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Short Communication

The mobile FOX AmpC beta-lactamases originated in *Aeromonas allosaccharophila*

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ABSTRACT

Objective: It is important to understand the origins of antibiotic resistance genes so that risks associated with the emergence of novel resistance genes can be assessed and managed. The chromosomal *ampC* gene (CAV-1) of *Aeromonas caviae* (*A. caviae*) has been reported as the origin of mobile FOX cephalosporinases. The recent identification of *A. caviae* as the origin of MOX-2 cephalosporinases and the comparably great sequence divergence between FOX and MOX genes makes it unlikely that both genes arose from the same species. Therefore, this study investigated the origin of FOX cephalosporinases using large-scale genomics.

Methods: Publicly available genomes and plasmids were searched for FOX-like genes. Synteny and nucleotide identities of the identified FOX-like genes and their genetic environments were compared and a phylogenetic tree was generated.

Results: FOX-like genes were identified in > 230 *Aeromonas* genomes and in 46 Enterobacteriaceae isolates. Analysis of the genomic context of CAV-1 revealed a truncated insertion sequence directly upstream of the *ampC* gene. The chromosomal *ampCs* of *A. caviae* (n = 31) were 75–78% identical to CAV-1. In contrast, CAV-1, mobile FOX genes and their context were 95–98% similar to the chromosomal *ampC*-locus of *Aeromonas allosaccharophila* (*A. allosaccharophila*) (n = 6). The *A. allosaccharophila ampCs* formed a monophyletic branch with mobile FOX genes, whereas the *A. caviae ampCs* clustered with mobile MOX genes.

Conclusions: These findings show that FOX cephalosporinases originate not in *A. caviae*, as previously reported, but in *A. allosaccharophila*, which is a fish pathogen. This finding agrees with the hypothesis that antibiotic use in aquaculture could have contributed to the emergence of FOX genes in human pathogens.

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Introduction

The plasmid-mediated FOX extended-spectrum cephalosporinases are AmpC β-lactamases, which have mostly been detected in the United States and Europe, that compromise the efficiency of β-lactam antibiotics, especially cefoxitin [1]. To date, eight FOX variants have been described in detail but more have been reported (https://card.mcmaster.ca/ontology/36206). To manage antibiotic use and assess risks associated with the emergence of novel antibiotic resistance genes (ARGs), it is important to understand how the mobile ARGs that cause treatment failure today ended up in clinically relevant bacteria. In 2003, Fosse et al. reported *Aeromonas caviae* (*A. caviae*) as the origin of mobile FOX β-lactamases, based on sequence similarity of the *ampC* gene (named CAV-1) obtained from the chromosome of *A. caviae* CIP 74.32 and mobile FOX genes [2] from Enterobacteriaceae pathogens. Other studies found the mobile FOX locus highly similar to the chromosomal *ampC*-loci of *Aeromonas veronii* and *Aeromonas salmonicida* [3]. More recently, research based on large-scale sequencing data revealed *A. caviae* as the origin of mobile MOX-2 *ampC* genes [4], which differ from the most closely related FOX genes by roughly 20% in nucleotide identity. This comparatively large degree of divergence makes it unlikely that both CMY-1/MOX and FOX genes are the mobilised *A. caviae ampC* gene. The rapidly rising available amount of sequenced bacterial genomes enables comparative

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genomic analysis on a large scale. The current study investigated the origin of mobile FOX cephalosporinases through screening of all available genomes and plasmids in Genbank followed by careful in silico comparison of their genetic environments (including synteny and presence/absence of mobile elements) and nucleotide identities in different isolates.

Materials and methods

Genome assemblies and plasmids available from Genbank (n = 184 755, downloaded March 2018, with updating of newly available genomes until January 2019) were screened for FOX genes (all variants downloaded from CARD, January 2019) using DIAMOND [5] v0.9.24.125 with a 70% identity cut-off and extracted up to 10 kbp upstream and downstream of identified genes. Such a relaxed cut-off was necessary to include the *A. caviae ampC* gene, which was the previously suggested origin of FOX genes, in the analysis. Since this cut-off also included CMY-1/MOX-1 genes, which are closely related to FOX genes, identified genes from this search will be referred to as 'FOX-like' in the following analysis. The 1861 bp CAV-1 sequence (Genbank accession: AF462690) reported by Fosse et al. (2003) was separately downloaded from Genbank, as it is believed that the whole genome of this isolate has not been sequenced. Cluster analysis of the identified genes and CAV-1 (95% amino acid identity to assess similarity of FOX-variants to one another, 99% nucleotide identity for phylogenetic analysis) was conducted using USEARCH [6] v8.0.1445. These sequences were annotated using PROKKA [7] v1.12, further annotating open reading frames (ORFs) annotated as 'hypothetical protein' using the Uniprot Knowledgebase (-id 50%). The ISFinder [8] database was used to identify insertion sequences and transposases.

Because this study aimed to investigate the genetic context of the identified genes, sequences encoding fewer than six genes were excluded from further analysis. Multiple sequence alignments for phylogenetic analysis were created using MAFFT [9] v7.310 (–maxiterate 1000 –globalpair). To avoid misleading results due to misannotated isolates (which is common in public databases), only *Aeromonas* spp. with three or more FOX-like-positive isolates were included in the synteny analysis and nucleotide comparison (Table 1). Due to known problems with assembly of complex mobile elements from short reads, unique mobile contexts were only included in the synteny analysis if generated by long-read or hybrid assembly (such as *Providencia rettgeri*, ASM322613v1). A phylogenetic tree of the identified FOX-like genes (clustered at 99% nucleotide identity) was generated using RAxML [10] v8.2.10 (-m GTRCAT) and 1000 times rapid bootstrapping. Alignments from the online available blastn [11] suite were used for comparative sequence analysis of all identified FOX-like loci (including CAV-1). ANIcalculator_v1 [12] was used to calculate the global average nucleotide identity and fraction of orthologous genes between genomes, as a measure of similarity between pairs of genomes.

Results

FOX-genes are associated with insertion sequences in Enterobacteriaceae but not in Aeromonas

Of the 283 identified FOX-positive genome assemblies and plasmids, 237 were found in *Aeromonas* spp. and 46 in Enterobacteriaceae (Table 1). Cluster analysis at 95% nucleotide identity resulted in two clusters, the first containing FOX-1, -2, -3, -4, -5, -7, -8, -10 and CAV-1, the second containing only FOX-9 (94% similar to FOX-7, centroid of the first cluster). In Enterobacteriaceae, FOX genes were always associated with mobile genetic elements (MGEs), either contained in a composite transposon consisting of two *ISAs2* flanking the genes *ampC* (FOX-like), *mdtL* and *yidZ* on either side or with unique *Tn3*-like mobile elements that also contained other insertion sequences (IS) (Fig. 1). FOX-5 (n = 36) and FOX-8 (n = 4) were the most frequently annotated variants in Enterobacteriaceae. The FOX-positive *Aeromonas* spp. generally lacked transposable elements or IS at the *ampC* locus. Though IS were occasionally identified close to the *ampC* gene in some *Aeromonas* isolates, all other genes in those sequences, with few exceptions, corresponded to the chromosomal *Aeromonas ampC*-locus. Of all *Aeromonas ampC*s, the *Aeromonas allosaccharophila* (*A. allosaccharophila*) ampC was most similar to mobile FOX genes (Table 1, Fig. 1). The synteny at the *ampC*-locus of *A. allosaccharophila, Aeromonas salmonicida, A. caviae* and *Aeromonas media* (*A. media*) matched the synteny of genes in the Enterobacteriaceae FOX-transposon, with the genes *mdtL* and *yidZ* being encoded downstream of the *ampC* gene.

Table 1
Comparison of *ampC* nucleotide identity between CAV-1 *ampC* gene and FOX-like genes identified with a 70% cut-off in different bacterial species.

Bacterial species	Number of isolates	Identity towards CAV-1 ampC region (1223 bp)	Alignment length
Enterobacter spp.	12	96–97%	1223 bp
Klebsiella spp.	18	79–97%	1072–1223 bp
Escherichia coli	6	96%	1223 bp
Citrobacter freundii	4	96%	1223 bp
Kluyvera ascorbata	1	97%	1216 bp
Salmonella enterica	1	96%	1223 bp
Pseudomonas spp.	2	78%	1073 bp
Serratia marcesecens	1	79%	1075 bp
Pantoea spp.	1	96%	1223 bp
A. allosaccharophila	6	95–97%	1223 bp
A. salmonicida	51	86–88%	1050–1068 bp
A. hydrophila	74	76–80%	1043–1053 bp
A. caviae	31	75–78%	1065–1072 bp
A. piscicola	2	88%	1068 bp
A. bestiarum	2	84–87%	1070–1079 bp
A. sobria	2	86%	1068 bp
A. dhakensis	13	77–78%	1042–1072 bp
A. media	6	79–80%	1040–1061 bp
A. enteropelogenes	5	77–81%	1024–1035 bp
A. jandaei	5	77–81%	1043–1135 bp
A. popoffii	1	88%	1061 bp
A. sanarellii	1	79%	1077 bp
A. veronii	3	79–81%	1069–1165 bp
A. encheleia	2	81%	1023 bp
A. rivipollensis	1	81%	1052
A. lacus	1	80%	1128 bp
A. diversa	1	80%	1130 bp
A. molluscorum	1	82%	1039 bp
A. bivalvium	3	77–78%	1065 bp

Aeromonas (*A.*) isolates without species annotation ($n = 24$) were excluded.
'*ampC region*' means all bases from the originally submitted sequence except for *mdtI*Δ and IS*Apu2*Δ. Thus, non-coding regions are included.

The chromosomal Aeromonas allosaccharophila *ampC-locus is highly similar to mobile FOX-loci*

Sequence comparison revealed 96–98% nucleotide identity over 2773–4188 bp between mobile FOX-loci from *Klebsiella pneumoniae* (18090_6#31, 12045_7#33), *Escherichia coli* (7996-1), *Enterobacter cloacae* (ASM95820v1), *Providencia rettgeri* (ASM322613v1) and the chromosomal *ampC*-locus (*ampC-mdtL-yidZ*) of *A. allosaccharophila* (PRJEB7019, ASM190854v1, ASM144689v1, PRJEB7045, ASM326549v1, Z9-6), all of which notably lacked IS or other indicators of mobility.

The alignment encompassed the complete sequence enclosed in the composite transposon, including all non-coding regions. The second copy of *yidZ* from *A. allosaccharophila* further downstream of the *ampC* locus was ca. 75% identical to the *yidZ* on

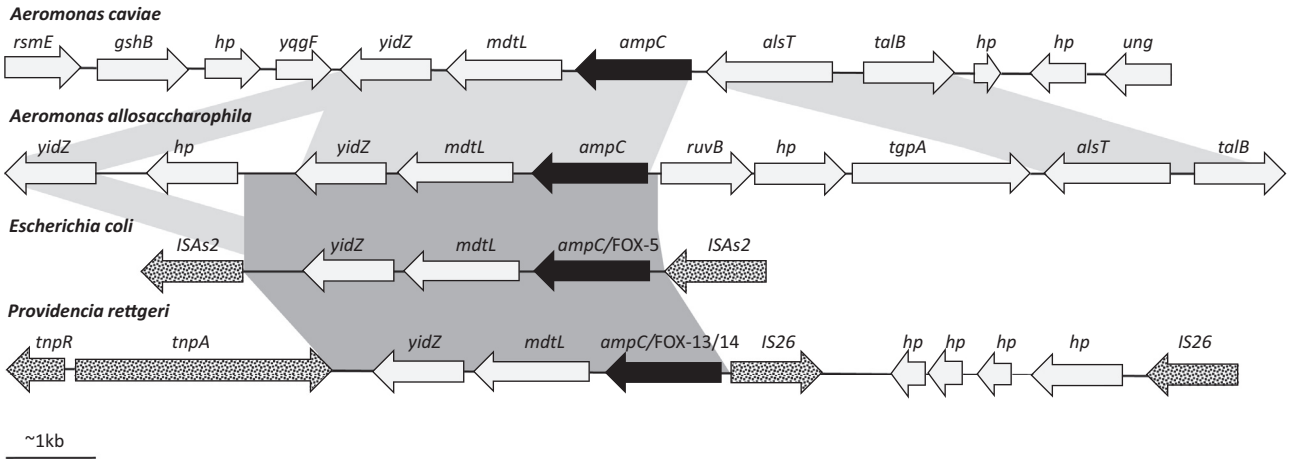

Fig. 1. Synteny comparison of the chromosomal *Aeromonas caviae*/*Aeromonas allosaccharophila ampC* loci and mobile FOX-type loci from *Providencia rettgeri* and *Escherichia coli*.
Dotted lines indicate transposases or ISs, the *ampC* gene is shown in black. Aligning regions are highlighted in grey, based on nucleotide identity (light grey: 75–85%, dark grey: 95–98%).

the MGEs. The alignments included (dependent on length) the genes *ampC-mdtL*Δ or *ampC-mdtL-yidZ* and the intergenic regions. Corresponding regions in *A. salmonicida* were 85–87% identical to the FOX-positive MGEs, and the same regions in *A. caviae* and *A. media* where 78–83% identical. A phylogenetic tree of the clustered FOX-like genes showed two monophyletic branches containing both chromosomal *Aeromonas ampC* genes and mobile FOX-like genes from Enterobacteriaceae, whereas the *ampC* genes of the other Aeromonads formed separate branches (Fig. 2). The distinct *Aeromonas* spp. formed well distinguishable branches, with the exception of the *A. media* branch, which contained two isolates with an average global nucleotide identity < 97% to the other *A. media* isolates. An *A. hydrophila* isolate (ZJ66-1) on the *A. caviae* branch was shown to be a misclassified *A. caviae* isolate (gANI towards *A. caviae* PRJEB7046, ASM329485v1, ASM78377v1 > 97%, gANI towards *A. hydrophila* ASM96364v1, ASM105711v1, ASM175634v1 < 90%). A similarity search showed that mobile FOX-like genes closely related to the *A. allosaccharophila ampC* were > 97% identical to FOX-5 and FOX-8. In contrast, mobile FOX-like genes from Enterobacteriaceae (*Pseudomonas* spp. isolates in Fig. 2) more closely related to the *A. caviae ampC* were ca. 79% identical to FOX-5/FOX-8. Instead, these genes were 98–99% identical to mobile CMY-1/MOX β-lactamases (Fig. 2).

CAV-1 shows signs of mobility in A. caviae *CIP74.23 and is different from the* ampC *of that species*

The 1861 bp DNA-fragment encoding CAV-1 reported by Fosse et al. in 2003 was found to encode three partially truncated ORFs, the first one being 96% identical to the *A. allosaccharophila* (PRJEB7019) *mdtL* gene (bp 1-176) over 176 bp, whereas the identity to the *A. caviae* (e.g. ASM326542v1, ASM173020v1, ASM20882v1) *mdtL* gene was 85% over 87 bp. The second ORF was 99% identical to *ISApu2* (bp 177–639), an insertion sequence originally described from an *A. caviae* plasmid. The third ORF (bp 640–1821) encoded the *ampC*, which was 95–97% identical to the chromosomal *ampC* of *A. allosaccharophila*, but 75–78% identical to the *ampC* of *A. caviae*.

Discussion

Large-scale screening of bacterial genomes allowed identification and comparison of a multitude of FOX-like genes in different genetic environments, both mobile and non-mobile, and study of their evolutionary relationship, including transfer events across species. Based on phylogenetic analysis, conserved synteny and nucleotide identities between FOX-carrying MGEs from Enterobacteriaceae and the *A. allosaccharophila ampC*-locus ≥ 97%, the chromosomal *ampC*-locus of *A. allosaccharophila* was identified as the origin of mobile FOX cephalosporinases. The very high sequence identities between the mobile and chromosomal FOX-loci suggest that the mobilisation of the *A. allosaccharophila ampC* to plasmids is an evolutionary recent event, likely promoted by human use of antibiotics. In silico approaches based on synteny and nucleotide identities are well suited to identify such 'recent origins', as often several thousand base pairs of the ARGs mobile and native locus are nearly identical, as presented here. However, when investigating the ancient (evolutionary) origins of an ARG, methods such as the search for ARG homologues using fragmented protein data [13] in combination with phylogenetic analysis may prove useful.

The current study furthermore showed that CAV-1, previously assumed to be derived from a chromosomal *A. caviae ampC,* is highly similar to mobile FOX variants, but comparably dissimilar to all analysed chromosomal *A. caviae ampC* genes. The identification of a truncated *ISApu2* next to the CAV-1 *ampC* further strengthens the assumption that the CAV-1 sequence might represent a mobile sequence in *A. caviae* CIP 74.32, and not, as reported by Fosse et al. in 2003, the chromosomal *ampC* gene. Alternatively, *A. caviae* CIP 74.32 could have been misclassified.

Although mostly associated with aquatic milieus or fish disease [14,15], isolates of *A. allosaccharophila* have been obtained from a multitude of environments such as human faeces or pig carcasses [16]. The species was classified as clinically non-significant in 2010 [17], based on its rare association with human disease, in contrast with several other *Aeromonas* spp.

It is tempting to hypothesise about the mobilisation of the *A. allosaccharophila ampC* in aquatic environments, such as aquaculture settings, were antibiotics are used. Although other classes of antibiotics dominate, β-lactams are regularly used in aquaculture of different species [18,19]. As a fish pathogen, *A. allosaccharophila* may be subjected to antibiotic selection pressure in such an environment, which is likely critical for maintenance and initial dissemination of a newly mobilised resistance gene. Cluster analysis showed that all mobile FOX-variants analysed in this study were > 94% similar in amino acid identity to each other, and more similar to the chromosomal *A. allosaccharophila ampC* gene than to the *ampC* of any other species. This strongly supports that all to date reported FOX-variants originated in *A. allosaccharophila*. Whether

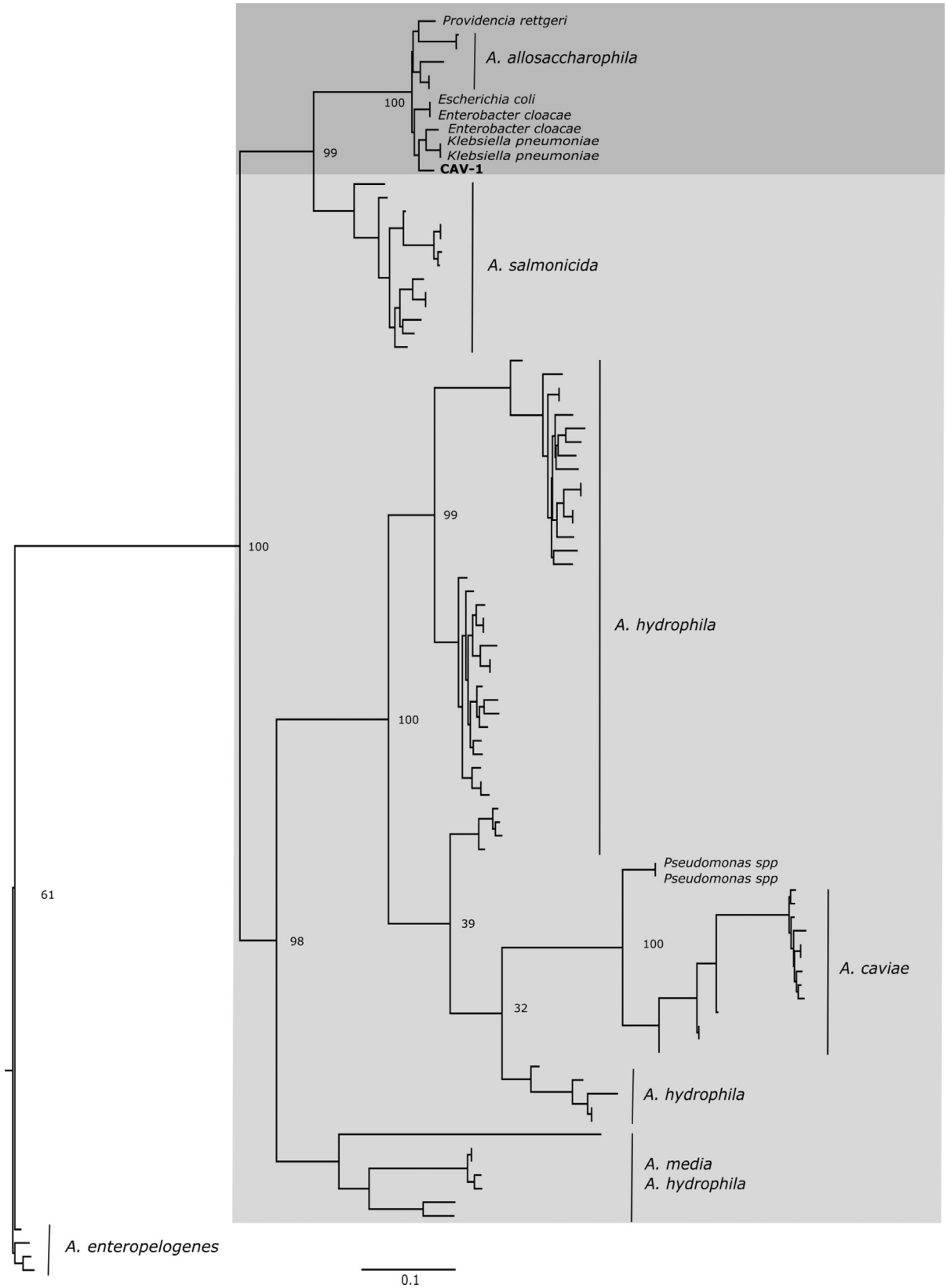

Fig. 2. Phylogenetic analysis with bootstrap support (for major branches) of clustered FOX-like genes.
Nucleotide identities towards mobile FOX-variants are indicated by background shade (light grey 75–88%, dark grey 95–100%).
Aeromonas (*A.*) sequences were derived from the chromosomal *ampC* gene.

the diversity between FOX-variants is a result of post-mobilisation evolution or if it reflects separate mobilisation events from different *A. allosaccharophila* strains remains unknown. The association of mobile FOX genes with different IS, such as ISAs2 and IS26, may indicate several mobilisation events mediated by different mobile elements but further research is needed to investigate the detailed mobilisation history of these genes.

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Ethical Approval: Not required.

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