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Fishy taint in chicken eggs is associated with a substitution within a conserved motif of the *FMO3* gene[B]

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Abstract

Fishy odor of urine and other secretions is a characteristic of trimethylaminuria in humans, resulting from loss-of-function mutations in the flavin-containing mono-oxygenase isoform *FMO3*. A similar phenotype exists in cattle, in which a nonsense mutation in the bovine orthologue causes fishy off-flavor in cow's milk. The fishy odor is caused by an elevated level of excreted odorous trimethylamine (TMA), due to deficient oxidation of TMA. We report the mapping of a similar disorder (fishy taint of eggs) and the chicken *FMO3* gene to chicken chromosome 8. The only nonsynonymous mutation identified in the chicken *FMO3* gene (T329S) changes an evolutionarily highly conserved amino acid and is associated with elevated levels of TMA and fishy taint in the egg yolk in several chicken lines. No differences in the expression of *FMO3* were found among individuals with different associated genotypes, indicating that the trait is not caused by a linked polymorphism causing altered expression of the gene. The results support the importance and function of the evolutionarily conserved motif FATGY, which has been speculated to be a substrate recognition pocket of N-hydroxylating siderophore enzymes and flavin-containing mono-oxygenases.

Keywords: Chicken; *FMO3* gene; Mutation; Trimethylaminuria; Linkage mapping

A fishy or crabby taint occurs occasionally in freshly laid chicken eggs. Especially brown layer lines like Rhode Island Red (RIR) are affected [1]. It was initially suggested that the tainting problem would be linked to shell color; however, it has been shown that this is not the fact [1,2]. Bolton et al. [1] deduced that the causative factor would be an autosomal semidominant mutant gene with variable expression. Hobson–Frohock et al. [3] demonstrated that fishy taint in eggs was a consequence of at least 10-fold concentration of trimethylamine (1.0 μg/g vs 0.1 μg/g), mostly in the egg yolk. It was hypothesized quite early that the trait is caused by mutation of a locus leading to a reduced capacity of trimethylamine (TMA) oxidation [4,5]. Pearson and Butler [6] showed that the "capacity for synthesizing TMA oxidase is strongly inherited" with no effect of gender.

In humans a similar autosomal recessive disorder is called trimethylaminuria (OMIM 602079). It is characterized by an odor (in breath, urine, sweat, or saliva) reminiscent of rotting fish, caused by defective N-oxidation of diet-derived TMA, leading to abnormal excreted amount of odorous trimethylamine. Therefore the disorder is sometimes referred to as the fish-like odor syndrome. Among others, choline, lecithin, and carnitine are known to be dietary precursors of TMA. Normally, the microsomal liver enzyme flavin-containing mono-oxygenase3 (FMO3) catalyzes oxidation of TMA to the nonodorous trimethylamine *N*-oxide. Trimethylaminuria has been shown to be due to mutations in the *FMO3* gene [7–

[☆] Sequence data from this article have been deposited with the GenBank Data Library under Accession Nos. AY239749–AY239753.

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11]. For an extensive review of the biochemical and clinical aspects of trimethylaminuria, see [12]. Recently, a similar disorder was described in cattle, in which a fishy off-flavor in cow's milk is caused by a nonsense mutation in the bovine *FMO3* gene [13,14].

We describe in this paper the mapping of the egg taint trait and the *FMO3* gene to chicken chromosome 8 and the variation in the chicken *FMO3* gene coding sequence associated with TMA content of egg yolk. Particularly, the only nonsynonymous polymorphism in the chicken *FMO3* gene associated with taint changes an evolutionarily highly conserved amino acid within the FMO-characteristic motif (FATGY). No differences in the expression of the gene were found among individuals with different genotypes for the polymorphism associated with taint, indicating that tainting is not caused by any linked polymorphism causing altered expression of the gene. The finding supports the suggested importance of the motif in the function of flavin-containing mono-oxygenases.

Results

Linkage mapping of the trait

We carried out a genome-wide search in five experimental half-sib progenies segregating for the egg taint trait. We pooled 20 individuals from both phenotypic extremes within each family and estimated the sire allele frequency distribution in the two pools (i.e., tainting vs nontainting) for 119 microsatellite loci covering 25 linkage groups. Four linkage groups, GGA4, GGA7, GGA8, and E46C08W18, showed skewed allele distribution among sire pools for one or more markers. Each region was genotyped with more markers. Only one region, GGA8, remained as a candidate site, giving consistent results within families for adjacent markers. We continued by genotyping individuals of the five segregating families for four microsatellite loci (MCW275, ADL322, LEI179, MCW305) at the distal end of chromosome 8.

Based on the hypothesis that the symptoms in poultry parallel those of human and cattle fish-odor syndromes and originate from a similar genetic disorder, we determined the linkage map position of chicken *FMO3* gene. To this aim, sequence variation was searched for in the chicken *FMO3* gene using published partial chicken *FMO3* cDNA sequences (gi: 13773452, gi: 14473472). The exon–intron boundaries were deduced by comparison to the published human genomic *FMO3* sequence (gi: 1209696). Primers were designed to sequence the introns between exon 5 and exon 6 (81 bp) and between exon 6 and exon 7 (110 bp). By sequencing several individuals of the mapping population, six polymorphic sites were identified in that area. By using one of these as a marker (SNP 13) in linkage analysis, it was shown that *FMO3* in chicken maps 5 cM (Kosambi, sex-averaged) distal to the end (MCW275) of our microsatellite linkage map of GGA8. The map position is compatible with the position of the human gene in the HSA 1q23–q25 region, based on comparative mapping information (http://www.zod.wau.nl/vf/), and it was later confirmed when the preliminary chicken genome sequence was published in March 2004 (http://www.ensembl.org/Gallus_gallus/).

Thereafter we analyzed the linkage of egg-tainting disorder to this chromosomal region assuming that the trait is inherited as an autosomal recessive. Two-point linkage analysis against the genotyped markers on the distal GGA8 gave the highest probability (lod score 37.62) of linkage to the marker *FMO3* (SNP 7) with the recombination fraction 0.0 (Table 1). The best multipoint location score for the trait against the fixed linkage map of the markers was close to the *FMO3* gene.

FMO3 polymorphisms in chicken

We determined the sequence of the chicken *FMO3* gene from genomic DNA of several individuals. The sequence information produced comprises the exon–intron structure of the chicken gene, the sequence of the entire coding sequence, and partial intron sequence (Fig. 1). The exon–intron structure is similar to that of the human gene [15], except for considerable variation in intron length. The entire coding sequence of the chicken *FMO3* gene was sequenced in both directions from at least two tainting and two nontainting individuals of the half-sib mapping population. Altogether 17 polymorphisms were detected, 9 of which occur in the coding sequence (Table 2A). Only one of the coding sequence polymorphisms leads to an amino acid change. It is due to a single base change (A to T) at position nt 1034 in the published cDNA sequence (gi: 18873598), leading to the codon change ACT to TCT and the corresponding T to S amino acid change at position aa 329 in the published amino acid sequence (GenBankAJ431390.1). The amino acid change resides within an evolutionarily conserved region containing the FMO-characteristic [16] pentapeptide motif FATGY (Fig. 2).

In addition, a part of the gene ranging from exon 5 to intron 8 was sequenced from a total of 48 individuals with known tainting status from three different brown layer populations and 22 additional individuals from the half-sib mapping population. Four additional polymorphisms (synonymous third position changes in exons 5, 6, and 7) were identified from the new populations (Table 2B). Among the 70

Table 1
Two-point lod scores for linkage between egg-taint disorder and markers on GGA8 (MLINK)

Locus	LOD	θ
FMO3	37.62	0.0
MCW275	5.95	0.05
ADL322	17.36	0.05
LEI179	8.67	0.1
MCW305	7.19	0.2

The highest lod score for each pair-wise combination at the most probable recombination fraction θ is shown.

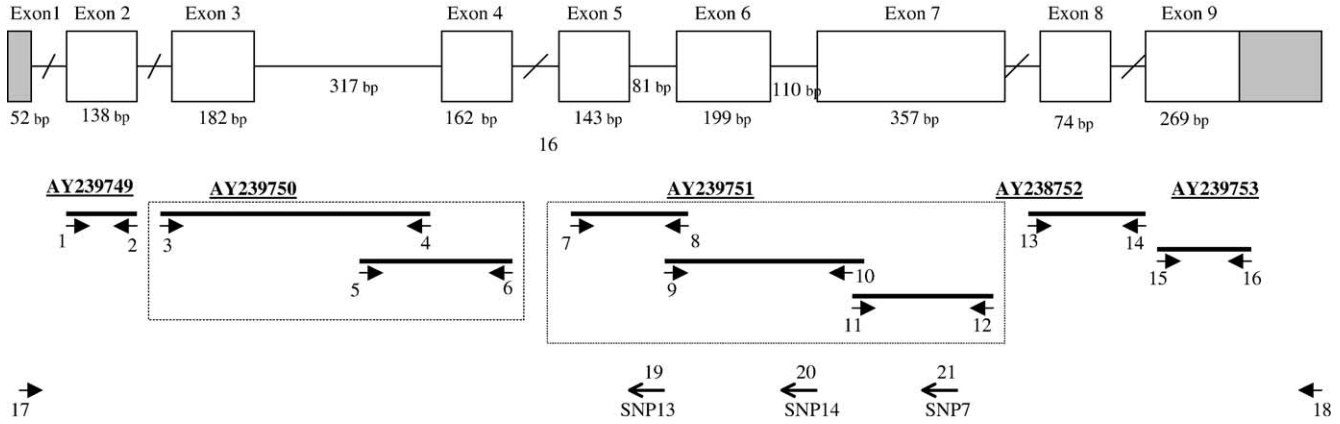

Fig. 1. A schematic presentation of the chicken *FMO3* gene. The exons are shown as open boxes and labeled by exon number following the numbering of human *FMO3* exons. Lines between the boxes indicate intron sequences: the line is interrupted when all of the intron was not sequenced and the length of the intron is undetermined. The primers used for amplification and sequencing are indicated by numbered arrows (numbers refer to Table 6). All sequence information is included in GenBank Accession Nos. AY239749–AY239753.

sequenced individuals, all 30 confirmed tainters showed identical homozygous haplotype for the 16 variant positions included. Five of the variant positions (1 in intron 5, 1 in exon 6, 2 in exon 7, including the T329S mutation, and 1 in intron 8) were fully associated with taint in the sense that no homozygous individuals for these positions were seen among the 40 nontainters. The result suggests a common origin for the tainting allele in present-day brown egg-layers, probably originating from their RIR background.

The occurrence of the T329S mutation in other chicken breeds was analyzed by SNP genotyping (Table 3). The results show that the mutation occurs in a wide variety of breeds. When the individuals homozygous for the mutation in the phylogenetically more distant breeds were sequenced from exon 5 to exon 7, different haplotypes were found, showing breakage of the linkage disequilibrium at the first polymorphic site upstream of T329S. The tainting status of these individuals remains unknown.

Table 2A
Polymorphic sites found in the chicken *FMO3* gene by sequencing from 4 to 10 individuals of the half-sib mapping population

SNP	GenBank accession No./ nucleotide position	Location	Wild-type chromosome	"Tainting" chromosome
1	AY239749/nt 90	Exon 2	C	T
2	AY239750/nt 86	Exon 3	C	T
3	AY239751/nt 98	Exon 5	T or C	C
4	AY239751/nt 101	Exon 5	T or C	C
5	AY239751/nt 249	Exon 6	C	T
6	AY239751/nt 615	Exon 7	C	T
7[a]	AY239752/nt 694	Exon 7	A	T
8	AY239753/nt 60	Exon 9	T	C
9	AY239753/nt 150	Exon 9	T	C
10	AY239750/nt 689	Intron 4	G	A
11	AY239751/nt 184	Intron 5	A or G	G
12	AY239751/nt 189	Intron 5	A or G	G
13	AY239751/nt 191	Intron 5	G	C
14	AY239751/nt 486	Intron 6	G or A	A
15	AY239751/nt 510	Intron 6	G or C	C
16	AY239752/nt 85	Intron 8	T	C
17	AY239752/nt 277	Intron 8	C	T

[a] The SNP for the T329S mutation.

Association of TMA content with FMO3 polymorphism

TMA phenotypes and the T329S substitution (SNP 7: A to T transition at nt 1034 of gi: 18873598 or nt 694 of AY239752) genotypes were scored in an independent F_2 population (Germany). Egg yolk TMA-N concentrations were measured by colorimetric analysis of TMA-N–picrate (see Methods). The phenotypes scored after dietary challenge with choline were classified into two groups, high and low, according to the TMA-N content in egg yolk. Hens in the low group had less than 2.1 μg TMA-N/g egg yolk, while high-group hens had more than 5.6 μg TMA-N/g egg yolk. All hens in the study produced eggs with low TMA content (average 0.9 μg TMA-N/g) before the dietary challenge. Genotyping SNP 7 (A/T) from 168 F_2 hens revealed that all TT homozygotes belonged to the high-TMA group, while heterozygotes AT and homozygotes AA expressed low TMA-N content in egg yolk (Table 4).

A χ^2 test revealed significant deviation of the observed genotype frequencies from the expected genotype frequencies among the two phenotype groups ($p < 0.0001$). The result showed that there was a highly significant association between the TMA content of the egg yolk and the DNA polymorphism leading to the T329S amino acid substitution.

Expression of FMO3

To analyze the possibility that the trait could be caused by aberrant splicing or transcription levels of *FMO3*, the expression of *FMO3* was analyzed in liver samples representing the different genotypes of T329S shown to be fully associated with the taint. No length variation was detected in the coding region cDNA among the different genotypes (Fig. 3), which eliminates splicing variation as a plausible cause of dysfunctioning of the enzyme. Analysis of the relative expression

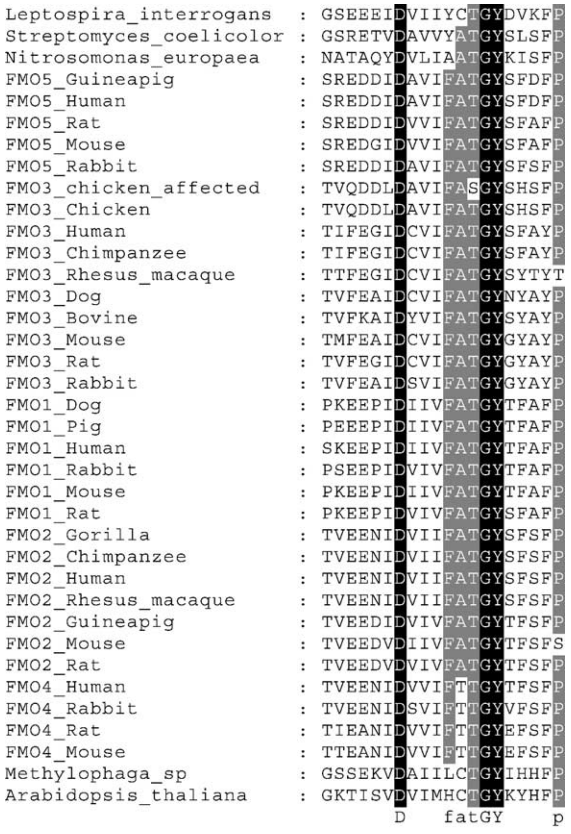

Fig. 2. Multiple alignment of protein sequences related to the chicken *FMO3* gene for the region of the T329S mutation. The following 36 amino acid sequences were used (Swiss-Prot/TrEMBL): tr|Q72LZ7| mono-oxygenase *Leptospira interrogans;* tr|Q9S204| *FMO Streptomyces coelicolor;* tr|Q82SV0| *FMO Nitrosomonas europaea;* sp|P36366| *FMO2,* sp|P49109| *FMO5 Cavia porcellus* (domestic guinea pig); sp|Q01740| *FMO1*, tr|Q86U73| *FMO2,* sp|P31513| *FMO3,* sp|P31512| *FMO4,* sp|P49326| *FMO5 Homo sapiens;* tr|Q8K4B8| *FMO2,* tr|Q8K4B7| *FMO4;* sp|P36365| *FMO1,* tr|Q9EQ76| *FMO3,* tr|Q8K4C0| *FMO5 Rattus norvegicus* (rat); sp|P50285| *FMO1,* tr|Q8K2I3| *FMO2,* sp|P97501| *FMO3,* tr|Q8VHG0| *FMO4,* tr|Q8R1W6| *FMO5 Mus musculus* (mouse); sp|P17636| *FMO1,* sp|P32417| *FMO3,* sp|P36367| *FMO4,* sp|Q04799| *FMO5 Oryctolagus cuniculus* (rabbit); tr|Q8QH01| *FMO3 Gallus gallus* (chicken); sp|Q8HZ69| *FMO2 Gorilla gorilla gorilla* (lowland gorilla); sp|Q28505| *FMO2,* sp|Q8SPQ7| *FMO3 Macaca mulatta* (rhesus monkey); sp|Q95LA2| *FMO1,* sp|Q95LA1| *FMO3 Canis familiaris* (dog); tr|Q8HYJ9| *FMO3 Bos taurus* (cattle); sp|P16549| *FMO1 Sus scrofa* (pig); sp|Q8HZ70| *FMO2,* sp|Q7YS44| *FMO3 Pan troglodytes* (chimpanzee); tr|Q83XK4| *Methylophaga sp.;* tr|Q8LFQ9| *Arabidopsis thaliana.* The alignment shows the evolutionary conservation of the FATGY motif in mammalian FMO isoforms. The chicken sequence containing the mutation T329S is indicated as chicken_affected.

levels of *FMO3* exon 7 showed no significant differences between the genotype groups (Table 5), indicating that tainting is not caused by any mutation affecting the transcript levels of *FMO3*.

Discussion

We have shown that both the egg-taint disorder and the chicken *FMO3* gene map to the same location on chicken chromosome 8 and have found an amino acid change in the *FMO3* gene associated with elevated TMA levels in the egg yolk. The identified amino acid change resides within an evolutionarily highly conserved region containing the FMO-characteristic pentapeptide motif FATGY in mammals [17], plants [18], and rhizobacteria [19]. Moreover, the dipeptide TG within this motif is conserved in related proteins since archaeal divergence. The specific function of the FATGY element is still unknown. It has been hypothesized that it may provide a hydrophobic pocket within a C-terminal substrate binding site of N-hydroxylating enzymes, such as amino acid hydroxylases in micro-organisms and flavin-containing dimethylaniline mono-oxygenases, including isozymes 1 to 5 of FMO [16,19]. Both the evolutionary conservation of the amino acid and the postulated function of the motif strongly suggest that the amino acid change itself may lead to a dysfunction in the chicken *FMO3* gene, thus leading to accumulation of TMA in the egg yolk in individuals homozygous for the mutation.

Three other commercial brown egg-layer stocks with different genetic backgrounds were tested for the association. The same A to T mutation was found to be fully associated with elevated TMA levels, TT homozygotes showing 10-fold higher TMA-N content in the egg yolk (see Supplementary material). No other mutations leading to amino acid changes

Table 2B
Additional polymorphic sites found by sequencing 48 individuals from three commercial brown egg-layer populations for a region ranging from exon 5 to intron 8

SNP	GenBank accession No./ nucleotide position	Location	Wild-type chromosome	"Tainting" chromosome
18	AY239751/nt 86	Exon 5	T or C	T
19	AY239751/nt 237	Exon 6	T or C	C
20	AY239751/nt 396	Exon 6	T or C	C
21	AY239751/nt 555	Exon 7	G or A[a]	G

[a] In only one individual.

Table 3
Frequency of the amino acid variants (T329S) in different chicken populations

Population	*N*	Allele frequency	
		T	S
Gallus gallus gallus	10	1.00	0.00
Fayoumi	10	1.00	0.00
Green-legged partridge	8	0.81	0.19
Icelandic landrace	10	1.00	0.00
ISA Brown	71	0.58	0.42
Lohmann Brown	67	0.62	0.38
Marans	111	0.96	0.04
TETRA	70	0.61	0.39
Transylvanian naked neck	8	0.69	0.31

ISA Brown, Lohmann Brown, and TETRA are commercial brown layer lines and Marans is an exotic chicken breed used for production in Europe. Samples of *G. gallus gallus* (red jungle fowl, feral), Icelandic landrace (native), and three standardized chicken breeds from Europe (Fayoumi, Green-legged partridge, and Transylvanian naked neck) were available through the EC-funded project AVIANDIV (http://w3.tzv.fal.de/aviandiv/index.html).

Table 4
Observed genotype frequencies of the A to T mutation leading to T329S amino acid substitution among groups of individuals expressing high or low TMA content in the egg yolk within the F_2 population (RIR × WL)

TMA content (μg TMA-N/g egg yolk)	Genotype			Σ
	AA	AT	TT	
High (>5.6)	0	0	54	54
Low (<2.1)	42	72	0	114

There is a significant difference between the observed and expected genotype frequencies (χ^2 test, $p < 0.0001$).

were observed. An identical haplotype was found for a studied stretch of the gene ranging from exon 5 to exon 7 in all known tainters included in the study, indicating a common origin for the mutation in current brown layer stocks. When a few individuals from breeds with more distant phylogenetic origin were sequenced for the same region, the common haplotype associated with T at nucleotide position 1034 in the cDNA was broken at the preceding variant site at nucleotide position 955 of the cDNA. The downstream breakpoint was not determined.

The presence of the T3295 variant in genetically divergent breeds suggests an old origin for the mutation. The reason for the high frequency of a probable loss-of-function mutation in certain chicken breeds remains unclear. No association with production traits has been observed (R. Preisinger, unpublished).

In humans, 12 missense mutations, 3 nonsense mutations, and 1 deletion of *FMO3* are currently known to cause TMAuria [11,12]. In the human *FMO3* mutation database (http://human-fmo3.biochem.ucl.acuk/Human_FMO3) a majority of the known TMAuria-causing mutations reside in exon 7, but none of these within the FATGY motif. In cattle, the causative mutation is a nonsense mutation in exon 6 of *FMO3* causing premature termination of the protein.

FMO3 has an important role in drug metabolism, and all occurring variants may have implications for the efficacy of drug treatment. Our results emphasize the importance of the FATGY motif for the proper function of the enzyme and provide a source for testing the enzymatic activity of the FASGY variant.

In chicken, the disorder is different from that in humans in the sense that the causative mutation is common in some egg-layer lines, but the tainting is observed only under certain conditions. The first "outbreak" of tainting was observed in the early 1970s when rapeseed was added to chicken feed. It seems that the FASGY variant is capable of oxidizing TMA under certain feeding regimes, but various factors can cause the enzymatic mechanism of the hen to be overloaded. Feed can affect the trait by providing increased dietary TMA, precursors for TMA formation by intestinal microbes or inhibitors of the oxidase. Known precursors in chicken are, e.g., TMA oxide, betaine, choline, and sinapine. Polyphenolic tannins and goitrins have been described as inhibitors of TMA oxidation. The intestinal microflora have also been shown to play an important role in tainting [20].

Organoleptic detection of tainting eggs (or chicken breath) has been used in breeding programs to reduce the occurrence of the trait. However, as mentioned, to be effective the method requires specific feeding challenges and is problematic because it is dependent on the observer as well as environmental factors. We present here a method to identify carriers and possible tainters by direct testing of *FMO3* genotype irrespective of the age, environmental factors, or gender of the tested individuals. This will help in eradicating the genetic factor behind egg taint from chicken breeding stocks.

Methods

Mapping populations

A half-sib mapping population was created at MTT (Finland) by an optimized mating plan. Two lines from the commercial Lohmann Brown breeding program (A and D) were crossed reciprocally as parental lines. In the F_1 generation, AD animals (from mating of line A males to line D females) included 122 males and 590 females, and the

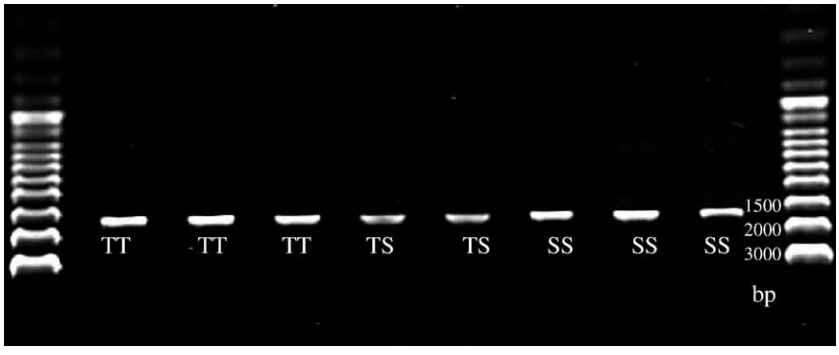

Fig. 3. The *FMO3* transcript is of similar length in liver of animals with different genotypes of the T329S mutation associated with tainting eggs (TT, TS, SS). Total RNA was reverse transcribed and the cDNA amplified with primers from exon 1 and exon 9. The products are visualized on ethidium–bromide-stained agarose gel. Quantitative expression levels that were measured for exon 7 by relative quantitative PCR (Table 5) showed no significant differences between the genotypes.

Table 5
Relative quantitation of *FMO3* expression in different genotypes for the T329S mutation

Genotype	*FMO3* average C_T	β-*actin* average C_T	ΔC_T *FMO3* − β-*actin*[a]	$\Delta\Delta C_T$ $\Delta C_T - \Delta C_{T\cdot TT}$	*FMO3* related to TT[b]
TT	22.96 ± 0.47	21.28 ± 0.54	1.68 ± 0.72	0 ± 0.72	1 (0.6–1.65)
TS	23.76 ± 0.33	22.52 ± 0.33	1.24 ± 0.46	−0.44 ± 0.46	1.36 (0.99–1.87)
SS	23.04 ± 0.56	21.46 ± 0.30	1.58 ± 0.63	−0.1 ± 0.63	1.07 (0.69–1.66)

No significant difference in expression level was found between genotype groups (F test, ANOVA, SAS Proc GLM, $p = 0.86$).
[a] The ΔC_T value is determined by subtracting the average β-*actin* C_T value from the average *FMO3* C_T value. The standard deviation of the difference is calculated from the standard deviations of the *FMO3* and β-*actin* values.
[b] The range (in parentheses) for each genotype class given for *FMO3* expression related to the genotype TT is determined by evaluating the expression $2^{-\Delta\Delta CT}$ with $\Delta\Delta C_T + s$ and $\Delta\Delta C_T - s$, where s is the standard deviation of the $\Delta\Delta C_T$ value.

reciprocal DA included 32 males and 163 females. The F_2 generation was generated from the most informative matings of F_1 individuals to maximize the power for linkage analysis. Twenty F_1 males were used (10 from each reciprocal line), each mated to 6 females (three different full sister pairs). The progeny from four hatches included from 90 to 120 female offspring in each half-sib family.

In an independent line cross design an F_2 population was produced at Cuxhaven (Germany) for confirmation and analysis of the association between TMA content in egg yolk, egg taint, and molecular markers. Twenty-one Rhode Island Red hens (known to produce tainted eggs by organoleptic scoring) were crossed in the parental generation with four White Leghorn cocks (a breed in which no tainted eggs have been observed). In the F_1 generation 9 males were mated to 45 females to produce an F_2 generation of 450 hens. From the F_2 hens, 84 tainters and 85 nontainters were selected by organoleptic scoring to be analyzed for TMA-N content in egg yolks after being fed a diet with added choline (6000 mg/kg feed).

Phenotypes

Organoleptic observation

Challenge tests were performed to get organoleptic estimates of egg taint in the Finnish half-sib mapping population. The F_2 hens were fed with a feed containing 10% rapeseed meal for 2 weeks at the age of 50–54 weeks. The taint was thereafter scored by olfactory tests for 3 consecutive days. Each egg was scored on the scale 1–5, according to the fishy odor, 1 being odorless and 2–5 expressing fishy odor with increasing intensity. For each individual hen, the scores of its eggs during the testing period were amalgamated to an average score. According to the results, 5 of the 20 half-sib families were found to segregate for egg taint after rapeseed challenge.

TMA-N content in egg yolk

Trimethylamine content in egg yolk was chemically determined from 169 hens from the German F_2 mapping population after two choline feeding challenge periods separated by a choline-free period (Reese et al., submitted for publication). Briefly, TMA was extracted from the egg yolk with 10% trichloroacetic acid (TCA) (15 ml TCA/15 g egg yolk). To separate the amine from its salts, 2 ml of extract was made alkaline by adding 1.5 ml 50% potassium hydroxide. Thereafter 0.5 ml 10% formaldehyde was added to fix other nitrogen-containing components. The free TMA was dissolved in 5 ml of toluene. An aliquot (2.5 ml) of the supernatant was mixed with an equal volume of 0.02% picric acid to form a yellow TMA-N–picrate complex, which was subsequently measured photometrically at 410 nm. Because the TMA-N–picrate complex is formed by the nitrogen in TMA, the TMA content is presented as TMA-N in the egg yolk.

Genome scan

For the initial genome scan (half-sib design) DNA pools were used to identify differences in sire allele frequencies between progeny groups sorted according to egg taint scores. Within each segregating half-sib (sire) family, two pools were formed, each containing 20 hens from the phenotypic extremes for egg taint. From each hen to be included in the pools 20 μl of whole blood was used. DNA was extracted from the pool with the same procedure as from individual samples [21]. Decision on which individuals were included in each pool was made according to their organoleptic taint values (average score per individual hen). The sire pools were genotyped for 119 microsatellite markers covering 25 chicken linkage groups. The sire allele frequencies in the two pools for each sire (tainting vs nontainting) were estimated directly from the height of peaks in chromatograms (ALF DNA Sequencer and Fragment Manager 1.1, Pharmacia, Sweden). A skewed allele distribution was taken as an indication of an association to be further analyzed.

Linkage map construction

Microsatellite markers MCW275, ADL322, LEI179, and MCW305 and two SNP markers within the *FMO3* gene (SNP 13 and SNP 7) were genotyped for all individuals in the five half-sib families segregating for taint. PCR primers and conditions for the microsatellite markers were as published (http://www.zod.wau.nl/vf/ and http://www.thearkdb.org/). SNP 13 (the G to C change within the intron between exons 5 and 6) and SNP 7 (the A to T change in exon 7) were detected using minisequencing (SnuPe Genotyping, Amersham Biosciences).

An amplified PCR product of the region (for SNP 13 the template was amplified with oligonucleotides 7 and 8, for SNP 7 with oligonucleotides 11 and 12; Table 6) was used as a template in a thermally cycled minisequencing reaction with dye-labeled terminators and sequence-specific primers ending at the polymorphic base (oligonucleotides 19 and 21; Table 6). Excess of deoxynucleotides and primers was removed with specific enzymatic treatment of the reaction product before minisequencing (ExoSapIT; Amersham Biosciences). Unincorporated dye-labeled terminators were subsequently removed by filtration (AutoSeq96 plates; Amersham Biosciences). Reaction products were detected on the capillary DNA electrophoresis instrument (MegaBACE; Amersham Biosciences). Analysis was based on allele signal intensity and relative allele mobility with MegaBACE SNP Profiler, version 1.0 (Molecular Dynamics 2001). The linkage map for the markers was constructed using the program package CRIMAP [22], options BUILD and FLIPS.

Linkage analysis of the tainting disorder

The linkage analysis of the trait was carried out with FASTLINK, version 4.1P [23,24] using the assumption of the disorder (tainting eggs after feeding challenge) being an autosomal recessive. All males and hens not scored for taint (dams) were coded unknown for their affection status. Hens with an average organoleptic score from 1 to 7 were classified as unaffected, and hens with the scores 9 to 15 were classified as affected (no hen had a score of 8). Options used for linkage analysis were MLINK for two-point analysis and ILINK for multipoint analysis.

Sequence analyses

We used information on the chicken cDNA and human genomic *FMO3* [15] to develop primers to amplify and sequence the entire *FMO3* coding sequence from chicken genomic DNA. Exonic primers were designed based on the complete chicken *FMO3* cDNA sequence (gi: 18873598) and the genomic sequence of the human *FMO3* gene (gi: 1209696), predicted either to flank exon–intron boundaries or to amplify exons assuming conservation of exon composition between human and chicken. Based on newly generated intron sequence we designed new primers to amplify and sequence the coding regions of chicken *FMO3* (Table 6). We sequenced the template fragments using the amplification primers as forward and reverse sequencing primers using standard procedures (DYEnamic ET Dyeterminator Kit and MegaBACE; Amersham Biosciences). The entire coding sequence of the chicken *FMO3* gene was sequenced from two tainting and two nontainting individuals. The sequences were analyzed with the Sequencher 3.1.1 analysis program (Gene Codes Corp., Ann Arbor, MI, USA). The multiple sequence alignment program ClustalW [25] was used for alignment of amino acid sequences to assess evolutionary conservation between FMO-related proteins originating from different organisms (Fig. 2).

RNA extraction

Liver samples (40 mg) were dissected immediately after decapitation from three hens of each genotype (TT, TS, SS) for the T329S mutation. The hens were taken at the age of 60 weeks from an F_5 generation of the half-sib mapping population used for linkage mapping of the trait. RNALater-

Table 6
The sequence information of oligonucleotides used for amplification and sequencing, including minisequencing primers

Analyzed DNA	Oligonucleotide	Direction	Sequence
AY239749	1	For	5′-ATGGTGCGACGCGTGGCTGT
AY239749	2	Rev	5′-TTCCGTGTAGCGCCAGAGCC
AY239750	3	For	5′-CAGAGGAAGGCAGAGCTAGC
AY239750	4	Rev	5′-GGGAGGTTTGGGTAGACATG
AY239750	5	For	5′-CGCTTCAAGACCACAGTCAC
AY239750	6	Rev	5′-GTAAAGTGCACACCTGGCTG
AY239751	7	For	5′-GGGATAGAGAAGTTTAAAGGTTG
AY239751	8	Rev	5′-ACACGGCTCATCACCCAGGA
AY239751	9	For	5′-TCCTGGGTGATGAGCCGTGT
AY239751	10	Rev	5′-GATGTTTCTCTGAATTCCTTCAC
AY239751	11	For	5′-GTGAAGGAATTCAGAGAAACA
AY239751	12	Rev	5′-CTGAAACACCTTGACTGCC
AY239752	13	For	5′-GGGCTCTGCACTCTCCCCTC
AY239752	14	Rev	5′-GATTTGCCCAATTCAACCAC
AY239753	15	For	5′-TGGTTCGGGACAAGCAACAC
AY239753	16	Rev	5′-TAGAGCACAGTGAGGAGGAG
cDNA	17	For	5’-CTCTCCTCCCACAGCTTCC
cDNA	18	Rev	5’-TGGAGAGGAGGAACCCTACC
Minisequencing			
SNP 13	19	Rev	5′-TGAGGCAGGGTTGAGCCACC
SNP 14	20	Rev	5′-CTGCTGGCCCTGTACAGGCT
SNP 7	21	Rev	5′-GGAAAGGAGTGAGAGTAACCAG

preserved tissue was disrupted by disposable grinders and homogenized by syringes and needles. Total RNA was isolated using the RNeasy Mini isolation kit (Qiagen) according to the manufacturer's protocol. The RNA preparations were treated with RNase-free DNase I and stored at −70°C.

Reverse transcription

Reverse transcription of total RNA was performed using the ImPromII Reverse Transcription System kit (Promega) according to the manufacturer's guidelines, with 1 μg of RNA in a total volume of 20 μl. The cDNA of the coding sequence of the gene was PCR amplified using oligonucleotides 17 and 18 (Table 6; annealing at 62°C) and analyzed by agarose gel electrophoresis.

Quantitative PCR

The *FMO3* expression in different T329S genotypes was analyzed by real-time quantitative PCR using a relative quantification method [26]. Gene expression levels of exon 7 were measured using the ABI Prism 7000 Sequence Detection System (Applied Biosystems) and ABsolute QPCR SYBR Green ROX Mix kit (ABgene). The sequences of the PCR primers used for RT quantification were β-actin (For, 5′-TGCGTGACATCAAGGAGAAGC; Rev, 5′-ATGCCAGGGTACATTGTGGT) and *FMO3* (oligonucleotides 11 and 12, Table 6). The PCR mixture (25 μl) contained 12.5 μl SYBR Green PCR mix (ABgene), 70 nM each primer, and about 100 ng cDNA template. The PCR protocol consisted of enzyme preactivation for 15 min at 95°C, denaturation at 95°C, annealing at 60°C, and extension at 72°C, total 40 cycles. Each sample was measured in triplicate. Melting curve analysis was used to check the specificity of amplified products. The expression of *FMO3* was normalized to the endogenous RNA control β-*actin*. For both genes, standard curves were obtained using serial twofold dilutions of cDNA. Data were analyzed according to the manufacturer's instructions (ABI Prism Sequence Detection System, User Bulletin 2).

Appendix A. Supplementary data

Supplementary data for this article may be found on ScienceDirect.

References

[1] W. Bolton, T.C. Carter, R. Morley Jones, The hen's egg: genetics of taints in eggs from hens fed on rapeseed meal, Br. Poult. Sci. 17 (1976) 313–320.
[2] E.J. Butler, A.W. Pearson, N.M. Greenwood, Trimethylamine taint in eggs: the occurrence of the causative metabolic defect in commercial hybrids and pure breeds in relation to shell colour, J. Sci. Food Agric. 35 (1984) 272–278.
[3] A. Hobson-Frohock, D.G. Land, N.M. Griffiths, R.F. Curtis, Egg taints: association with trimethylamine, Nature 243 (1973) 304–305.
[4] A.W. Pearson, et al., Rapeseed meal and egg taint: demonstration of the metabolic defect in male and female chicks, Vet. Rec. 7 (1979) 318–319.
[5] W. Pearson, et al., Effect of rapeseed meal on hepatic trimethylamine oxidase activity in the domestic fowl in relation to egg taint, J. Sci. Food Agric. 30 (1979) 291–298.
[6] A.W. Pearson, E.J. Butler, Effects of selective breeding and age on the ability of domestic fowl (*Gallus domesticus*) to oxidase trimethylamine, Comp. Biochem. Physiol. 76 (1983) 67–74.
[7] C.T. Dolphin, A. Janmohamed, R.L. Smith, E.A. Shephard, I.R. Phillips, Missense mutation in flavin-containing mono-oxygenase gene, FMO3, underlies fish-odour syndrome, Nat. Genet. 17 (1997) 491–494.
[8] J.R. Cashman, et al., Human flavin-containing monooxygenase form 3: cDNA expression of the enzymes containing amino acid substitutions observed in individuals with trimethylaminuria, Chem. Res. Toxicol. 10 (1997) 837–841.
[9] E.P. Treacy, et al., Mutations of the flavin-containing monooxygenase gene (*FMO3*) cause trimethylaminuria, a defect in detoxification, Hum. Mol. Genet. 7 (1998) 839–845.
[10] B.R. Akerman, et al., Trimethylaminuria is caused by mutations of the *FMO3* gene in a North American cohort, Mol. Genet. Metab. 68 (1999) 24–31.
[11] D. Hernandez, et al., Trimethylaminuria and a human FMO3 mutation database, Hum. Mutat. 22 (2003) 209–213.
[12] J.R. Cashman, et al., Biochemical and clinical aspects of the human flavin-containing monooxygenase form 3 (FMO3) related to trimethylaminuria, Curr. Drug Metab. 4 (2003) 151–170.
[13] A. Lundén, V. Gustafsson, M. Imhof, R. Gauch, J.-O. Bosset, High trimethylamine concentration in milk from cows on standard diets is expressed as fishy off-flavor, J. Dairy Res. 69 (2002) 383–390.
[14] A. Lundén, S. Marklund, V. Gustafsson, L. Andersson, A nonsense mutation in the *FMO3* gene underlies fishy off-flavor in cow's milk, Genome Res. 12 (2002) 1885–1888.
[15] C.T. Dolphin, J.H. Riley, R.L. Smith, E.A. Shephard, I.R. Phillips, Structural organization of the human flavin-containing monooxygenase 3 gene (*FMO3*), the favored candidate for fish-odor syndrome, determined directly from genomic DNA, Genomics 46 (1997) 260–267.
[16] M. Stehr, et al., A hydropholic sequence motif common to N-hydroxylating enzymes, Trends Biochem. Sci. 23 (1998) 56–57.
[17] V. Lattard, et al., Cloning, sequencing, tissue distribution and heterologous expression of rat flavin-containing monooxygenase 3, Arch. Biochem. Biophys. 391 (2001) 30–40.
[18] Y. Zhao, et al., A role for flavin-monooxygenase-like enzymes in auxin biosynthesis, Science 291 (2001) 306–309.
[19] C. Ambrosi, L. Leoni, L. Putignani, N. Orsi, P. Visca, Pseudobactin biogenesis in the plant growth-promoting *Rhizobacterium pseudomonas* strain B10: identification and functional analysis of the L-ornithine, J. Bacteriol. 182 (2000) 6233–6238.
[20] J. Zentek, Egg taint—A problem of practical importance, Lohmann Inf. 28 (2003) 1–4.
[21] M. Tuiskula-Haavisto, et al., Mapping of quantitative trait loci affecting quality and production traits in egg layers, Poultry Sci. 81 (2002) 919–927.
[22] P. Green, K. Falls, S. Crooks. CRI-MAP documentation, version 2.4. http://linkage.rockefeller.edu/soft/crimap/, 1990.
[23] R.W. Cottingham Jr., R.M. Idury, A.A. Schäffer, Faster sequential genetic linkage computations, Am. J. Hum. Genet. 53 (1993) 252–263.
[24] A.A. Schäffer, S. Gupta, K. Shriram, R.W. Cottingham Jr., Avoiding recomputation in linkage analysis, Hum. Hered. 44 (1994) 225–237.
[25] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice, Nucleic Acids Res. 22 (1994) 4673–4680.
[26] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, Methods 25 (2001) 402–408.