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Inbreeding of house dust mites, a tool for genomic studies and allergy-related applications

To the Editor,

Draft assemblies of the genomes of the house dust mites (HDM) *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, and *Euroglyphus maynei* have been recently published.^{1,2} The information on HDM genomics and proteomics that is being generated will be of great value for acarology studies and will contribute to better understand mite allergens, and to optimize allergy diagnosis and immunotherapy. However, the quality of genomic assemblies published so far has been challenged by the high level of heterozygosity of the sequenced HDM colonies,^{2,3} thus undermining our ability to finely interrogate the genomes and integrate transcriptomics and proteomics investigations. By contrast, a paradigmatic example of a high quality Acari genomic assembly and annotation is that of the red spider mite (640 scaffolds, N50 = 10 Mb, 1.3% gaps), for which sequencing was conducted on an iso-female line (namely, a line obtained from descendants of a single mother) that underwent eight generations of sib-mating (mating between siblings; SM) to maximize homozygosity⁴ (<http://bioinformatics.psb.ugent.be>). Here, we describe the generation of inbreeding lines of the diploid species *D. pteronyssinus* for use in genomic and proteomic studies, and of interest for allergy-related applications. Prior to this investigation, the tolerance of *D. pteronyssinus* to inbreeding was unknown. A first attempt to inbreed our laboratory colony (derived from a European commercial stock culture) by consecutive backcrossing of male offspring with their corresponding mothers failed to produce stable inbred lines. Up to four generations were achieved by this method, but all lines were finally lost due to low developmental rate. In a second attempt, a less intensive inbreeding scheme was performed starting from iso-female lines and performing brother-sister crosses at each generation. Following this sib-mating strategy, a number of

inbred lines were successfully selected by their optimal growth allowing a sequential escalation of the culture. The time between subcultures was systematically recorded for each line to assess culture performance (Appendix S1). Including the 11 starting couples from the stock colony, the inbreeding program comprised a total of 117 crosses. Approximately one-third of the crosses ($n = 38$) produced viable/stable lines of different inbreeding levels, reaching the 5th SM generation from one of the iso-female lines (see genealogical tree in Figure S1). Failure to propagate the remaining crosses ($n = 79$) may have been caused by problems of fecundity (laid eggs), fertility (hatched eggs), immature mortality, population collapse after adulthood, or eventually by the use of all individuals to conduct further SM crosses. Culture performance varied significantly between inbred lines. On average, a pattern of longer subculture time was observed as inbreeding progressed (Figure 1, Figure S2). Finally, our approach led to a number of stable lines of reasonable inbreeding level: F inbreeding coefficient of 0.469 and 0.484 for SM-4 and SM-5, respectively. The high degree of extinction and the delay in culture performance of most inbred lines suggest that the SM strategy induced *inbreeding depression* in *D. pteronyssinus*. Namely, a decline in fitness (eg, survival, fecundity, growth, etc.) of the offspring associated to deleterious recessive mutations emerging in homozygosis during the inbreeding program.⁵ However, differences within each SM generation could be remarkable, with some highly inbred lines showing performance close to the range of the F₀ generation (43–49 days) and the stock colony (approximately 42 days). Thus, genetic purging of deleterious mutations may have occurred in the selected progeny, while divergence in the remaining non-purged (non-lethal) mutations could explain differences among inbred lines (Figure 1).

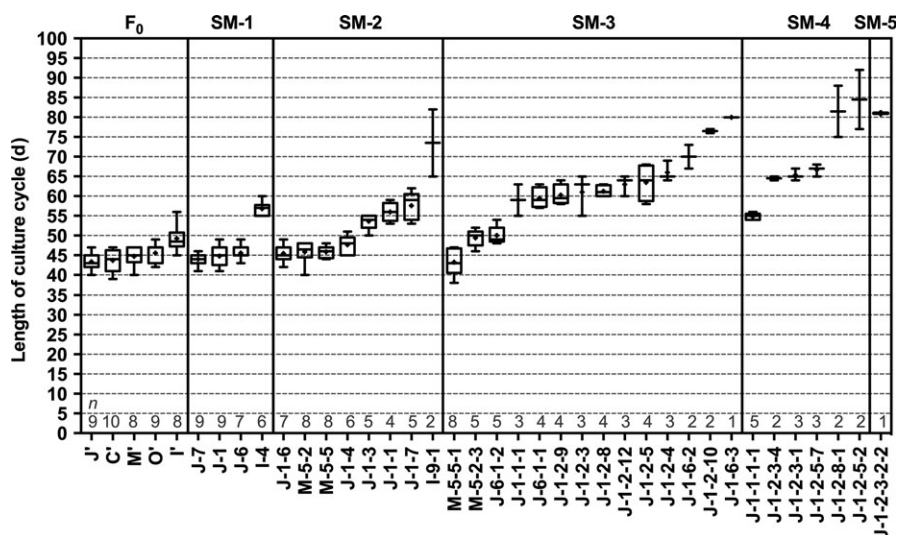


FIGURE 1 Culture performance of *Dermatophagoides pteronyssinus* iso-female lines and sib-mating inbred lines. Vertical axis indicates the average duration of a regular culture cycle (2 g; n denotes the number of cycles per mite line). Data are represented as min to max Box & Whisker plots; average is indicated as a cross. F₀, iso-female lines; SM-#, sib-mating inbred lines from consecutive generations

TABLE 1 Characterization of major isoallergen variants in selected *Dermatophagoides pteronyssinus* inbred lines

Der p 1 ^b			Der p 2 ^e			Der p 23 ^f										
Mite line ^a	Sib-mating generation	Isoallergen variants	Polymorphic substitutions ^d		Polymorphic substitutions ^d					Polymorphic substitutions ^d					Insertions	
			124	139	57	64	128	131	Isoallergen variants	34	39	44	49	31-32	37-38	
Stock colony		n.r. ^c	AV	A/S	n.r.	V>L	S>T	L>M	N>D	Der p 23.0101 ALA22869	H	E	K	S	IDVKTS	VT
C'	F ₀	Der p 1.0102	V	A	n.r.	V>L	S>T	L>M	N>D	Der p 23.0101 ALA22869	H	E	K	S	IDVKTS	VT
O'	F ₀	n.r.	V>A	A>S	n.r.	V	S>T	L>M	N>D	Der p 23.0101 ALA22868	H	E	K	S	IDVKTS	VT
I'	F ₀	n.r.	A>V	A/S	Der p 2.0109	V	S	L	N	Der p 23.0101 ALA22869	H	E	K	S	IDVKTS	VT
I-4	1	n.r.	A>V	A/S	Der p 2.0109	V	S	L	N	Der p 23.0101 ALA22869	H	E	K	S	IDVKTS	VT
I-9-1	2	Der p 1.0102 Der p 1.0105	A>V	A	Der p 2.0109	V	S	L	N	Der p 23.0101 ALA22868	H	E	K	S	IDVKTS	VT
M'	F ₀	n.r.	AV	A/S	n.r.	V	S>T	L>M	N>D	Der p 23.0101 ALA22869	H	E	K	S	IDVKTS	VT
M-5-2-3	3	Der p 1.0105	A	A	n.r.	V	S>T	L>M	N>D	Der p 23.0101 ALA22869	H	E	K	S	IDVKTS	VT
M-5-5-1	3	Der p 1.0102 Der p 1.0105	V>A	A	Der p 2.0109	V	S	L	N	Der p 23.0101 ALA22869	H	E	K	S	IDVKTS	VT
J'	F ₀	Der p 1.0102 Der p 1.0105	V>A	A	Der p 2.0101	V	T	M	D	Der p 23.0101	H	E	K	S	-	-
J-1	1	Der p 1.0102 Der p 1.0105	V>A	A	Der p 2.0101	V	T	M	D	Der p 23.0101	H	E	K	S	-	-
J-7	1	Der p 1.0102 Der p 1.0105	V/A	A	Der p 2.0101	V	T	M	D	Der p 23.0101	H	E	K	S	-	-
J-6-1-2	3	Der p 1.0102	V	A	Der p 2.0101	V	T	M	D	Der p 23.0101	H	E	K	S	-	-
J-1-1-1-1	4	Der p 1.0102	V	A	Der p 2.0101	V	T	M	D	Der p 23.0101	H	E	K	S	-	-
J-1-2-5-7	4	Der p 1.0102	V	A	Der p 2.0101	V	T	M	D	Der p 23.0101	H	E	K	S	-	-
J-1-2-3-2-2	5	Der p 1.0102	V	A	Der p 2.0101	V	T	M	D	Der p 23.0101	H	E	K	S	-	-

^aMite lines as in supplementary Figure S1.^bThe reference amino acid sequence is the Der p 1.0102 mature peptide (www.allergen.org).^cNot resolved, mixed variants.^dResidue positions in the reference sequence; ">" denotes higher proportion based on chromatogram intensity; "/" indicates similar proportion. Results for Der p 23 variant-specific primers are shown in two independent rows (">") denotes that sequencing with specific primers was negative).^eThe reference amino acid sequence is the Der p 2.0101 full peptide (www.allergen.org).^fThe reference amino acid sequence is the Der p 23.0101 full peptide (www.allergen.org).

As compiled in the WHO/IUIS Allergen Nomenclature database (www.allergen.org), HDM allergens may show great sequence variability. The segregation of alleles for the three major allergens Der p 1, Der p 2, and Der p 23 during the inbreeding process was followed by sequencing genomic DNA on the stock colony, five iso-female lines, and ten SM-inbred lines (Figure S1, Appendix S1). Our stock colony was polymorphic for the three major allergen genes, whereas fixation of certain alleles increased as inbreeding progressed (Table 1). Four of the characterized lines were homozygous for the three genes simultaneously, all coding for isoallergen variants Der p 1.0102, Der p 2.0101, and Der p 23.0101. Altogether, two Der p 1 variants were unequivocally identified, with at least an extra non-resolved variant bearing a newly described substitution (S-139); two Der p 2 variants, with at least an extra non-segregated variant; and three Der p 23 variants, canonical Der p 23.0101, and two variants not included in the WHO/IUIS database, but reported in GenBank, ALA22869 and ALA22868. Noteworthy, the variants detected in our study fairly match the combination of variants that, as for their immunogenic profile and prevalence in natural mite populations, have been proposed as suitable for recombinant allergen-based products.⁶ Der p 23 has recently been identified as a major and serodominant allergen, yet results on its IgE reactivity titers have been variable among studies and geographical locations.⁷ Interestingly, the two isoforms found in our lines contain insertions and polymorphic substitutions compared to the canonical variant, although, for their location at the N-terminal region of the protein, they are not expected to significantly affect IgE recognition⁸ (Table 1; Figures S3 and S4).

In this report, we show the limitations arising when an inbreeding program of *D. pteronyssinus* is undertaken, most probably associated to inbreeding depression. Still, we were able to obtain stable SM-inbred lines selected for their optimal culture that will contribute to generate a high quality genomic assembly of use in genomic and proteomic studies. Also, the fact that certain lines such as J-1-1-1-1 and M-5-5-1 (Figure 1) exhibited good performance in spite of the high level of inbreeding opens the possibility of using inbred lines for industrial/pharmacological interests. On the one hand, inbred lines subjected to genetic purging of deleterious recessive mutations could be used to refresh old stock cultures for improvement/maintenance of optimal growth, or for the creation of new hybrid lines to potentially maximize heterosis (ie, hybrid vigor). On the other hand, since variability on the allergen sequence is significant toward immunoreactivity to IgE, IgG, and monoclonal antibodies, as shown using recombinant allergens,⁹ inbred lines could be selected for a specific allergenic profile (eg, isoallergen variant of interest, native hypoallergenic variant, high allergen expression, etc.) to be applied as reference stock colonies for immunology research, or purification of unique natural (properly folded) allergens. Our inbreeding approach would provide a method to determine the importance of isoallergen variants in natural conditions. Thus, differences in immunoreactivity reported between recombinant Der p 2.0101 and Der p 2.0104-like variants⁸ could be confirmed using mite lines homozygous for Der p 2.0101 and Der p 2.0109, as those obtained in this study. Finally, mite lines obtained by our approach (used alone, or in combination

after intercrossing selected lines) could eventually be employed for commercial production, providing better standardization of the isoallergen variants contained in detection kits, diagnosis reagents, or immunotherapy vaccines.

CONFLICTS OF INTEREST

PHC has led research contracts between CSIC and ALK-Abelló. The rest of authors declare that they have no relevant conflict of interests.

Keywords

Dermatophagoides pteronyssinus, genomic assembly, inbreeding depression, isoallergen variants, sib-mating

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

DOI: 10.1111/all.13611

Breathing pattern disorder in difficult asthma: Characteristics and improvement in asthma control and quality of life after breathing re-training

To the Editor,

Breathing pattern disorder (BPD), or dysfunctional breathing, encompasses heterogeneous disorders characterized by abnormal breathing patterns resulting in symptoms that mimic asthma.¹ BPD affects 24%-29% of patients with asthma and 30%-64% of patients with difficult asthma.¹⁻⁴ Diagnosis is generally based on Nijmegen score, a questionnaire that detects hyperventilation, just one BPD form.^{1,4,5} Difficult asthma is distinct from severe asthma,⁶ and patient factors including comorbidities such as BPD are thought to be particularly important in patients with difficult asthma.

In primary care, breathing retraining for patients with asthma improves quality of life (QOL).⁷ Patients with difficult asthma present a much greater health burden, and BPD is independently associated with worse asthma control and QOL; therefore, treatment may deliver a greater benefit.⁸

This study, in patients with difficult asthma, examines BPD characteristics and reports outcomes following breathing retraining.

We undertake systematic evaluation for difficult asthma, where patients with suspected BPD undergo a multidimensional physiotherapist assessment. Ethics approval was obtained (HREC 185/15); requirement for informed consent was waived.

Nijmegen, Asthma Control Test (ACT), and Asthma QOL Questionnaire (AQLQ) were administered at baseline and 6 months. Asthma exacerbations were defined as ≥ 3 days systemic corticosteroids. Inhaler technique and adherence were assessed and optimized.

Asthma comorbidities were sought; allergic rhinitis—symptoms and atopy; chronic rhinosinusitis—clinical symptoms with abnormal sinus CT or rhinological examination; anxiety and depression—physician diagnosis or elevated Hospital Anxiety and Depression score (HADS); and vocal cord dysfunction—paroxysmal vocal cord movement on nasoendoscopy.

Patients with suspected BPD were assessed by a specialized physiotherapist for mouth breathing, thoracic dominance, hyperventilation (elevated respiratory rate at rest), thoraco-abdominal asynchrony, accessory muscle use and musculoskeletal abnormalities. Breathing retraining was delivered over one or more sessions. A

general therapeutic approach comprised promotion of abdominal and nose breathing, postural training, relaxation and feedback to consciously decrease the respiratory rate tailored to individual BPD features detected at assessment.

Of 109 consecutive patients assessed between June 2015 and December 2017, 35 underwent BPD evaluation. Twenty-nine of 35 patients were diagnosed with BPD (patient characteristics Table 1). Twenty-five had documented variable airflow obstruction (Table 1); three had a clinical asthma diagnosis, and one had fixed airflow obstruction (never smoker).

The mean number (and standard deviation) of exacerbations in the prior 6 months was 3.1 ± 2.3 ; ACT score, 13.1 ± 4.3 ; AQLQ score, 3.9 ± 1.1 ; and reliever use over 7 days, 24 ± 43 actuations.

TABLE 1 Baseline demographic data

Age (mean $\pm$ standard deviation)	49 $\pm$ 12 years
Sex (% female)	59%
BMI (mean $\pm$ standard deviation)	31 $\pm$ 6.7 kg/m ²
FEV1% predicted (mean $\pm$ standard deviation)	71 $\pm$ 22
Variable airflow obstruction	25/29 (86%)
Bronchodilator reversibility	16/29 (55%)
Peak flow variability	6/29 (21%)
Bronchial hyperreactivity	3/29 (10%)
Years since asthma diagnosis (mean $\pm$ standard deviation)	30 $\pm$ 17.8
Serum eosinophils (mean $\pm$ standard deviation)	0.19 $\pm$ 0.16 $\times 10^9$ /L
Total IgE (mean $\pm$ standard deviation)	428 $\pm$ 997 kU/L
Smoking status (%)	
Never	69
Ex	24
Current	7
Employed	59%

Supporting information

Appendix S1. Methods

Mite culture & inbreeding

The mites used in this study came from a *D. pteronyssinus* laboratory culture kept in the lab for more than 5 years and originated from a commercial culture (1). The stock colony was reared as previously described (2) in 50 ml wide-mouth sterile Erlenmeyer flasks containing 2 g of a pre-heated (60°C for 6 h) 1:1 mixture of fish food flakes (Vipan Sera, Heinberg, Germany) and brewer's yeast flakes (Santiveri, Barcelona, Spain) covered with sterile hydrophilic cotton caps. Environmental conditions in all experiments were $24 \pm 0.3^\circ\text{C}$ and $76 \pm 2\%$ relative humidity (RH) in the dark. RH control was achieved by placing culture units into sealed boxes with a saturated NaCl solution. The inbreeding strategy consisted in sequential rounds of sibling mating (sib-mating) brother-sister. Briefly, the breeding program started from different couples of a virgin female (obtained from individually isolated nymphs) and a single male (randomly collected from the stock colony) reared on concave microscope slides containing a fish food flake of approximately 2 mg and confined using a cover slip that allowed gas exchange, as previously described (3). After approximately 2 weeks, adult couples were removed and, once the tritonymphal stage was reached, part of the progeny was individually isolated in new slides until adulthood. For each subsequent sib-mating generation, new couples of virgin females and brothers were paired. When possible, part of the progeny derived from each cross was maintained in the original slide to establish the corresponding inbred line. These cultures were sequentially upscaled: first, by letting mites consume the food in the slides, and transferring them to a ventilated 1.5 ml microtube containing 35 mg of fish food until culture saturation. The whole content of the microtube was then used to inoculate 1 g of 1:1 (fish food : brewer's yeast) standard medium into a 50 ml flask, maintained as described above. Once 1 g cultures were saturated, the regular rearing protocol of the stock colony was employed (2 g of fresh medium were sub-cultured using 150 mg of thriving culture). The culture saturation point was defined by stereoscope inspection as when population was thriving, medium flakes were fully disaggregated but still some unconsumed fragments could be seen. Thus, the sub-culture time was not fixed as for the stock culture (42 days) but it was rather dependent on the progress of each inbred line. The duration of all culture phases was systematically recorded. The genealogy of inbred lines was codified using a capital letter (denoting the original couple) followed by one number per sib-mating generation, and separated by hyphens (e.g. J-1-2-3-2-2, denoting a 5th sib-mating generation line; Fig. S1).

The level of inbreeding was estimated by the Wright's inbreeding coefficient (F) (4) based on a sib-mating brother-sister pedigree using the web application <http://www.insilicase.com/Web/WebC of I.aspx>. Note that the inbreeding coefficient of F_0 ancestors was estimated as being 0, although it could be higher considering they derived from a laboratory colony.

Sequencing Der p 1, Der p 2 and Der p 23 genes

D. pteronyssinus from iso-female lines and inbred lines ($n > 200$ mixed-stage mites) were isolated following the paper disk method (2). Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Mite manipulation was performed avoiding cross-contamination of different lines. Partial sequences of the major allergen genes were obtained by PCR, followed by purification (QIAquick PCR Purification Kit, Qiagen) and Sanger sequencing. The PCR master mix contained 2.5 μ l of PCR buffer II, 1.5 μ l $MgCl_2$ (25 mM), 2.5 μ l dNTPs mix (2 mM), 0.5 μ l of primers (10 μ M of each forward and reverse), 0.2 μ l of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Austin, USA) (5U/ μ l) and 5 μ l of DNA template (12.5 ng gDNA/ μ l), the total volume was made up to 25 μ l with sterile MilliQ water. The thermocycler protocol was as follows: 95°C, 10 min; [95°C, 30 sec; 50°C, 30 sec; 72°C, 1 min and 30 sec] 40 cycles; 72°C, 10 min. PCR & sequencing specific primers used in this study are listed in Table S1. Amplification of *Der p 23* with specific primers designed from the canonical sequence *Der p 23.0101* revealed the existence of two possible amplicons (of the expected size and larger; Fig. S3). Direct and reverse-sequencing chromatograms using these primers showed overlapping peaks, indicating the presence of at least an extra DNA template, and consistent with Genbank accessions KP895832 and/or KP895831. Accordingly, two new sequencing primers were designed to specifically amplify either *Der p 23.0101* (short variant) or KP895832 (long variant) (Table S1). These were subsequently employed to characterize the inbred mite lines under study. Inspections of sequencing chromatograms (e.g. detection of double peaks revealing single nucleotide polymorphisms, SNPs), alignments, and *in silico* translation were carried out using the software package Geneious 4.8.5 (Biomatters Ltd, Auckland, New Zealand). Reference *Der p 1*, *Der p 2* and *Der p 23* sequences were retrieved from the WHO/IUIS Allergen Nomenclature Sub-committee database (www.allergen.org).

Supplementary references

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Fig. S1. Genealogical tree of *D. pteronyssinus* iso-female lines (F_0 , denoted by a capital letter followed by a prime symbol) and inbred lines (denoted by a capital letter referring to the iso-female line of origin, and followed by one Arabic numeral per sib-mating generation, separated by hyphens). Each Arabic numeral indicates a particular brother-sister couple isolated from the preceding sib-mating cross (note that, unless specified by a crossed circle, only those lines from which a stable culture was obtained are included). Mite lines that were characterized by sequencing of *Der p 1*, *Der p 2* and *Der p 23* genes are highlighted (by a black circle, as opposed to an empty circle).

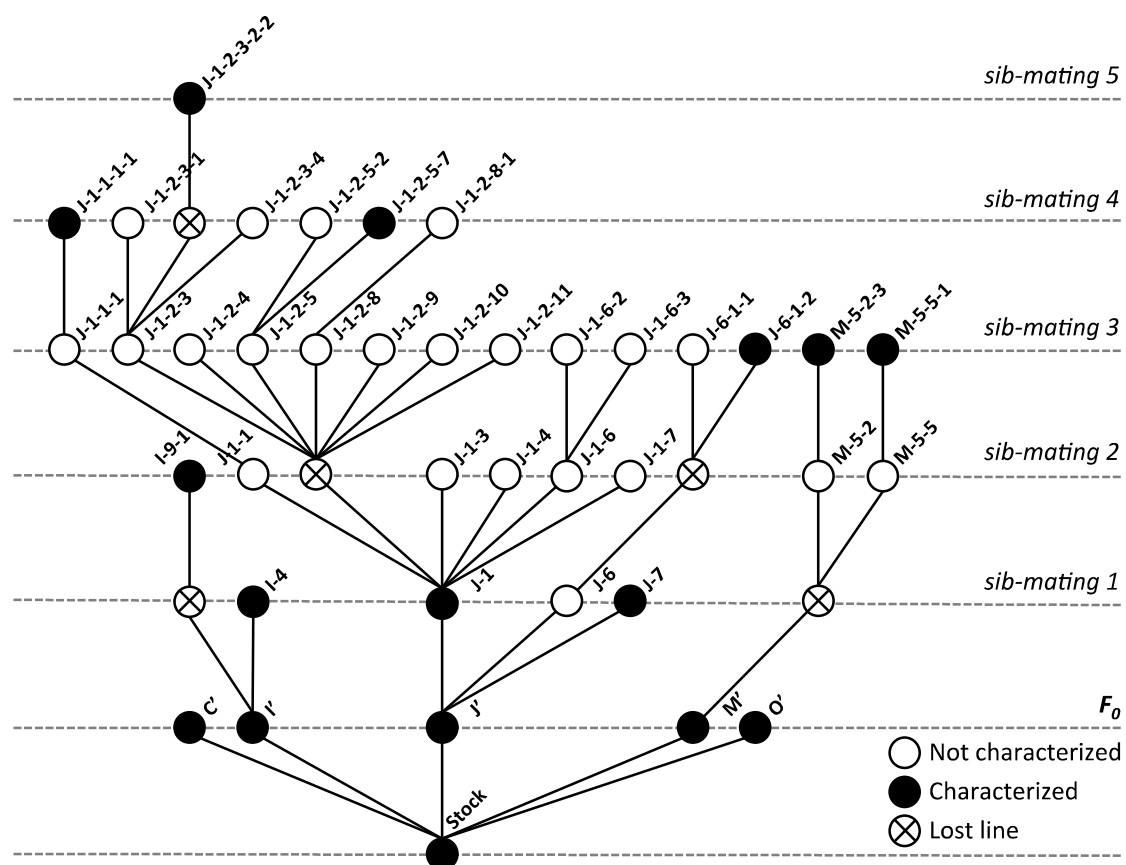


Fig. S2. Culture performance of *D. pteronyssinus* iso-female lines and sib-mating inbred lines. Vertical axis indicates the time from the establishment of initial couples on slides till the first time a saturated culture is reached on Erlenmeyer flask containing 1 g of medium. F₀, iso-female lines; SM-#, sib-mating inbred lines from consecutive generations (bars from consecutive SM generations alternate black/grey color).

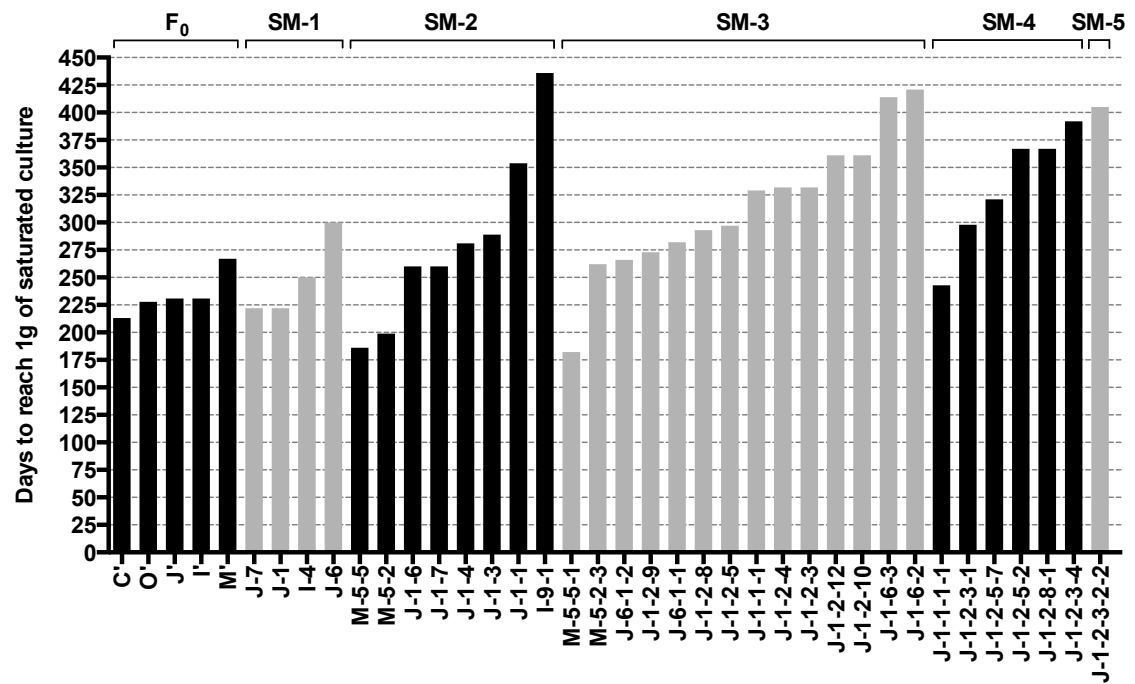
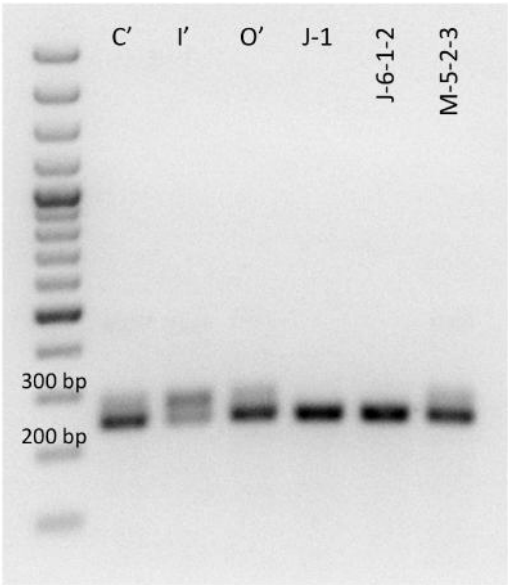


Fig. S3. Agarose (2%) electrophoresis of PCR products using *Der p 23* specific primers and gDNA from selected *D. pteronyssinus* inbred lines as template. Iso-female lines C', I' and O', and the inbred line M-5-2-3 are heterozygous for *Der p 23.0101* and one of the two isoform variants ALA22869 or ALA22868; inbred lines J-1 and J-6-1-2 are homozygous for the isoform *Der p 23.0101*.



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Der p 23.0101      DDPPT-----TVHPTT--TEQPDDKFECPSRFGYFADPKDPHKFYICSNWEAVHKDCPGNTR
ALA22869.1        DDPTTIDVKTSTVLPTTVTTKQPDEFCPTRFGYFADPKDPHKFYICSNWEAVHKDCPGNTR
ALA22868.1        DDPTTIDVKTSTVQPTTVTTKQPDDKFECPSTRFGYFADPKDPHKFYICSNWEAVHKDCPGNTR
*****           **          * *****
*****           **          *
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Reference sequences					
Accession (GenBank)	gDNA ^a	PCR primers (5'-3')	Amplicon length gDNA (bp)	Sequencing primers (5'-3')	Coverage ^b
U0195; DQ185508; 77224	MQNO02000003.1	F: GGTGCCATCAACCATTTGTCCGATTT R: TTTGTTTAGAAGATGACAACATATGG	971	F: idem as PCR R: idem as PCR	81.0%
U06239; FM177223; 63560	MQNO02000067.1	F: TTTCATTGTTGGTGCAGCC R: AAATTTTGTTGATTTAATCGCGG	516/524	F: idem as PCR R: idem as PCR	95.0%
U04751; KP895832	MQNO02000153.1	F: TTATTTCTGTTGGCCATTTGGTCC R: GTGCATGTTTCTTCATCTTCATTCC	244/271	F: ATCATATGCCGCCAATGATAATG F: TCGTATGCCGCCAATGATAATAATA	79.1% (EU414751) 80.7% (KP895832)

^b Maximum sequencing coverage with respect to the complete coding sequence (cDNA), including primer sequences

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Mite culture & inbreeding

The mites used in this study came from a *D. pteronyssinus* laboratory culture kept in the lab for more than 5 years and originated from a commercial culture (1). The stock colony was reared as previously described (2) in 50 ml wide-mouth sterile Erlenmeyer flasks containing 2 g of a pre-heated (60°C for 6 h) 1:1 mixture of fish food flakes (Vipan Sera, Heinberg, Germany) and brewer's yeast flakes (Santiveri, Barcelona, Spain) covered with sterile hydrophilic cotton caps. Environmental conditions in all experiments were $24 \pm 0.3^\circ\text{C}$ and $76 \pm 2\%$ relative humidity (RH) in the dark. RH control was achieved by placing culture units into sealed boxes with a saturated NaCl solution. The inbreeding strategy consisted in sequential rounds of sibling mating (sib-mating) brother-sister. Briefly, the breeding program started from different couples of a virgin female (obtained from individually isolated nymphs) and a single male (randomly collected from the stock colony) reared on concave microscope slides containing a fish food flake of approximately 2 mg and confined using a cover slip that allowed gas exchange, as previously described (3). After approximately 2 weeks, adult couples were removed and, once the tritonymphal stage was reached, part of the progeny was individually isolated in new slides until adulthood. For each subsequent sib-mating generation, new couples of virgin females and brothers were paired. When possible, part of the progeny derived from each cross was maintained in the original slide to establish the corresponding inbred line. These cultures were sequentially upscaled: first, by letting mites consume the food in the slides, and transferring them to a ventilated 1.5 ml microtube containing 35 mg of fish food until culture saturation. The whole content of the microtube was then used to inoculate 1 g of 1:1 (fish food : brewer's yeast) standard medium into a 50 ml flask, maintained as described above. Once 1 g cultures were saturated, the regular rearing protocol of the stock colony was employed (2 g of fresh medium were sub-cultured using 150 mg of thriving culture). The culture saturation point was defined by stereoscope inspection as when population was thriving, medium flakes were fully disaggregated but still some unconsumed fragments could be seen. Thus, the sub-culture time was not fixed as for the stock culture (42 days) but it was rather dependent on the progress of each inbred line. The duration of all culture phases was systematically recorded. The genealogy of inbred lines was codified using a capital letter (denoting the original couple) followed by one number per sib-mating generation, and separated by hyphens (e.g. J-1-2-3-2-2, denoting a 5th sib-mating generation line; Fig. S1).

The level of inbreeding was estimated by the Wright's inbreeding coefficient (F) (4) based on a sib-mating brother-sister pedigree using the web application <http://www.insilicase.com/Web/WebC of I.aspx>. Note that the inbreeding coefficient of F_0 ancestors was estimated as being 0, although it could be higher considering they derived from a laboratory colony.

Sequencing Der p 1, Der p 2 and Der p 23 genes

D. pteronyssinus from iso-female lines and inbred lines ($n > 200$ mixed-stage mites) were isolated following the paper disk method (2). Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Mite manipulation was performed avoiding cross-contamination of different lines. Partial sequences of the major allergen genes were obtained by PCR, followed by purification (QIAquick PCR Purification Kit, Qiagen) and Sanger sequencing. The PCR master mix contained 2.5 μ l of PCR buffer II, 1.5 μ l $MgCl_2$ (25 mM), 2.5 μ l dNTPs mix (2 mM), 0.5 μ l of primers (10 μ M of each forward and reverse), 0.2 μ l of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Austin, USA) (5U/ μ l) and 5 μ l of DNA template (12.5 ng gDNA/ μ l), the total volume was made up to 25 μ l with sterile MilliQ water. The thermocycler protocol was as follows: 95°C, 10 min; [95°C, 30 sec; 50°C, 30 sec; 72°C, 1 min and 30 sec] 40 cycles; 72°C, 10 min. PCR & sequencing specific primers used in this study are listed in Table S1. Amplification of *Der p 23* with specific primers designed from the canonical sequence *Der p 23.0101* revealed the existence of two possible amplicons (of the expected size and larger; Fig. S3). Direct and reverse-sequencing chromatograms using these primers showed overlapping peaks, indicating the presence of at least an extra DNA template, and consistent with Genbank accessions KP895832 and/or KP895831. Accordingly, two new sequencing primers were designed to specifically amplify either *Der p 23.0101* (short variant) or KP895832 (long variant) (Table S1). These were subsequently employed to characterize the inbred mite lines under study. Inspections of sequencing chromatograms (e.g. detection of double peaks revealing single nucleotide polymorphisms, SNPs), alignments, and *in silico* translation were carried out using the software package Geneious 4.8.5 (Biomatters Ltd, Auckland, New Zealand). Reference *Der p 1*, *Der p 2* and *Der p 23* sequences were retrieved from the WHO/IUIS Allergen Nomenclature Sub-committee database (www.allergen.org).

Supplementary references

1. Dermauw W, Van Leeuwen T, Vanholme B, Tirry L. The complete mitochondrial genome of the house dust mite *Dermatophagoides pteronyssinus* (Trouessart): a novel gene arrangement among arthropods. *BMC Genomics* 2009;**10**:107.

2. Vidal-Quist JC, Ortego F, Lombardero M, Castañera P, Hernández-Crespo P. Allergen expression in the European house dust mite *Dermatophagoides pteronyssinus* throughout development and response to environmental conditions. *Med Vet Entomol* 2015;**29**(2):137-46.
3. Vidal-Quist JC, Ortego F, Lambrecht BN, Castañera P, Hernández-Crespo P. Effects of domestic chemical stressors on expression of allergen genes in the European house dust mite. *Med Vet Entomol* 2017;**31**(1):97-101.
4. Wright S. Evolution in Mendelian populations. *Genetics* 1931;**16**(2):97-159.

Fig. S1. Genealogical tree of *D. pteronyssinus* iso-female lines (F_0 , denoted by a capital letter followed by a prime symbol) and inbred lines (denoted by a capital letter referring to the iso-female line of origin, and followed by one Arabic numeral per sib-mating generation, separated by hyphens). Each Arabic numeral indicates a particular brother-sister couple isolated from the preceding sib-mating cross (note that, unless specified by a crossed circle, only those lines from which a stable culture was obtained are included). Mite lines that were characterized by sequencing of *Der p 1*, *Der p 2* and *Der p 23* genes are highlighted (by a black circle, as opposed to an empty circle).

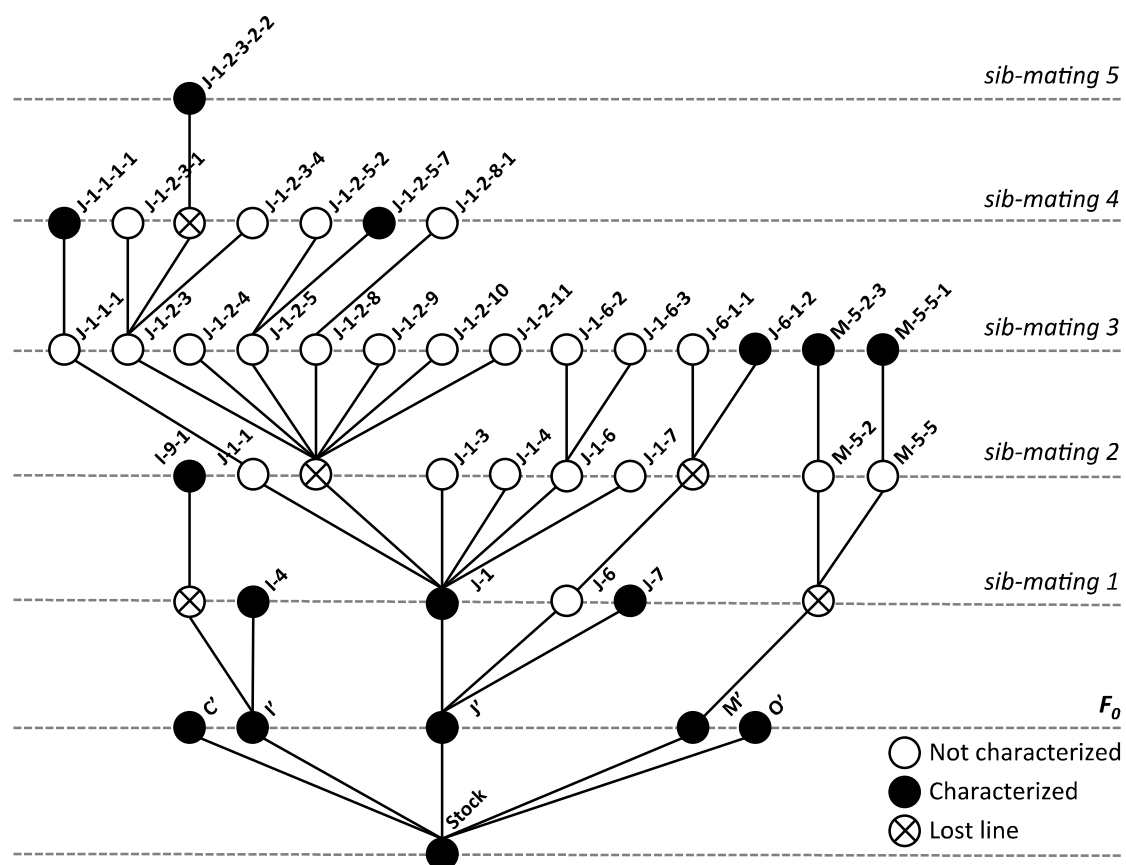


Fig. S2. Culture performance of *D. pteronyssinus* iso-female lines and sib-mating inbred lines. Vertical axis indicates the time from the establishment of initial couples on slides till the first time a saturated culture is reached on Erlenmeyer flask containing 1 g of medium. F₀, iso-female lines; SM-#, sib-mating inbred lines from consecutive generations (bars from consecutive SM generations alternate black/grey color).

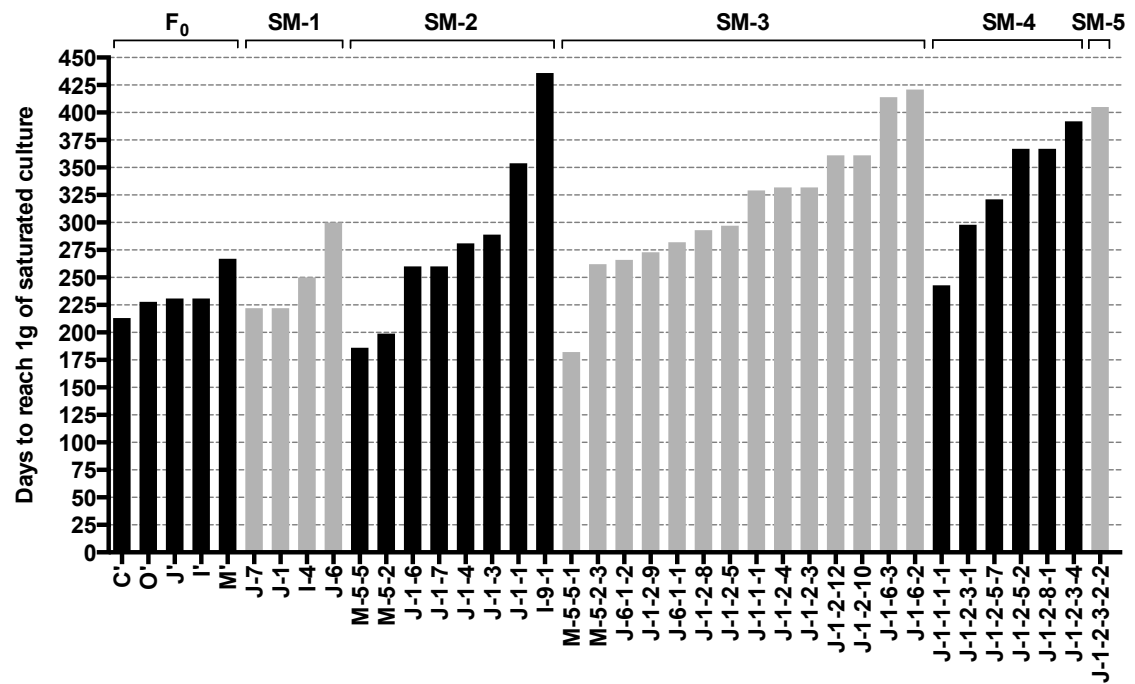


Fig. S3. Agarose (2%) electrophoresis of PCR products using *Der p 23* specific primers and gDNA from selected *D. pteronyssinus* inbred lines as template. Iso-female lines C', I' and O', and the inbred line M-5-2-3 are heterozygous for *Der p 23.0101* and one of the two isoform variants ALA22869 or ALA22868; inbred lines J-1 and J-6-1-2 are homozygous for the isoform *Der p 23.0101*.

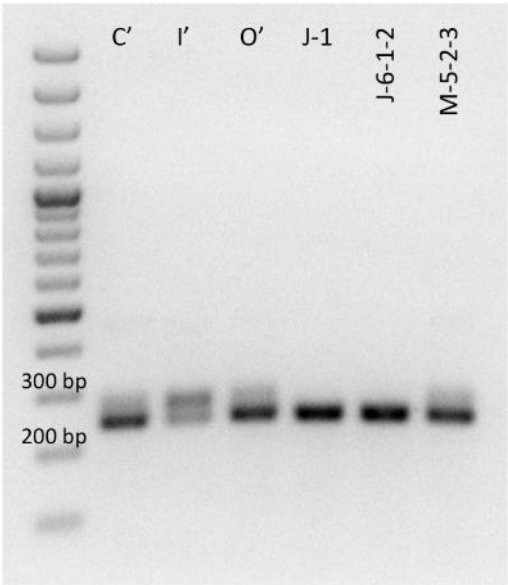


Fig. S4. Alignment of predicted partial protein sequences of the Der p 23 isoforms identified in this report. Amino acid residues corresponding to the annealing region of sequencing/PCR primers have been removed. In bold, insertions and amino acid substitutions compared to the canonical isoform Der p 23.0101.

Der p 23.0101
ALA22869.1
ALA22868.1

DDPTT-----TVHPTT--TEQPDDKFECPSRFGYFADPKDPHKFYICSNWEAVHKDCPGNTR
DDPTT**IDVKT**STVLPTT**VTTK**QPDD**EF**ECPT**R**FGYFADPKDPHKFYICSNWEAVHKDCPGNTR
DDPTT**IDVKT**STV**Q**PTT**VTTK**QPDDKFECPT**R**FGYFADPKDPHKFYICSNWEAVHKDCPGNTR

Table S1. List of primers used in this study.

Gene	Reference sequences		PCR primers (5'-3')	Amplicon length gDNA (bp)	Sequencing primers (5'-3')	Coverage ^b
	cDNA (GenBank accessions)	gDNA ^a				
<i>Der p 1</i>	U11695; DQ185508; FM177224	MQNO0200003.1	F: GGTGCCATCAACCATTTGTCCGATT	971	F: idem as PCR	81.0%
			R: TTTGTTTAGAGAATGACAACATATGG		R: idem as PCR	
<i>Der p 2</i>	AF276239; FM177223; AM263560	MQNO02000067.1	F: TTTCATTGTTGGTCGCAGCC	516/524	F: idem as PCR	95.0%
			R: AAATTTTGTTTGATTTAATCGCGG		R: idem as PCR	
<i>Der p 23</i>	EU414751; KP895832	MQNO02000153.1	F: TTATTCGTGGCCATTTTGTCC	244/271	F: ATCATATGCCGCCAATGATAATG	79.1% (EU414751)
			R: GTGCATGTTCTTCATCTCATTCC		F: TCGTATGCCGCCAATGATAATAATA	80.7% (KP895832)

^a Genome scaffold nr. in assembly GCA_001901225.2, GenBank

^b Maximum sequencing coverage with respect to the complete coding sequence (cDNA), including primer sequences