tool

Bowtie is one of the most widely used tools for aligning millions of short nucleotide sequences, or short reads, to large genomes since it is much faster and more memory-efficient than the other alignment programs (Trapnell and Salzberg 2009). By using a different indexing scheme, named Burrows-Wheeler Transform (BWT) and Index, Bowtie has demonstrated its advantages over other short-read mapping tools. According to Langmead *et al.* (2009), an index is generally built from oligomers originating from either the query sequence or the reference genome. Therefore, for a large genome and a huge number of query sequences, it requires much memory for storing the index and time for searching within it. The BWT-based index used in Bowtie does not contain this large number of oligomers. Thus, it uses much less memory space. Also, there is an efficient algorithm helping Bowtie to search for the matches within this index in much less time than other mapping tools. The procedure to create a BTW-based index from a reference genome, and how to search for location of a short RNA sequence in the genome are illustrated in **Figure 10**.

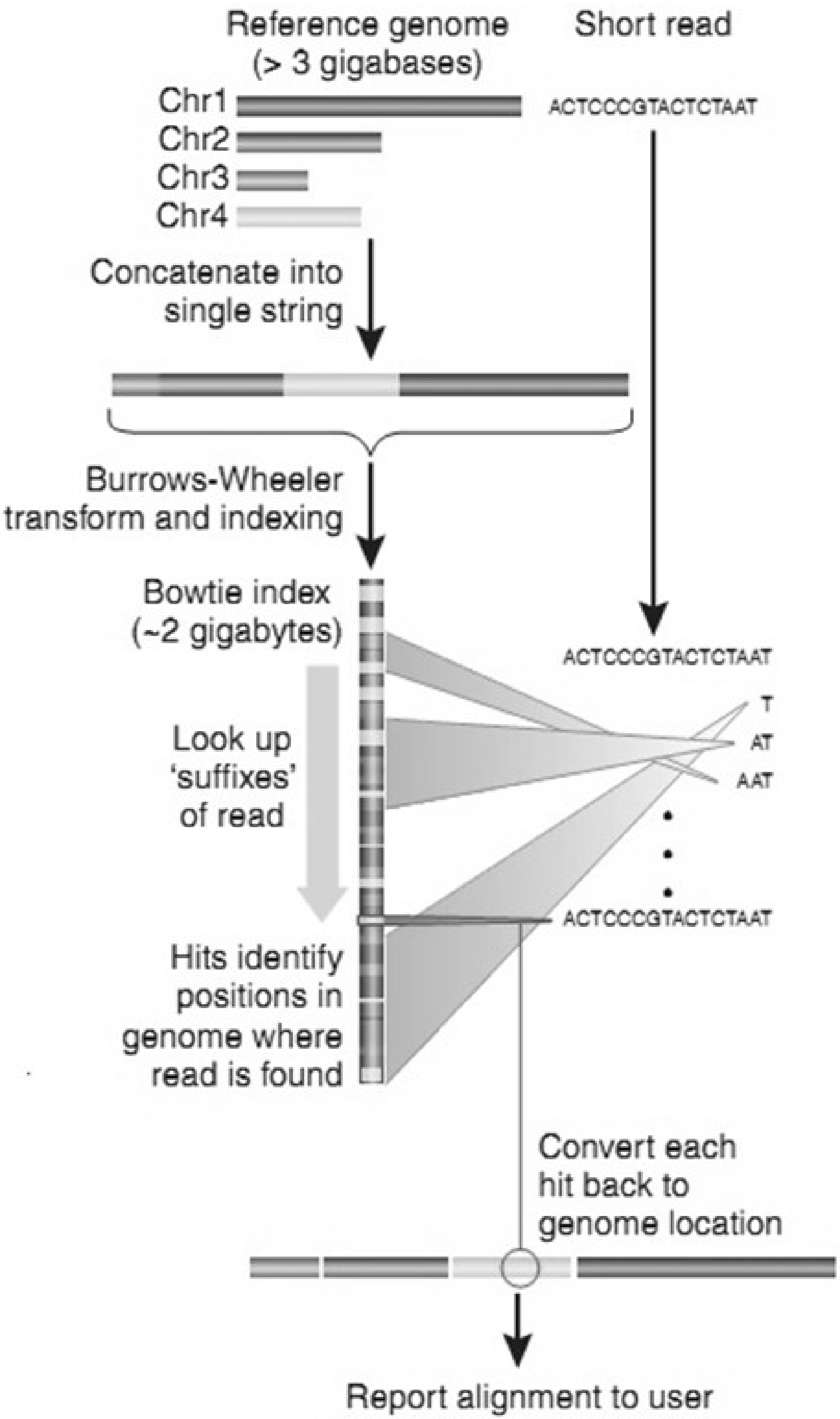

Reference genome
(> 3 gigabases)
Short read
Chr1
Chr2
Chr3
Chr4
ACTCCCGTACTCTAAT
Concatenate into single string
Burrows-Wheeler transform and indexing
Bowtie index (~2 gigabytes)
ACTCCCGTACTCTAAT
T
AT
AAT
Look up 'suffixes' of read
ACTCCCGTACTCTAAT
Hits identify positions in genome where read is found
Convert each hit back to genome location
Report alignment to user

Figure 10. Overview of the short-read mapping using Bowtie

(reproduced from Trapnell and Salzberg 2009)

As described by Langmead *et al.* (2009), the BWT indexing strategy considers the genome sequence as a string "T" which is then subjected to the Burrows-Wheeler transformation. For example, if the string "T" is "acaacg", the transformation is carried out by firstly adding a character "$" to the end of the string T to form "acaacg$". Characters in the new string are then permuted to form an initial matrix as in **Figure 11**. Indexes of rows in the initial matrix, which are called initial indexes, are kept before the rows are alphabetically sorted. The sorted matrix is called the Burrows-Wheeler (BW) matrix. The first column of this matrix is the "genome dictionary", and the last one is called the BWT.

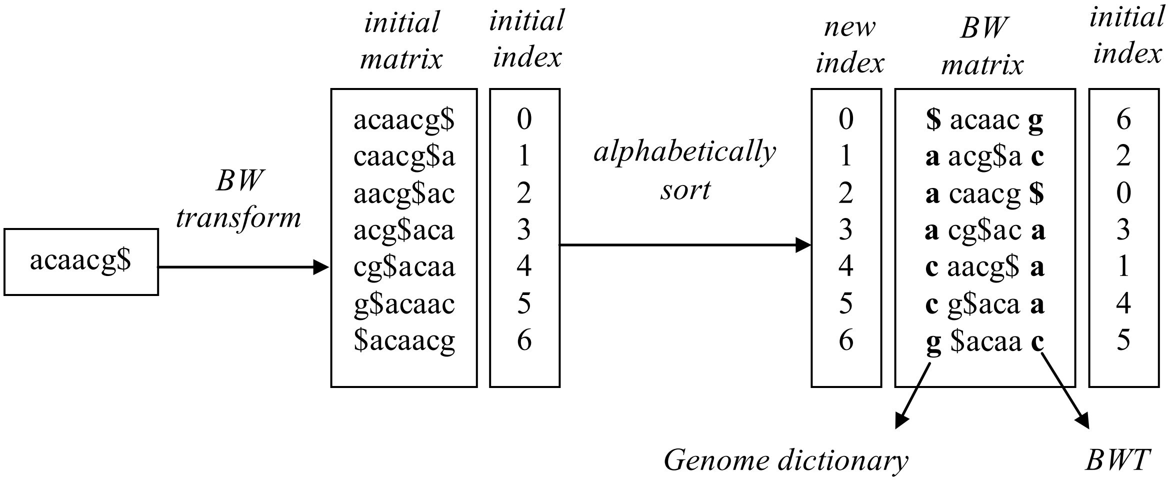
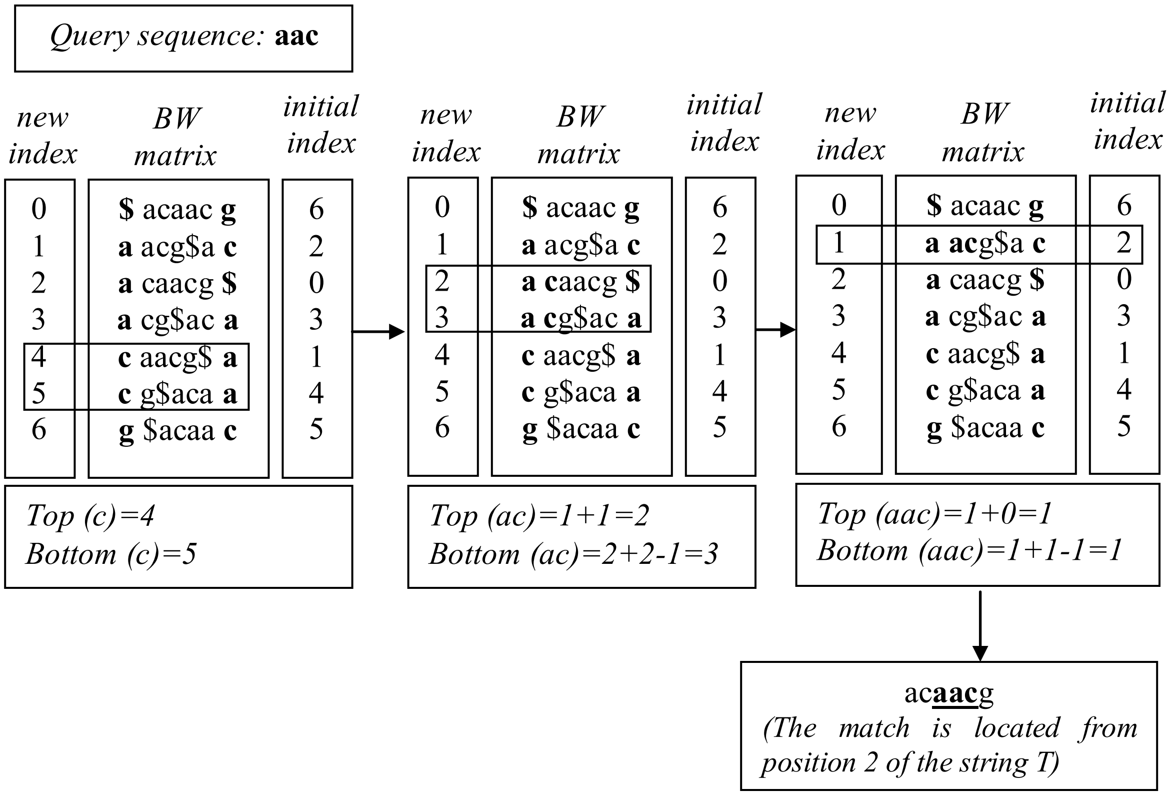


Figure 11. Building Burrows-Wheeler Transform based index of the string "acaacg"

(adapted from Langmead *et al.* 2009)



Query sequence: **aac**

new index	*BW matrix*	*initial index*
0	**$** acaac **g**	6
1	**a** acg$a **c**	2
2	**a** caacg **$**	0
3	**a** cg$ac **a**	3
4	**c** aacg$ **a**	1
5	**c** g$aca **a**	4
6	**g** $acaa **c**	5

Top (c)=4
Bottom (c)=5

new index	*BW matrix*	*initial index*
0	**$** acaac **g**	6
1	**a** acg$a **c**	2
2	**a c**aacg **$**	0
3	**a c**g$ac **a**	3
4	**c** aacg$ **a**	1
5	**c** g$aca **a**	4
6	**g** $acaa **c**	5

Top (ac)=1+1=2
Bottom (ac)=2+2-1=3

new index	*BW matrix*	*initial index*
0	**$** acaac **g**	6
1	**a ac**g$a **c**	2
2	**a** caacg **$**	0
3	**a** cg$ac **a**	3
4	**c** aacg$ **a**	1
5	**c** g$aca **a**	4
6	**g** $acaa **c**	5

Top (aac)=1+0=1
Bottom (aac)=1+1-1=1

ac**aac**g
(The match is located from position 2 of the string T)

Figure 12. Description of EXACTMATCH algorithm used in Bowtie

(adapted from Langmead *et al.* 2009)

To search for exact matches of a query sequence "aac", the EXACTMATCH algorithm is used as outlined in **Figure 12** (Langmead *et al.* 2009):

(i) The last character "c" of the query string is searched within the "genome dictionary" to determine the range of rows beginning with "c". In this case, the indexes of the top and the bottom rows of this c-block are 4 (top (c) = 4) and 5 (bottom (c) = 5), respectively.

(ii) In this step, the algorithm searches for a range of rows beginning with "ac", the two last characters of the query string. Initially, from the c-block, the characters in the

BWT column are checked. If there is “a” among these characters, it means that “ac” exists in the original string “T”, and the exact-match search is continued by looking up the dictionary to find the first row beginning with “a” (top (a)). Finally, the indexes of the top and the bottom rows of the “ac-block” are then calculated as followed:

Top (ac) = top (a) + number of “a” above the c-block in BWT

Bottom (ac) = top (ac) + number of rows beginning with “ac” -1

(iii) Similarly, the range of rows beginning with the query sequence “aac” is determined. These rows show the matches found in the reference string “T”. Since the initial indexes of these rows from the unsorted matrix are kept, they can be used to determine the positions of the matches in the original string “T”.

In case there is no exact match found, a modified EXACTMATCH algorithm is applied so that Bowtie can also search for alignments with a few mismatches (Langmead *et al.* 2009). In this inexact alignment strategy, when the range of rows becomes empty, which means that no exact match is found, the search comes back to the previous range that perfectly matches with a substring of the query sequence. Then, Bowtie substitutes a new base for the different one so that it can find a new range that exactly matches with the modified query sequence. A substitution is an introduction of a mismatch. There can be many possible base substitutions to search for the inexact alignments. Because each base in the short read sequences has a value showing the sequencing quality, each possible substitution has a value calculated from the sum of the quality values of the substituted bases. The one having the lowest value is selected since the mismatches should be from the low-quality bases. This modified algorithm can help Bowtie deal with the problem of sequencing errors. In brief, by using the above indexing strategy and algorithms, Bowtie can considerably reduce computational cost and efficiently map billions of short reads.

1.5. Overview of aminoacyl-tRNA synthetases

1.5.1. Functions of aminoacyl-tRNA synthetases

Aminoacyl-tRNA synthetases (AARSs) are indispensable enzymes in the translation process since they play a crucial role in the attachment between the tRNA adaptors and their corresponding amino acids. These enzymes are also called tRNA ligases (e.g., leucine tRNA ligase, serine tRNA ligase, etc.) or translases (e.g., leucine translase, serine translase, etc.). There should be at least 20 types of AARS proteins corresponding to the 20 standard amino acids (Arnez and Moras 2009). Moreover, in eukaryotic cells, some organelles such as the chloroplasts in plants and the mitochondria have their own AARS enzymes functioning in their translation systems (Sissler *et al.* 2000). The main role of all aminoacyl-tRNA synthetases is to catalyze the aminoacylation reaction, which is specific for each amino acid. As reviewed by Arnez and Moras (2009), the aminoacylation reaction generally occurs in two steps (**Figure 13**). First, the amino acid activation is carried out with the participation of the adenosine triphosphate (ATP) molecule and Mg^{2+} ions to form aminoacyl-adenylate (AA-AMP) and pyrophosphate (PPi). The latter is released while the former is still bound to the active site of AARS. The role of Mg^{2+} ions is to stabilize the conformation of the ATP molecule. It is also involved in the formation of AA-AMP (Arnez and Moras 1997). Second, the amino acid from the AA-AMP molecule is transferred onto either the 2’ or 3’ sugar hydroxyl group of the adenosine nucleotide at the 3’terminal of its cognate tRNA. After the second step, an adenosine monophosphate (AMP) molecule and the amino acid charged tRNA are released from the AARS enzyme.

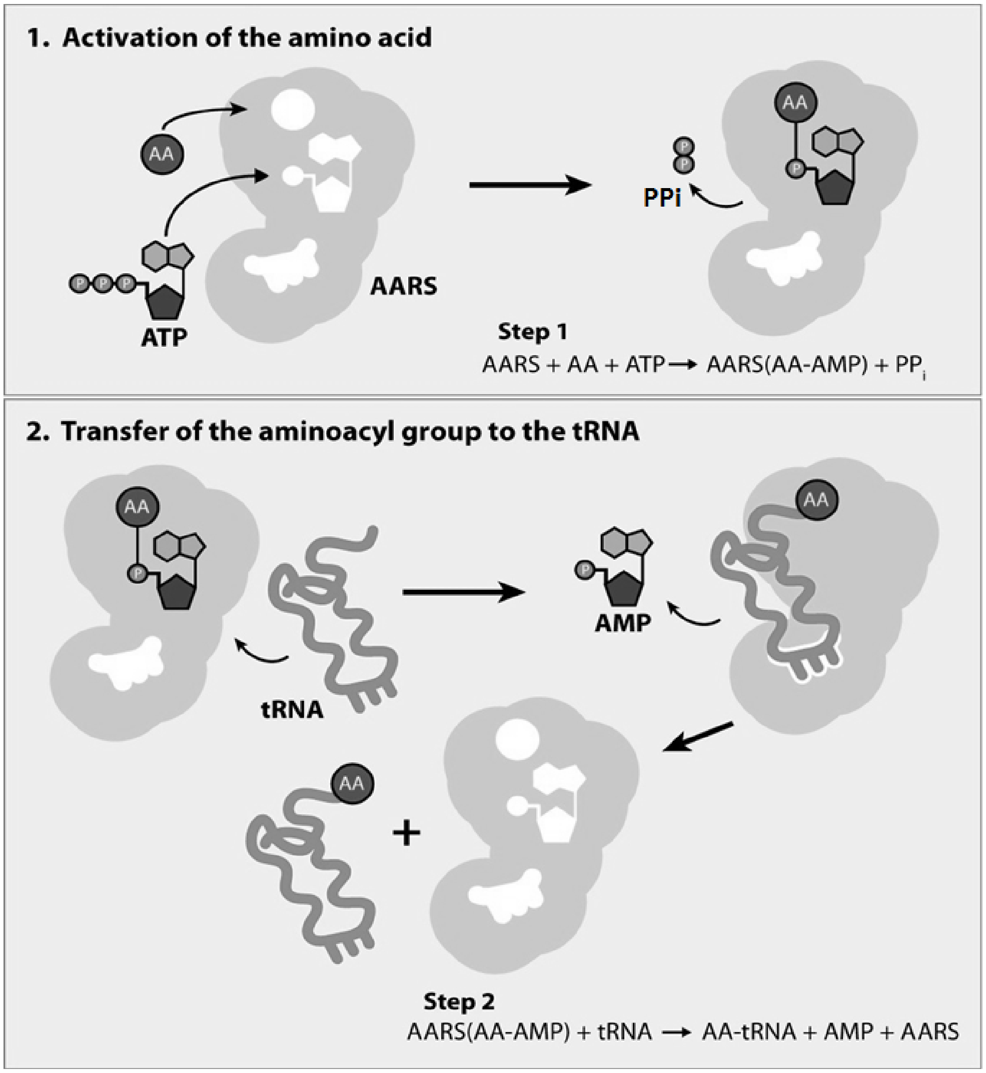

1. Activation of the amino acid
AA
P P P
ATP
AARS
P
P
PPi
AA
P
Step 1
AARS + AA + ATP → AARS(AA-AMP) + PP$_i$
2. Transfer of the aminoacyl group to the tRNA
AA
P
tRNA
P
AMP
AA
AA
+
Step 2
AARS(AA-AMP) + tRNA → AA-tRNA + AMP + AARS

Figure 13. The two-step aminoacylation reaction

(reproduced from Antonellis and Green 2008)

Generally, amino acids are bound to their corresponding tRNAs directly after the aminoacylation reaction. However, in several archaeal and bacterial species, an alternative way, which is called the indirect aminoacylation pathway, is used for Asn and Gln (**Figure 14**) (Ibba *et al.* 2000; Arnez and Moras 2009). In the indirect aminoacylation pathway, Glu and Asp are first attached to tRNA^{Gln} and tRNA^{Asn} by the activities of glutamyl-tRNA synthetase and aspartyl-tRNA synthetase, respectively. Then, these incorrect attachments are recognized and repaired by the enzymes glutamyl-tRNA^{Gln} aminotransferase and aspartyl-tRNA^{Asn} aminotransferase respectively to form the correct aminoacylated tRNAs (Ibba *et al.* 2000).

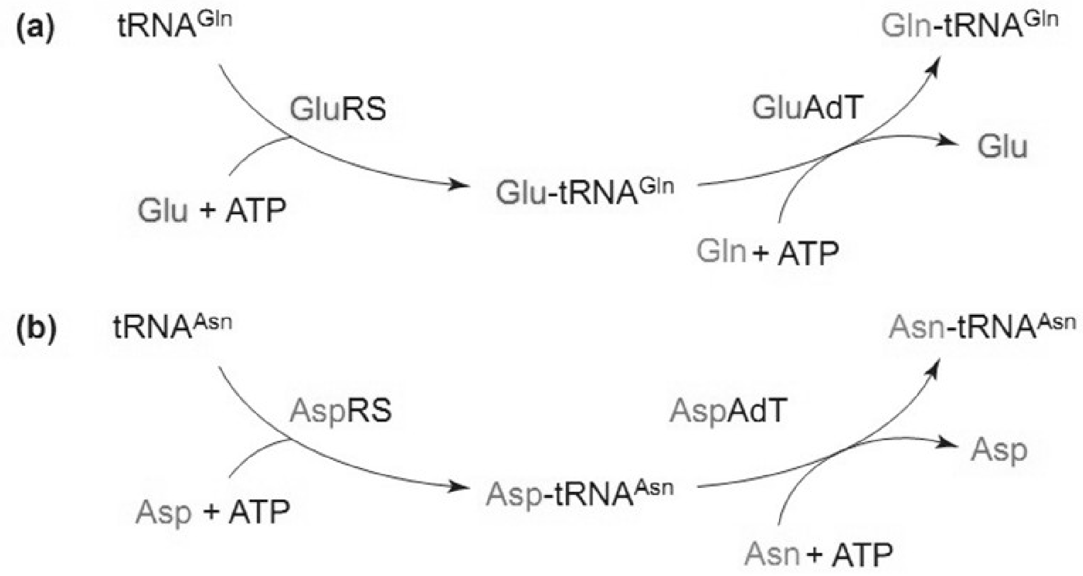


Figure 14. Indirect aminoacylation pathways

(reproduced from Ibba *et al.* 2000)

((a) formation of Asn-tRNA^{Asn}; (b) formation of Gln-tRNA^{Gln})

(GluRS and AspRS: glutamyl- and aspartyl-tRNA synthetases;

GluAdT and AspAdT: glutamyl-tRNA^{Gln} and aspartyl-tRNA^{Asn} aminotransferases)

In addition to their primary function in protein synthesis, aminoacyl-tRNA synthetases are also involved in many other activities of the cell. Some of those include the tRNA processing, RNA splicing, RNA trafficking, rRNA synthesis, apoptosis, transcriptional and translational regulation (reviewed by Szymanski, Deniziak and Barciszewski 2000; Ibba and Soll 2001).

1.5.2. General characteristics of aminoacyl-tRNA synthetases and their genes

Like the cytoplasmic AARS genes, the ones encoding organellar AARSs are also located in the nuclear genome (Sissler *et al.* 2000). Due to the indirect aminoacylation pathway, some bacterial and archaeal species do not have a complete set of 20 canonical types of AARSs. In contrast, in eukaryotic species, it is reported that cytoplasmic AARS enzymes of the 20 specificities are found in all of these organisms (Ibba and Soll 2001). However, similar to prokaryotic species, their set of organellar AARSs may lack some enzymes. For example, the mitochondrial glutaminyl-tRNA synthetase is not found in *S. cerevisiae*, it is likely that Gln is attached to tRNA^{Gln} through the aminoacylation utilizing the amidotransferase enzyme (Sissler *et al.* 2000). This observation is consistent with the endosymbiosis hypothesis in which the mitochondrion had a bacterial origin (Arnez and Moras 1997). Moreover, there may be multiple genes encoding AARS enzymes of the same specificity. For instance, two cytoplasmic glycyl-tRNA synthetases were detected in *S. cerevisiae* (Turner, Lovato and Schimmel 2000). In addition, there are genes encoding both cytoplasmic and mitochondrial AARSs of the same specificity or coding for bifunctional enzymes specific for two amino acids (Ibba and Soll 2001). Also, in different organisms, organellar and cytoplasmic AARSs of the same amino acid can be encoded by either the same gene or separate genes (Sissler *et al.* 2000). Therefore, the number of cytoplasmic and organellar AARS genes varies across different organisms.

For the genes encoding both cytoplasmic and mitochondrial AARSs of the same specificity, they are usually found to contain two open reading frames (ORFs) in the same reading frame and only differing in their start codon positions. The longer ORF encodes the mitochondrial enzyme while the shorter one encodes the cytoplasmic enzyme. The extra sequence at the 5’ end of the mitochondrial ORF codes for the mitochondrial targeting peptide which is necessary for the gene product to be imported into the mitochondrion. One example of these genes is the one encoding cytoplasmic and mitochondrial histidyl-tRNA synthetase in *S. cerevisiae* (Natsoulis, Hilger and Fink 1986). Moreover, there is another type of these bifunctional AARS genes, e.g. the cytoplasmic and mitochondrial lysyl-tRNA synthetase gene in human, which forms the two enzymes by alternative splicing patterns from the same primary transcript. Protein products from these different splicing processes only differ by their short regions at the amino terminus (Ambrogelly *et al.* 2010).

Protein sequences of AARSs of the 20 specificities vary in their lengths and amino acid compositions (Arnez and Moras 2009). Aminoacyl-tRNA synthetases of the same amino acid in eukaryotic organisms are usually larger than those of prokaryotic species since they often have extended sequences at both the N- and C- termini. Functions of these extra sequences are not fully understood. It has been shown that they play a role in increasing the enzyme stability and are not involved in the aminoacylation reaction (Szymanski *et al.* 2000).

When considering the primary structures of aminoacyl-tRNA synthetase enzymes, the mitochondrial AARSs show low similarities with their cytoplasmic counterparts while they are highly similar to those in prokaryotes. Moreover, the mitochondrial signal peptides of mitochondrial AARSs have various lengths and amino acid compositions (Sissler *et al.* 2000).

The quaternary structures of different AARSs are also diverse since some have a single subunit while others have multiple ones, including homodimers (α_2), homotetramers (α_4) and heterotetramers ($\alpha_2\beta_2$) (Arnez and Moras 2009). The similar oligomeric structures are found from AARSs of the same specificities. However, there are some exceptions. For example, both cytoplasmic and mitochondrial glycyl-tRNA synthetases in eukaryotic organisms contain two identical subunits (α_2) while those in prokaryotic species consist of four polypeptide chains ($\alpha_2\beta_2$). Also, cytoplasmic phenylalanine-tRNA ligases in eukaryotes are composed of four subunits ($\alpha_2\beta_2$) while those from mitochondria comprise only a single subunit (α) (Sissler *et al.* 2000).

As reported by Guo *et al* (2010), AARS enzymes are composed of multiple domains. The central one, also called the active-site domain or the aminoacylation domain, is present in all of the enzymes and functions in specifically identifying and binding to amino acids, tRNAs and ATP molecules. Some AARSs have editing domains, which can recognize the inappropriate attachment between an amino acid and a tRNA, then remove the incorrect amino acid. It is also reported that some novel domains, which are not related to the aminoacylation reaction, have been gradually added to eukaryotic AARSs during evolution. These new domains are involved in various functions of AARSs in addition to their role in protein synthesis.

According to the review of Arnez and Moras (2009), AARSs are classified into two classes according to the difference in the structures of their active-site domains. Each class includes 10 enzymes. The only exception is LysRS, which is generally a class I AARS; however, those in archaea belong to class II (Sissler *et al.* 2000). The class I enzymes share the same active site structure, which is a conventional nucleotide binding fold, namely the Rossmann fold, and consists of parallel β sheets surrounding α helices. Within the active site, two highly conserved motifs, "HIGH" and "KMSKS", are found in the ATP-binding region (Arnez and Moras 1997). The histidines (H) and lysines (K) in these two signature sequences are important since they interact with the triphosphate of the ATP molecule. In the aminoacylation reactions catalyzed by class I AARSs, amino acids are transferred to the 2'OH of the 3' terminal adenosine of the tRNA. Most of the class I enzymes are monomeric, and only two of them have homodimers in their quaternary structures. In contrast to class I AARSs, all class II enzymes contain multiple subunits, which are mostly homodimers. Two of them, alanyl-tRNA synthetase and phenylalanyl-tRNA synthetase are homotetrameric and heterotetrameric, respectively. Most of them attach amino acids to their cognate tRNAs at the 3'OH group of the 3' terminal adenosine. Only phenylalanyl-tRNA synthetase forms the bond between the amino acid and tRNA at the 2'OH group. The active site structure of the class II AARSs include seven-stranded antiparallel β sheets surrounded by α helices. Within this structure, three conserved motifs are found. The motif 1 sequence, which contains a strictly conserved proline (P), primarily forms the dimer interface. The motif 2 and motif 3 also contain some conserved amino acids and are involved in positioning the amino acid and the ATP molecule.

1.6. Rationale for this study

Myceliophthora thermophila has drawn much attention because its ability to work efficiently at elevated temperatures, an environment suitable for many industrial processes. This organism is currently being used as a protein-production host in the enzyme manufacturing industry (Hans *et al.* 2011). Moreover, *M. thermophila* is the first filamentous fungus with a finished genome sequence and the first thermophilic eukaryote sequenced (Berka *et al.* 2011). The availability of a complete genome sequence provides us with the opportunity to study the gene complements related to specific cellular processes systematically. To gain insights into the translation system of *M. thermophila* and find out why this fungus is such a good host organism for protein production, this study focuses on analyzing the genes encoding tRNAs and AARS enzymes, two indispensable components of the protein synthesis machinery in all organisms. Firstly, these genes are predicted in the genome sequence by different bioinformatics tools. Then, the reliability of the prediction is evaluated by comparing the predicted gene models to the sequenced transcriptome data as well as to sequences of experimentally characterized tRNAs and AARSs collected from the literature. From the complete set of reliable cytoplasmic tRNA genes, the optimal codons encoding each amino acid can be deduced, which can potentially be used for the codon optimization process to improve the protein production capacity of *M. thermophila*. Moreover, when the complete set of tRNA genes is found, its relationship with the tRNA abundance as well as the codon usage can be examined. Furthermore, genes detected from the nuclear genome of *M. thermophila* in this study can be added to the reliable data sets of tRNA and AARS genes.

CHAPTER 2. MATERIALS AND METHODS

2.1. tRNA gene prediction, curation and annotation procedure

The overall procedure to detect the complete set of cytoplasmic tRNA genes in *M. thermophila* comprises four main stages (**Figure 15**). First, tRNA genes were predicted from the nuclear genome by using different tRNA gene prediction tools. Transfer RNA genes detected from the predictors were then compared to discard the duplicates. Second, sequenced short RNAs were mapped onto the genome. Third, experimentally characterized fungal tRNAs and tRNA genes were collected. Their primary and secondary structures were analyzed to deduce the common features and conserved regions of fungal tRNAs. Finally, the predicted tRNA genes were compared to the mapped short RNA reads and the experimentally characterized fungal tRNAs to select the most reliable tRNA genes.

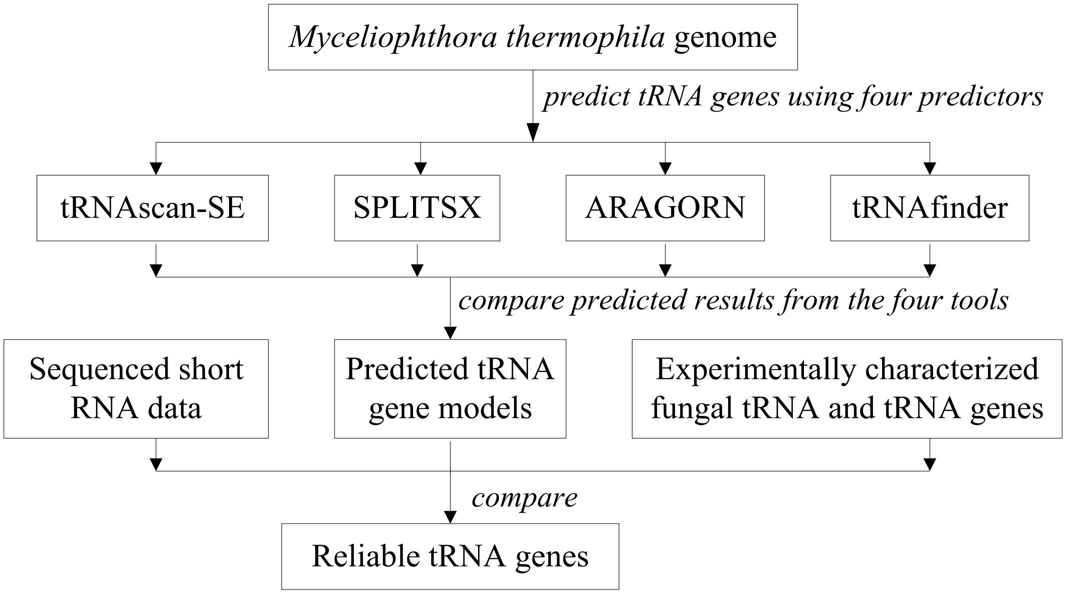

Myceliophthora thermophila genome
predict tRNA genes using four predictors
tRNAscan-SE
SPLITSX
ARAGORN
tRNAfinder
compare predicted results from the four tools
Sequenced short RNA data
Predicted tRNA gene models
Experimentally characterized fungal tRNA and tRNA genes
compare
Reliable tRNA genes

Figure 15. The tRNA gene prediction, curation and annotation workflow

2.1.1. Predicting tRNA genes

Transfer RNA genes were detected from the nuclear genome of *M. thermophila* which was the unmasked genome assembly version 2 and was downloaded from the website of the United States Department of Energy (DOE) Joint Genome Institute (JGI) (http://genome.jgi.doe.gov/Spoth2/Spoth2.download.html). To reduce the possibility of missing true tRNA genes, all of the four most widely used tRNA gene predictors – tRNAscan-SE version 1.3 (Lowe and Eddy 1997), SPLITSX (Sugahara *et al.* 2007), ARAGORN (Laslett and Canback 2004) and tRNAfinder (Kinouchi and Kurokawa 2006) – were used in this study. Since tRNAscan-SE program can predict tRNA genes in eukaryotic, prokaryotic and archaeal genomes, the parameter specific for eukaryotic genomes was used when predicting tRNA genes from *M. thermophila*. All of the other parameters were set to default values. When running SPLITSX, the default values for parameters were selected except for "`-d`", the possible number of introns in each tRNA gene. In this study, "`-d`" was set to 3, which was the maximal number of introns that SPLITSX could detect. The parameters used when running ARAGORN were as followed:

"`-t -gcstd -i -io -rp -seq -br`".

where `-t` : search for tRNA genes

`-gcstd` : use standard genetic code

`-i` : search for tRNA genes containing introns (When this parameter is selected, ARAGORN detects tRNA genes having intron lengths from 0 to 3000 nucleotides. Otherwise, ARAGORN only predicts tRNA genes not containing introns.)

`-io` : allow tRNA genes with long introns to overlap the shorter ones

`-rp` : report possible pseudogenes

`-seq` : print out primary sequence

`-br` : show secondary structure of the tRNA genes using round brackets

When running tRNAfinder, the parameters were set to default values except for the maximum length of introns. The default value of intron length parameter is zero. Therefore, it is necessary to increase this value so that tRNAfinder can detect intron-containing tRNA genes. The intron length parameter strongly affects the time of the prediction process as well as the memory for storing output data. The cost of time and memory increases with the length of introns. Since intron lengths of the fungal cytoplasmic tRNA genes in tRNAdb database do not exceed 100 nucleotides (Juhling *et al.* 2009), the maximum intron length parameter was set to 100. Finally, tRNA genes predicted from the four programs were compared by using a Perl script to discard identical results. The final predicted data set contains unique tRNA genes which may differ in their gene positions, intron positions, anticodons or assigned amino acids. The pseudogenes reported from the tools were also included in the predicted data set for further analyses.

2.1.2. Processing and mapping sequenced short RNA data

2.1.2.1. Processing the sequenced short RNA data

To obtain the tRNA transcripts for sequencing, 2.5µg of total RNAs extracted from *M. thermophila* were resolved by gel electrophoresis, and short RNA molecules (~ 40-114 base pairs) were excised. This sample containing short RNAs was sequenced by using the Illumina RNA-Seq sequencing method in the University of Missouri DNA Core Facility (http://biotech.rnet.missouri.edu/dnacore/).

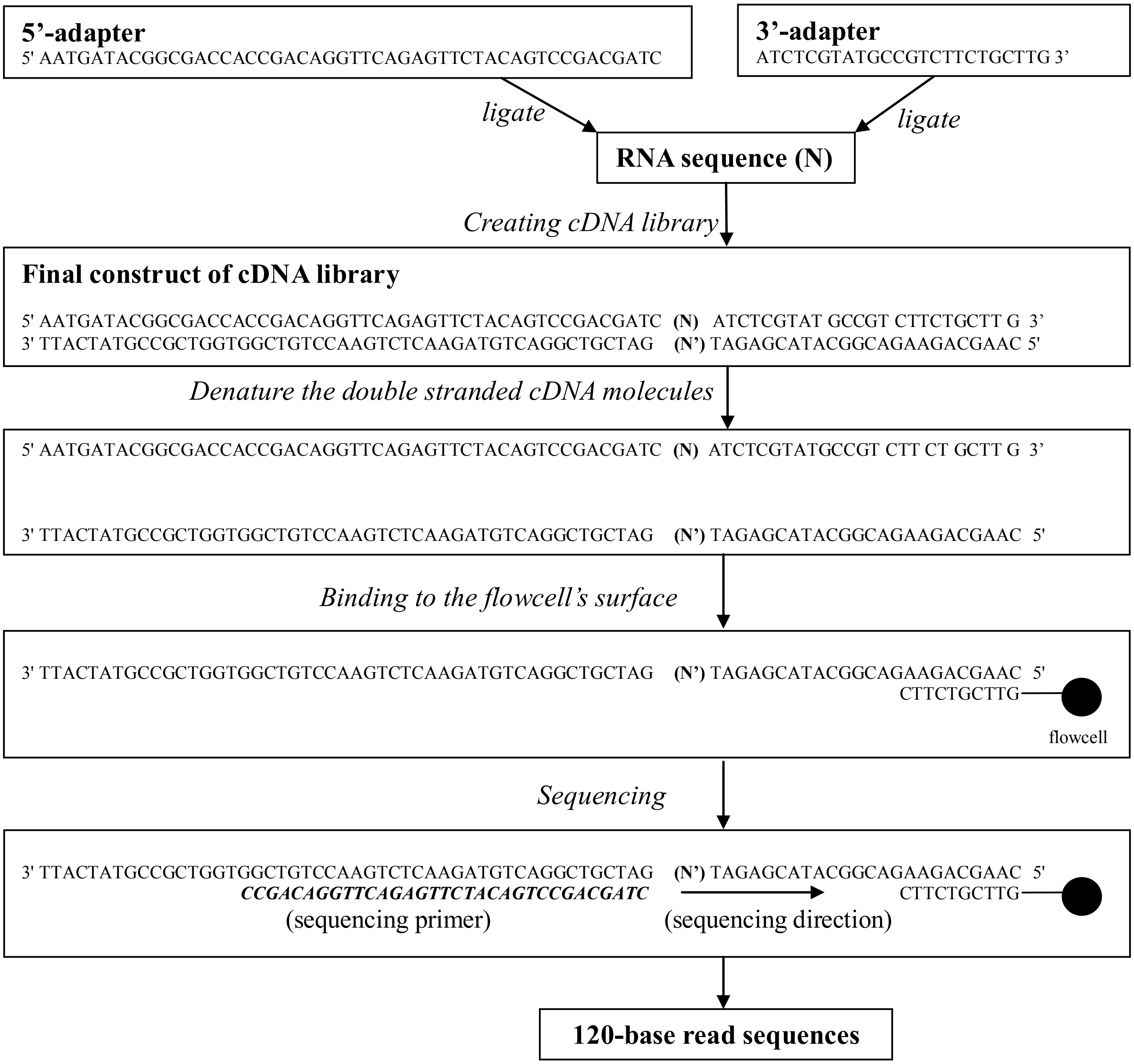

5'-adapter
5' AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACAGTCCGACGATC
3'-adapter
ATCTCGTATGCCGTCTTCTGCTTG 3'
ligate
ligate
RNA sequence (N)
Creating cDNA library
Final construct of cDNA library
5' AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACAGTCCGACGATC (N) ATCTCGTAT GCCGT CTTCTGCTT G 3'
3' TTACTATGCCGCTGGTGGCTGTCCAAGTCTCAAGATGTCAGGCTGCTAG (N') TAGAGCATACGGCAGAAGACGAAC 5'
Denature the double stranded cDNA molecules
5' AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACAGTCCGACGATC (N) ATCTCGTATGCCGT CTT CT GCTT G 3'
3' TTACTATGCCGCTGGTGGCTGTCCAAGTCTCAAGATGTCAGGCTGCTAG (N') TAGAGCATACGGCAGAAGACGAAC 5'
Binding to the flowcell's surface
3' TTACTATGCCGCTGGTGGCTGTCCAAGTCTCAAGATGTCAGGCTGCTAG (N') TAGAGCATACGGCAGAAGACGAAC 5'
CTTCTGCTTG
flowcell
Sequencing
3' TTACTATGCCGCTGGTGGCTGTCCAAGTCTCAAGATGTCAGGCTGCTAG (N') TAGAGCATACGGCAGAAGACGAAC 5'
CCGACAGGTTCAGAGTTCTACAGTCCGACGATC
CTTCTGCTTG
(sequencing primer)
(sequencing direction)
120-base read sequences

Figure 16. Overview of the short RNA sequencing process

(*(N): sequence of the short RNA; (N'): complementary sequence of the short RNA*)

Figure 16 illustrates an overview of the sequencing process. First, 5'-adapters and 3'-adapters were ligated at the two endings of short RNAs. After the cDNA library was established, each double stranded DNA molecule in the library was denatured to form two single stranded DNAs. The strand complementary to the original short RNA sequence bound to the flowcell while the other strand was discarded. The flowcell contained a short oligonucleotide that hybridized to a part of the 3' adapter. The sequencing was carried out by using a sequencing primer complementary to a fragment at the 3' end of the 5'-adapter. Next, the sequence of 120 nucleotides downstream of the 5' adapter was sequenced. Therefore, the short read data contained sequences of short RNAs and 3'-adapters. For example, if the length of the short RNA was 100 nucleotides in length, and we obtain a 120-nucleotide read, the extra 20 nucleotides at the 3' end of the read are from the 3'-adapter. Accordingly, sequences of the 3'-adapters and any nucleotides downstream of them were trimmed from the sequenced RNAs before using the sequenced short RNA data for the tRNA gene curation and annotation.

2.1.2.2. Mapping short RNA data to the genome of *M. thermophile*

After trimming the adapter sequences, the short reads were aligned to the genome of *M. thermophila* using Bowtie (Langmead *et al.* 2009) and BLAT (Kent 2002) programs successively. Reads already mapped to the genome by Bowtie were not used in the mapping using BLAT. Since Bowtie can map millions of short reads to a large genome much faster than the other sequence alignment programs, it is used in the first stage of the mapping. However, since the version of Bowtie used in this study does not support gapped alignment, many of the reads containing the splice-junctions between introns and exons may not be mapped correctly. For that reason, BLAT is used in the second stage of the mapping because this tool can deal with the gapped alignment.

BLAT uses an index comprising non-overlapping oligomers from the reference genome and linearly scans the query sequence to find the matching regions. Then, it extends these matches to obtain larger alignments (Kent 2002). Compared to the index building strategy and method used for searching the matches from Bowtie, BLAT requires more memory space and time. However, since the majority of the reads were mapped by Bowtie, the number of the remaining reads was small enough for BLAT to work well. The advantage of BLAT is that it allows alignments with large gaps, which is helpful for the identification of intron regions.

2.1.3. Collecting and analyzing experimentally characterized fungal tRNAs and tRNA genes

2.1.3.1. Selecting databases to collect experimentally characterized fungal tRNAs and tRNA genes

Several databases of tRNA and tRNA gene sequences have been published and used in the study of tRNAs. Those include the "Compilation of tRNA sequences and sequences of tRNA genes", also called Sprinzl database (Sprinzl and Vassilenko 2005), the Genomic tRNA database (GtRNAdb) (Chan and Lowe 2009), the tRNA gene database curated manually by experts (tRNADB-CE) (Abe *et al.* 2011), and the transfer RNA database (tRNAdb) (Juhling *et al.* 2009). First established in 1978, many updated versions of the Sprinzl database have been published (Sprinzl *et al.* 1982; 1983a, 1983b; 1984; 1985; 1987; 1989; 1991; 1993; 1996; 1998; 2005), and this database has been widely used as a reliable dataset of "true positives" in tRNA gene detection research

(Eddy and Durbin 1994; Pavesi *et al.* 1994; Lowe and Eddy 1997; Laslett and Canback 2004; Kinouchi and Kurokawa 2006). However, the website provided in the publication of the latest release of this compilation, which was published in 2005, is not accessible. For the GtRNAdb, tRNA genes from this database are all automatically predicted by tRNAscan-SE program (Chan and Lowe 2009). Similarly, most of the tRNA genes from tRNADB-CE are predicted by tRNAscan-SE, ARAGORN and tRNAfinder. Only tRNA genes that are differently predicted by the three tools are curated manually by experts; however, they are still predicted genes (Abe *et al.* 2011). Therefore, GtRNAdb and tRNADB-CE are not good resources for experimentally identified tRNAs. The tRNAdb database is, in fact, a new version of the Sprinzl compilation updated in 2009 (Juhling *et al.* 2009). It contains both experimentally and automatically identified tRNA and tRNA gene sequences. The sequences in this database are phylogenetically categorized so that users can easily select those belonging to fungi as well as other group of species for further study. For these reasons, tRNAdb (http://trnadb.bioinf.uni-leipzig.de/) is selected as a good source for the experimentally characterized fungal tRNAs and tRNA genes.

Each entry in tRNAdb provides the literature on the identification of the corresponding tRNA sequence. Hence, from these papers, along with the related ones from the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/), the fungal tRNA sequences were examined to determine which ones are the most reliable and can be used in the “true positive” dataset for the tRNA gene annotation.

2.1.3.2. Assigning GO evidence codes

Properties of each tRNA gene product collected from the literature were assigned by using the terminologies established from the Gene Ontology (GO) Project

(Ashburner *et al.* 2000). These properties refer to three aspects: the Molecular Function, the Biological Process and the Cellular Component. When a GO term is assigned, a GO evidence code is given to show the available evidence supporting the assignment. The terms deduced from experimental assays are the most reliable. Their GO evidence codes are usually IDA (Inferred from Direct Assay), IMP (Inferred from Mutant Phenotype), IGI (Inferred from Genetic Interaction), IEP (Inferred from Expression Pattern) and IPI (Inferred from Physical Interaction). For those inferred from computational experiments and statements from the authors in the literature, the ones having the GO evidence codes TAS (Traceable Author Statement) and IGC (Inferred from Genomic Context) are considered to be reliable. For those having the code ISS (Inferred from Sequence Similarity), the reference sequence is checked to see whether it is trusted or not. The GO evidence codes determined in this study were according to the decision tree in **Figure 17**. Based on the GO terms for the three aspects, and their corresponding GO evidence codes, each tRNA gene collected from the literature was determined whether it is experimentally characterized or not. For example, if a tRNA^{Lys} gene has the GO terms "AAG codon-amino acid adaptor activity" for the Molecular Function aspect, "Lysyl-tRNA aminoacylation" for the Biological Process aspect and "cytoplasm" for Cell Component aspect, and the corresponding evidence codes are IDA, IDA, IGC respectively, it is considered to be a reliable gene since the three GO terms demonstrate the properties of a cytoplasmic tRNA^{Lys} gene and the evidence codes indicate that these properties are supported by experimental data. After collecting the fungal tRNA genes from the literature, only the experimentally characterized ones which were located in the cytoplasm were kept in the "true positive" dataset.

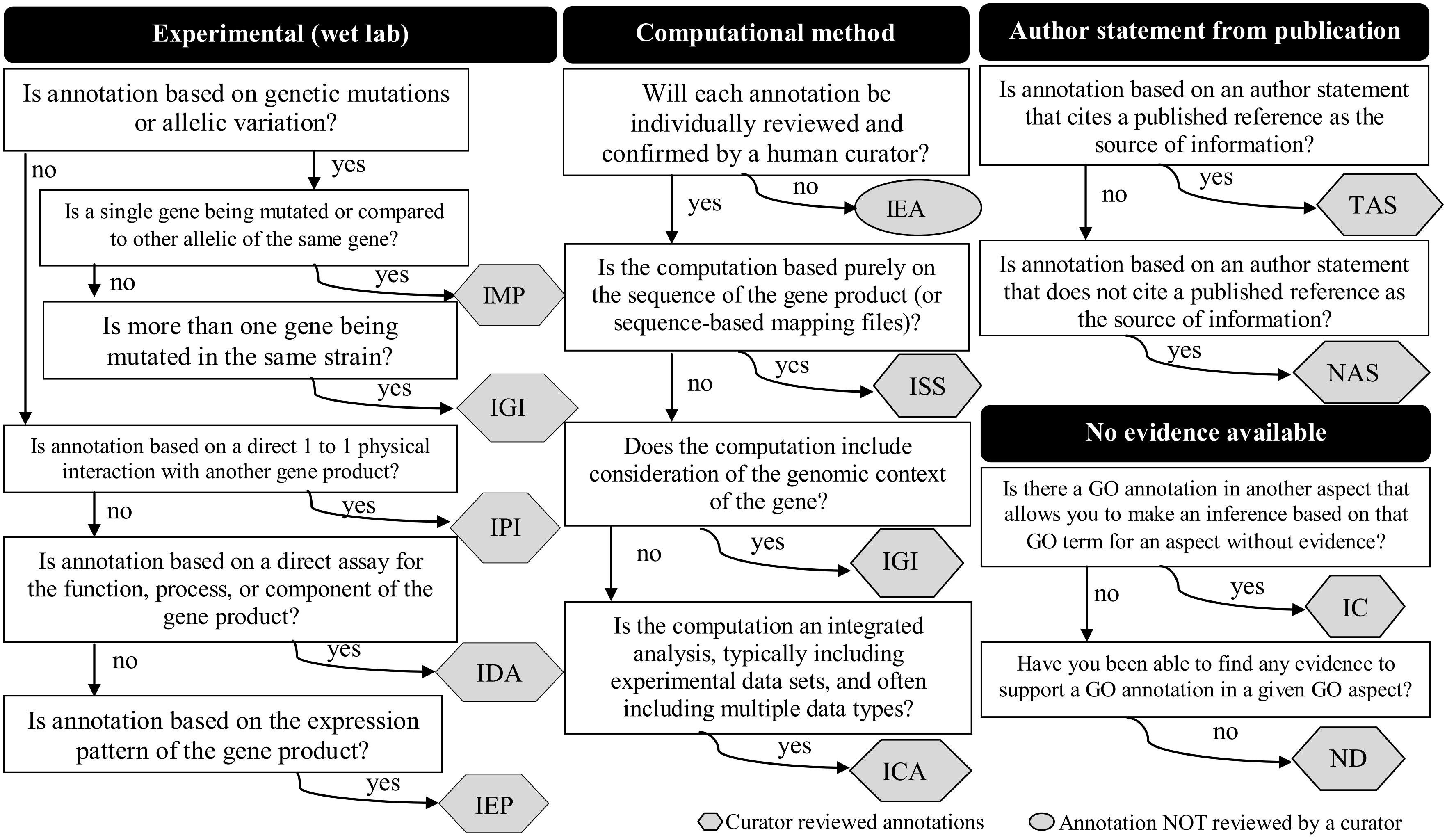

Experimental (wet lab)
Is annotation based on genetic mutations or allelic variation?
no
yes
Is a single gene being mutated or compared to other allelic of the same gene?
no
yes
IMP
Is more than one gene being mutated in the same strain?
yes
IGI
Is annotation based on a direct 1 to 1 physical interaction with another gene product?
no
yes
IPI
Is annotation based on a direct assay for the function, process, or component of the gene product?
no
yes
IDA
Is annotation based on the expression pattern of the gene product?
yes
IEP
Computational method
Will each annotation be individually reviewed and confirmed by a human curator?
no
IEA
yes
Is the computation based purely on the sequence of the gene product (or sequence-based mapping files)?
yes
ISS
no
Does the computation include consideration of the genomic context of the gene?
no
yes
IGI
Is the computation an integrated analysis, typically including experimental data sets, and often including multiple data types?
yes
ICA
Author statement from publication
Is annotation based on an author statement that cites a published reference as the source of information?
no
yes
TAS
Is annotation based on an author statement that does not cite a published reference as the source of information?
yes
NAS
No evidence available
Is there a GO annotation in another aspect that allows you to make an inference based on that GO term for an aspect without evidence?
no
yes
IC
Have you been able to find any evidence to support a GO annotation in a given GO aspect?
no
ND
⬡ Curator reviewed annotations
⬭ Annotation NOT reviewed by a curator

Figure 17. GO evidence code decision tree (reproduced from The Gene Ontology 1999)

2.1.3.3. Collecting and analyzing primary and secondary structures of the experimentally characterized tRNAs and tRNA genes

Transfer RNA sequences in tRNAdb contain modified nucleotides represented by the characters such as K, L, D, I, etc., which are different from the normal nucleotides (A, U, G, C). Therefore, the unmodified tRNA sequences obtained from GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) were collected and used in the sequence analysis. After removing CCA at the 3'end, the collected tRNA sequences were used as query sequences in the BLAST searches against the corresponding fungal genomes to find their gene locations and their paralogs. The genomes used in this analysis were from the following fungal organisms: *Saccharomyces cerevisiae S288c*, *Candida albicans SC5314*, *Neurospora crassa OR74A*, *Candida guilliermondii*, *Podospora anserina S mat+* and *Schizosaccharomyces pombe 972h-*. These genome sequences were downloaded from the GenBank database, the Broad Institute's website (http://www.broadinstitute.org/) and the Candida Genome Database (CGD) (http://www.candidagenome.org/). From the gene locations found in the BLAST search results, the regions downstream of the genes were manually examined to determine the transcriptional termination sites, which should contain at least four consecutive thymidines.

For the primary sequence analysis, unique tRNA gene sequences were subjected to the multiple sequence alignment (MSA) to create an MSA profile which was then used to build a phylogenetic tree to show the relationships between the same type of tRNA genes from different fungal species, and between different types of tRNA genes from the same species. The ClustalW program integrated in the MEGA (Molecular

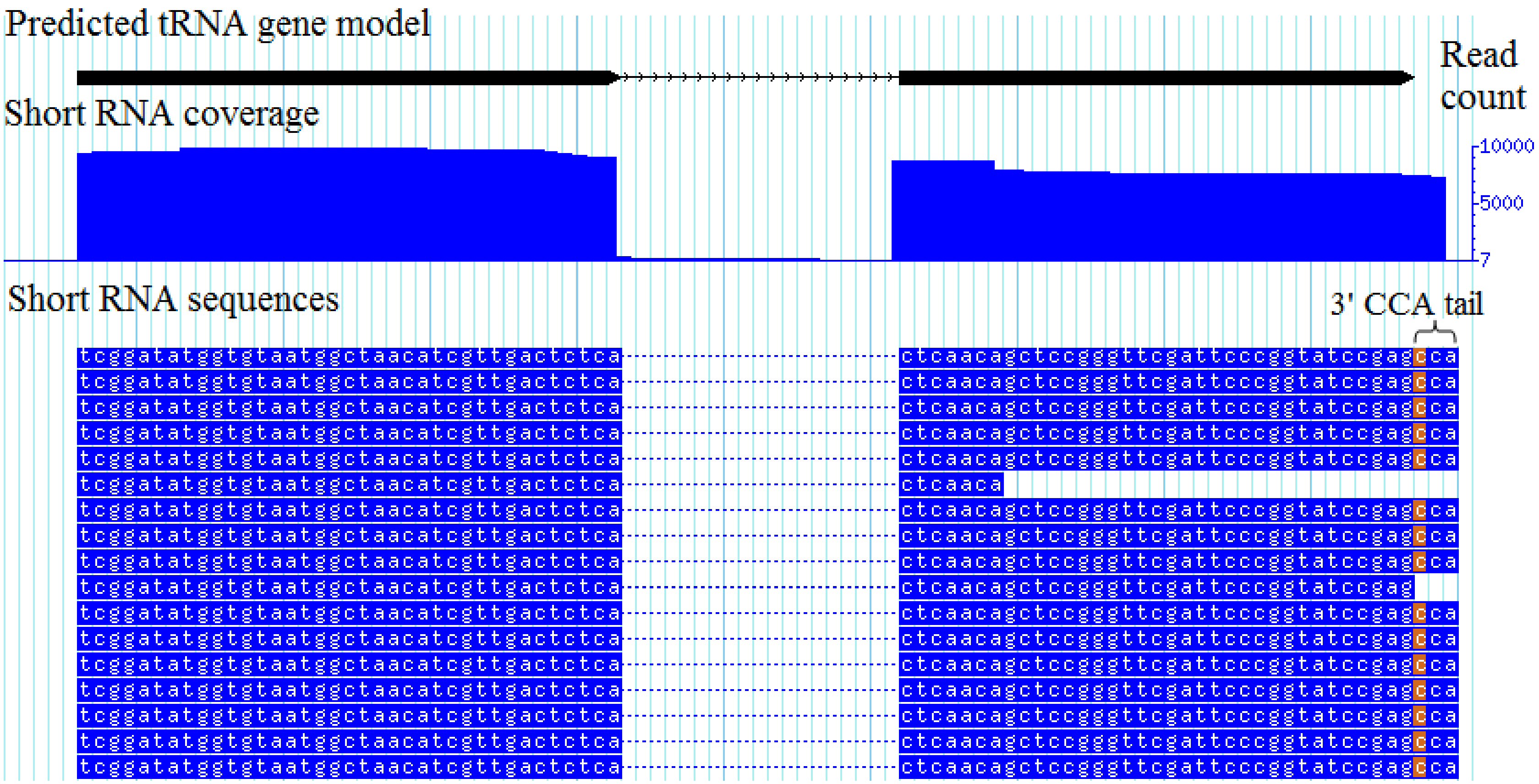
Evolutionary Genetics Analysis) program was used for the alignment (Thompson, Gibson and Higgins 2002; Tamura *et al.* 2011). The tree was constructed and evaluated by using the Maximum Likelihood method and the bootstrap analysis (Felsenstein 1981, 1985). The number of bootstrap replications was 500. Moreover, sequences of the two internal promoters of the characterized tRNA genes were compared to determine the consensus sequences by running the Weblogo program (Crooks *et al.* 2004). The A box and B box sequences were trimmed from position 8 to 19 from the 5' end, and from position 12 to 22 from the 3' end of each tRNA gene, respectively.

For the secondary structure analysis, cloverleaf models of the experimentally identified tRNAs were collected from the literature. Subsequently, separate sets of oligonucleotides of each of the stems and loops were extracted from the cloverleaf structures and subjected to the Weblogo program (Crooks *et al.* 2004) to find conserved positions and consensus sequences.

To investigate the relationship between the four transposable elements (TE) (*delta*, *sigma* and *tau* elements) and tRNA genes, these TEs were used as query sequences in the BLAST search against the six fungal genomes mentioned above. Next, all of the TE locations reported from the BLAST search results were analyzed to examine how many of the tRNA genes were located adjacent to them. If a majority of the tRNA genes are close to the TE regions in the genomes, these elements can be used as good indicators for the tRNA gene annotation.

2.1.4. Manual curation and annotation of tRNA genes in *M. thermophila*

The manual curation and annotation procedure consists of three main stages in which the gene models, the primary and secondary structures of predicted tRNA genes are examined. In the first stage, tRNA gene models predicted by the four tRNA gene prediction tools were compared with the sequenced short RNAs that were mapped onto the genome. To visualize the gene models and short read data, the Generic Genome Browser (GBrowse) (https://reviewers.fungalgenomics.ca/gene_model_pages/tRNA_Table.html), a web-based application commonly used for showing features and annotations of genomes (Stein *et al.* 2002), was employed (**Figure 18**). A tRNA gene is more likely to be correctly predicted if its gene model is well-matched with the read coverage. Besides, the more reads mapped onto a predicted tRNA gene region, the higher likelihood that this gene is a true tRNA gene. Also, the number of reads mapped onto the exons should be much higher than those mapped onto the introns. In addition to the read coverage, sequences of the reads aligned to the predicted tRNA genes are also examined. If the 3'-CCA tail is found in the short RNA sequences, it is a good indicator for a true tRNA gene since CCA is added at the 3' end of tRNAs after the transcription process (Phizicky and Hopper 2010). Moreover, some genes predicted from the four tools are different only from their ending positions. The 3'-CCA tail indicator can help to determine which tool reports the most accurate results in these cases. After the first stage, tRNA genes that have gene models matching with the short RNA coverage are selected for further analyses. For the gene models that do not perfectly match with the short read coverage, they are subjected to the second stage of the tRNA gene annotation if there is evidence found for the expression of these genes in the transcriptome data such as the high number of mapped reads or the presence of the 3'-CCA tail.



```
tcggatatggtgtaatggctaacatcgttgactctca    ctcaacagctccgggttcgattcccggtatccgagcca
tcggatatggtgtaatggctaacatcgttgactctca    ctcaacagctccgggttcgattcccggtatccgagcca
tcggatatggtgtaatggctaacatcgttgactctca    ctcaacagctccgggttcgattcccggtatccgagcca
tcggatatggtgtaatggctaacatcgttgactctca    ctcaacagctccgggttcgattcccggtatccgagcca
tcggatatggtgtaatggctaacatcgttgactctca    ctcaacagctccgggttcgattcccggtatccgagcca
tcggatatggtgtaatggctaacatcgttgactctca    ctcaaca
tcggatatggtgtaatggctaacatcgttgactctca    ctcaacagctccgggttcgattcccggtatccgagcca
tcggatatggtgtaatggctaacatcgttgactctca    ctcaacagctccgggttcgattcccggtatccgagcca
tcggatatggtgtaatggctaacatcgttgactctca    ctcaacagctccgggttcgattcccggtatccgagcca
tcggatatggtgtaatggctaacatcgttgactctca    ctcaacagctccgggttcgattcccggtatccgag
tcggatatggtgtaatggctaacatcgttgactctca    ctcaacagctccgggttcgattcccggtatccgagcca
tcggatatggtgtaatggctaacatcgttgactctca    ctcaacagctccgggttcgattcccggtatccgagcca
tcggatatggtgtaatggctaacatcgttgactctca    ctcaacagctccgggttcgattcccggtatccgagcca
tcggatatggtgtaatggctaacatcgttgactctca    ctcaacagctccgggttcgattcccggtatccgagcca
tcggatatggtgtaatggctaacatcgttgactctca    ctcaacagctccgggttcgattcccggtatccgagcca
tcggatatggtgtaatggctaacatcgttgactctca    ctcaacagctccgggttcgattcccggtatccgagcca
tcggatatggtgtaatggctaacatcgttgactctca    ctcaacagctccgggttcgattcccggtatccgagcca
```

Figure 18. Comparing predicted tRNA gene models and sequenced short RNA data on GBrowse

(Nucleotides highlighted in red color are mismatches when mapping short RNA sequences onto the genome. Since nucleotides of the 3'-CCA tail are not encoded by the genome, they are frequently found as mismatches in the mapped read sequences.)

In the second stage, the primary structures of tRNA genes were examined by comparing sequences of the A box and B box with the consensus ones deduced from the experimentally characterized fungal tRNA genes. Moreover, the genomic region downstream of each tRNA gene is checked to see if the transcriptional termination site can be found within this region. In the third stage, the predicted secondary structure of each tRNA gene was examined by checking the conserved and semi-conserved positions in stems and loops of the cloverleaf model. These conserved and semi-conserved nucleotides were found from the secondary structure analysis of the experimentally identified fungal tRNAs. The predicted intron regions were also considered in this step. The tRNA genes having introns, which are located one nucleotide downstream of the anticodon, are likely to be more reliable since all of the introns in the fungal tRNA genes collected from the literature in this study are located in this position. Finally, by combining evidence from the transcriptome data, the primary and secondary structure analyses, the data set of reliable tRNA genes from the *M. thermophila* genome was obtained.

2.2. AARS gene curation and annotation procedure

An overview of the AARS gene detection procedure is illustrated in **Figure 19**. First, reliable aminoacyl-tRNA synthetases from different databases were collected and analyzed to determine the common features of the AARS gene family. Then, the collected AARSs were used as query sequences in the BLASTP search against the 11,342 protein sequences which were predicted from the nuclear genome of *M. thermophila* and were provided by the Genozymes project. Significant hits found from the BLASTP search were considered as AARS gene candidates. Next, the gene models of these candidates were examined and compared to the transcriptome data on GBrowse. Moreover, protein sequences of the AARS candidates were analyzed to see whether they satisfied features specific for either cytoplasmic or mitochondrial AARSs. Finally, by combining results from the BLASTP search, transcriptome data analysis and protein sequence analysis, the most reliable AARS genes of *M. thermophila* were determined.

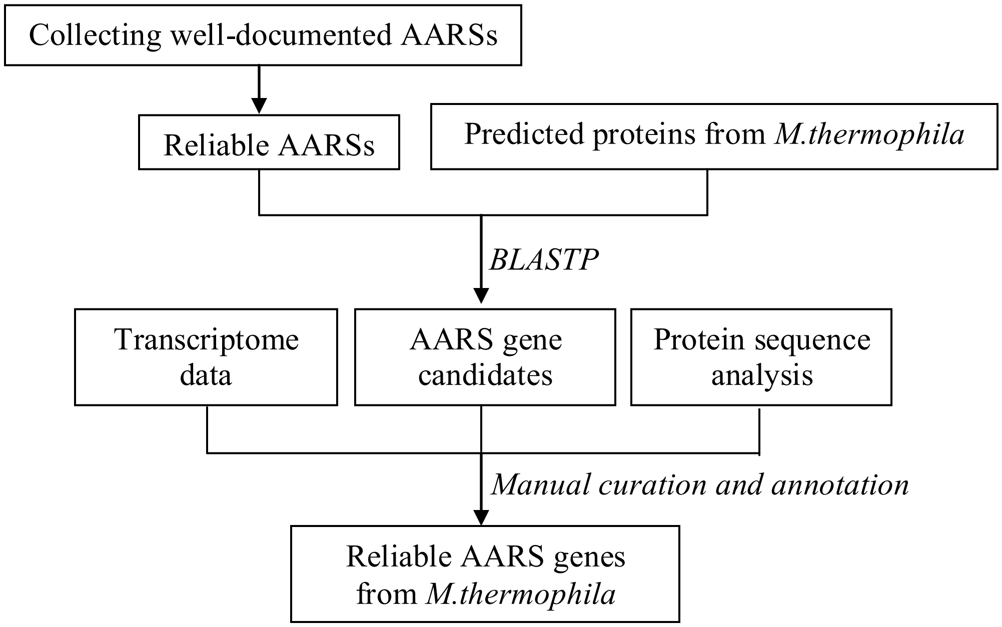

Collecting well-documented AARSs
Reliable AARSs
Predicted proteins from *M.thermophila*
BLASTP
Transcriptome data
AARS gene candidates
Protein sequence analysis
Manual curation and annotation
Reliable AARS genes from *M.thermophila*

Figure 19. Overall procedure of the AARS gene detection in *M. thermophila*

2.2.1. Collecting and analyzing reliable aminoacyl-tRNA synthetases

2.2.1.1. Collecting experimentally characterized fungal cytoplasmic AARSs

The experimentally characterized fungal cytoplasmic AARSs were collected from various databases presented in **Table 1**. References related to each of the collected

AARS sequences were examined to select only the AARSs that were experimentally characterized.

Table 1. Databases used in the search for experimentally characterized fungal cytoplasmic AARSs

Database name	Websites
Saccharomyces Genome Database (SGD)	http://www.yeastgenome.org/
Candida Genome Database (CGD)	http://www.candidagenome.org
UniProt	http://www.uniprot.org/
GenBank and GenPept databases from NCBI (National Center for Biotechnology Informatio	http://www.ncbi.nlm.nih.gov/
GeneDB	http://www.genedb.org/
BRENDA	http://www.brenda-enzymes.info/
Aminoacyl-tRNA synthetase database	http://biobases.ibch.poznan.pl/aars/

2.2.1.2. Collecting other reliable AARSs

Since there were few fungal cytoplasmic AARSs experimentally identified in the databases, it was necessary to search for other reliable AARSs. This second search was done within the UniProt database. Entries of all aminoacyl-tRNA synthetases in UniProt were downloaded. The GO terms and GO evidence codes of these AARSs were checked. Only the ones having GO terms specific for AARSs and having reliable GO evidence codes at the three aspects (Molecular Function, Biological Process and Component) were selected. This search included both cytoplasmic and mitochondrial AARSs from all organisms.

2.2.1.3. Sequence analysis of the characterized AARSs

Sequences of all characterized AARSs were subjected to the multiple sequence alignment using ClustalW (Thompson *et al.* 2002). The MSA profile was then used to build a phylogenetic tree which showed the similarity between different types of AARSs from various species. The Neighbor–Joining method (Saitou and Nei 1987) and the bootstrap analysis (Felsenstein 1985) were applied for constructing and evaluating the tree.

To search for the signature motif regions of the characterized AARSs, amino acid sequences of the same AARS type were aligned to obtain MSA profiles of different specificities. Then, based on these MSA profiles and the AARS signature motifs found by Eriani *et al.* (1995), the motifs of the characterized AARSs were inferred.

To check if all of the characterized mitochondrial AARSs contain the mitochondrial targeting peptide (mTP) at the N-terminus of their amino acid sequences, the mTP detection was performed on all characterized mitochondrial AARS sequences by the TargetP program (Nielsen *et al.* 1997; Emanuelsson *et al.* 2000)

2.2.2. Manual curation and annotation of AARS genes in *M. thermophila*

Significant hits found from the BLASTP search which used the characterized AARSs as queries and searched against predicted protein sequences of *M. thermophila* were manually examined by checking their transcriptome data and their sequence characteristics. First, the gene models of these hits were compared to the sequenced mRNAs that were previously mapped onto the genome (**Figure 20**). The gene models and the mapped mRNA data were provided by the Genozymes project and were displayed on GBrowse (https://reviewers.fungalgenomics.ca/gene_model_pages/AARS_Table.html). The gene models from the Joint Genome Institute (JGI) were also used in this manual curation. Only gene models well-matched with the transcript coverage were selected. There should be many more mRNA sequences mapped to the exon regions as compared to those mapped to the intron regions.

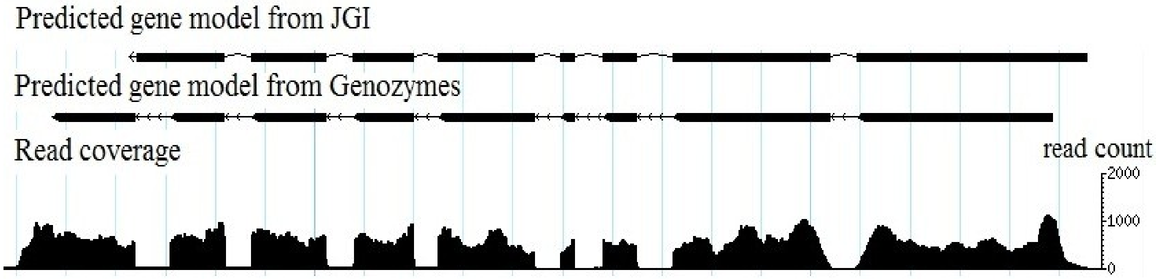


Figure 20. Comparing AARS gene models and transcriptome data on GBrowse

Secondly, the nucleotide sequences of these gene models were examined by checking their start and stop codons as well as the intron splicing sites. There should not be any stop codons present within the exon regions. Also, the introns should begin with “GT” at the 5’ end and end with “AG” at the 3’end according to the “GT-AG” rule suggested by Chambon (Breathnach and Chambon 1981). Next, protein sequences of the genes selected from the first two steps were checked to see whether they had the signature motifs specific for AARS enzymes. Moreover, the mTPs were searched within amino acid sequences of the AARS candidates to determine whether they had cytoplasmic or mitochondrial functions or both of these functions. It should be noted that some AARS genes may encode both mitochondrial and cytoplasmic AARS enzymes of the same specificity such as the genes encoding mitochondrial and cytoplasmic valyl-tRNA synthetase and histidyl-tRNA synthetase in *S. cerevisiae* (Natsoulis *et al.* 1986; Chatton *et al.* 1988). Hence, if the protein sequence of an AARS gene contains an mTP at its N-terminus, and it is more similar to cytoplasmic AARSs than to the mitochondrial AARSs, it is likely that the protein has both cytoplasmic and mitochondrial functions.

Since the BLASTP search is based on the predicted proteins of *M. thermophila*, it may be possible that some genes are missed during the protein-encoding gene prediction. Accordingly, some AARS genes may also be missed due to the incorrect gene prediction. To address this issue, the TBLASTN search was carried out against the whole genome of *M. thermophila* using the characterized AARSs as query sequences. Results from the BLASTP and TBLASTN searches were compared to ensure that no genomic region possibly encoding AARSs is missed.

2.3. Calculating the effective number of codons (N_c)

The method to calculate the effective number of codons was proposed by Wright (1990). When calculating N_c values, 20 amino acids are classified into five different synonymous families (SF), each of which consists of the amino acids having the same number of synonymous codons (**Table 2**).

Table 2. The synonymous families and the amino acids in each family

Family name(*)	Number of synonymous codons	Amino acids	Number of amino acids
SF1	1	Met, Trp	2
SF2	2	Asn, Asp,Cys, Gln, Glu, His, Lys, Phe, Tyr	9
SF3	3	Ile	1
SF4	4	Ala, Gly, Pro, Thr, Val	5
SF6	6	Arg, Leu, Ser	3

(*) The number in the family name shows the number of synonymous codons of amino acids in each family

Contribution of an amino acid *j* (F_j) to the overall codon usage of a gene *X* is estimated according to the formula (1).

$$F_j = (n \sum_{i=1}^{k} p_i - 1)/(n-1) \qquad (1)$$

where n : the number of amino acid *j* in the sequence of gene *X*

k : the number of synonymous codons of amino acid *j* (e.g. $k_{Ala} = 4$)

p_i : frequency of codon *i* among its synonymous codons (if n_i is the number of codon *i* in gene *X*, then $p_i = n_i/n$)

The N_c value of the gene *X* is calculated as followed:

$$N_c = 2 + \left(\frac{9}{\overline{F_2}}\right) + \left(\frac{1}{F_3}\right) + \left(\frac{5}{\overline{F_4}}\right) + \left(\frac{3}{\overline{F_6}}\right) \qquad (2)$$

where $\overline{F_2}$, $\bar{F}_4$, $\overline{F_6}$: the average of F_j values of the amino acids in the family SF2, SF4 and SF6, respectively

If an amino acid does not exist in the gene, the calculation only considers the ones that are present. If Ile is absent or rarely used in the gene, the value of F_3 is calculated as the average of $\overline{F_2}$ and $\bar{F}_4$.

An N_c of a gene takes the values from 20 to 61. When N_c is equal to 20, it indicates that the codon usage of the gene is extremely biased since it utilizes only one codon to code for each amino acid. In contrast, when N_c is equivalent to 61, the gene does not have codon bias (Wright 1990). In this study, the N_c values were calculated by using the codonW 1.4.2 program (Peden 2005).

2.4. Determining preferred codons

The preferred codons were deduced by combining the results from three methods. The first method was based on 420 protein-encoding genes having the highest gene expression levels in *M. thermophila*. The 101,727 codons from the mRNA sequences of these genes were classified into groups according to the amino acid specificity. The codon having the highest percentage in each group was considered as the preferred codon of the corresponding amino acid. For instance, if the percentages of four codons coding for Ala (GCT, GCC, GCA and GCG) are 10%, 20%, 5% and 65%, respectively, the preferred codon of Ala would be GCG. The second method was based on the complete set of tRNA genes. Transfer RNA genes in each isoacceptor family were classified into anticodon specific groups. The group having the highest number of tRNA genes in each isoacceptor family was selected for further analysis. It has been reported that although a tRNA can decode multiple codons due to the Wobble base pairing, it mainly decodes the codon which it perfectly matches (Percudani *et al.* 1997). Hence, among codons recognized by tRNAs in a selected group, the perfectly matched codon was considered as the preferred codon of the corresponding amino acid. It should be noted that in tRNAs having nucleotide "A" at their anticodon wobble position, this nucleotide is frequently converted into "I" during the post-transcriptional modification (Percudani *et al.* 1997). Unlike "A", which substantially forms base pairing with "U", "I" favourably matches with either "U" or "C" (Ikemura and Ozeki 1983). Therefore, when searching for the codon well-matched with a tRNA having "A" at its wobble position, the codons terminated by either "C" or "U" were preferentially considered. The third method was based on a set of protein-encoding genes having N_c values lower than 31. Due to the low N_c values, these genes obviously show that they have bias in their codon usage. Similar to the first method, codons from this gene set were classified into amino acid specific groups, and percentages of codons in each group were calculated. The codon having the highest percentage in each group was selected as a preferred codon. Finally, results from the three methods were compared to select the final set of preferred codons. Since Met and Trp are encoded by only one codon, they are not included in the preferred codon selection.

2.5. Analyzing relationships between tRNA gene number, tRNA abundance and codon usage

The relationships between tRNA gene number, tRNA abundance and codon usage are examined by using the Pearson correlation analysis. A correlation coefficient value (*r*), which ranges from -1 to 1, is calculated for each of the relationships. If the value of *r* is less than zero, it means that the two entities in the analysis are negatively correlated. Otherwise, they are positively correlated. The higher the absolute value of *r* is, the stronger the correlation of the two entities is. Along with the *r* value, the *p* value, which shows the probability that the correlation is found by chance, is calculated. Generally, a correlation is considered to be statistically significant when its *p* value is lower than 0.05. To calculate the *r* and *p* values, the SPSS v.20 (Statistical Package for the Social Sciences) program (http://www-01.ibm.com/software/analytics/spss/) is used. In all of the correlation analyses in this study, the selenocystein tRNA (tRNA^{SeC}) was not included.

2.5.1. Relationship between tRNA gene number and tRNA abundance

The tRNA abundance was calculated from the number of short RNAs mapped onto exon regions of the tRNA genes. One problem of this calculation is that it depends on the lengths of the tRNAs because a long tRNA may have more reads mapped onto it than a shorter one although their gene expression levels are similar. To solve this problem, the original read count number was transformed into the FPKM value, which was the number of Fragments Per Kilobase of exon per Million fragments mapped (Mortazavi *et al.* 2008). Each tRNA gene has an FPKM value calculated from its mapped reads.

To determine the relationship between tRNA gene number and tRNA abundance, tRNA genes detected from *M. thermophila* were grouped into anticodon specific families. The initiator tRNA ($tRNA_i^{Met}$) and the elongator tRNA specific for methionine (tRNA^{Met}) were considered as two different families. The tRNA abundance of each family is the total FPKM values of all tRNA genes in the family. Finally, the numbers of tRNA genes and the tRNA abundance values of all of the anticodon specific families were subjected to the Pearson correlation analysis.

2.5.2. Relationship between tRNA gene number, tRNA abundance and codon usage

Two strategies were applied to determine the relationship between tRNA availability and codon usage. In the first approach, the relationship was examined at the amino acid level. First, tRNA genes were classified into isoacceptor families, each of which is specific for a given amino acid. The tRNA abundance of an isoacceptor family was calculated as the sum of FPKM values of tRNA genes in that family. Subsequently, the Pearson correlations between frequencies of amino acids and either tRNA abundance values or tRNA gene numbers of the isoacceptor families were calculated. In the second approach, the relationship between tRNA availability and codon usage was examined at codon level. The method of Percudani *et al.* (1997) was applied to determine this relationship. Firstly, each anticodon specific tRNA family has a “tRNA usage” value, which is calculated from the frequencies of codons that tRNA genes in the family can recognize. Due to the Wobble base paring between the first nucleotide of the anticodon and third nucleotide of the codon (**Table 3**), a tRNA can code for more than one codon (Crick 1966). In this study, “A” in the first position of the anticodons is considered as “I” and can form base pairing with “T”, “C” and “A” because adenosine (A) is usually converted into inosine (I) in eukaryotic nuclear tRNAs. Since each tRNA primarily codes for its perfectly matching codon, the codons that are recognized by the Wobble base pairing are not included in the tRNA usage calculation except for the cases in which the perfect matching does not exist. For example, if there are three anticodon-specific tRNA families of the tRNA^{Ala} with the anticodons AGC, CGC, TGC, and there are four codons GCT, GCG, GCA and GCC, the tRNA usage of these three families is calculated as presented in **Table 4**. Secondly, the tRNA abundance of each anticodon specific family was calculated as the total FPKM values of all tRNA genes in the family. Next, the tRNA abundance and tRNA usage values of all anticodon specific families were subjected to the Pearson correlation analysis to determine the relationship between tRNA abundance and codon usage. Similarly, the correlation between tRNA gene numbers and codon usage was also calculated. In the second approach, initiator tRNAs and the first codons of all protein-coding genes were not included in the analysis. The frequencies of codons and amino acids were calculated from the coding sequences of all predicted protein-encoding genes in *M. thermophila* which were provided by the Genozymes projects.

Table 3. Base pairing at the third position of the codon according to Wobble theory (Crick 1966)

First base of anticodon	Bases recognized on the codon
T	A, G
C	G
A	T
G	T, C
I	T, C, A

Table 4. Calculating tRNA usage values for three tRNA gene families specific for the anticodons AGC, CGC and TGC

Amino acid	Anticodon	Codons*	tRNA usage**
Ala	AGC	GCT, GCA, GCC	F(GCT) + F(GCC)
Ala	CGC	GCG	F(GCG)
Ala	TGC	GCA, GCG	F(GCA)

((*) Codon: all possible codons that the tRNAs in each family can decode according to the Wobble theory (Crick 1966);(**) F(X): frequency of codon X)

CHAPTER 3. RESULTS

3.1. tRNA gene prediction results

3.1.1. tRNAscan-SE

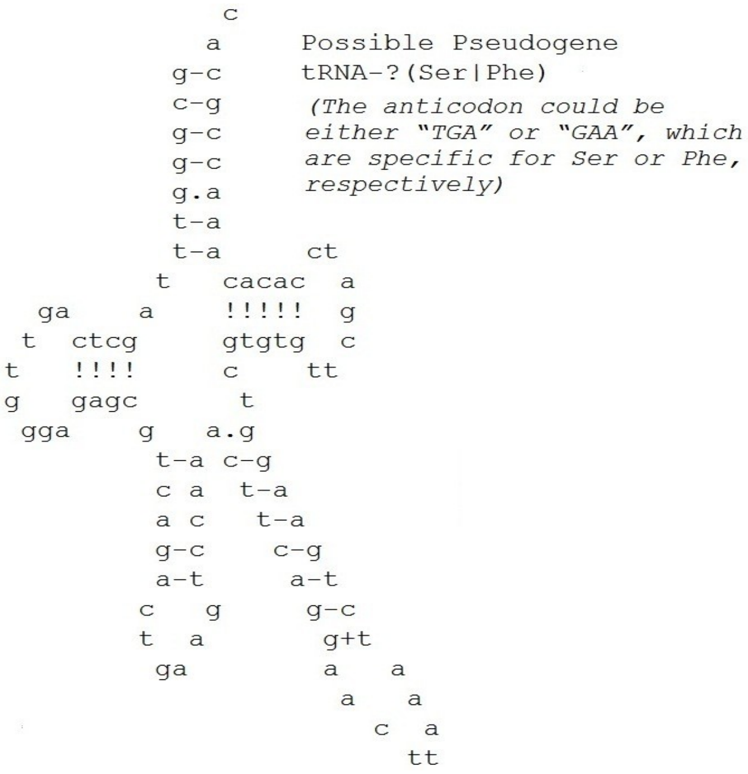
tRNAscan-SE detected 194 genes including 46 anticodon-specific gene families (**Supplementary table 1**). Although not all tRNA genes for the 61 anticodons were predicted, these 194 genes were still able to decode the 20 standard amino acids. Moreover, one tRNA gene specific for SeC and two pseudogenes corresponding to $tRNA^{Ile}$ and $tRNA^{Pro}$ were found from the predicted result. More than half of these predicted genes (65.46%) contain intervening sequences. These genes have only one intron located in the anticodon-loop region. The intron lengths are from 7 to 57 nucleotides.

3.1.2. SPLITSX

There are 195 tRNA genes predicted by SPLITSX. Results from SPLITSX were almost identical to those of tRNAscan-SE program except that one more pseudogene which had the anticodon “TCT” and was specific for Arg was found. There was no tRNA genes containing multiple introns detected from the genome of *M. thermophila*.

3.1.3. ARAGORN

Unlike tRNAscan-SE and SPLITSX, ARAGORN predicts any possible tRNA gene region, which may overlap each other or contain a very long intron sequence. Therefore, the number of genes that it predicted was much higher than the other predictors. The 349 tRNA genes predicted by ARAGORN comprised most of the 61 anticodon specific tRNA gene families, except for one family specific for the anticodon “ACA” (**Supplementary table 2**). In addition to tRNA genes coding for the 20 universal amino acids, there were one and four tRNA genes specific for Pyl and SeC, respectively. Besides, ARAGORN reported the genomic regions that might contain tRNA genes although the program was not able to determine their anticodons, or their predicted anticodons matched with stop codons. There were six tRNA genes having anticodons matching with stop codons, and four pseudogenes whose anticodons were not identified. An example of these pseudogenes was illustrated in **Figure 21**. The anticodon region proposed from the predicted secondary structure of this pseudogene contains only two nucleotides (“GA”); hence, the normal 3-nucleotide anticodon sequence could not be identified. Since these pseudogenes still have some features of a tRNA gene, they were included in further analyses to select the most reliable tRNA gene candidates.



```
             c
            a     Possible Pseudogene
          g-c     tRNA-?(Ser|Phe)
          c-g     (The anticodon could be
          g-c     either “TGA” or “GAA”, which
          g-c     are specific for Ser or Phe,
          g.a     respectively)
          t-a
          t-a     ct
         t   cacac  a
  ga    a    !!!!!  g
 t  ctcg     gtgtg  c
t   !!!!     c    tt
g   gagc      t
 gga    g   a.g
         t-a c-g
         c a  t-a
         a c   t-a
         g-c    c-g
         a-t     a-t
        c   g     g-c
        t  a       g+t
         ga        a   a
                    a   a
                      c  a
                        tt
```

Figure 21. Secondary structure of a pseudogene predicted by ARAGORN

More than 75% of the tRNA genes predicted by ARAGORN contain intervening sequences. The intron lengths were from 1 to 2991 nucleotides. While tRNA genes predicted from the other tools do not contain introns longer than 100 nucleotides, there were 36.4% of the tRNA genes from ARAGORN having introns longer than 100 nucleotides.

3.1.4. tRNAfinder

tRNAfinder predicted 190 tRNA genes including 47 anticodon-specific gene families. These genes can code for 19 universal amino acids (**Supplementary table 3**). Transfer RNA genes coding for the amino acid "Tyr" were not detected. There were 64.2% of the genes containing one intron. The intron lengths range from 7 to 94 nucleotides. The program also found one tRNA gene specific for the amino acid SeC.

3.1.5. Compare predicted tRNA genes from the four tools

The total number of tRNA genes predicted from the four programs was 928 genes. After removing the duplicates, the predicted data set contained 465 unique tRNA genes. As shown in **Figure 22**, results from the four tools are not in good agreement since the same results from all of these programs made up only 18% of the non-redundant predicted gene set. More than half of the predicted tRNA genes (56%) were from ARAGORN but not from the other predictors. When manually examining the genes only predicted by ARAGORN, it revealed that, except for those having introns longer than 100 nucleotides in length, most of the remaining genes were similar to the genes reported by the other predictors. However, their intron positions and ending positions were not exactly the same as from the other programs. **Figure 22** also showed that results from tRNAscan-SE, SPLITSX and tRNAfiner are highly consistent since the same results from these tools made up a large proportion in the non-redundant predicted data set. As mentioned above, results from tRNAscan-SE and SPLITSX are almost identical except for one pseudogene from SPLITSX. Among the total 190 genes from tRNAfinder, only ten genes were not predicted by tRNAscan-SE and SPLITSX.

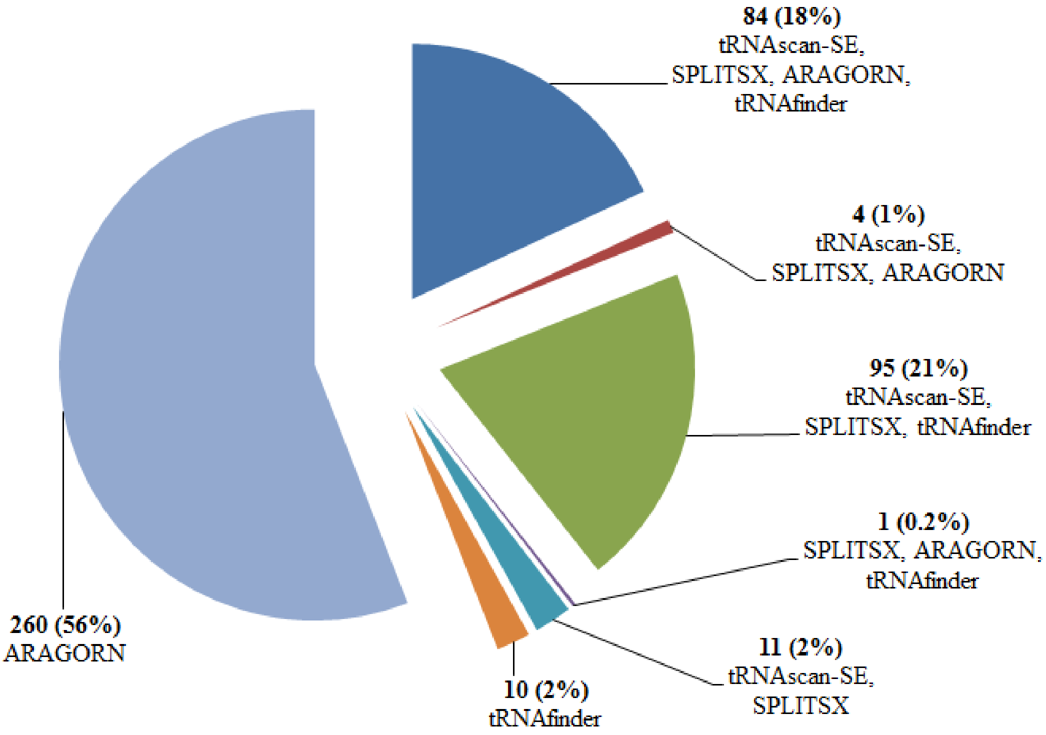


Figure 22. Numbers and percentages of tRNA genes predicted by different tools in the non-redundant predicted tRNA gene set

3.2. Mapping of short RNAs

After trimming the adapter sequences and discarding reads having more than five consecutive unidentified nucleotides, over 14 million reads were obtained from sequencing the short RNAs. 87.3% of them were mapped to the genome of *M.*

thermophila using Bowtie. The remaining 12.7% of the short RNAs were then subjected to the mapping process using BLAT. Finally, by combining the two programs, 94% of the total reads were mapped to the genome. 13.36% of them were mapped to the predicted tRNA gene regions. Although the number of short RNA sequences mapped by BLAT was fewer than those mapped by Bowtie, they were improtant for the manual curation and annotation process on GBrowse, especially for the intron-containing tRNA genes. As illustrated in **Figure 23**, when using only Bowtie, the read coverage does not match with the predicted tRNA gene model. However, when combining with BLAT program, the mapping result is much improved.

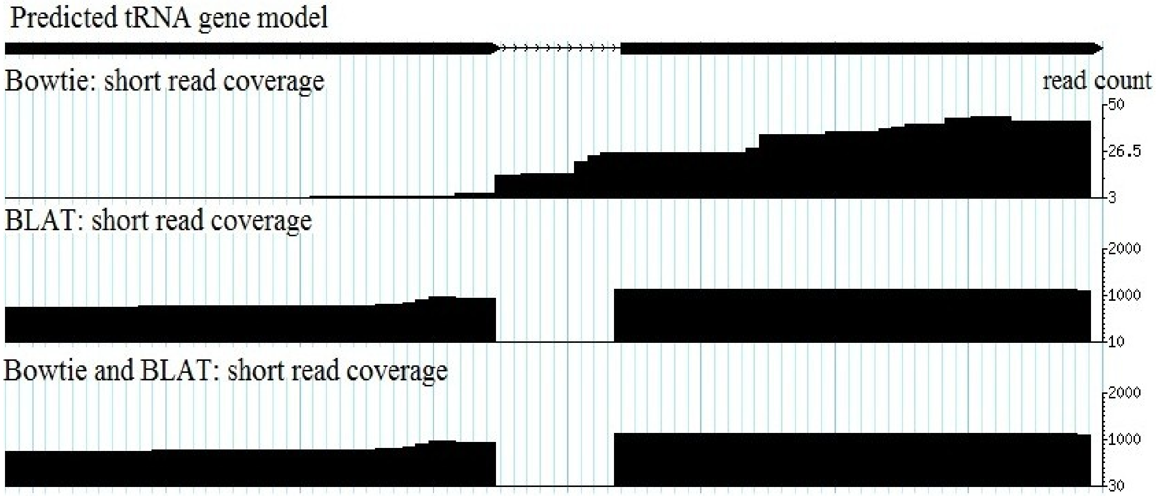


Figure 23. The short read mapping was improved when using BLAT

(The tRNA gene model in this example is located in the plus strand of chromosome 1 of the genome, from position 10293658 to position 10293739)

3.3. Summary of characterized fungal tRNAs and tRNA genes

There were 56 tRNA sequences and 322 tRNA gene sequences from 16 fungal species in tRNAdb database. After examining the published papers related to each of these sequences, there were 55 tRNAs and 53 tRNA genes found to be experimentally characterized. Moreover, four new experimentally characterized fungal tRNA genes were found from the literature. Their sequences were extracted from the papers since they were not available in either GenBank or tRNAdb. After discarding the duplicates, the final data set contained 85 unique tRNA sequences from 14 fungal species (**Supplementary table 4**), 43 of them were from *S. cerevisiae* (**Table 5**).

Table 5. Number of sequences of each fungal species in the experimentally characterized tRNA sequence data set

Fungal species	Number of tRNA sequences
Candida albicans	4
Candida cylindracea	8
Candida guilliermondii	1
Candida lusitaniae	1
Candida melibiosica	1
Candida parapsilosis	1
Candida rugosa	1
Candida tropicalis	1
Candida utilis	7
Candida zeylanoides	1
Neurospora crassa	3
Podospora anserine	2
Saccharomyces cerevisiae	43
Schizosaccharomyces pombe	11

Transfer RNA genes of the 20 isoacceptor families were included in the experimentally characterized fungal tRNAs collected in this study. Moreover, five tRNA initiators ($tRNA_i^{Met}$), which were used specifically in the translation initiation process, were found. However, this reliable data set did not comprise tRNAs from all 61 anticodon-specific families (**Table 6**). There are nine tRNAs specific for Ser, which have the anticodons “CAG” that generally decodes Leu. Their tRNA sequences are more similar to the sequences of tRNA^{Ser} than those of tRNA^{Leu} (**Figure 24**). All of these $tRNA_{CAG}^{Ser}$ come from organisms of the *Candida* genus. It has been reported that the codon “CUG”, which perfectly matches with the anticodon “CAG”, is used to encode Ser instead of Leu in many *Candida* species (Ohama *et al.* 1993; Santos, Keith and Tuite 1993; Suzuki *et al.* 1994). However, not all organisms in the *Candida* group have this non-universal coding system (Ohama *et al.* 1993).

Table 6. Number of experimentally characterized fungal tRNAs in the isoacceptor families and anticodon-specific families

AA* : # of tRNAs	Anticodon: number of tRNAs						
Ala: 4	AGC:3	CGC:0	GGC:0	TGC:1			
Arg: 6	ACG:3	CCG:0	CCT:1	GCG:0	TCG:0	TCT:2	
Asn: 1	ATT:0	GTT:1					
Asp: 3	ATC:0	GTC:3					
Cys: 1	ACA:0	GCA:1					
Gln: 2	CTG:1	TTG:1					
Glu: 4	CTC:1	TTC:3					
Gly: 2	ACC:0	CCC:0	GCC:1	TCC:1			
His: 3	ATG:0	GTG:3					
Ile: 3	AAT:3	GAT:0	TAT:0				
Leu: 8	AAG:3	CAA:3	CAG:0	GAG:0	TAA:1	TAG:1	
Lys: 3	CTT:2	TTT:1					
Met: 1	CAT:1						
Met$_i$**: 5	CAT:1						
Phe: 5	AAA:0	GAA:5					
Pro: 2	AGG:0	CGG:0	GGG:0	TGG:2			
Ser: 19	ACT:0	AGA:3	CGA:2	GCT:1	GGA:0	TGA:4	CAG:9
Thr: 3	AGT:2	CGT:1	GGT:0	TGT:0			
Trp: 1	CCA:1						
Tyr: 4	ATA:0	GTA:4					
Val: 5	AAC:3	CAC:1	GAC:0	TAC:1			

((*)AA: Amino acid; (**)Met$_i$: the first methionine in the protein sequence; The underlined codon generally codes for Leu; however, it codes for Ser in some Candida species)

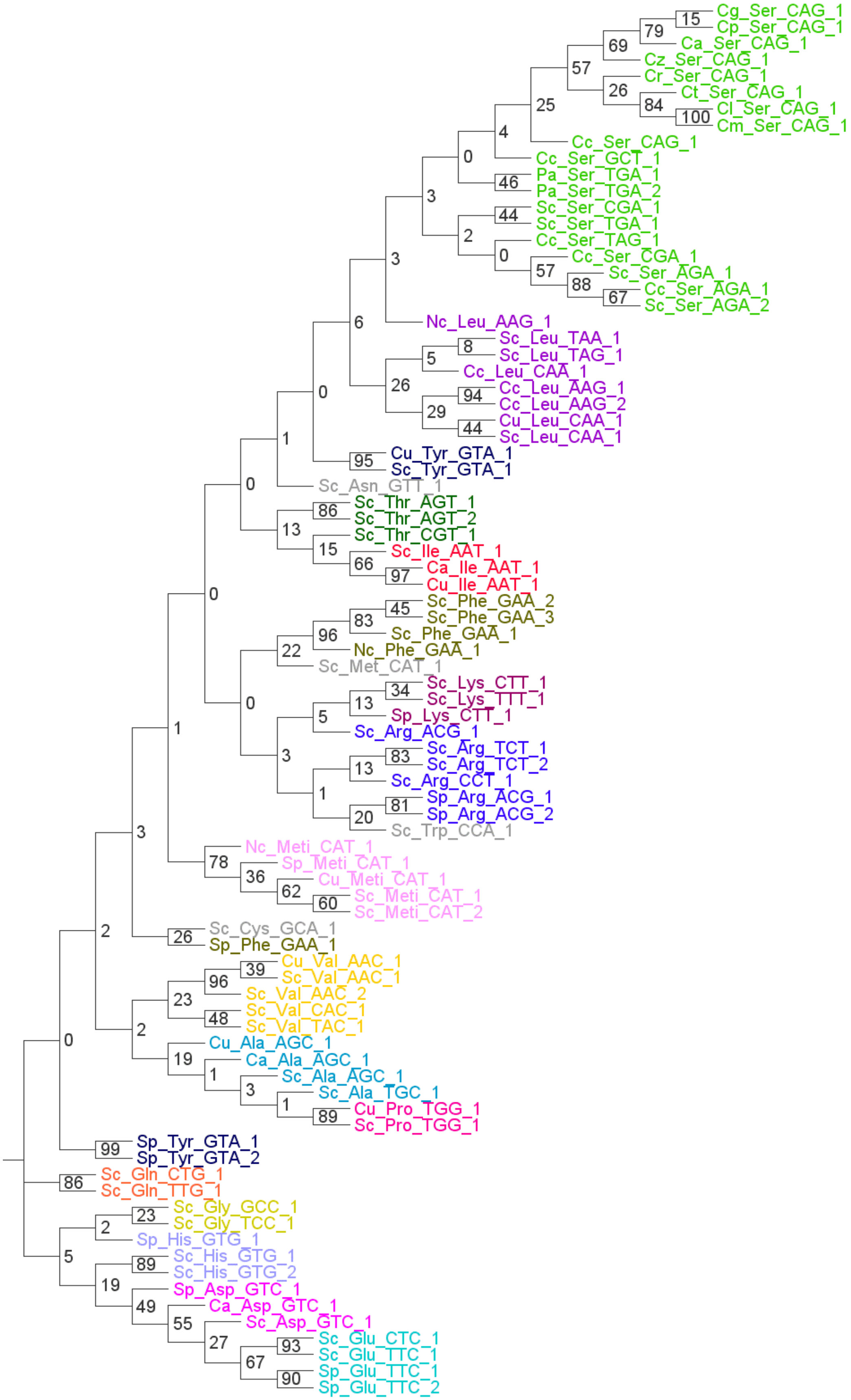

15
Cg_Ser_CAG_1
79
Cp_Ser_CAG_1
69
Ca_Ser_CAG_1
57
Cz_Ser_CAG_1
26
Cr_Ser_CAG_1
25
Ct_Ser_CAG_1
84
100
Cl_Ser_CAG_1
Cm_Ser_CAG_1
4
Cc_Ser_CAG_1
0
Cc_Ser_GCT_1
46
Pa_Ser_TGA_1
Pa_Ser_TGA_2
3
44
Sc_Ser_CGA_1
Sc_Ser_TGA_1
2
Cc_Ser_TAG_1
0
Cc_Ser_CGA_1
3
57
Sc_Ser_AGA_1
88
Cc_Ser_AGA_1
67
Sc_Ser_AGA_2
Nc_Leu_AAG_1
6
8
Sc_Leu_TAA_1
5
Sc_Leu_TAG_1
Cc_Leu_CAA_1
26
94
Cc_Leu_AAG_1
Cc_Leu_AAG_2
29
44
Cu_Leu_CAA_1
Sc_Leu_CAA_1
0
95
Cu_Tyr_GTA_1
Sc_Tyr_GTA_1
1
Sc_Asn_GTT_1
0
86
Sc_Thr_AGT_1
Sc_Thr_AGT_2
13
Sc_Thr_CGT_1
15
Sc_Ile_AAT_1
66
Ca_Ile_AAT_1
97
Cu_Ile_AAT_1
0
45
Sc_Phe_GAA_2
Sc_Phe_GAA_3
83
96
Sc_Phe_GAA_1
22
Nc_Phe_GAA_1
Sc_Met_CAT_1
34
Sc_Lys_CTT_1
Sc_Lys_TTT_1
13
0
Sp_Lys_CTT_1
5
Sc_Arg_ACG_1
1
83
Sc_Arg_TCT_1
Sc_Arg_TCT_2
13
3
Sc_Arg_CCT_1
1
81
Sp_Arg_ACG_1
Sp_Arg_ACG_2
20
Sc_Trp_CCA_1
3
Nc_Meti_CAT_1
78
Sp_Meti_CAT_1
36
Cu_Meti_CAT_1
62
Sc_Meti_CAT_1
60
Sc_Meti_CAT_2
Sc_Cys_GCA_1
26
Sp_Phe_GAA_1
2
Cu_Val_AAC_1
39
Sc_Val_AAC_1
96
Sc_Val_AAC_2
23
Sc_Val_CAC_1
48
Sc_Val_TAC_1
2
Cu_Ala_AGC_1
19
Ca_Ala_AGC_1
1
Sc_Ala_AGC_1
0
3
Sc_Ala_TGC_1
1
Cu_Pro_TGG_1
89
Sc_Pro_TGG_1
99
Sp_Tyr_GTA_1
Sp_Tyr_GTA_2
86
Sc_Gln_CTG_1
Sc_Gln_TTG_1
23
Sc_Gly_GCC_1
Sc_Gly_TCC_1
2
Sp_His_GTG_1
5
89
Sc_His_GTG_1
Sc_His_GTG_2
19
Sp_Asp_GTC_1
49
Ca_Asp_GTC_1
55
Sc_Asp_GTC_1
27
93
Sc_Glu_CTC_1
Sc_Glu_TTC_1
67
Sp_Glu_TTC_1
90
Sp_Glu_TTC_2

Figure 24. Phylogenetic tree built from the experimentally characterized fungal tRNA sequences

(Transfer RNAs from the same isoacceptor family are presented in the same color. Transfer RNAs specific for the first methionine (Meti) in the protein sequence and those specific for the other methionines are classified in two different isoacceptor families. For the isoacceptor families that have only one tRNA sequence in the experimentally characterized tRNA data set, their tRNAs are presented in grey color. The numbers on the tree are bootstrap values, which show the probability of clades on the tree. The higher the probability is, the more likely the corresponding branch or clade is. Name of each tRNA on the tree includes abbreviations of the corresponding species name, the assigned amino acid, the anticodon and the order number of that tRNA in its anticodon-specific family. For example, there are two $tRNA_{GTA}^{Tyr}$ *from S. pombe. Thus, names of these two tRNAs are Sp_Tyr_GTA_1 and Sp_Tyr_GTA_2. Abbreviations:* ***Ca****, Candida albicans;* ***Cc****, Candida cylindracea;* ***Cg****, Candida guilliermondii;* ***Cl****, Candida lusitaniae;* ***Cm****, Candida melibiosica;* ***Cp****, Candida parapsilosis;* ***Cr****, Candida rugosa;* ***Ct****, Candida tropicalis;* ***Cu****, Candida utilis,* ***Cz****, Candida zeylanoides;* ***Nc****, Neurospora crassa;* ***Pa****, Podospora anserine;* ***Sc****, Saccharomyces cerevisiae;* ***Sp****, Schizosaccharomyces pombe*)

The relationships among the 85 experimentally characterized fungal tRNAs are displayed in the phylogenetic tree constructed from their sequences (**Figure 24**). Although there are some exceptions, the tree generally shows that tRNAs in the same isoacceptor family are grouped together. These clusters indicate that tRNAs specific for the same amino acid from different species are more similar to each other than those from different isoacceptor families from the same organism. There are several significant clades having bootstrap values equal or greater than 0.7. These clades demonstrate the close relationship between tRNAs having the same anticodons or decoding the same amino acids although they originated from different species. However, due to the low bootstrap values of a majority of clades, it may require additional evidence, such as the ability to form cloverleaf structure as well as the conserved positions within the stems and loops, in addition to the primary sequence similarity, to detect new tRNAs.

3.4. Results from the manual curation and annotation of tRNA genes in *M. thermophila*

After manually examining the transcriptome data as well as the primary and secondary structures of all 465 unique tRNA genes predicted by the four prediction tools, 194 genes are found to be correctly predicted (https://reviewers.fungalgenomics.ca/gene_model_pages/tRNA_Table.html). Some of these genes do not have good matchings between their gene models and the short read coverage. However, they are considered as reliable tRNA genes since they have common features of the experimentally characterized fungal tRNA genes. Also, there is evidence in the sequenced data, such as the high number of mapped reads or the 3'-CCA tail, which demonstrates that these predicted genes are true positive results. Most of the 194 tRNA genes are predicted by more than one tool, except for one gene only predicted by ARAGORN (**Table 7**). The tRNA genes identically predicted by all four tools are all true tRNA genes. Most of the incorrectly predicted genes come from ARAGORN. Some of them are from tRNAfinder, and only two of them are from tRNAscan-SE and SPLITSX. As shown in **Figure 25**, tRNAscan-SE and SPLITSX are the most reliable programs among the four predictors.

Results from these two tools are almost the same since SPLITSX is basically an extended version of tRNAscan-SE with some modifications to detect tRNA genes containing multiple introns. It should be noted that all three pseudogenes predicted by tRNAscan-SE and SPLITSX are found to be true tRNA genes. tRNAfinder is also a reliable tool for tRNA gene detection although it is not as good as tRNAscan-SE and SPLITSX. It cannot find several true tRNA genes and has a higher number of false positive results. As compared to the others, ARAGORN has the lowest rate of true positive outcomes. This tool reported the highest number of tRNA gene locations since it predicted tRNA genes having introns longer than 100 nucleotides while the other tools did not. However, all genes containing introns longer than 100 nucleotides from ARAGORN were incorrectly predicted genes. On the other hand, although a number of tRNA gene locations reported by ARAGORN did contain the true genes, their ending positions or intron positions are not accurate (**Figure 26**). All of the pseudogenes and tRNA genes having anticodons matching with stop codons reported by ARAGORN are not true positives.

The 194 reliable tRNA genes found in *M. thermophila* are from 46 anticodon specific families. Although these tRNA genes do not comprise all of the 61 anticodon specific families, they can code for all of the 20 universal amino acids. In addition, genes of the tRNAs specifically used for translational initiation were found among the tRNA$^{\mathrm{Met}}$ genes. There is one tRNA gene found to be specific for SeC as determined by its anticodon. This gene is predicted by all of the tRNA predictors. According to the transcriptome data, it is a reliable gene. However, its secondary structure is more similar to those of normal elongator tRNAs than the special one of tRNA$^{\mathrm{SeC}}$, which is described previously. Therefore, to ensure whether this tRNA actually carries SeC to the polypeptide chain in the translation process or not, experimental evidence may be required. According to the numbers of tRNA genes in the anticodon specific families presented in **Table 8**, it is likely that *M. thermophila* does not use the synonymous codons equally. Instead, its codon usage tends to bias toward a subset of the 61 codons since there is frequently one anticodon specific family has many more tRNA genes than the others in the same isoacceptor family.

Table 7. Number of correctly and incorrectly predicted tRNA genes as revealed by manual curation and annotation

Predictor(s) that reports unique tRNA genes	**Total # of predicted genes**	**Correctly predicted genes**	**Incorrectly predicted genes**
tRNAscan-SE, SPLITSX, ARAGORN, tRNAfinder	84	84	0
tRNAscan-SE, SPLITSX, ARAGORN	4	3	1
tRNAscan-SE, SPLITSX, tRNAfinder	95	94	1
SPLITSX, ARAGORN, tRNAfinder	1	1	0
tRNAscan-SE, SPLITSX	11	11	0
tRNAfinder	10	0	10
ARAGORN	260	1	259

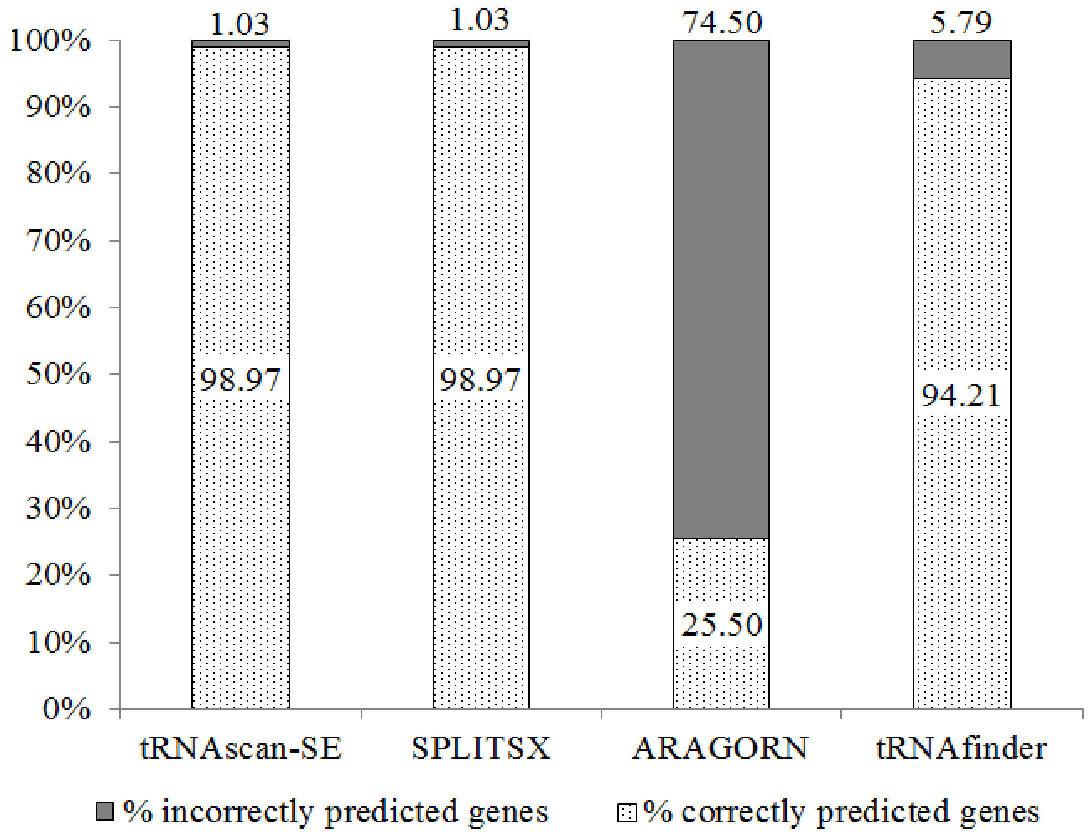


Figure 25. Comparing results from the four tRNA gene prediction tools

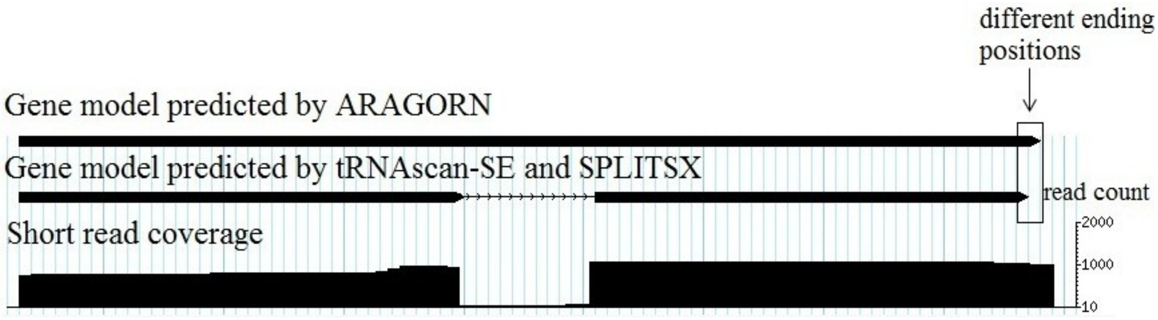

different ending positions
Gene model predicted by ARAGORN
Gene model predicted by tRNAscan-SE and SPLITSX
read count
Short read coverage
2000
1000
10

Figure 26. Example of a tRNA gene from ARAGORN having incorrect ending and intron positions

(The gene model predicted by tRNAscan-SE and SPLITSX is considered by manual curation and annotation as a true tRNA gene. It is located in the plus strand of chromosome 5 of the genome, from position 513153 to position 513236. Because the 3'-CCA tail is added to the tRNA transcript after the transcription process, the short RNA sequences may contain the three nucleotides "CCA" at their 3' end in addition to the tRNA gene sequences. Therefore, the short read coverage may extend longer than the tRNA gene model.)

Table 8. Number of tRNA genes of *M. thermophila* in isoacceptor families and anticodon specific families

AA : Gene number	Anticodon: Gene number					
Ala: 12	AGC:7	CGC:3	GGC:0	TGC:2		
Arg: 14	ACG:7	CCG:2	CCT:2	GCG:0	TCG:1	TCT:2
Asn: 6	ATT:0	GTT:6				
Asp: 10	ATC:0	GTC:10				
Cys: 3	ACA:0	GCA:3				
Gln: 7	CTG:5	TTG:2				
Glu: 10	CTC:8	TTC:2				
Gly: 16	ACC:0	CCC:2	GCC:11	TCC:3		
His: 4	ATG:0	GTG:4				
Ile: 10	AAT:8	GAT:1	TAT:1			
Leu: 15	AAG:7	CAA:2	CAG:4	GAG:0	TAA:1	TAG:1
Lys: 12	CTT:9	TTT:3				
Met: 4	CAT:4					
Met$_i$: 4	CAT:4					
Phe: 5	AAA:0	GAA:5				
Pro: 11	AGG:5	CGG:3	GGG:0	TGG:3		
Ser: 17	ACT:0	AGA:5	CGA:3	GCT:6	GGA:0	TGA:3
Thr: 11	AGT:6	CGT:3	GGT:0	TGT:2		
Trp: 4	CCA:4					
Tyr: 6	ATA:0	GTA:6				
Val: 12	AAC:7	CAC:3	GAC:0	TAC:2		
SeC: 1	TCA:1					

As stated earlier, the number of short RNA reads covering a predicted tRNA gene demonstrates its reliability. The graph in **Figure 27** shows that a large fraction of the reliable tRNA genes have a high number of mapped reads. More than half of them have at least 1000 mapped reads. Most of the remaining genes had more than 100 read sequences mapped onto them.

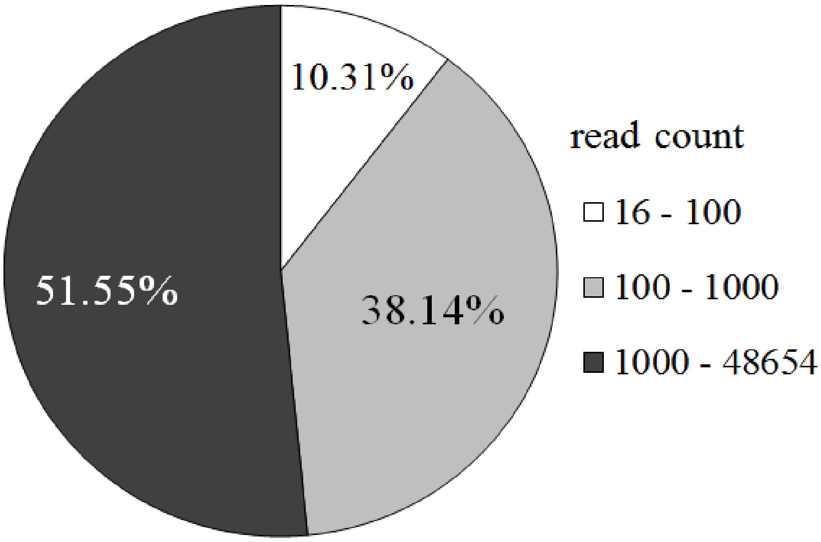


Figure 27. The amount of short reads mapped onto the reliable tRNA genes

In addition, there are many genomic regions in the genome of *M. thermophila* that match with a large number of the sequenced short RNAs. However, no tRNA gene is predicted within these regions (**Figure 28**). Their lengths are consistent with those of tRNA genes. Therefore, to ensure that all tRNA genes were detected, the tRNA gene prediction was carried out again on nucleotide sequences of the genomic regions highly covered by the short reads and having at least 70 nucleotides in length. No new reliable tRNA gene was found in this second prediction.

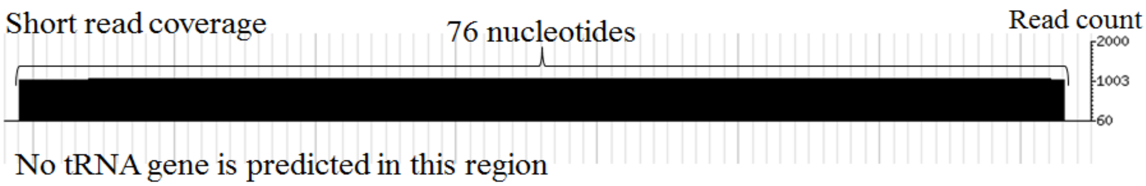


Figure 28. Example of a genomic region highly covered by the short reads and having no predicted tRNA gene

(The genomic region in this example is in chromosome 1 of the genome, from position 7512588 to position 7512663)

3.5. Characteristics of experimentally characterized fungal tRNA genes and tRNA genes found in *M. thermophila*

Lengths of tRNAs found in *M. thermophila* are similar to those of the characterized fungal tRNAs. Lengths of the class II tRNAs (tRNA^{Leu} and tRNA^{Ser}) in *M. thermophila* and in the experimentally characterized tRNA data set are 82-91 nucleotides and 83-87 nucleotides, respectively. For the class I tRNAs, their lengths range from 74 to 77 nucleotides in *M. thermophila*, and from 74 to78 nucleotides in the characterized tRNA data set. The difference between the class I and class II tRNA lengths is mostly due to their different V-loop lengths. Lengths of the V-loop are from 11 to 15 nucleotides for the class II tRNAs, and from 3 to 5 nucleotides for the class I tRNAs. The length range of all fungal tRNAs found in this study is in agreement with the commonly known tRNA lengths which are from 74 to 98 nucleotides (Westhof and Auffinger 2001).

Nucleotides at the conserved and semi-conserved positions in tRNAs of *M. thermophila* are consistent with those found in the characterized fungal tRNAs. Within the A box region which is from position 8 to 19 in the cloverleaf structure, four strictly conserved nucleotides “T”, “G”, “A” and “G” are found at positions 8, 10, 14, and 18, respectively. Furthermore, at position 9 and 15, only purine nucleotides (A, G) are observed. Also, only pyrimidine nucleotides (C, T) are found at position 16. Within the B box region which is from position 52 to 62 in the secondary structure, the nucleotides “G”, “T”, “C”, “A” and “C” are always found at positions 53, 55, 56, 58 and 61, respectively. At position 57, only purine nucleotides are observed. At position 54 and 60, there is a distinction between initiator and elongator tRNAs. The nucleotide “T” is always located at position 54 of all elongator tRNAs while it is always “A” at this position in initiator tRNAs. Moreover, at position 60 of all elongator fungal tRNAs, it is either “T” or “C” while it is always “A” in initiator tRNAs. In addition to the conserved and semi-conserved positions of the A box and B box, which are located within the D and T stem and loop regions, there are three more semi-conserved positions found within the anticodon loop. Pyrimidine and purine nucleotides are always observed at position 32 and 37, respectively. The nucleotides at position 33 are frequently either “T” or “G”. The conserved and semi-conserved nucleotides of all fungal tRNAs in this study are presented in **Figure 29**. Besides the invariant nucleotides, the base pairings within the stems in the cloverleaf structures of the fungal tRNAs were considered. It showed that in addition to the normal base pairs (A-T, G-C), several unusual pairs (T-G, T-T, C-T and C-A) were found in the A-stem. Among those, T-G is the most frequently observed.

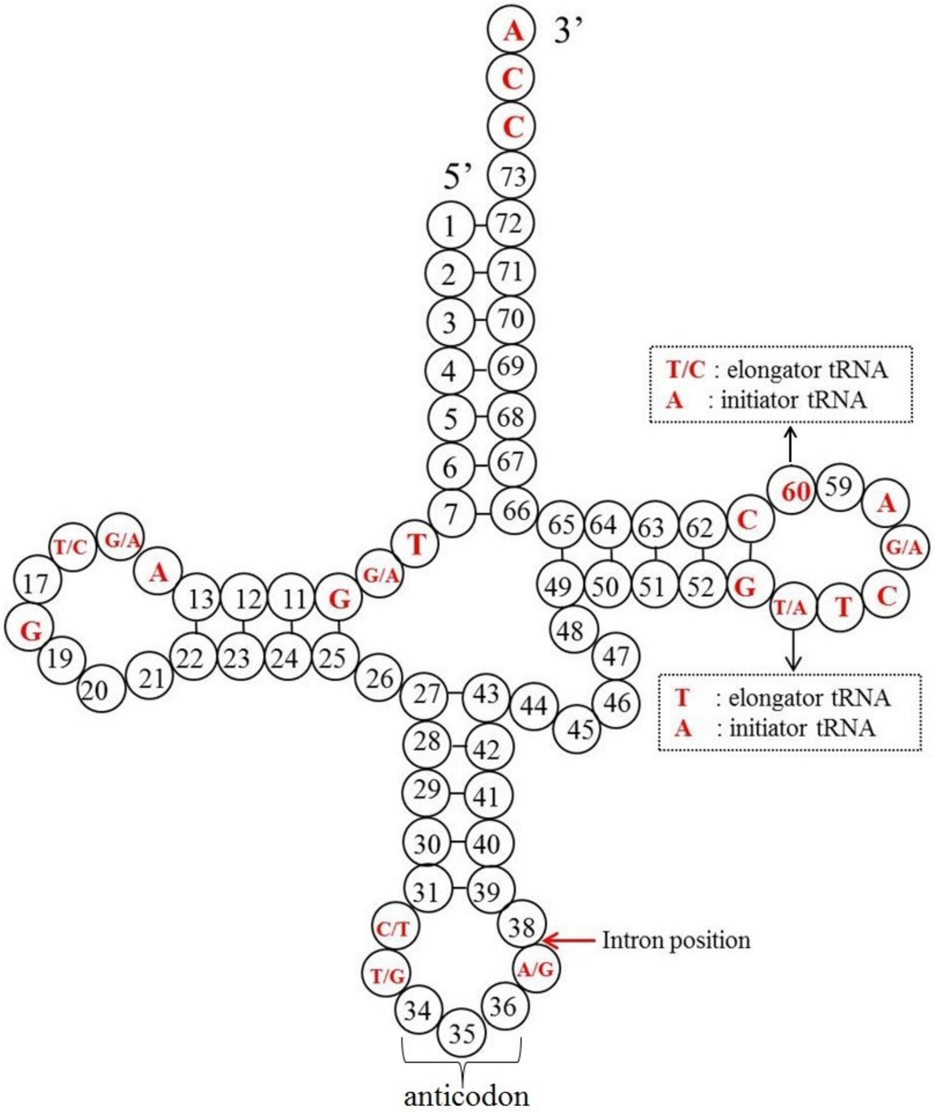
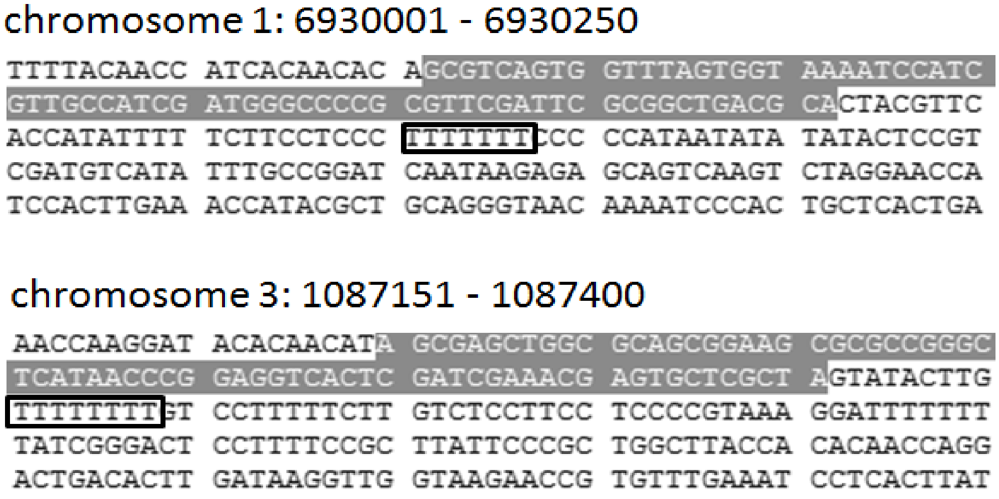


Figure 29. Conserved and semi-conserved nucleotides as well as the intron position of the fungal tRNAs in this study

In *M. thermophila*, 65% of the tRNA genes contain introns. All of the intron-containing genes in *M. thermophila* as well as in the characterized fungal tRNA data set have only one intron. All of the introns are located one nucleotide downstream of the anticodon (**Figure 29**). Intron lengths of the tRNAs in *M. thermophila* and the experimentally identified tRNAs range from 7-57 nucleotides and 8-34 nucleotides, respectively. The intron-containing tRNA genes are specific for most of the 20 amino acids, except for Asn and Cys. Transfer RNA genes in the same isoacceptor family or in the same anticodon specific family may or may not have introns. Therefore, introns are not specific for any isoacceptor family or anticodon-specific family.

The downstream regions of all of the experimentally identified tRNA genes and their paralogs as well as the 194 tRNA genes found in *M. thermophila* contain a stretch of consecutive thymidines (poly-T), which has been proposed to be the transcriptional termination site (Schmidt and Söll 1981; Hamada *et al.* 2000) (**Figure 30**). Lengths of the poly T are frequently from 5 to 22 nucleotides. Also, the distance between the 3' end of the tRNA genes and the poly T regions were from 0 to 84 nucleotides. For a majority of the tRNA genes, this distance is from 0 to 13 nucleotides. This provides further evidence that the tRNA genes curated in this study are correct.



chromosome 1: 6930001 - 6930250

```
TTTTACAACC ATCACAACAC AGCGTCAGTG GTTTAGTGGT AAAATCCATC
GTTGCCATCG ATGGGCCCCG CGTTCGATTC GCGGCTGACG CACTACGTTC
ACCATATTTT TCTTCCTCCC TTTTTTTCCC CCATAATATA TATACTCCGT
CGATGTCATA TTTGCCGGAT CAATAAGAGA GCAGTCAAGT CTAGGAACCA
TCCACTTGAA ACCATACGCT GCAGGGTAAC AAAATCCCAC TGCTCACTGA
```

chromosome 3: 1087151 - 1087400

```
AACCAAGGAT ACACAACATA GCGAGCTGGC GCAGCGGAAG CGCGCCGGGC
TCATAACCCG GAGGTCACTC GATCGAAACG AGTGCTCGCT AGTATACTTG
TTTTTTTTGT CCTTTTTCTT GTCTCCTTCC TCCCCGTAAA GGATTTTTTT
TATCGGGACT CCTTTTCCGC TTATTCCCGC TGGCTTACCA CACAACCAGG
ACTGACACTT GATAAGGTTG GTAAGAACCG TGTTTGAAAT CCTCACTTAT
```

Figure 30. Examples of poly-T regions found downstream of the tRNA genes

(The genomic regions shown in these examples are in the nuclear genome of M. thermophila. The tRNA gene sequences are highlighted. The poly-T regions, proposed to be the transcription termination signals, are boxed.)

The *delta*, *tau* and *sigma* elements are only found in the genome of *S. cerevisiae*, and the *beta* element is only detected in the genome of *C. albicans*. These transposable elements (TEs) are not found in *M. thermophila* as well as in the other fungal genomes which harbour the characterized tRNA genes. Therefore, the four TEs could not be used to support the tRNA gene annotation in *M. thermophila*. There might be other TEs specific for *M. thermophila*; however, in the scope of this study, only the *delta*, *tau*, *sigma* and *beta* elements which are commonly reported to be located adjacent to tRNA genes are investigated. There is no clear relationship found between the four TEs and tRNA genes in the genomes of *S. cerevisiae* and *C. albicans*. As shown in **Table 9**, for each of the four TEs, not all of its copies are located close to tRNA genes. For the ones positioned adjacent to tRNA genes, they are located upstream of the tRNA genes. The distances between the *sigma* elements and their adjacent tRNA genes are very similar to those reported by Del Rey *et al.* (1982). The transfer RNA genes located nearby the TEs are from all of the 20 isoacceptor families. Some of them contain introns while some do not. For the gene copies of the same tRNA gene, some of them are located near the TEs, while the others are not. It indicates that the four TEs may be located adjacent to various types of tRNA genes, and there is no relationship between these TEs and a specific group of tRNA genes.

Table 9. Numbers of the four TEs found in the genomes of *S. cerevisiae* and *C. albicans*

Species	TE name	Number of TE copies	Number of TEs adjacent to tRNA genes	Distance[(*)] (nucleotides)
S. cerevisiae	*delta*	157	49	80-928
S. cerevisiae	*sigma*	24	14	14-18
S. cerevisiae	*tau*	18	6	147-925
C. albicans	*beta*	4	1	36

[(*)] *the distances between the TEs and their adjacent tRNA genes*

3.6. Results from collecting and analyzing reliable AARSs

3.6.1. Summary of characterized AARSs

Results from the searches from different databases showed that only 27 fungal cytoplasmic AARSs have been experimentally characterized (**Supplementary table 5**). Most of them are from *S. cerevisiae*. These cytoplasmic fungal AARSs do not include all of the enzymes of the 20 universal AARS families since the prolyl-tRNA synthetase and arginyl-tRNA synthetase are still missing. Although these 27 enzymes are well-documented, some of them still have the unreliable GO evidence code "IEA" (Inferred from Electronic Annotation) at the three GO aspects. Therefore, their new GO terms and GO evidence codes were assigned by the decision tree in **Figure 17**.

In addition to the 27 fungal cytoplasmic AARSs, 52 other aminoacyl-tRNA synthetases obtained from UniProt are found to be reliable according to their assigned GO terms and GO evidence codes at the three GO aspects (**Supplementary table 6**). Most of these evidence codes were IDA (Inferred from Direct Assay), TAS (Traceable Author Statement), IMP (Inferred from Mutant Phenotype) and IGI (Inferred from Genetic Interaction). They included both mitochondrial and cytoplasmic AARSs from bacterial, fungal and other eukaryotic species.

In summary, the reliable AARS data set in this study consists of 79 enzymes from 11 organisms. Among those, there are 22 mitochondrial proteins, 49 cytoplasmic ones and 8 enzymes having both mitochondrial and cytoplasmic functions (**Table 10**). For the AARS genes encoding both mitochondrial and cytoplasmic enzymes, their transcriptional products comprised two alternative mRNAs coding for two protein isoforms. The longer isoform encodes the mitochondrial AARS while the shorter one encodes the cytoplasmic enzyme. The two isoforms are mostly identical except that the longer one has an extra sequence at its N-terminus which codes for the mitochondrial targeting peptide. The cytoplasmic compilation comprises enzymes from each of the 20 universal AARS families. For the mitochondrial AARS collection, enzymes specific for Gln, Pro and Cys have not been found yet. Since phenylalanyl-tRNA synthetase (PheRS) has two subunits (α and β), there are two sequences found to encode PheRSα and PheRSβ. Moreover, there is an AARS from *H. sapiens* functioning as both prolyl-tRNA synthetase and glutamyl-tRNA synthetase.

Table 10. Number of characterized AARSs collected in this study

Species group	Species name	Number of AARSs[*]
Bacteria	*Escherichia coli*	**8**
	Mycobacterium tuberculosis	**1**
	Thermus thermophilus	**1**
Fungi	*Candida albicans*	**4** (3 cyt, 1 cyt and mit)
	Neurospora crassa	**2** (1 cyt, 1 cyt and mit)
	Saccharomyces cerevisiae	**33** (16 cyt, 4 cyt and mit, 13 mit)
	Schizosaccharomyces pombe	**2** (1 cyt, 1 mit)
Other eukaryotic organisms	*Arabidopsis thaliana*	**1** (1 mit)
	Bos Taurus	**1** (1 mit)
	Homo sapiens	**25** (17 cyt, 2 cyt and mit, 6 mit)
	Rattus norvegicus	**1** (1 cyt)

[*] *The eukaryotic AARSs are either cytoplasmic (cyt) or mitochondrial (mit). Also, some of them have both cytoplasmic and mitochondrial functions (cyt and mit). Those in*

brackets describe in detail the numbers of cytoplasmic, mitochondrial and bifunctional enzymes of each organism.

3.6.2. Results from analyzing the characterized AARS sequences

The relationship between different types of AARSs is shown in the phylogenetic tree built from all reliable AARS sequences collected in this study. As presented in **Figure 31**, AARSs specific for the same amino acid tend to cluster together on the tree. The high bootstrap values of the clades within each cluster demonstrate that these groupings are not random events. It indicates that AARSs of the same amino acid from different organisms are more similar to each other than AARSs having different specificities from the same species. Moreover, for the enzymes specific for the same amino acid, the mitochondrial and cytoplasmic AARSs form two distinct groups. Therefore, when using an AARS sequence as a query in the BLAST search to detect AARS enzymes in a new sequenced genome, the most similar hit found from the BLAST search would be the AARS that have the same specificity with the query AARS. Also, if the query enzyme has the cytoplasmic function, it is more likely that the AARS found from the BLAST search is a cytoplasmic enzyme than a mitochondrial enzyme and vice versa. For AARSs of Met, Thr and Asp, as shown on the tree, the mitochondrial enzymes are more similar to the bacterial ones than to their cytoplasmic counterparts, which is consistent with those reported by Sissler *et al.* (2000). However, for AARSs of Ser, Asn and Lys, the cytoplasmic enzymes are more similar to the bacterial ones than the mitochondrial enzymes. Also, for alanyl-tRNA synthetase, the cytoplasmic enzymes are more similar to their mitochondrial counterparts than the bacterial ones. Hence, when using a bacterial

AARS as a query sequence to search for AARS enzymes in a eukaryotic genome, further analysis would be required to determine whether the most similar hit found from the BLAST search is a cytoplasmic or mitochondrial AARS. For the phenylalanyl-tRNA synthetase (PheRS), the tree showed that the mitochondrial PheRSs tend to cluster with the alpha subunit of the cytoplasmic enzymes.

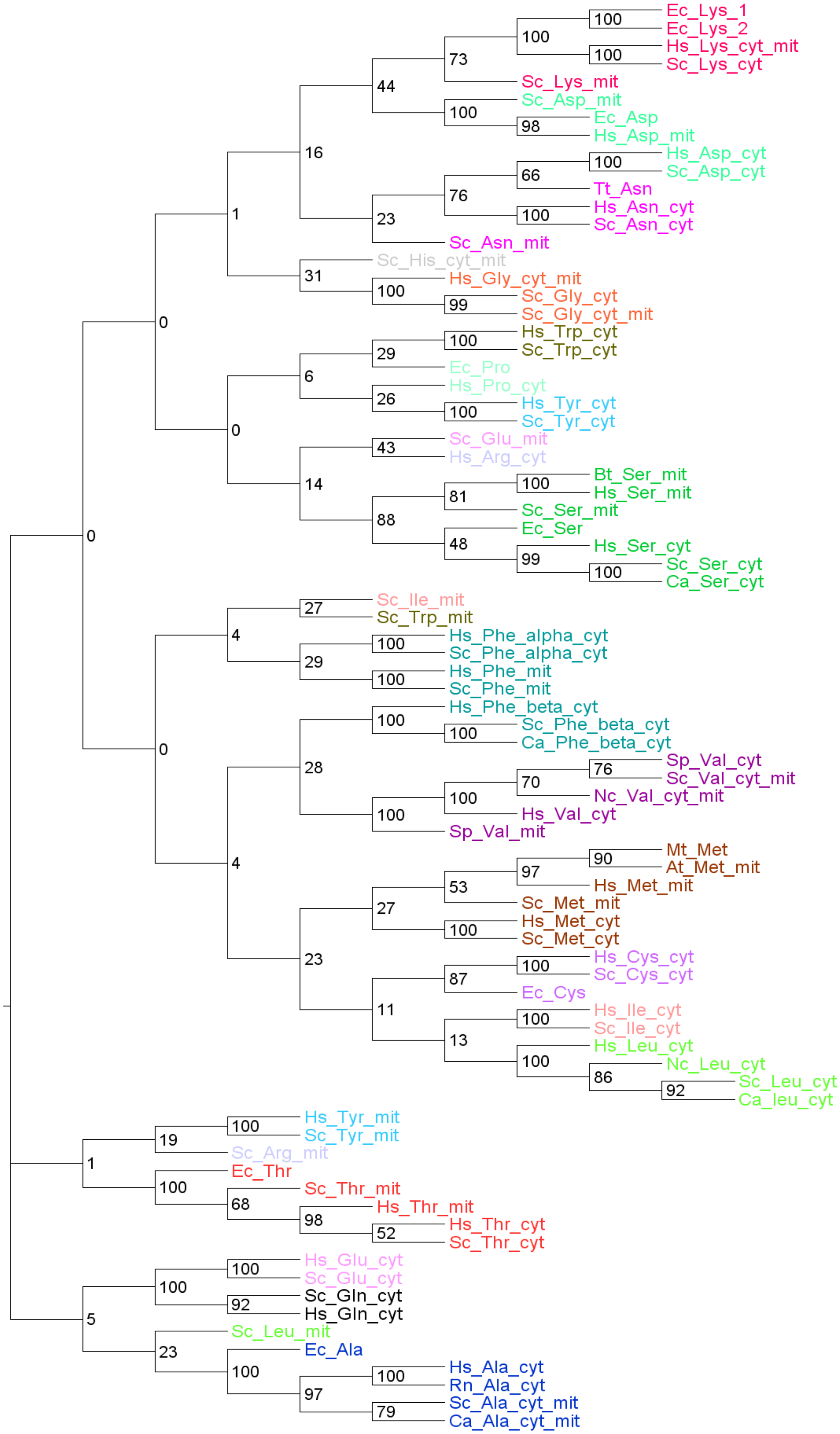

Ec_Lys_1
100
Ec_Lys_2
100
Hs_Lys_cyt_mit
100
73
Sc_Lys_cyt
Sc_Lys_mit
44
Sc_Asp_mit
100
Ec_Asp
98
Hs_Asp_mit
16
Hs_Asp_cyt
100
Sc_Asp_cyt
66
Tt_Asn
76
Hs_Asn_cyt
1
100
23
Sc_Asn_cyt
Sc_Asn_mit
Sc_His_cyt_mit
31
Hs_Gly_cyt_mit
100
Sc_Gly_cyt
99
Sc_Gly_cyt_mit
0
Hs_Trp_cyt
100
Sc_Trp_cyt
29
Ec_Pro
6
Hs_Pro_cyt
26
Hs_Tyr_cyt
100
Sc_Tyr_cyt
0
Sc_Glu_mit
43
Hs_Arg_cyt
Bt_Ser_mit
100
14
Hs_Ser_mit
81
Sc_Ser_mit
88
Ec_Ser
0
48
Hs_Ser_cyt
99
Sc_Ser_cyt
100
Ca_Ser_cyt
Sc_Ile_mit
27
Sc_Trp_mit
4
Hs_Phe_alpha_cyt
100
Sc_Phe_alpha_cyt
29
Hs_Phe_mit
100
Sc_Phe_mit
Hs_Phe_beta_cyt
100
Sc_Phe_beta_cyt
100
Ca_Phe_beta_cyt
0
Sp_Val_cyt
28
76
Sc_Val_cyt_mit
70
Nc_Val_cyt_mit
100
Hs_Val_cyt
100
Sp_Val_mit
Mt_Met
90
4
At_Met_mit
97
Hs_Met_mit
53
Sc_Met_mit
27
Hs_Met_cyt
100
Sc_Met_cyt
Hs_Cys_cyt
23
100
Sc_Cys_cyt
87
Ec_Cys
11
Hs_Ile_cyt
100
Sc_Ile_cyt
13
Hs_Leu_cyt
100
Nc_Leu_cyt
86
Sc_Leu_cyt
92
Ca_leu_cyt
Hs_Tyr_mit
100
Sc_Tyr_mit
19
Sc_Arg_mit
1
Ec_Thr
100
Sc_Thr_mit
68
Hs_Thr_mit
98
Hs_Thr_cyt
52
Sc_Thr_cyt
Hs_Glu_cyt
100
Sc_Glu_cyt
100
Sc_Gln_cyt
92
Hs_Gln_cyt
5
Sc_Leu_mit
Ec_Ala
23
100
Hs_Ala_cyt
100
Rn_Ala_cyt
97
Sc_Ala_cyt_mit
79
Ca_Ala_cyt_mit

Figure 31. Phylogenetic tree built from the characterized AARS protein sequences (*AARSs having the same specificity are presented in the same colour. The numbers shown on the tree are the bootstrap values. Name of each AARS on the tree consists of its species name, its amino acid specificity and the cellular component in which the enzyme primarily functions. Each AARS is either cytoplasmic (cyt) or mitochondrial (mit). Some of them are bifunctional (cyt_mit). Abbreviations of the species names are:* ***Ec****, Escherichia coli;* ***Mt****, Mycobacterium tuberculosis;* ***Tt****, Thermus thermophilus;* ***Ca****, Candida albicans;* ***Sc****, Saccharomyces cerevisiae;* ***Sp****, Schizosaccharomyces pombe;* ***At****, Arabidopsis thaliana;* ***Bt****, Bos Taurus;* ***Hs****, Homo sapiens;* ***Rn****, Rattus norvegicus*)

The signature motifs specific for class I and class II AARSs are observed in most of the characterized AARS enzymes except for two class I AARSs which are the cytoplasmic arginyl-tRNA synthetase (ArgRS) from human and the mitochondrial ArgRS from *S. cerevisiae*. As previously mentioned, class I AARSs have two conserved motifs: "HIGH" and "KMSKS". The two characterized ArgRSs from human and *S. cerevisiae* do not harbour the "KMSKS" motif. It has been reported that there are two groups of ArgRS enzymes. Proteins in the first group have the canonical "KMSKS" motif while those of the second one do not; yet, they have a conserved lysine (K) upstream of the "HIGH" motif (Sekine *et al.* 2001). This invariant K is observed in the upstream region of the "HIGH" motif of the two characterized ArgRSs. When comparing sequences of the class I AARS signature motifs of all characterized AARSs, it showed that only the first histidine of the "HIGH" motif is strictly conserved (**Figure 32 (A)**). The second amino acid of this motif is diverse. The other residues of the two class I motifs are highly conserved; however, they are substituted by alternative amino acids in some cases. For the three motifs of the class II AARSs, the multiple sequence alignment profiles of these motif sequences reveal some invariant and highly conserved residues which are in agreement with the ones discovered earlier by Eriani *et al*. (1995) (**Figure 32 (B), (C), (D)**).

Hs_Arg_cyt	HVGH	
Sc_Arg_mit	HAGH	
Hs_Cys_cyt	HMGH	KMSKS
Sc_Cys_cyt	HMGH	KMSKS
Ec_Cys	HIGH	KMSKS
Hs_Gln_cyt	HIGH	VVSKR
Sc_Gln_cyt	HIGH	VLSKR
Hs_Glu_cyt	HIGH	VLSKR
Sc_Glu_cyt	HIGH	LLSKR
Sc_Glu_mit	HLGS	KLSKR
Hs_Ile_cyt	HYGH	KMSKR
Sc_Ile_cyt	HYGH	KMSKS
Sc_Ile_mit	HLGH	KMSKS
Ca_Leu_cyt	HAGH	KMSKS
Hs_Leu_cyt	HLGH	KMSKS
Nc_Leu_cyt	HAGH	KMSKS
Sc_Leu_cyt	HAGH	KMSKS
Sc_Leu_mit	HIGH	KMSKS
Hs_Met_cyt	HLGN	KFSKS
Sc_Met_cyt	HLGN	KFSKS
At_Met_mit	HMGS	KMGKS
Hs_Met_mit	HIGH	KMSKS
Sc_Met_mit	HLGH	KMSKS
Mt_Met	HVGH	KMSKS
Hs_Trp_cyt	HVGH	KMSAS
Sc_Trp_cyt	HLGH	KMSAS
Sc_Trp_mit	HLGN	KMSKS
Hs_Tyr_cyt	HVAY	KMSSS
Sc_Tyr_cyt	HCGY	KMSAS
Hs_Tyr_mit	HVGH	KLGKS
Sc_Tyr_mit	HLGN	KFGKS
Hs_Val_cyt	HLGH	KMSKS
Nc_Val_cyt_mit	HCGH	KMSKS
Sc_Val_cyt_mit	HIGH	KMSKS
Sp_Val_cyt	HIGH	KMSKS
Sp_Val_mit	HIGH	KMSKS
Consensus	**H GH**	**KMSKS**

(A) "HIGH" and "KMSKS" motifs of the class I AARSs

Ca_Ala_cyt_mit	--QHTYVPS--SSVVPHND---PTL
Hs_Ala_cyt	--EHTYVHS--SATIPLDD---PTL
Rn_Ala_cyt	--EHTYVHS--SATIPLDD---PTL
Sc_Ala_cyt_mit	--EHKFVKS--SPVVPFDD---PTL
Ec_Ala	--GHQVVAS--SSLVPHND---PTL
Hs_Asn_cyt	--FRDHFFD--RGYYEVTP---PTL
Sc_Asn_cyt	--VRRVYDE--EHLTEVTP---PCM
Sc_Asn_mit	--FMLYFQK--NHFTKVSP---PIL
Tt_Asn	--IHEFFGE--RGFLRFDA---PIL
Hs_Asp_cyt	--FRETLIN--KGFVEIQT---PKI
Sc_Asp_cyt	--FREYLAT--KKFTEVHT---PKL
Hs_Asp_mit	--MREYLCNL-HGFVDIET---PTL
Sc_Asp_mit	--IRNSFNN--FDFTEVET---PML
Ec_Asp	--VRRFMDD--HGFLDIET---PML
Hs_Gly_cyt_mit	---NYNVKSP-ITGNDLSP---PVS
Sc_Gly_cyt	---EYNINDP-VTNDVLDA---LTS
Sc_Gly_cyt_mit	---KYDIGNP-VTGETLES---PRA
Sc_His_cyt_mit	--LSGLFKK--HGGVTIDT---PVF
Ec_Lys_1	--IRQFMVN--RGFMEVET---PMM
Ec_Lys_2	--IRQFMVA--RGFMEVET---PMM
Hs_Lys_cyt_mit	--IRSFLDE--LGFLEIET---PMM
Sc_Lys_cyt	--IRRFLDQ--RKFIEVET---PMM
Sc_Lys_mit	--LRKFLDD--RNFVEVET---PIL
Hs_Phe_α_cyt	--FRQIFLE--MGFTEM-----PTD
Sc_Phe_α_cyt	--FRQIFFS--MGFTEM-----PSN
Hs_Phe_mit	--YKQYVGRFGTPLFSVYDNLSPVV
Sc_Phe_mit	----NSVDN---TFKIFNN-FKPVV
Hs_Pro_cyt	--FDAEIKK--LGVENCYF---PMF
Ec_Pro	--VREEMNN--AGAIEVSM---PVV
Ca_Ser_cyt	--GLSFLSS--KGYVPLQA---PVM
Hs_Ser_cyt	--ALRTLGS--RGYIPIYT---PFF
Sc_Ser_cyt	--GLQFLAA--KGYIPLQA---PVM
Bt_Ser_mit	---LNKLIH--RGFTPMTV---PDL
Hs_Ser_mit	---FNKLLR--RGFTPMTV---PDL
Sc_Ser_mit	--LKKANEN---GFSSCVP---PSI
Ec_Ser	--LDLHTEQ--HGYSENYV---PYL
Hs_Thr_cyt	--IRSEYRK--RGFQEVVT---PNI
Sc_Thr_cyt	--LRTEYRK--RGYEEVIT---PNM
Hs_Thr_mit	--IRAEYAH--RGFSEVKT---PTL
Sc_Thr_mit	MKLQQKFKF---GFNEVVT---PLI
Ec_Thr	--VRSKLKE--YQYQEVKG---PFM
Consensus	**P**

(B) Motif 1 of class II AARSs

Figure 32. Multiple sequence alignment profiles of the signature motif sequences of the characterized AARSs

(Invariant amino acids are highlighted in grey color. Highly conserved residues are in bold)

```
Ca_Ala_cyt_mit    KRAANSQKCIRAGGKHNDLEDVGRDSYHHTFFEMLGNWSFGDY  TLPNKHIDTGMG-FERLVSILQNK-YSNYDTDVFLP
Hs_Ala_cyt        SRAANTQKCIRAGGKHNDLDDVGKDVYHHTFFEMLGSWSFGDY  PLPKKSIDTGMG-LERLVSVLQNK-MSNYDTDLFVP
Rn_Ala_cyt        SRAANTQKCIRAGGKHNDLDDVGKDVYHHTFFEMLGSWSFGDY  PLPKKSIDTGMG-LERLVSVLQNK-MSNYDTDLFVP
Sc_Ala_cyt_mit    KRAYNSQKCIRAGGKHNDLEDVGKDSYHHTFFEMLGNWSFGDY  PLPAKHIDTGMG-FERLVSVLQDV-RSNYDTDVFTP
Ec_Ala            SRATTSQRCVRAGGKHNDLENVGYTARHHTFFEMLGNFSFGDY  PLPKPSVDTGMG-LERIAAVLQHV-NSNYDIDLFRT
Hs_Asn_cyt        GDVFCIAQSYRAE------QSRT—RRHLAEYTHVEAECPFLT   YGTCPHGGYGLG-LERFLTWILNR-YHIRDV-CLYP
Sc_Asn_cyt        GDVYTIQESFRAE------KSHT—RRHLSEYTHIEAELAFLT   YGTCPHGGYGIG-TERILAWLCDR-FTVRDC-SLYP
Sc_Asn_mit        SRCWTLSPCFRAE------KSDT—PRHLSEFWMLEVEMCFVN   EGSAPHGGFGLG-FERFISYLYGN-HNIKDA-IPFY
Tt_Asn            AKVYTFGPTFRAE------RSKT—RRHLLEFWMVEPEVAFMT   FGSVPHSGFGLG-LERTVAWICGL-AHVREA-IPFP
Hs_Asp_cyt        EKVFSIGPVFRAE------DSNT—HRHLTEFVGLDIEMAFNY   FGAPPHAGGGIG-LERVTMLFLGL-HNVRQT-SMFP
Sc_Asp_cyt        ERVYEIGPVFRAE------NSNT—HRHMTEFTGLDMEMAFEE   YGCPPHAGGGIG-LERVVMFYLDL-KNIRRA-SLFP
Hs_Asp_mit        DRYFQVARCYRDE------GSRP--DRQPEFTQIDIEMSFVD   YGAPPHGGIALG-LDRLICLVTGS-PSIRDV-IAFP
Sc_Asp_mit        NKYYQMARCFRDE------DLRA--DRQPEFTQVDMEMAFAN   MGTPPHAGFAIG-FDRMCAMICET-ESIRDV-IAFP
Ec_Asp            DRYYQIVKCFRDE------DLRA--DRQPEFTQIDVETSFMT   YGTPPHAGLAFG-LDRLTMLLTGT-DNIRDV-IAFP
Hs_Gly_cyt_mit    FAAAQIGNSFRNEISP---RS-G—LIRVREFTMAEIEHFVDP   EVVPNVIEPSFG-LGRIMYTVFEHTFHVREG-DEQR
Sc_Gly_cyt        FASASIGKSFRNEISP---RS-G—LLRVREFLMAEIEHFVDP   EYIPNVIEPSFG-LGRIIYCIFDHCFQVRVD-SESR
Sc_Gly_cyt_mit    FASASIGKSFRNEISP---RA-G—LLRVREFLMAEIEHFVDP   EYVPSVIEPSFG-IGRIIYSVFEHSFWNRPE-DNAR
Sc_His_cyt_mit    IKRYHIAKVYRRDQ-----PAMT—KGRMREFYQCDFDVAGTF   STQIPCVGISFG-VERIFSLIKQR---INSS-TTIK
Ec_Lys_1          ERVFEINRNFRNE------GIS--VRHNPEFTMMELYMAYAD   HGLPPTAGLGIG-IDRMVMLFTNS-HTIRDV-ILFP
Ec_Lys_2          ERVFEINRNFRNE------GIS--VRHNPEFTMMELYMAYAD   YGLPPTAGLGIG-IDRMIMLFTNS-HTIRDV-ILFP
Hs_Lys_cyt_mit    DRVYEIGRQFRNE------GID--LTHNPEFTTCEFYMAYAD   YGLPPTAGWGMG-IDRVAMFLTDS-NNIKEV-LLFP
Sc_Lys_cyt        DRVYEIGRQFRNE------GID--MTHNPEFTTCEFYQAYAD   YGLPPTGGWGCG-IDRLAMFLTDS-NTIREV-LLFP
Sc_Lys_mit        QKVYEIGKVFRNE------GID--STHNAEFSTLEFYETYMS   YGMPPVGGFGLG-IDRLCMLFCDK-KRIEEV-LPFG
Hs_Phe_α_cyt      VKYFSIDRVFRNE------TLD--ATHLAEFHQIEGVVADHG   PENVSVIAWGLS-LERPTMIKYGI-NNIREL-VGHK
Sc_Phe_α_cyt      TRLFSIDRVFRNE------AVD--ATHLAEFHQVEGVLADYN   PKDLRVLGWGLS-LERPTMIKYKV-QNIREL-LGHK
Hs_Phe_mit        DAFLVVGDVYRRD------QID--SQHYPIFHQLEAVRLFSK   AQDRIGWAFGLG-LERLAMILYDI-PDIRLF-WCED
Sc_Phe_mit        SGFLISADVYRRD------EID--KTHYPVFHQMEGATIWKR   PSETIGWAFGLG-LDRIAMLLFEI-PDIRLL-WSRD
Hs_Pro_cyt        IKLNQWCNVVRWEFK----HPQP—FLRTREFLWQEGHSAFAT   EKQFAYQN-SWGLTTRTIGVMTMV-HGDNMG-LVLP
Ec_Pro            LNFYQIQTKFRDEVRP---RF-G—VMRSREFLMKDAYSFHTS   NQILTMGCYGIG-VTRVVAAAIEQNYD-ERG-IVWP
Ca_Ser_cyt        VRYAGYSSCFRREAGSHGKDAWG—IFRVHAFEKIEQFVLTEP   KYVHCLNSTLSA-TERTICCILEN-YQKEDG-LVIP
Hs_Ser_cyt        IKYAGLSTCFRQEVGSHGRDTRG—IFRVHQFEKIEQFVYSSP   EFVHMLNATMCA-TTRTICAILEN-YQTEKG-ITVP
Sc_Ser_cyt        IHYVGYSSCFRREAGSHGKDAWG—VFRVHAFEKIEQFVITEP   KYVHCLNSTLAA-TQRALCCILEN-YQTEDG-LVVP
Bt_Ser_mit        IRMVCSSTCYRAETDT-GKEPWG—LYRVHHFTKVEMFGVTGP   QFAHTVNATGCA-VPRLLIALLES-YQQKDGSVLVP
Hs_Ser_mit        VRMVCSSTCYRAETNT-GQEPRG—LYRVHHFTKVEMFGVTGP   QFAHTVNATACA-VPRLLIALLES-NQQKDGSVLVP
Sc_Ser_mit        KKLVGVSRCYRAEAGARGKDTKG—LYRVHEFTKVELFCWSKP   EYVHTLNGTAMA-IPRVIVALVENFYDPSTGKISVP
Ec_Ser            IKMTAHTPCFRSEAGSYGRDTRG—LIRMHQFDKVEMVQIVRP   RLVHTLNGSGLA-VGRTLVAVMEN-YQQADGRIEVP
Hs_Thr_cyt        LRLADFGVLHRNELSG---ALTG—LTRVRRFQQDDAHIFCAM   -RPVIVHRAILGSVERMIAILTEN-----YG-GKWP
Sc_Thr_cyt        WRVADFGVIHRNEFSG---ALSG—LTRVRRFQQDDAHIFCTH   -RPVMIHRAILGSVERMTAILTEH-----FA-GKWP
Hs_Thr_mit        LRLADFGALHRAEASG---GLGG—LTRLRCFQQDDAHIFCTT   -RPVLIHRAVLGSVERLLGVLAES-----CG-GKWP
Sc_Thr_mit        LRFSDFSPLHRNEASG---ALSG—LTRLRKFHQDDGHIFCTP   -RPIMIHRATFGSIERFMALLIDS-----NE-GRWP
Ec_Thr            LRMAEFGSCHRNEPSG---SLHG—LMRVRGFTQDDAHIFCTE   -VPVMIHRAILGSMERFIGILTEE-----FA-GFFP
Consensus                  FR E                 F                         G  ER                   P
```

(C) Motif 2 of class II AARSs

(D) Motif 3 of class II AARSs

Fig. 32. Continued.

3.7. Results from the manual curation and annotation of AARS genes in *M. thermophila*

In the BLASTP search against all 11,342 predicted proteins of *M. thermophila* using the characterized AARSs as query sequences, 36 significant hits were found. These hits were considered as AARS candidates for *M. thermophila* and subjected to further analysis in which their gene models and sequences were manually examined. When comparing the AARS gene models, which were predicted by either JGI or the Genozymes project, and the sequenced transcript data on GBrowse (https://reviewers.fungalgenomics.ca/gene_model_pages/AARS_Table.html), gene models of the 36 AARS candidates match well to their corresponding transcript coverage. In some cases, the genes models proposed by the Genozymes project are better than those from JGI and vice versa. As presented in **Figure 33**, more correct gene models are predicted from the Genozymes project.

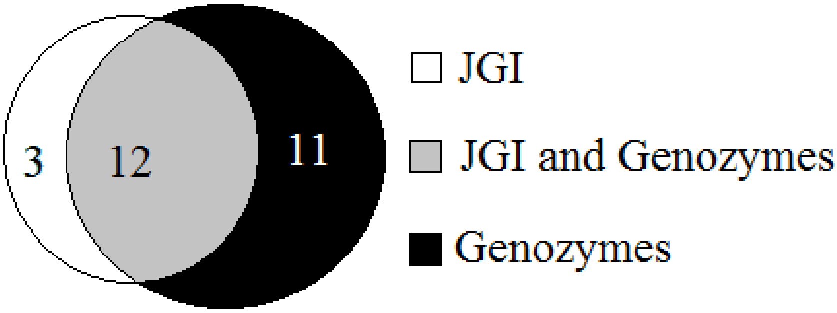


Figure 33. The numbers of correct gene models from JGI and the Genozymes project when comparing the gene models of 36 AARS candidates and the transcriptome data

The nucleotide sequences of the 36 selected gene models were examined by checking their start and stop codons as well as the intron splice sites for the genes containing introns. The start and stop codons of all of the 36 gene models are the same as the universal ones. There are no stop codons within their exon regions. Most of the intron splice sites follow the “GT-AG” rule except for three introns of three gene models. These three exceptions have “GC” at the 5’ splice site instead of “GT” as frequently observed. However, it has been observed that in addition to a number of introns beginning with “GT”, there exist a small proportion of introns starting with “GC”. These non-canonical 5’ splice sites occupy 0.5% of the annotated 5’ spice sites in SpliceDB database (Burset, Seledtsov and Solovyev 2001; Kitamura-Abe *et al.* 2004). Therefore, the gene models containing the 5’ splice site “GC” are also considered as correct. When analyzing the protein sequences, AARS signature motifs were found in most of the 36 AARS candidates except for two candidates which were proposed to be the arginyl-tRNA synthetase (ArgRS) and the histidyl-tRNA synthetase (HisRS). The protein sequence of the predicted ArgRS, a class I AARS, does not harbour the “KMSKS” motif; however, it contains lysine (K) in the upstream region of the “HIGH” motif. As mentioned earlier, some well-documented ArgRSs which lack the canonical “KMSKS” motif have a conserved K in the upstream region of the “HIGH” motif. Therefore, it is possible that this candidate is a true ArgRS. For the proposed HisRS, a class II AARS, the three signature motifs specific for class II AARSs were not found. In fact, only the HisRS having both mitochondrial and cytoplasmic functions in *S. cerevisiae* shows the sequence similarity with this candidate among all of the characterized AARSs. However, this similarity (37%) is much lower than the similarities of the other AARS candidates with the experimentally characterized AARSs. Although the query coverage from the BLASTP search is high (82%), the target coverage is very low (26%). Therefore, it is likely that this candidate is not an AARS enzyme. Hence, it was eliminated from AARS gene annotation. The remaining 35 AARS candidates are considered to be true AARS enzymes due to the sequence similarities from the BLASTP search and the presence of the signature motifs. The amino acid specificities as well as the cytoplasmic and mitochondrial functions of these 35 candidates were inferred from their most similar proteins in the characterized AARS data set and the results from the mTP detection. After assigning the specificities and functions of these 35 candidates, it showed that the mitochondrial AARSs of Ala, Gly, Cys, Arg and Gln were not detected. The missing of mitochondrial glutaminyl-tRNA synthetase (GlnRS) in *M. thermophila* is due to the lack of mitochondrial GlnRS in the query sequences in the BLASTP search. Also, it has been reported that mitochondrial GlnRS can be absent due to the indirect aminoacylation pathway (Sissler *et al.* 2000). For Ala, Gly, Cys and Arg, when using the bacterial, mitochondrial and cytoplasmic AARSs specific for these amino acids in the well-documented compilation as query sequences in the BLASTP search, only one AARS candidate found for each of these amino acids. On the other hand, when considering results of the TBLASTN search, there were no other genomic regions found to potentially code for AARSs in addition to those found from the BLASTP search. Therefore, it is likely that the unique AARSs of Ala, Gly, Cys and Arg found in *M. thermophila* function in both the cytoplasm and mitochondrion. However, the protein sequences of AARSs specific for these amino acids do not contain mitochondrial targeting peptide identified by the mitochondrial signal peptide detection. As previously mentioned, an AARS gene encoding both mitochondrial and cytoplasmic enzymes frequently has two alternative start codons, forming two ORFs on the same reading frame. The longer ORF encodes the mitochondrial AARS, and the shorter one encodes the cytoplasmic counterpart. Therefore, upstream regions of the four genes encoding AARSs specific for Ala, Gly, Cys and Arg in *M. thermophila* were examined. The result showed that another start codon was present in the upstream region of each of these genes. In each gene, the two alternative codons are located close to each other. When replacing the start codons of the four AARS genes with the new ones found in their upstream regions, the mitochondrial signal peptides were detected in the N-termini of the corresponding protein gene products. This demonstrates that the mitochondrial and cytoplasmic enzymes of the AARSs specific for Ala, Gly, Cys and Arg in *M. thermophila* are encoded by a single gene by using alternative start codons.

In summary, based on the transcriptome data, the sequence similarity with the well-documented AARSs and the presence of the signature motifs, 35 reliable AARS enzymes are found in *M. thermophila*. According to results from the BLASTP and TBLASTN searches, it is likely that most of the complete set of AARSs in *M. thermophila*, except for mitochondrial GlnRS, is detected. The numbers of mitochondrial, cytoplasmic and bifunctional enzymes are 12, 15 and 8, respectively. As presented in **Table 11**, the cytoplasmic AARSs found in *M. thermophila* include those of all of the 20 amino acids. For the mitochondrial AARSs, only the enzyme specific for Gln is missing. For the cytoplasmic phenylalanyl-tRNA synthetase, two genes encoding two subunit types (α and β) of this enzyme were detected.

Table 11. Sequence similarity between the reliable AARSs of *M. thermophila* and the experimentally characterized AARSs from the BLASTP search result

Target ID	Annotation	mTP	E-value	%Id	%Si	Tcov (%)	Qcov (%)
Spoth2p4_001239	cyt and mit AlaRS	M	0	59	73	90	99
Spoth2p4_006882	cyt and mit ArgRS	M	0	44	63	88	92
Spoth2p4_005588	cyt AsnRS	0	e-152	48	62	99	97
Spoth2p4_002032	cyt AspRS	0	e-123	46	60	88	88
Spoth2p4_003336	cyt and mit AspRS	M	e-107	42	61	86	53
Spoth2p4_006955	cyt and mit CysRS	M	0	38	56	95	95
Spoth2p4_003148	cyt GlnRS	0	0	56	69	73	86
Spoth2p4_004343	cyt GluRS	0	e-171	50	63	87	98
Spoth2p4_006221	cyt and mit GlyRS	M	0	62	77	94	89
Spoth2p4_000464	cyt and mit HisRS	M	e-151	54	69	90	83
Spoth2p4_010270	cyt IleRS	0	0	59	74	99	100
Spoth2p4_011163	cyt LeuRS	0	0	79	87	100	100
JGI|2306339	cyt LysRS	0	0	60	74	96	94
Spoth2p4_000073	cyt and mit LysRS	M	3e-82	36	54	80	83
Spoth2p4_005998	cyt MetRS	0	0	52	67	75	88
Spoth2p4_005952	cyt PheRS α	0	e-154	54	71	95	95
Spoth2p4_004338	cyt PheRS β	0	0	55	71	100	100
Spoth2p4_009767	cyt ProRS	0	e-153	51	67	100	93
Spoth2p4_004145	cyt SerRS	0	e-162	60	75	96	96
Spoth2p4_008920	cyt ThrRS	0	0	54	69	94	92
JGI|2133586	cyt TrpRS	0	2e-145	58	75	93	84
Spoth2p4_007753	cyt TyrRS	0	e-105	52	74	88	87
Spoth2p4_002418	cyt and mit ValRS	M	0	80	89	90	90
Spoth2p4_008564	mit AsnRS	M	5.00E-94	39	57	96	93
Spoth2p4_001247	mit AspRS	M	4.00E-95	40	54	77	70
Spoth2p4_005979	mit GluRS	M	6.00E-80	46	60	63	54
Spoth2p4_000038	mit IleRS	M	0	36	53	99	97
Spoth2p4_004687	mit LeuRS	M	0	46	61	90	84
Spoth2p4_004749	mit MetRS	M	e-111	42	57	92	88
Spoth2p4_011074	mit PheRS	M	e-121	47	65	95	88
JGI|2296525	mit ProRS	M	2.00E-88	33	49	96	95
Spoth2p4_010261	mit SerRS	M	3.00E-74	38	55	95	79
Spoth2p4_001274	mit ThrRS	M	e-107	45	60	85	80
Spoth2p4_006334	mit TrpRS	M	2.00E-78	43	62	91	73
Spoth2p4_000276	mit TyrRS	M	9.00E-68	29	50	99	81

*(This table only presents the sequence comparison results between each of the 35 AARSs from M. thermophila and its most similar AARS among the query sequences which comprise all of the characterized AARSs collected from the databases. The “**Target ID**”*

*column shows the identification numbers of the 35 AARSs in the predicted protein set of M. thermophila. The IDs that begin with "JGI" indicate that the corresponding proteins are from JGI. The others are from Genozymes project. The "**Annotation**" column presents the annotated functions of these 35 proteins. They can have either cytoplasmic (cyt) or mitochondrial (mit) function, or both of them (cyt and mit). The "**mTP**" column has two values, which is zero if there is no mitochondrial targeting peptide detected in the protein sequence or "M" if the mitochondrial signal peptide is found. Other columns are: **%Id**, percentage of sequence identity; **%Si**, percentage of sequence similarity; **Tcov**, target coverage; **Qcov**, query coverage)*

3.8. Preferred codons in *M. thermophila*

When considering the codon occurrences in the coding regions of protein-encoding genes, among the codons of the same tRNA which has an anticodon beginning with "A", the codon having "C" at its 3' end has the highest frequency. For example, the $tRNA_{AGC}^{Ala}$ can encode three codons GCC, GCT and GCA, and frequencies of these codons are 56.45%, 20.29% and 6.29%, respectively (**Supplementary table 7**). It shows that when the nucleotide "A" is located at the Wobble position of an anticodon, it preferentially binds to the nucleotide "C" instead of "T". It may be because the nucleotide "A" at the Wobble position is usually modified into "I" (Percudani *et al.* 1997), and this modification causes some changes in its binding property. Therefore, when selecting preferred codons according to tRNA genes, among the codons encoded by the same tRNA that has an anticodon beginning with "A", the codon having "C" at its 3' end should be selected.

As shown in **Table 12**, preferred codons deduced from the three methods which are based on the tRNA gene set, the highly expressed genes and the genes having N_c values lower than 31 are almost identical, except for the case of Ser. Among tRNA genes coding for Ser, the group of tRNA genes having the anticodon "GCT" has the highest tRNA gene number, and the codon perfectly matching with this anticodon is AGC. In the sets of highly expressed genes and genes having low N_c values, the codons having the highest frequencies are TCG and ACG, respectively. It is noticed that three of the six codons coding for Ser (AGC, TCG and TCC) have similar frequencies. In the highly expressed gene set, frequencies of these three codons are 26.93%, 25.56% and 25.31%, respectively. In the set of genes having low N_c values, their frequencies are 32.52%, 32.33% and 28.55%, respectively. Because frequencies of these three codons are similar and low, and because the numbers of tRNA genes of different anticodon-specific groups in the $tRNA^{Ser}$ gene family are not distinctly different (**Supplementary table 7**), it reveals that no codon is clearly found to be preferentially used among the codons coding for Ser. Therefore, no codon is selected for Ser in the preferred codon selection. In summary, using the three methods which are based on the tRNA gene numbers, the highly expressed genes and the genes having low N_c values, 17 optimal codons were determined for 17 amino acids. Among the three methods, the method based on genes having low N_c values is the simplest one since it does not need the sequenced RNA data. Hence, it can be used to quickly determine the preferred codons of an organism.

Table 12. Preferred codons in *M. thermophila* found from the three methods which are based on the tRNA genes, the highly expressed genes and the genes having N_c values lower than 31

Amino acid	Method based on highly expressed genes	Method based on tRNA gene numbers	Method based on genes having N_c <31	Combined three methods
Ala	GCC	GCC	GCC	GCC
Arg	CGC	CGC	CGC	CGC
Asn	AAC	AAC	AAC	AAC
Asp	GAC	GAC	GAC	GAC
Cys	TGC	TGC	TGC	TGC
Gln	CAG	CAG	CAG	CAG
Glu	GAG	GAG	GAG	GAG
Gly	GGC	GGC	GGC	GGC
His	CAC	CAC	CAC	CAC
Ile	ATC	ATC	ATC	ATC
Leu	CTC	CTC	CTC	CTC
Lys	AAG	AAG	AAG	AAG
Phe	TTC	TTC	TTC	TTC
Pro	CCC	CCC	CCC	CCC
Ser	TCG	AGC	AGC	None
Thr	ACC	ACC	ACC	ACC
Tyr	TAC	TAC	TAC	TAC
Val	GTC	GTC	GTC	GTC

3.9. Correlation between tRNA gene number, tRNA abundance and codon usage in *M. thermophila*

To determine the relationship between the number of tRNA genes in the genome and the amount of tRNAs in the cell, tRNA genes are classified into anticodon specific groups. Each group has a FPKM value which shows the estimated tRNA abundance and is calculated from the sequenced short RNAs. Subsequently, the number of tRNA genes and FPKM values of all of the anticodon specific groups are subjected to the Pearson correlation analysis. The correlation coefficient value of this correlation is 0.51, which indicates a moderate correlation between the two entities. Moreover, since the *p* value is zero, the relationship between tRNA gene number and tRNA abundance is considered to be statistically significant.

As previously mentioned, the relationship between tRNA availability and codon usage is examined at the amino acid level and the codon level. At the amino acid level, tRNA genes are classified into amino acid specific groups. The FPKM value, or the tRNA abundance, of each group is calculated as the sum of FPKM values of all tRNA genes in the group. After using Pearson correlation to analyze the relationship between the FPKM values of all of the groups and the amino acid frequencies, no significant correlation between these two objects is found according to the low correlation coefficient value (r=0.097) and the high *p* value (*p*= 0.675). In contrast, when analyzing the connection between tRNA gene numbers of the amino acid specific groups and amino acid frequencies, a strong and statistically significant correlation is observed (r=0.901, *p*=0) (**Figure 34**). When examining the correlations between tRNA availability and codon usage at the codon level, tRNA genes are grouped together according to their anticodons. Each anticodon specific group has a tRNA gene number value, a FPKM value and a tRNA usage value. The tRNA usage is the frequency of codons that are encoded by tRNA genes in the group. The codons encoded by the Wobble base pairing are not included when calculating the tRNA usage except for some Wobble codons that do not have perfectly matching tRNA genes. After using Pearson correlation analysis, a strong relationship (r=0.8) between tRNA gene number and tRNA usage, or codon frequency, is observed (**Figure 35**). For the relationship between tRNA abundance and codon frequency, the correlation coefficient value is 0.4, which indicates a weak correlation. The *p* values of these two correlations are zero and 0.006, respectively. Since these p values are lower than 0.05, the relationships observed between tRNA gene number and codon frequency as well as between tRNA abundance and codon frequency are statistically significant.

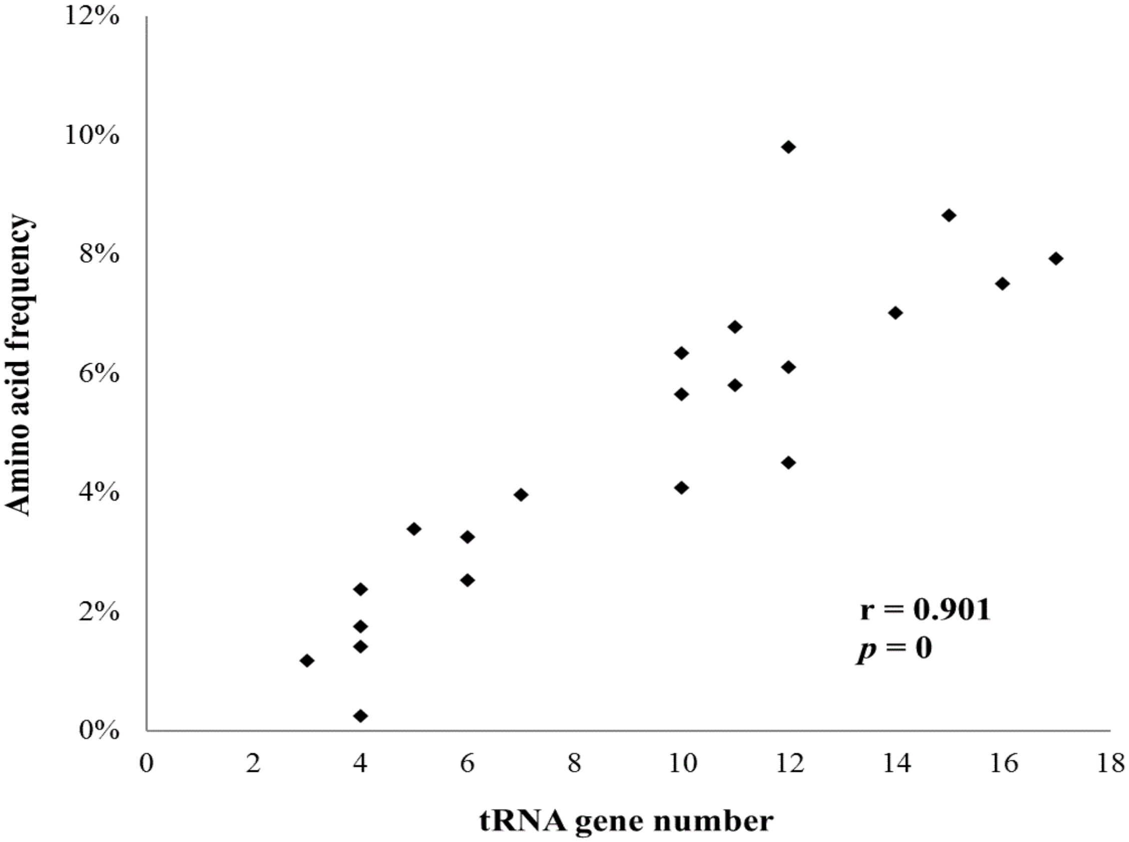


Figure 34. Correlation between tRNA gene number and amino acid frequency

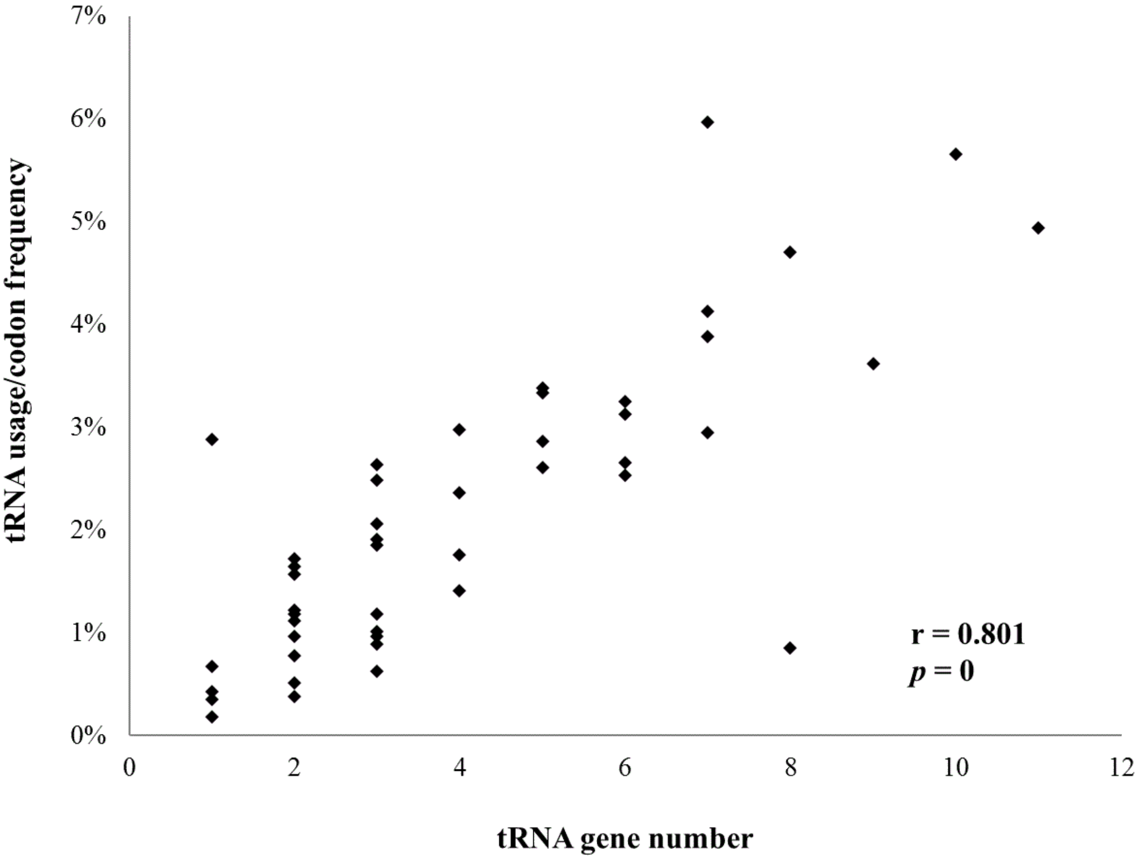


Figure 35. Correlation between tRNA gene number and codon frequency

CHAPTER 4. DISCUSSION

4.1. Reliable tRNA gene detection in *M. thermophila*

Along with the recognition of various and crucial roles of the non-coding RNAs (Mattick and Makunin 2006), tRNA gene detection has been an important part to complete a genome project. Generally, tRNA genes are detected by using the tRNA gene prediction tools. In this thesis, tRNA genes in *M. thermophila* are detected by combining both predicted results from different tRNA gene predictors and the experimental data. Therefore, they are expected to be more reliable than tRNA genes from other genome projects. In the tRNA gene annotation of *C. albicans* (Fan *et al.* 2007), *A. niger* (Pel *et al.* 2007), cow (D. T. Tang *et al.* 2009) and *O. sativa* (Itoh *et al.* 2007), tRNA genes were identified by the tRNAscan-SE program. The number of tRNA gene sets in different organisms has been increasing considerably in the GtRNAdb database by using this tRNA gene predictor (Chan and Lowe 2009). Yet, it has been reported that this tool has some limitations in finding the complete set of tRNA genes in a genome (Bakke *et al.* 2009). Some attempts have been made to improve the result by combining tRNAscan-SE with other bioinformatics programs, such as Sim4 alignments or ARAGORN for the tRNA gene detection in *D. melanogaster* and mouse, respectively (Misra *et al.* 2002; Coughlin *et al.* 2009). However, tRNA genes reported from most of the genomes are predicted genes and require supporting experimental data. There has been very limited experimental evidence confirming the reliability of predicted tRNA genes so far. The tRNA gene detection in mouse is among few efforts to make the tRNA gene finding more reliable by verifying the expression of predicted tRNA genes from microarray data. In this attempt, as described by Coughlin *et al.* (2009), predicted tRNA genes were classified into different families according to their sequence similarity. Then, oligonucleotides specific for each family were determined and used as probes in the microarray analysis. The hybridization of RNAs extracted from mouse cells and the probes confirmed the expression of the tRNA gene families. Although this procedure can examine the expression levels of the tRNA groups, it is not able to confirm the reliability of individual genes. In this thesis, the expression of tRNA genes predicted from the genome of *M. thermophila* is verified by examining the sequenced short RNAs mapped onto its predicted gene model. Besides, the reliability of each tRNA gene is confirmed by comparing its primary and secondary structures to those of the experimentally characterized fungal tRNAs. Furthermore, the prediction stage is carried out by not only tRNAscan-SE but also three other widely used tools - tRNAfinder, SPLITSX and ARAGORN. These additional predictors can complement the tRNA genes that may be missed by tRNAscan-SE. In addition, when repeating the tRNA gene prediction on the genomic regions that are mapped with a high number of short RNAs, no new tRNA gene is found. Based on these reasons, it is likely that the complete set of tRNA genes in *M. thermophila* has been detected, and the tRNA genes determined in this study are reliable.

4.2. Reliable tRNA data for the tRNA gene annotation in fungal genomes

The manually curated and annotated tRNA genes in *M. thermophila* as well as the experimentally characterized fungal tRNAs and tRNA genes collected in this thesis can be used as a reliable data set for the tRNA gene annotation in fungal genomes. Although tRNAdb is a good source of tRNA data, it contains both automatically predicted and experimentally identified tRNAs. Moreover, tRNA sequences provided in tRNAdb database contain modified nucleotides while the unmodified sequences would be more convenient for the tRNA sequence analysis in the tRNA gene annotation process. In this thesis, the reliability of tRNA and tRNA genes extracted from tRNAdb is ensured by examining their related literatures. Also, the unmodified tRNA sequences are provided by using sequences from the GenBank database. For the tRNA genes of *M. thermophila*, their reliability is confirmed according to the sequenced RNA data and the comparison to the experimentally characterized fungal tRNA sequences. In addition to the reliable tRNA sequences, the strictly conserved and semi-conserved nucleotides in the stems and loops of cloverleaf structures found from analyzing the reliable tRNA sequences in this thesis provide useful information for the tRNA gene detection in fungal genomes. Besides, information of the transcriptional termination sites can support the tRNA annotation results.

4.3. Comparing different tRNA gene predictors

When using the four widely used tRNA gene predictors (tRNAscan-SE, SPLITSX, tRNAfinder and ARAGORN) in the tRNA gene detection in *M. thermophila*, tRNAscan-SE and SPLITSX perform better than the others since they have the highest percentages of correctly predicted genes. However, they did not find the complete set of tRNA genes. There is one reliable tRNA gene uniquely predicted by ARAGORN. Therefore, to reduce the possibility of missing genes, results from different predictors should be combined. Advantages and better results obtained from combining multiple tRNA gene-finders have been mentioned previously (Ardell 2010). Although the tools could detect the initiator $tRNA^{Met}$, none of them is able to distinguish between the initiator and the elongator $tRNA^{Met}$. Hence, the tools should be improved by adding features differentiating between these two types of tRNA^{Met}. The typical characteristics of initiator tRNAs found from this study can be used in this improvement.

4.4. Reliable AARS gene detection in *M. thermophila*

Like the tRNA gene detection process, the AARS gene finding procedure in this thesis combines predicted and experimental data to increase the reliability of the detected AARSs. The expression of each AARS gene is confirmed by examining the sequenced transcripts. The cytoplasmic and mitochondrial functions as well as the amino acid specificities of the AARS enzymes are assigned from the high sequence similarities with the well-documented AARSs, the occurrences of the signature motifs and results from the mitochondrial targeting peptide detection. According to results from the BLASTP search against all predicted protein-encoding genes in *M. thermophila* and from the TBLASTN search against the complete sequenced genome, it is likely that most of the AARS genes of *M. thermophila* have been determined. Only mitochondrial glutaminyl-tRNA synthetase is not detected. One possible explanation of this gene missing is that the mitochondrion uses the indirect aminoacylation pathway which does not required glutaminyl-tRNA synthetase to synthesize the $\text{Gln-tRNA}^{\text{Gln}}$ complex (**Figure 14**). This explanation has been proposed for the missing mitochondrial glutaminyl-tRNA synthetase in *S. cerevisiae* (Sissler *et al.* 2000). In brief, based on the sequenced RNA data and results from the sequence comparison to the characterized aminocyl-tRNA synthetases, the AARS enzymes found in *M. thermophila* are considered to be reliable. These manually curated and annotated AARSs as well as the characterized AARSs collected from the databases can be used as a reliable source of AARS data for the AARS gene annotation in other genomes.

4.5. Genes encoding both mitochondrial and cytoplasmic AARSs

In *M. thermophila*, eight AARS genes specific for Ala, Arg, Asp, Cys, Gly, Lys, His and Val encode both cytoplasmic and mitochondrial enzymes. Like the bifunctional AARS genes that have been reported so far, those found in *M. thermophila* encode the mitochondrial and cytoplasmic enzymes by harbouring two alternative start codons which form two open reading frames (ORF) in the same frame. The longer ORF codes for the mitochondrial AARS, and the shorter one codes for the cytoplasmic counterpart. The bifunctional AARS genes have been found to encode AARSs of His, Val, Gly, Lys and Ala (Natsoulis *et al.* 1986; Chatton *et al.* 1988; Kubelik, Turcq and Lambowitz 1991; Tolkunova *et al.* 2000; Turner *et al.* 2000; H. L. Tang *et al.* 2004). This study provides the first examples of bifunctional AARS genes of Asp, Cys and Arg.

4.6. Correlation between tRNA gene number, tRNA abundance and codon usage

Along with evidence previously reported in *S. cerevisiae* and *B. subtilis*, a significant correlation between the numbers of tRNA genes and their expression levels is also observed in *M. thermophila* although it is not as strong as those found previously. The correlation coefficient values are 0.91 in *S. cerevisiae* (Percudani *et al.* 1997) and 0.86 in *B. subtilis* (Kanaya *et al.* 1999) while this value is only 0.51 in *M. thermophila*. When comparing the detected tRNA genes with the codon usage in *M. thermophila*, strong correlations between tRNA gene numbers and codon frequencies as well as amino acid frequencies are observed. This result is similar to those previously found in *S. cerevisiae* (Percudani *et al.* 1997), *C. elegans* (Duret 2000) and *C. albicans* (Fan *et al.* 2007). This evidence indicates that the number of tRNA genes is obviously a factor in the codon selection of each organism. This relationship supports the hypothesis that natural selection participates in the codon selection process since the adaptation of codon usage to the tRNA availability would result in increasing either efficiency or fidelity of the translation (Hershberg and Petrov 2008).

4.7. Potential of codon optimization in *M. thermophila* and a quick method for determining preferred codons

Based on the complete set of tRNA genes found in *M. thermophila*, the optimal codons, which match well with the tRNAs having the highest gene numbers, are determined. These preferred codons can be applied in the codon optimization of the protein production using *M. thermophila* as a host. The genes harbouring the preferred codons may be translated more accurately and efficiently due to the high availability of the corresponding tRNAs. In addition to using the number of tRNA genes to determine the preferred codons, two other methods, which were based on the data sets of highly expressed genes and the genes having low N_c values, were used and gave similar results. Among the three methods, the one based on N_c values is the simplest method since it does not need the complete tRNA gene set or the highly expressed protein encoding genes. It suggests that this method can be used to quickly estimate the preferred codons of an organism.

CHAPTER 5. CONCLUSIONS

By comparing the predicted gene models to the sequenced transcriptome data and the characterized tRNA and AARS genes collected from the literature and different databases, the exhaustive sets of tRNA and AARS genes were determined from the nuclear genome of *M. thermophila*. The 194 tRNA genes found in this study can code for all 20 universal amino acids. The 35 AARSs consist of both cytoplasmic and mitochondrial aminoacyl-tRNA synthetases in *M. thermophila*. The cytoplasmic enzymes comprise AARSs of all of the 20 amino acid specificities. For the mitochondrial AARSs, only mitochondrial glutaminyl-tRNA synthetase is missing. The lack of this gene in *M. thermophila* supports for the hypothesis that mitochondria use the indirect aminoacylation path which does not require glutaminyl-tRNA synthetase for the synthesis of Gln-tRNA^{Gln}. The characterized tRNA and AARS genes collected from the literature and the databases as well as those found in *M. thermophila* in this study can be used as reliable tRNA and AARS data sets for the detection of these genes in other genomes.

When comparing the four commonly used tRNA gene predictors – tRNAscan-SE, SPLITSX, ARAGORN and tRNA finder, the tRNA gene detection results in this study showed that predicted results from tRNAscan-SE and SPLITSX are better than those from the other tools. However, to detect the complete set of tRNA genes in the genome, multiple tRNA gene prediction programs should be combined to reduce the possibility of missing genes.

There is a moderate correlation between tRNA gene number and tRNA abundance in *M. thermophila*. Besides, strong correlations are observed between tRNA gene number and codon frequency as well between tRNA gene number and amino acid frequency in this organism. Evidence of the relationship between tRNA gene availability and codon usage in *M. thermophila* indicates that natural selection plays a role in the codon selection process. By analyzing the tRNA gene number and the codon frequencies, 17 preferred codons of 17 multiple codon coded amino acids, except for Ser, were determined and could be applied to improve the protein production in *M. thermophila*. Different methods which are based on the complete tRNA gene set, the highly expressed genes and the genes having low N_c values are used to deduce the preferred codons and give similar results. Among them, the method based on the genes having low N_c values is the simplest one and could be used to quickly determine preferred codons in an organism.

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APPENDICES

Supplementary table 1. tRNA gene prediction results from tRNAscan-SE

AA* : Gene number	Anticodon: Gene number					
Ala: 12	AGC:7	CGC:3	GGC:0	TGC:2		
Arg: 13	ACG:7	CCG:2	CCT:2	GCG:0	TCG:1	TCT:1
Asn: 6	ATT:0	GTT:6				
Asp: 10	ATC:0	GTC:10				
Cys: 3	ACA:0	GCA:3				
Gln: 7	CTG:5	TTG:2				
Glu: 10	CTC:8	TTC:2				
Gly: 17	ACC:0	CCC:2	GCC:11	TCC:4		
His: 4	ATG:0	GTG:4				
Ile: 9	AAT:8	GAT:1	TAT:1			
Leu: 15	AAG:7	CAA:2	CAG:4	GAG:0	TAA:1	TAG:1
Lys: 12	CTT:9	TTT:3				
Met: 8	CAT:8					
Phe: 5	AAA:0	GAA:5				
Pro: 11	AGG:6	CGG:3	GGG:0	TGG:3		
Ser: 16	ACT:0	AGA:5	CGA:3	GCT:5	GGA:0	TGA:3
Thr: 11	AGT:6	CGT:3	GGT:0	TGT:2		
Trp: 4	CCA:4					
Tyr: 6	ATA:0	GTA:6				
Val: 12	AAC:7	CAC:3	GAC:0	TAC:2		
SeC: 1	TCA:1					
Pseudo:2	GAT:1	TGG:1				

(*) AA: amino acid

Supplementary table 2. tRNA genes predicted by ARAGORN

AA : Gene number	**Anticodon: Gene number**					
Ala: 25	AGC:4	CGC:9	GGC:4	TGC:8		
Arg: 34	ACG:6	CCG:7	CCT:4	GCG:4	TCG:7	TCT:6
Asn: 9	ATT:1	GTT:8				
Asp: 14	ATC:1	GTC:13				
Cys: 3	ACA:0	GCA:3				
Gln: 10	CTG:6	TTG:4				
Glu: 19	CTC:16	TTC:3				
Gly: 25	ACC:2	CCC:3	GCC:14	TCC:6		
His: 10	ATG:1	GTG:9				
Ile: 10	AAT:7	GAT:2	TAT:1			
Leu: 23	AAG:8	CAA:4	CAG:6	GAG:3	TAA:1	TAG:1
Lys: 19	CTT:12	TTT:7				
Met: 7	CAT:7					
Phe: 4	AAA:3	GAA:1				
Pro: 21	AGG:6	CGG:6	GGG:3	TGG:6		
Ser: 38	ACT:3	AGA:5	CGA:9	GCT:12	GGA:4	TGA:5
Thr: 28	AGT:7	CGT:6	GGT:8	TGT:7		
Trp: 5	CCA:5					
Tyr: 10	ATA:1	GTA:9				
Val: 20	AAC:8	CAC:5	GAC:5	TAC:2		
SeC: 4	TCA:4					
Pyl: 1	CTA:1					
Stop: 6	TTA:6					
Pseudo: 4						

Supplementary table 3. tRNA genes predicted by tRNAfinder

AA : Gene number	**Anticodon: Gene number**					
Ala: 11	AGC:6	CGC:3	GGC:0	TGC:2		
Arg: 15	ACG:7	CCG:2	CCT:2	GCG:0	TCG:2	TCT:2
Asn: 6	ATT:0	GTT:6				
Asp: 11	ATC:1	GTC:10				
Cys: 3	ACA:0	GCA:3				
Gln: 7	CTG:5	TTG:2				
Glu: 10	CTC:8	TTC:2				
Gly: 16	ACC:0	CCC:2	GCC:11	TCC:3		
His: 4	ATG:0	GTG:4				
Ile: 10	AAT:8	GAT:1	TAT:1			
Leu: 15	AAG:7	CAA:2	CAG:4	GAG:0	TAA:1	TAG:1
Lys: 12	CTT:9	TTT:3				
Met: 8	CAT:8					
Phe: 7	AAA:0	GAA:7				
Pro: 11	AGG:6	CGG:3	GGG:0	TGG:2		
Ser: 16	ACT:0	AGA:5	CGA:3	GCT:5	GGA:0	TGA:3
Thr: 11	AGT:6	CGT:3	GGT:0	TGT:2		
Trp: 4	CCA:4					
Tyr: 0	ATA:0	GTA:0				
Val: 12	AAC:7	CAC:3	GAC:0	TAC:2		
SeC: 1	TCA:1					

Supplementary table 4. Experimentally characterized fungal tRNAs and tRNA genes collected in this study

Species	Strain	Amino acid	Anticodon	Gene ID (tRNAdb)	Gene ID (Genbank)	tRNA ID (tRNAdb)	tRNA ID (Genbank)	Literature (PMIDs)
Candida albicans	unknown	Ser	CAG	tdbD00001753 tdbD00002874	X64564	tdbR00000618	none	8440250
Candida albicans	2005E	Ala	AGC	tdbD00000217	Y08493	none	none	9282735
Candida albicans	2005E	Asp	GUC	tdbD00000499	Y08491	none	none	9282735
Candida albicans	2005E	Ile	AAU	tdbD00001319	Y08492	none	none	9282735
Candida cylindracea	unknown	Leu	CAA	none	none	tdbR00000253	D14894	8121794
Candida cylindracea	unknown	Leu	IAG	none	none	tdbR00000254	D14895	8121794
Candida cylindracea	unknown	Leu	IAG	none	none	tdbR00000255	D14896	8121794
Candida cylindracea	MS_5	Ser	CAG	tdbD00002886	S42192	tdbR00000409	D12939 S42406	8121794 1502151
Candida cylindracea	unknown	Ser	UGA	none	none	tdbR00000410	D14897	8121794
Candida cylindracea	unknown	Ser	IGA	none	none	tdbR00000411	D14898	8121794
Candida cylindracea	unknown	Ser	CGA	none	none	tdbR00000412	D14899	8121794
Candida cylindracea	unknown	Ser	GCU	none	none	tdbR00000413	D14900	8121794
Candida guilliermondii	JCM1539	Ser	CAG	tdbD00001759	D17533	none	none	7748957
Candida lusitaniae	JCM5936	Ser	CAG	tdbD00001758	D17534	none	none	7748957
Candida melibiosica	JCM 1613	Ser	CAG	none	none	none	none	8371978
Candida parapsilosis	JCM 1785	Ser	CAG	none	none	none	none	8371978
Candida rugosa	JCM 1619	Ser	CAG	none	none	none	none	8371978
Candida tropicalis	JCM1541	Ser	CAG	tdbD00001757	D17535	none	none	7748957
Candida utilis	unknown	Ala	IGC	none	none	tdbR00000013	M35061	4796875
Candida utilis	unknown	Ile	IAU	none	none	tdbR00000171	K01061	5817084
Candida utilis	unknown	Met	CAU	none	none	tdbR00000528	K00323	573264
Candida utilis	TFO 1086	Leu	CAA	none	none	tdbR00000252	K00232	350863
Candida utilis	unknown	Pro	UGG	none	none	tdbR00000325	K00357	6920386

Species	**Strain**	**Amino acid**	**Anticodon**	**Gene ID (tRNAdb)**	**Gene ID (Genbank)**	**tRNA ID (tRNAdb)**	**tRNA ID (Genbank)**	**Literature (PMIDs)**
Candida utilis	unknown	Tyr	GUA	none	none	tdbR00000557	M24830	4308683
Candida utilis	unknown	Val	IAC	none	none	none	X02700	4305011
Candida zeylanoides	JCM 1627	Ser	CAG	none	none	none	none	8371978
Neurospora crassa	OR23_1A	Met	CAU	none	none	tdbR00000525	K00316	146192
Neurospora crassa	unknown	Phe	GAA	tdbD00000784	J01251	tdbR00000082	X02710	6449692 6449691
Neurospora crassa	74A	Leu	AAG	tdbD00001752	X00736	none	none	6235483
Podospora anserina	unknown	Ser	UGA	tdbD00002875	X05227	none	none	3937728
Podospora anserina	unknown	Ser	UGA	tdbD00002876	X05226	none	none	3937728
Saccharomyces cerevisiae	VP64_9A	Ala	IGC	tdbD00000219	X13793	tdbR00000012	K01059	2648329 1089070
Saccharomyces cerevisiae	unknown	Arg	UCU	tdbD00002586	X00375 J01378 J01377	tdbR00000370	K00158	6321234 6253814 6986864
Saccharomyces cerevisiae	unknown	Arg	UCU	none	none	tdbR00000371	K00159	6986864
Saccharomyces cerevisiae	unknown	Arg	ICG	tdbD00002585	V01330	tdbR00000369	K00157	16453416 1100396
Saccharomyces cerevisiae	unknown	Arg	CCU	tdbD00002587	V01326	none	none	16453444
Saccharomyces cerevisiae	J24_24A	Asn	GUU	tdbD00002076	X58787	tdbR00000300	M26099	1840659 6395902
Saccharomyces cerevisiae	unknown	Asp	GUC	tdbD00000502	M10994	tdbR00000035	K00171	3886659 4551153
Saccharomyces cerevisiae	unknown	Ala	UGC	tdbD00000218	M10958	none	none	3886659
Saccharomyces cerevisiae	unknown	Cys	GCA	tdbD00000372	K02653	tdbR00000021	X01939	6088502 819006
Saccharomyces cerevisiae	unknown	Glu	UUC	tdbD00000644	K02652 K02652 K02651 Z00039	tdbR00000054	K00191	6088502 4376021
Saccharomyces cerevisiae	C836	Glu	CUC	tdbD00000645	X06132	none	none	2833361
Saccharomyces cerevisiae	unknown	His	GUG	none	none	tdbR00000144	M26097	6370316

Species	Strain	Amino acid	Anticodon	Gene ID (tRNAdb)	Gene ID (Genbank)	tRNA ID (tRNAdb)	tRNA ID (Genbank)	Literature (PMIDs)
Saccharomyces cerevisiae	S288c	His	GUG	tdbD00001112	K01597 K01596 K01595	tdbR00000145	M26098	6305975 6370316
Saccharomyces cerevisiae	unknown	Pro	UGG	none	none	tdbR00000323	M26096	6370316
Saccharomyces cerevisiae	X2180	Gly	GCC	tdbD00000951	X05272 U19971 X05269 X05270 M10995	tdbR00000129	K00204	3039147 3886659 4569887
Saccharomyces cerevisiae	unknown	Gly	UCC	none	X05271 X05265	tdbR00000130	none	3039147
Saccharomyces cerevisiae	ATCC 25657	Ile	IAU	tdbD00001320	X05058	tdbR00000170	K02004	3547324 6370253
Saccharomyces cerevisiae	DBY939	Met	CAU	tdbD00003496	M22701 M22702	tdbR00000526	K00321	3011608 4344891
Saccharomyces cerevisiae	unknown	Met	CAU	tdbD00003495	J01373	none	none	6278434
Saccharomyces cerevisiae	unknown	Leu	CAA	tdbD00001754	J01370	tdbR00000249	K00228	387786 4574158
Saccharomyces cerevisiae	unknown	Leu	UAA	tdbD00001756	X69765	tdbR00000251	X63952	8394042 1799629
Saccharomyces cerevisiae	unknown	Leu	UAG	none	none	tdbR00000250	K00229	374075
Saccharomyces cerevisiae	unknown	Lys	UUU	tdbD00001463	none	tdbR00000193	K00287 M36150	4589316 17778175
Saccharomyces cerevisiae	alpha S288c	Lys	CUU	none	none	tdbR00000192	K00286	4576137
Saccharomyces cerevisiae	Y185, C836	Met	CAU	tdbD00001919	Z00037	tdbR00000284	M10268	6253952 826525
Saccharomyces cerevisiae	5178 IC2X2B2	Phe	GAA	tdbD00000785	J01369 J01368 J01367	tdbR00000083	K01553 X00655 M14856	343104 5637712 3545201
Saccharomyces cerevisiae	unknown	Phe	GAA	none	none	tdbR00000084	M14867	3545201
Saccharomyces cerevisiae	unknown	Phe	GAA	tdbD00000786	M17332	none	none	3308382
Saccharomyces cerevisiae	C836	Thr	IGU	tdbD00003079	X56504	tdbR00000443	K00278	2011498 992092

Species	**Strain**	**Amino acid**	**Anticodon**	**Gene ID (tRNAdb)**	**Gene ID (Genbank)**	**tRNA ID (tRNAdb)**	**tRNA ID (Genbank)**	**Literature (PMIDs)**
Saccharomyces cerevisiae	unknown	Thr	IGU	none	none	tdbR00000444	K00279	992092
Saccharomyces cerevisiae	C836	Thr	CGU	tdbD00003080	none	none	none	2011498
Saccharomyces cerevisiae	unknown	Trp	CCA	tdbD00003403	S48358	tdbR00000494	M35060	1398091 4350483
Saccharomyces cerevisiae	unknown	Ser	IGA	none	none	tdbR00000407	K01557	5991670
Saccharomyces cerevisiae	unknown	Ser	IGA	tdbD00002877	V01329	tdbR00000407	K01558	16453416 5991670
Saccharomyces cerevisiae	unknown	Ser	CGA	tdbD00002879	none	none	K00367	355641
Saccharomyces cerevisiae	unknown	Ser	UGA	tdbD00002880	K00572	tdbR00000406	K00368	6262655 355641 389430
Saccharomyces cerevisiae	unknown	Tyr	GUA	tdbD00003654	J01383 J01382 J01380	tdbR00000555	M10266	341157 5938777
Saccharomyces cerevisiae	unknown	Val	UAC	none	none	tdbR00000466	M35070	4606852
Saccharomyces cerevisiae	unknown	Val	CAC	none	none	tdbR00000465	K00249	410008
Saccharomyces cerevisiae	unknown	Val	IAC	tdbD00003245	V01331	tdbR00000464	K00248	16453416 4375496
Saccharomyces cerevisiae	unknown	Val	AAC	tdbD00003246	X54514	none	none	2235498
Saccharomyces cerevisiae	unknown	Gln	CUG	tdbD00002387	M29654	none	none	3295253
Saccharomyces cerevisiae	unknown	Gln	UUG	tdbD00002386	X02445 V01295	none	none	2989539
Schizosaccharomyces pombe	unknown	Glu	UUC	none	none	tdbR00000055	X01325	379816
Schizosaccharomyces pombe	unknown	Glu	UUC	tdbD00000647	X00239	none	none	6561518
Schizosaccharomyces pombe	unknown	Lys	CUU	tdbD00001466	X00283	none	none	6561518
Schizosaccharomyces pombe	unknown	Arg	ACG	tdbD00002588	X00239	none	none	6561518
Schizosaccharomyces pombe	unknown	Arg	ACG	tdbD00002589	X00240	none	none	6561518
Schizosaccharomyces pombe	unknown	Asp	GUC	tdbD00000503	K00570	none	none	6561518
Schizosaccharomyces pombe	unknown	His	GUG	tdbD00001113	X00241	none	none	6561518

Species	Strain	Amino acid	Anticodon	Gene ID (tRNAdb)	Gene ID (Genbank)	tRNA ID (tRNAdb)	tRNA ID (Genbank)	Literature (PMIDs)
Schizosaccharomyces pombe	unknown	Met	CAU	tdbD00003497	V01360	tdbR00000527	X69095	7407924 8332511
Schizosaccharomyces pombe	unknown	Phe	GAA	tdbD00000789	X00242	tdbR00000085	K00344	6561518 247991
Schizosaccharomyces pombe	972h_	Tyr	GUA	none	none	tdbR00000556	K00273	116193
Schizosaccharomyces pombe	972h_	Tyr	GUA	none	none	none	K00272	116193

Supplementary table 5. Experimentally characterized fungal cytoplasmic AARSs collected in this study

Species	Strain	Gene name	Enzyme name	Function (*)	Protein length	Gene ID (Genbank)	Protein ID (UniProt)	Literature (PMIDs)
Saccharomyces cerevisiae	GRF88	ALA1	Alanyl-tRNA synthetase	cyt + mit	958	U18672	P40825	7761427
Saccharomyces cerevisiae	S288c	DED81	Asparaginyl-tRNA synthetase	cyt	554	NC_001140	P38707	9605503
Saccharomyces cerevisiae	unknown	DPS1	Aspartyl-tRNA synthetase	cyt	557	X03606	P04802	3513127
Saccharomyces cerevisiae	X2180	YNL247W	Cysteinyl-tRNA synthetase	cyt	767	Z71523	P53852	9523015
Saccharomyces cerevisiae	S288c	GLN4	Glutaminyl-tRNA synthetase	cyt	809	J02784	P13188	3301841
Saccharomyces cerevisiae	unknown	GUS1	Glutamyl-tRNA synthetase	cyt	708	U32265	P46655	11069915
Saccharomyces cerevisiae	S288c	GRS1	Glycyl-tRNA Synthase	cyt + mit	667	Z35990 X78993 BK006936	P38088	10874035
Saccharomyces cerevisiae	S288c	GRS2	Glycyl-tRNA Synthase	cyt	618	U51033	Q06817	10874035
Saccharomyces cerevisiae	unknown	HTS1	Histidine-Trna Synthetase	cyt + mit	526	M14048	P07263	3521891
Saccharomyces cerevisiae	S288c	ILS1	Isoleucine-tRNA Synthetase	cyt	1072	M19992	P09436	3311074
Saccharomyces cerevisiae	S288c	CDC60	Leucyl-tRNA synthetase	cyt	1090	X62878	P26637	1398122
Saccharomyces cerevisiae	X2180	KRS1	Lysyl-tRNA synthetase	cyt	591	J04186	P15180	2903861
Saccharomyces cerevisiae	FL100	MES1	Methionyl-tRNA synthetase	cyt	751	V01316	P00958	6341994

Species	Strain	Gene name	Enzyme name	Function (*)	Protein length	Gene ID (Genbank)	Protein ID (UniProt)	Literature (PMIDs)
Saccharomyces cerevisiae	FL100	FRS1	Phenylalanyl-tRNA synthetase (β)	cyt	595	J03964	P15624	3049607
Saccharomyces cerevisiae	FL100	FRS2	Phenylalanyl-tRNA synthetase (α)	cyt	503	J03965	P15625	3049607
Saccharomyces cerevisiae	X2180	SES1	Seryl-tRNA synthetase	cyt	462	X04884	P07284	3031581
Saccharomyces cerevisiae	D273-10B	THS1	Threonyl-tRNA synthetase	cyt	734	X02906	P04801	2995918
Saccharomyces cerevisiae	unknown	WRS1	Tryptophanyl-tRNA synthetase	cyt	432	Z48149	Q12109	9046085
Saccharomyces cerevisiae	unknown	TYS1	Tyrosyl-tRNA synthetase	cyt	394	L12221	P36421	8509419
Saccharomyces cerevisiae	FL100	VAS1	Valyl-tRNA Synthetase	cyt + mit	1058	J02719	P07806	3275649
Neurospora crassa	OR47A	leu-6	leucyl-tRNA synthetase	cyt	1123	M30473	P10857	2532300
Neurospora crassa	74-OR23-1A	cyt-20	Valyl-tRNA Synthetase	cyt + mit	1050	M64703	P28350	1830127
Candida albicans	CBS 5736	SES1	Seryl-tRNA synthetase	cyt	462	AF290915	Q9HGT6	11223940
Candida albicans	2005E	CDC60	Leucyl-tRNA synthetase	cyt	1097	AF293346	Q9HGT2	11574161
Candida albicans	SC5314	ALA1	Alanyl-tRNA synthetase	cyt + mit	969	none	Q5A8K2	16928688
Candida albicans	ATCC 26555	FRS1	Phenylalanyl-tRNA synthetase (β)	cyt	593	Y12589	O13432	9561746
Schizosaccharomyces pombe	unknown	VAS1	Valyl-tRNA Synthetase	cyt	980	none	O75005	20106903

Supplementary table 6. Reliable AARSs collected from UniProt database according to their GO terms and GO evidence codes

Species	Strain	Gene name	Enzyme name	Function (*)	Protein length	Protein ID (UniProt)
Arabidopsis thaliana	unknown	T22E16.60	Methionyl-tRNA synthetase	mit	616	Q9M2T9
Bos taurus	unknown	SARS2	Seryl-tRNA synthetase	mit	518	Q9N0F3
Escherichia coli	K12	alaS	Alanyl-tRNA synthetase	cyt	876	P00957
Escherichia coli	K12	aspS	Aspartyl-tRNA synthetase	cyt	590	P21889

Species	**Strain**	**Gene name**	**Enzyme name**	**Function (*)**	**Protein length**	**Protein ID (UniProt)**
Escherichia coli	K12	cysS	Cysteinyl-tRNA synthetase	cyt	461	P21888
Escherichia coli	K12	lysU	Lysyl-tRNA synthetase	cyt	505	P0A8N5
Escherichia coli	K12	lysS	Lysyl-tRNA synthetase	cyt	505	P0A8N3
Escherichia coli	K12	proS	Prolyl-tRNA synthetase	cyt	572	P16659
Escherichia coli	K12	serS	Seryl-tRNA synthetase	cyt	430	P0A8L1
Escherichia coli	K12	thrS	Threonyl-tRNA synthetase	cyt	642	P0A8M3
Homo sapiens	unknown	AARS	Alanyl-tRNA synthetase	cyt	968	P49588
Homo sapiens	unknown	RARS	Arginyl-tRNA synthetase	cyt	660	P54136
Homo sapiens	unknown	NARS	Asparaginyl-tRNA synthetase	cyt	548	O43776
Homo sapiens	unknown	DARS	Aspartyl-tRNA synthetase	cyt	501	P14868
Homo sapiens	unknown	DARS2	Aspartyl-tRNA synthetase	mit	645	Q6PI48
Homo sapiens	unknown	EPRS	Bifunctional aminoacyl-tRNA synthetase (ProRS (class II) and GluRS (class II))	cyt	1512	P07814
Homo sapiens	unknown	CARS	Cysteinyl-tRNA synthetase	cyt	748	P49589
Homo sapiens	unknown	QARS	Glutaminyl-tRNA synthetase	cyt	775	P47897
Homo sapiens	unknown	GARS	Glycyl-tRNA Synthase	cyt + mit	739	P41250
Homo sapiens	unknown	IARS	Isoleucine-tRNA Synthetase	cyt	1262	P41252
Homo sapiens	unknown	LARS	Leucyl-tRNA synthetase	cyt	1176	Q9P2J5
Homo sapiens	unknown	KARS	Lysyl-tRNA synthetase	cyt + mit	597	Q15046
Homo sapiens	unknown	MARS	Methionyl-tRNA synthetase	cyt	900	P56192
Homo sapiens	unknown	MARS2	Methionyl-tRNA synthetase	mit	593	Q96GW9
Homo sapiens	unknown	FARS2	Phenylalanyl-tRNA synthetase	mit	451	O95363
Homo sapiens	unknown	FARSA	Phenylalanyl-tRNA synthetase (alpha subunit)	cyt	508	Q9Y285
Homo sapiens	unknown	FARSB	Phenylalanyl-tRNA synthetase (beta subunit)	cyt	589	Q9NSD9
Homo sapiens	unknown	SARS	Seryl-tRNA synthetase	cyt	514	P49591
Homo sapiens	unknown	SARS2	Seryl-tRNA synthetase	mit	518	Q9NP81

Species	**Strain**	**Gene name**	**Enzyme name**	**Function (*)**	**Protein length**	**Protein ID (UniProt)**
Homo sapiens	unknown	TARS	Threonyl-tRNA synthetase	cyt	723	P26639
Homo sapiens	unknown	TARS2	Threonyl-tRNA synthetase	mit	718	Q9BW92
Homo sapiens	unknown	WARS	Tryptophanyl-tRNA synthetase	cyt	471	P23381
Homo sapiens	unknown	YARS	Tyrosyl-tRNA synthetase	cyt	528	P54577
Homo sapiens	unknown	YARS2	Tyrosyl-tRNA synthetase	mit	477	Q9Y2Z4
Homo sapiens	unknown	VARS	Valyl-tRNA Synthetase	cyt	1264	P26640
Mycobacterium tuberculosis	unknown	metG	Methionyl-tRNA synthetase	cyt	519	O05593
Rattus norvegicus	unknown	Aars	Alanyl-tRNA synthetase	cyt	968	P50475
Saccharomyces cerevisiae	S288c	MSR1	Arginyl-tRNA synthetase	mit	643	P38714
Saccharomyces cerevisiae	S288c	SLM5	Asparaginyl-tRNA synthetase	mit	492	P25345
Saccharomyces cerevisiae	S288c	MSD1	Aspartyl-tRNA synthetase	mit	658	P15179
Saccharomyces cerevisiae	S288c	MSE1	Glutamyl-tRNA synthetase	mit	536	P48525
Saccharomyces cerevisiae	S288c	ISM1	Isoleucine-tRNA synthetase	mit	1002	P48526
Saccharomyces cerevisiae	S288c	NAM2	Leucyl-tRNA synthetase	mit	894	P11325
Saccharomyces cerevisiae	S288c	MSK1	Lysyl-tRNA synthetase	mit	576	P32048
Saccharomyces cerevisiae	S288c	MSM1	Methionyl-tRNA synthetase	mit	575	P22438
Saccharomyces cerevisiae	S288c	MSF1	Phenylalanyl-tRNA synthetase	mit	469	P08425
Saccharomyces cerevisiae	S288c	DIA4	Seryl-tRNA synthetase	mit	446	P38705
Saccharomyces cerevisiae	S288c	MST1	Threonyl-tRNA synthetase	mit	462	P07236
Saccharomyces cerevisiae	S288c	MSW1	Tryptophanyl-tRNA synthetase	mit	379	P04803
Saccharomyces cerevisiae	S288c	MSY1	Tyrosyl-tRNA synthetase	mit	492	P48527
Schizosaccharomyces pombe	972h-	vas1	Valyl-tRNA Synthetase	mit	950	O14160
Thermus thermophilus	HB8	asnS	Asparaginyl-tRNA synthetase	cyt	438	P54263

(*) Each AARS gene encodes either a cytoplasmic (cyt) or a mitochondrial (mit) enzyme, or both of them (cyt + mit)

Supplementary table 7. Number of tRNA genes in each anticodon specific family and codon frequencies calculated from the highly expressed genes and the genes having N_c less than 31 in *M. thermophila*

AA	Anti-codon	Decoded codons [(*)]	tRNA gene number	Codon frequency calculated from highly expressed genes (%)		Codon frequency calculated from genes having $N_c < 31$ (%)	
Ala	AGC	GCT,GCA,GCC	7	GCT (20.29)	**GCC (56.45)**	GCT (6.12)	**GCC (71.98)**
	CGC	GCG	3	GCG (16.97)		GCG (19.37)	
	TGC	GCA,GCG	2	GCA (6.29)		GCA (2.54)	
Arg	ACG	CGT,CGA,CGC	7	CGT (17.93)	**CGC (43.66)**	CGT (4.37)	**CGC (63.05)**
	CCG	CGG	2	CGG (15.28)		CGG (20.88)	
	CCT	AGG	2	AGG (11.68)		AGG (8.79)	
	TCG	CGA,CGG	1	CGA (5.28)		CGA (1.71)	
	TCT	AGA,AGG	2	AGA (6.16)		AGA (1.2)	
Asn	GTT	AAC,AAT	6	**AAC (84.28)**	AAT (15.72)	**AAC (94.76)**	AAT (5.24)
Asp	GTC	GAC,GAT	10	**GAC (72.5)**	GAT (27.5)	**GAC (90.81)**	GAT (9.19)
Cys	GCA	TGC,TGT	3	**TGC (81.55)**	TGT (18.45)	**TGC (96.37)**	TGT (3.63)
Gln	CTG	CAG	5	**CAG (79.48)**		**CAG (92.77)**	
	TTG	CAA,CAG	2	CAA (20.52)		CAA (7.23)	
Glu	CTC	GAG	8	**GAG (84.86)**		**GAG (93.32)**	
	TTC	GAA,GAG	2	GAA (15.14)		GAA (6.68)	
Gly	CCC	GGG	2	GGG (7.77)		GGG (10.75)	
	GCC	GGC,GGT	11	**GGC (59.02)**	GGT (25.51)	**GGC (78.58)**	GGT (7.32)
	TCC	GGA,GGG	3	GGA (7.7)		GGA (3.35)	
His	GTG	CAC,CAT	4	**CAC (76.52)**	CAT (23.48)	**CAC (94.02)**	CAT (5.98)
Ile	AAT	ATT,ATA,ATC	8	ATT (24.55)		ATT (7.99)	
	GAT	ATC,ATT	1	**ATC (72.26)**		**ATC (90.68)**	
	TAT	ATA	1	ATA (3.18)		ATA (1.33)	
Leu	AAG	CTT,CTA,CTC	7	CTT (12.98)	**CTC (43.9)**	CTT (2.99)	**CTC (51)**
	CAA	TTG	2	TTG (8.77)		TTG (2.87)	
	CAG	CTG	4	CTG (29.81)		CTG (41.99)	
	TAA	TTA,TTG	1	TTA (1.72)		TTA (0.27)	
	TAG	CTA,CTG	1	CTA (2.82)		CTA (0.88)	
Lys	CTT	AAG	9	**AAG (90.36)**		**AAG (96.7)**	
	TTT	AAA,AAG	3	AAA (9.64)		AAA (3.3)	
Phe	GAA	TTC,TTT	5	**TTC (78.59)**	TTT (21.41)	**TTC (89.04)**	TTT (10.96)
Pro	AGG	CCT,CCA,CCC	5	CCT (18.6)	**CCC (44.11)**	CCT (5.18)	**CCC (52.96)**
	CGG	CCG	3	CCG (27.48)		CCG (39.32)	
	TGG	CCA,CCG	3	CCA (9.81)		CCA (2.54)	
Ser	AGA	TCT,TCA,TCC	5	TCT (12.39)	TCC (25.31)	TCT (3.96)	TCC (28.55)
	CGA	TCG	3	**TCG (26.93)**		TCG (32.33)	
	GCT	AGC,AGT	6	AGC (25.56)	AGT (4.15)	**AGC (32.52)**	AGT (1.26)
	TGA	TCA,TCG	3	TCA (5.66)		TCA (1.38)	
Thr	AGT	ACT,ACA,ACC	6	ACT (15.18)	**ACC (55.18)**	ACT (4.54)	**ACC (63.4)**
	CGT	ACG	3	ACG (20.59)		ACG (29.64)	
	TGT	ACA,ACG	2	ACA (9.05)		ACA (2.42)	
Tyr	GTA	TAC,TAT	6	**TAC (80.25)**	TAT (19.75)	**TAC (93.56)**	TAT (6.44)
Val	AAC	GTT,GTA,GTC	7	GTT (19.08)	**GTC (58.14)**	GTT (4.62)	**GTC (69.21)**
	CAC	GTG	3	GTG (19.18)		GTG (25.1)	
	TAC	GTA,GTG	2	GTA (3.6)		GTA (1.07)	

((*) Decoded codons: possible codons that can match with the corresponding anticodons in the table, according to the Wobble theory. The bold codons are the ones having the highest frequencies among the synonymous ones.)