Received: 27 July 2018 | Revised: 19 October 2018 | Accepted: 21 October 2018

DOI: 10.1111/are.13900

ORIGINAL ARTICLE

WILEY Aquaculture Research

Using genetic methods for analysis of fish meals and feeds employed in Greek mariculture

Antonis Vlachavas[1] | Nikoleta Karaiskou[1] | Lambros Kokokiris[2] | Foteini-Izampela Zampeta[1] | Elena Drosopoulou[1] | Alexander Triantafyllidis[1]

[1]Department of Genetics, Developmental and Molecular Biology, School of Biology, AUTH, Thessaloniki, Macedonia, Greece

[2]Department of Human Nutrition and Dietetics, Alexander Technological and Educational Institute of Thessaloniki, Thessaloniki, Sindos, Greece

Correspondence
Nikoleta Karaiskou, Department of Genetics, Developmental and Molecular Biology, School of Biology, Aristotle University of Thessaloniki, Thessaloniki, Macedonia, Greece.
Email: nikolbio@bio.auth.gr

Abstract

Large quantities of high protein fish meals are needed to sustain cultured species and thus the impact to marine ecosystem has been highly discussed. The aim of this study was to apply a PCR-cloning methodology for a robust insight into the composition of commercial fish meals and feeds for farmed species of the Greek mariculture, assessing the risk posed by aquaculture to marine ecosystems but also the risk posed by commercial fish feeds to the increase in trophic level of species farmed in Greece. 89% of the sequences were identified to species level and only 11% to genus/family level. Overall, a total of 49 taxa were identified (44 fish species/taxon, five non-fish species/taxon). Even though small pelagic fish like *Engraulis* sp. were the main portion, a wide range of species constituted the fish meals and feeds. Plant and animal species were also detected as an alternative protein source. Feed products employed in Greek mariculture still contain large portions of fish meals which increase the mean trophic level of farmed species causing a farming up trend. The results emphasize that such molecular methodologies are needed to certify aquafeeds allowing fish feed producers to demonstrate their commitment to sustainable aquaculture.

KEYWORDS
16s rRNA gene, fish feed, fish meal, PCR-cloning

1 | INTRODUCTION

Fish farming has grown to augment the supply of fresh fish to western markets and it is one of the fastest growing sectors of food production constituting an important industry in many countries worldwide (Trujillo, Piroddi, & Jacquet, 2012). Fish meals (and oils) are key components of aquafeeds. They are good sources of essential amino acids, vitamins, phospholipids, fatty acids and energy (Farajollahi et al., 2009). Based on marine ingredient organization (IFFO), Fish meals are used both in aquaculture production and animal feed production (75% and 25% respectively). Fish meals can be made from almost any type of seafood, particularly small sized marine fish which contain a high percentage of bones and oil (Farajollahi et al., 2009). The impact of this industry on marine ecosystems has raised concern during the last years and created much debate among researchers, because farmed fish require large quantities of high-quality protein for their growth. Thus, the risk of overfishing wild fish for the production of fish meal (Naylor et al., 2000) is considerable.

Mediterranean mariculture industry is shifting from culturing low-trophic-level species to culturing high-trophic-level species, a phenomenon known as farming up (Stergiou, Tsikliras, & Pauly, 2009) and this transition to farming high-trophic-level fish species has consequences for fisheries because it increases the demand for fish-meal-and-oil based aquafeeds (Stergiou et al., 2009). Many concerns have been raised about the impact of fish farming on marine ecosystems, taking into account that farmed fish require large quantities of

Antonis Vlachavas and Nikoleta Karaiskou contributed equally.

fishery resources (fish meals [and oils] for the production of fish-meal-and-oil based aquafeeds (Naylor et al., 2000)). Attempts to reverse the farming up trend can be achieved, among various manners, by promoting high efficiency in the use of living marine resources in aquafeeds (Tsikliras, Stergiou, Adamopoulos, Pauly, & Mente, 2014). However, the lack of appropriate methods for an efficient identification of the living marine resources used to produce aquafeeds is holding back promotion of high efficiency in the use of living marine resources in aquafeeds.

Moreover, in an attempt to minimize the impact of using fishery production for feeding farmed fish, the fish meals in feeds are progressively substituted by plant (Allan et al., 2000; Gomes, Rema, & Kaushik, 1995) or even meat proteins of non ruminant species (EU legislation 58/2013). Thus, pork and poultry proteins are legally mixed in fish meals with cereal grains and other plant proteins (e.g. Ardura et al., 2012; Doosti, Ghasemi Dehkordi, & Rahimi, 2014; Sitja-Bobadilla et al., 2005). However, the quality of fish meals is often questioned due to presence of undesirable substances (adulteration) like those from ruminant species (sheep and cattle, e.g., Doosti et al. (2014) totally banned as components of animal feeds (EU 999/2001)).

Several methods have been developed to screen animal feed composition. Classical microscopy is the only official methodology (EU legislation 126/2003) to screen animal feed since now. However, recent collaborative studies (von Holst et al., 2008) revealed great deviation in the results between laboratories and resulted in significant differences in sensitivity, specificity and accuracy of the method. Molecular techniques have proven capable to overpass problems related to the above methodology (Farajollahi et al., 2009; LUO et al., 2008; Santaclara, Espiñeira, Cabado, & Vieites, 2007). PCR-based methods have played a central position in fish species identification in aquafeeds due to the extraordinary power of PCR to increase target DNA copy numbers from few copies to easily detectable quantities (Teletchea, 2009). PCR amplification techniques are therefore particularly useful when the product to be authenticated contains fragmented DNA, like the case of highly processed fish meal and other fish derivatives that undergo autoclaving following European regulations.

In spite of the priority for high efficiency in the use of living marine resources but also the need to regulate undesirable substances in fish feeds, the analysis and control of species content in fish meals and feeds used to produce farmed fish, are not routinely carried out in Greece. It is well known that Greece constitutes one of the most important countries in fish aquaculture sector. It is dominated by the farming of marine finfish, specifically of gilthead sea bream and European sea bass. The amount of feed required for growing fish in Greece is estimated to be approximately 10,000–12,000 t/year. Most of this is pelleted feed of which 70% is imported and 30% is produced locally. Additionally, it has been estimated (Tacon & Metian, 2008) that Greek fish feed consists of around 11% of fish oils and 35% of fish meal imported mainly from Europe and not produced locally. The analysis and control of content in aquafeed products is of crucial importance to protect human health, animal health and the environment (Nagase, Maeta, Aimi, Suginaka, & Morinaga, 2009).

The aim of this study was to apply a DNA analysis method for a robust insight into the composition of commercial fish meals and feeds for farmed species of the Greek mariculture. Additionally, we try to assess the risk posed by aqua feed items in the aquaculture systems in respect to trophic chain by estimating possible change of the natural trophic level of farmed species after the use of the specific fish feeds.

2 | MATERIAL AND METHODS

2.1 | Aquafeed products

Four types of imported fish meals (M1–M4 meals, in fine powder form) employed to formulate feeds for marine fish and two commercial types of feeds for marine fish (F1 feed, produced in Greece, dry pellets and F2 feed, imported from Thailand) were analyzed. M1–M4 meals and F1 and F2 feeds were provided by aquafeed companies. Any of the tested samples had any kind of labelling indicating their composition.

2.2 | DNA analysis

DNA extraction was carried from 15 mg of commercial product. For all samples, it was first necessary to get rid of fats and oil since they could interfere in DNA extraction process. The fat and oils extraction process, was carried out by resuspending the samples in methanol–chloroform–water (2:1:0.8) for 24 hr and then washing them in distilled water, and PBS 1× buffer so as to eliminate the remains of the solution used previously. DNA was extracted with silica gel columns (QIAamp® DNA Mini Kit, Qiagen, Germany) following manufacturer instructions. A fragment of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the primers described by Horreo, Ardura, Pola, Martinez, & Garcia-Vazquez, (2013). PCR amplification conditions were described by Ardura et al., (2012).

The amplified products were purified with PureLink PCR purification kit and then cloned with Qiagen PCR Cloning kit, Germany, following manufacturer instructions. Plasmid DNAs were extracted with alkaline extraction method (Birnboim, 1983) and the insert fragment was amplified with PCR using the universal plasmid primers SP6 (5′-ATTTAGGTGACACTATAG-3′) and T7 (5′-TAATACGACTCACTATAGGG-3′). The amplification reaction was performed in total volume of 30 μl including 15 U KAPA Taq DNA polymerase (KAPA Biosystem, Boston), 15 pmol of each primer and 20 ng of DNA template. PCR conditions were: denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 1 min and extension at 72°C for 30 s and a final extension at 72°C for 5 min. PCR products with positive visualized bar were purified and capillary sequenced using SP6 primer was conducted with an ABI 3730xl DNA Analyzer (Applied Biosystems Inc., Foster City, CA, USA).

Sequences obtained were analyzed employing the program BLAST within NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) database. BLAST finds similarity between sequences and can therefore be employed for identifying the species of unknown sequences. Sequence similarity >98% was considered reliable. An alternative analysis was followed with the MEGAN 4 program (MEta Genome ANalyzer, (Huson, Auch, Qi, & Schuster, 2007)). MEGAN 4 is used to estimate and interactively explore the taxonomical content of the data set, using the NCBI taxonomy to summarize and order the results against one or more reference databases, typically using BLASTN. The program uses a simple algorithm that assigns each sequence of the data set to the lowest common ancestor (LCA) of the set of taxa that it hit in the comparison. As a result, species-specific sequences are assigned to taxa near the leaves of the NCBI tree, whereas widely conserved sequences are assigned to high-order taxa closer to the root. The software can also compare two or more data sets and performs comparative taxonomic analysis. Additionally, plots of the number of distinct species against the number of clones sequenced were used to assess whether most species present in the sample had been discovered. The proportional number of clones containing a given species/taxon was used to describe the abundance of each species to the tested sample.

2.3 | Trophic level

We calculated the mean weighted trophic level of each aquafeed product (feed or meal, TL_A) on the basis of their species composition (i.e., the fraction of each species in the product and the trophic level of the given species in the wild (TL_N).

The TL_A was calculated as

$$TL_A = \sum_{j=1}^{G} DC_{ij} * troph_j$$

where $troph_j$ is the trophic level of each species j, DC_{ij} is the fraction of each species j in the aquafeed product and G is the total number of species detected in each aquafeed product.

We also calculated the mean trophic level of five farmed fish species (TL_Fs), assuming that they are fed with aquafeeds containing the fish meals analysed in this study. The fish we considered were gilthead seabream (*Sparus aurata*), European seabass (*Dicentrarchus labrax*), common pandora (*Pagellus erythrinus*), common dentex (*Dentex dentex*) and red porgy (*Pagrus* sp.). The corresponding TL_Fs were calculated based on the current percentages of fishmeal (and oil) and plant materials included in their aquafeeds, as reported by (Tsikliras et al., 2014).

The TL_F was calculated as

$$TL_F = 1 + \sum_{j=1}^{G} DC_{ij} * troph_j$$

where $troph_j$ is the trophic level of aquafeed ingredient *j*, DC_{ij} is the fraction of *j* in the aquafeed *i*, and G is the total number of aquafeed ingredients.

For all aquafeeds for which we estimated TL_F, we used the control diet formulations of replacement experiments, with the exception of seabream, for which the diet formulation of the commercial aquafeed was known and used. The trophic level of oil fraction assumed to be equal with the TL of fish meal fraction, assuming that both fractions are derived from the same group of fish. However, as the production of 1 kg of fish oil requires around 20 kg of whole fish (Shepherd & Jackson, 2013) and 1 kg of fishmeal requires 4.5 kg of whole fish, the TL of oil fraction was multiplied with the coefficient 4.44. The trophic level of plant material was taken as 1.0, which is the trophic level of photosynthetic plants. The TL_F was estimated using the fishmeal and oil and plant material, whereas the remaining ingredients were not taken into account in the analysis. The natural trophic level of each species in the wild (TL_N) was taken from FishBase (www.fishbase.org; Froese & Pauly 2018), which provides estimates of trophic levels based on diet composition data.

3 | RESULTS

DNA was successfully extracted from samples and the PCR products were purified and cloned. A number of 95 clones (n = 95) per aquafeed product were successfully amplified. In most cases the number of species detected reached a plateau after 45–70 clones indicating that all species are likely to have been sampled (Figure 1), except of M2 meal and F1 feed for which more clones had to be analyzed (98 or 110; Table 1). The vast majority of the sequences were identified to species level (528/591) and only 63 ones matched to more than two species and were assigned either to genus or family level.

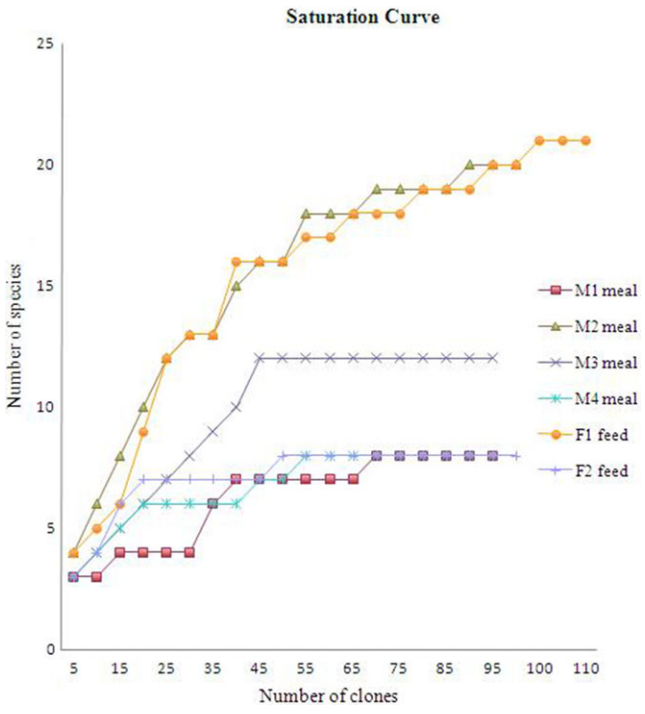

FIGURE 1 Saturation curves comparing sampling effort (number of clones sequenced) with number of different species/taxon identified [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Species/taxon genetically identified, number of clones containing a given species/taxon (proportional number of clones, %) detected in fish feeds (F1: feed for marine fish, F2: aquarium feed) and fish meals (M1–M4) analyzed

		Aquafeed products						
		Meals				Feeds		
a/a	Species/Taxon	M1	M2	M3	M4	F1	F2	TMP_c (%)
1	*Ammodytes* sp.			4 (4.2)			15 (15.3)	9.8
2	*Anchoa nasus*						1 (1)	1
3	*Auxis* sp.	1 (1.1)	3 (3.1)			1 (0.9)		1.7
4	*Boops boops*		3 (3.1)					3.1
5	*Brama brama*		1 (1)					1
6	*Bulla* sp.[a]				2 (2.1)			2.1
7	*Capros aper*			7 (7.4)				7.4
8	*Centengraulis mysticetus*				18 (18.9)			18.9
9	*Chelidonichthys* sp.	1 (1.1)						1.1
10	*Chloroscombrus chrysurus*		1 (1)					1
11	*Clupea harengus*			4 (4.2)	10 (10.5)	2 (1.8)		5.5
12	*Coryphaena* sp		1 (1)					1
13	*Diplodus bellotii*					1 (0.9)		0.9
14	*Draconetta xenica*		1 (1)					1
15	*Engraulis encrasicolus or Engraulis* sp.	87 (91.6)	13 (13.3)	3 (3.2)		2 (1,8)	4 (4.1)	22.8
16	*Engraulis ringens*	1 (1.1)	2 (2.0)	64 (67.4)	53 (55.8)	24 (21.8)	46 (46.9)	32.5
17	*Ethmolosa fimbritiata*					1 (0.9)		0.9
18	Gadidae						1 (1)	1
19	*Galeichthys peruvianus*			1 (1.1)				1.1
20	*Gallus* sp.[a]					2 (1.8)		1.8
21	*Gasterosteus aculeatus*			1 (1.1)				1.1
22	*Glycine max*[a]					1 (0.9)		0.9
23	*Glyptocephalus cynoglossus*		2 (2)	1 (1.1)		1 (0.9)		1.3
24	*Lepidotrigla grandis*		1 (1)					1
25	*Mallotus villosus*			3 (3.2)	4 (4.2)	12 (10.9)	27 (27.6)	11.5
26	*Merluccius polli*		1 (1)					1
27	*Merluccius senegalesis*		1 (1)					1
28	*Merluccius* sp.				4 (4.2)			4.2
29	*Micromesistius* sp.					2 (1.8)		1.8
30	*Opisthonema libertate*				1 (1.1)			1.1
31	Pleuronectinae			1 (1.1)				1.1
32	*Pomadasys incisus*		1 (1)					1
33	*Rheocles* sp *or R.lateralis*		4 (4.1)					4.1
34	*Salmo salar*					5(4.5)		4.5
35	*Sardina pilchardus*		3 (3.1)			10 (9.1)		6.1
36	*Sardinella aurita*	1 (1.1)	23 (23.3)		3 (3.2)	31 (28.2)		14
37	*Sardinella longicepcs*					1 (0.9)		0.9
38	*Sardinops* sp.	1 (1.1)						1.1
39	*Scatophagus argus*		1 (1)					1
40	*Scomber* sp. *or S. australasicus*					3 (2.7)		2.7
41	*Sprattus sprattus*	1 (1.1)		4 (4.2)				2.6
42	*Sus scrofa domesticus*[a]			2 (2.1)		2 (1.8)		2
43	*Tetragonurus cuvieris*		1 (1)					1

(Continues)

TABLE 1 (Continued)

		Aquafeed products						
		Meals				Feeds		
a/a	Species/Taxon	M1	M2	M3	M4	F1	F2	TMP_c (%)
44	*Thunus* sp						1 (1)	1
45	*Trachurus longimanus*		2 (2)					2
46	*Trachurus mediterraneus*		13 (13.3)			2 (1.8)		7.5
47	*Trichiurus lepturus*	2 (2.1)	20 (20.4)			2 (1.8)		8.1
48	*Trisopterus esmarkii*					2 (1.8)		1.8
49	*Triticum* sp. *or T. aestivum or T. urartu* [a]					3 (2.7)	3 (3.1)	2.9
	Total number of clones	95	98	95	95	110	98	
	Total number of species/taxon	8	21	12	8	21	8	

Note. Total mean proportional number of clones (TMP_c, %) containing a given species/taxon.
Shading: the most abundant fish species.
[a]Non-fish species.
Means marked with grey colour.

The number of taxa detected ranged from 8 to 21 depending on type of fish feed product (Table 1). M2 meal and F1 feed were the most diverse with 21 taxa contained. Overall, a total of 49 taxa detected in all fish feed products (44 fish species/taxon, five non-fish species/taxon). Twenty-four taxa (*n* = 24, 49%) identified in meals (M1-M4), 14 in feeds (*n* = 14, 28.5%) but 12 species/taxon identified in both meals and feeds (*n* = 11, 22.5%).

A high majority of species/taxa (*n* = 29, 58%) detected in less than 2% and only two species (4%) identified in frequencies higher than 20% of the clones (32.5%, *Engraulis ringens*, 22.8%, *E. encrasicolus*; Table 1). The proportion of clones containing a given species/taxon was also different depending on the type of fish feed product. Peruvian anchovy *Engraulis ringens*, European anchovy *E. encrasicolus*, Pacific anchoveta *Centengraulis mysticetus*, capelin *Mallotus villosus*, sand lance *Ammodytes* sp., round sardinella *Sardinella aurita*, Largehead hairtail *Trichiurus lepturus*, Mediterranean horse mackerel *Trachurus mediterraneus*, boarfish *Capros aper*, European pilchard *Sardina pilchardus*, Atlantic herring *Clupea harengus* were the 11 most abundant species amongst the six products (detected in more than 5% of the clones from all products containing these species Table 1). The anchovies (*E. ringens and E. encrasicolus*), the capelin (*M. villosus*), the round sardine (*S. aurita*), the largehead hairtail (*T. lepturus*), were highly abundant (e.g. *E. encrasicolus* was detected in 91.5% of analysed cloned of M1) and present from six (i.e., the *E. ringens*) to three (i.e., the *T. lepturus*) types of products confirming their importance as constituents of aquafeeds (Table 1).

However, five non-fish taxa detected as constituents of M3 and M4 meals but also of F1 and F2 feeds had frequencies from 0.9% to 3.1%. The domestic pig *Sus scrofa domesticus* was detected as constituent of M3 meal (2.1%) and the marine gastropod, of the genus *Bulla* sp. as constituent of the M4 meal (2.1%). As far as the feeds are concerned, the plant of the genus *Triticum* sp., constituted 2.7% of the clones in F1 feed but 3.1% in F2 feed and the plant *Glycine max* detected in 0.9% of the clones in F1 feed. The domestic pig *Sus scrofa domesticus* and the bird junglefowl, of the genus *Gallus* sp. were detected as constituents of F1 feed (1.8% each; Table 1).

The data sets from the six feed products were compared and the phylogenetic trees were constructed based on MEGAN (Figure 2). There were cases that some sequence data matched more than two to three different species and thus they were assigned to higher taxonomic level (i.e., the case of *Carangiformes*) as it is the lowest-common taxonomical ancestor of the matched species and they were placed to internal node. Clupeiformes were the main constituent of three fish feed meals (95.7% of M1, 89.4% of M4, 76.5% of M3) but Clupeiformes and Percomorpha were almost evenly recorded as main constituents of M2 meal (41.8% and 56.1%, respectively; Figure 2). The F1 and F2 feeds had a more mixed representation of fish taxa with Clupeiformes representing around 50% of their composition.

The comparison of the four meals (M1-4) revealed seven shared fish species with different frequencies (Table 1): *E. ringens* and *E. encrasicolus* (*Engraulis* sp.), *S. sprattus*, *T. lepturus*, *S. aurita*, *C. harengus*, and *M. villosus*. The F1, F2 feeds shared only three species, i.e., the fish species *M. villosus*, and *E. ringens* and the cereal, *Triticum* sp.

The fish species contained in the six products belong to trophic levels from 2.4 to 3 (*n* = 9 species, 20.5%), from 3 to 4 (*n* = 24, 54.5%) and from 4 to 4.5 (*n* = 11, 25%). This means that the role in the trophic web of the marine ecosystem of the vast majority of species is as predators (*n* = 35, 79.5%, trophic indice: 3–4, 54.5%) or as higher predators (25%, trophic indice: 4–4.5). The trophic level of fish meals (TL_M) ranged from 2.90 (M3, *n* = 11 ingredients) to 3.63 (M2, *n* = 21 ingredients; Table 2).

The trophic levels for five farmed species (TL_Fs, *S. aurata*, *D labrax*, *P. erythrinus*, *D. dentex*, *P. pagrus*) feeding on feeds containing M1–4 meals (and oils from the same constituent fish species) ranged from 3.6 (i.e., TL_F-M3 meal-*P. erythrinus*) to 5.58 (i.e., TL_F-M2-*D. dentex*; Table 3). Overall, the mean TL_F of each farmed species was higher than its ttrophic level in the natural environment (TL_N), that is

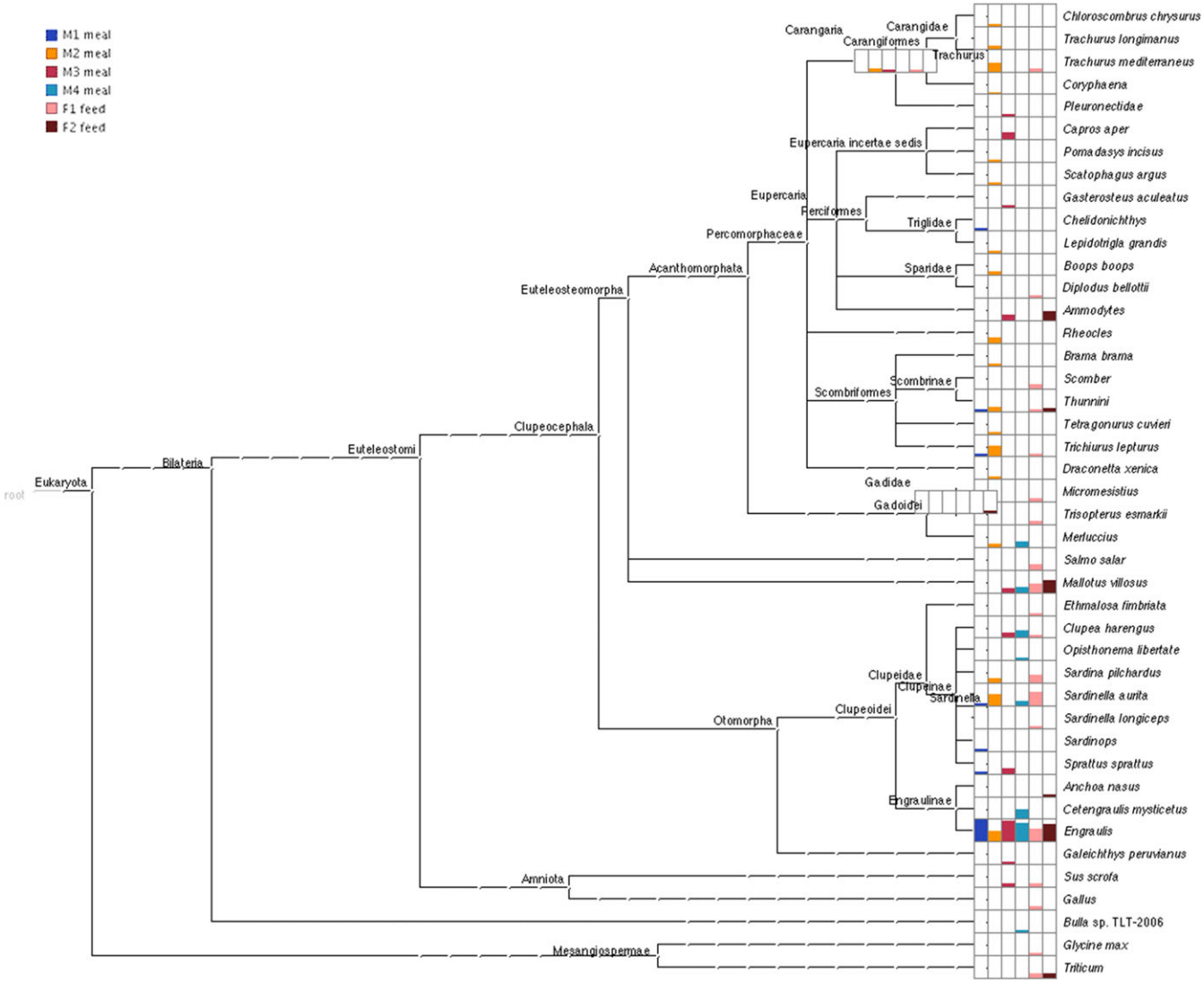

FIGURE 2 Taxonomic analysis of the six aquafeed products studied based on MEGAN4. Each node represents a taxon and is drawn as a gray box that contains a bar chart indicating how many reads were assigned to the corresponding taxon, for each of the data sets, on a logarithmic scale. Different colours refer to different samples [Colour figure can be viewed at wileyonlinelibrary.com]

mean TL_F was from 2.4% (i.e., sea bass, *D. labrax*) to 36.4% higher than TL_N (i.e., sea bream, *S. aurata*; Table 3). Thus, aquafeeds formulation based on M1-M4 meals (and corresponded oils) analyzed are supposed to increase the trophic level of the main farmed fish of Greek mariculture.

Most of the species identified are of commercial interest for fisheries with a diverse geographic distribution from the north and south Hemispheres and from the Atlantic and Pacific oceans. Most abundant fish species constituted F1 feed, caught mainly in Atlantic east and Pacific south oceans (30.0% and 28.1% respectively; Figure 3). However, those constituted F2 feed, caught mainly in Pacific south (46.9%), Pacific north and Atlantic north (27.6%) oceans. Most abundant species constituted M1 meal, caught mainly in Atlantic north, Mediterranean and Africa south fishing areas (91.6%), those constituted M2 meal in Atlantic east (29.6%) and all world fishing areas (world distribution, 23.5%). Finally, the abundant species constituted M3 meal originated mainly from Pacific south (68.4%) and those of M4 meal from Pacific south, east ocean (75.8%).

4 | DISCUSSION

4.1 | Aquafeeds composition and sustainability of fish meal production practices

This study represents the first attempt to deeply monitor fish feed products (meals and feeds) employed in Greek mariculture by means of a DNA analysis. The big advantage of the present study was the screening of high number of clones from each type of fish feed provided a robust coverage of species present, resulting to the identification of 49 species/taxa. However, a similar analysis to identify species in feeds employed in Spanish aquaculture resulted in a low number (n = 8, (Ardura et al., 2012)). The low number of clones sequenced by the authors (20–25) from each fish feed or/and the

TABLE 2 Species/taxon genetically identified, trophic level of the species in their natural environment (TL_N), mean weighted trophic level of aquafeed products (TL_A, feeds, meals) on the basis of their species composition (i.e., the fraction of a given species in the product and the trophic level of the given species in the wild (TL_N)

			TLA of aquafeed products					
			Meals				Feeds	
a/a	Species/Taxon	TLN	M1	M2	M3	M4	F1	F2
1	*Ammodytes* sp.	3.10			0.13			0.47
2	*Anchoa nasus*	3.40						0.03
3	*Auxis* sp.	4.27	0.04	0.13			0.04	
4	*Boops boops*	3.00		0.09				
5	*Brama brama*	4.10		0.04				
6	*Bulla* sp.	2.00				0.04		
7	*Capros aper*	3.10			0.23			
8	*Centengraulis mysticetus*	2.50				0.47		
9	*Chelidonichthys* sp.	3.98	0.04					
10	*Chloroscombrus chrysurus*	3.20		0.03				
11	*Clupea harengus*	3.38			0.14	0.36	0.06	
12	*Coryphaena* sp	4.40		0.04				
13	*Diplodus bellotii*	3.60					0.03	
14	*Draconetta xenica*	3.30		0.03				
15	*Engraulis encrasicolus or Engraulis* sp.	3.10	2.84	0.41	0.10		0.06	0.13
16	*Engraulis ringens*	2.86	0.03	0.06	1.93	1.60	0.62	1.34
17	*Ethmolosa fimbritiata*	2.50					0.02	
18	Gadidae	4.40						0.04
19	*Galeichthys peruvianus*	3.70			0.04			
20	*Gallus* sp.	2.50					0.05	
21	*Gasterosteus aculeatus*	3.50			0.04			
22	*Glycine max*	1.00					0.01	
23	*Glyptocephalus cynoglossus*	3.60		0.07	0.04		0.03	
24	*Lepidotrigla grandis*	3.40		0.03				
25	*Mallotus villosus*	3.20			0.10	0.13	0.35	0.88
26	*Merluccius polli*	4.50		0.05				
27	*Merluccius senegalesis*	4.50		0.05				
28	*Merluccius* sp.	4.40				0.19		
29	*Micromesistius* sp.	4.00					0.07	
30	*Opisthonema libertate*	2.90				0.03		
31	Pleuronectinae	3.30			0.03			
32	*Pomadasys incisus*	3.80		0.04				
33	*Rheocles* sp *or R.lateralis*	3.00		0.12				
34	*Salmo salar*	4.40					0.20	
35	*Sardina pilchardus*	3.06		0.09			0.28	
36	*Sardinella aurita*	3.40	0.04	0.80		0.11	0.96	
37	*Sardinella longicepcs*	2.41					0.02	
38	*Sardinops* sp.	3.30	0.03					
39	*Scatophagus argus*	3.00		0.03				
40	*Scomber* sp. *or S. australasicus*	4.20					0.11	
41	*Sprattus sprattus*	3.00	0.03		0.13			
42	*Sus scrofa domesticus*	2.50			0.05		0.05	

(Continues)

TABLE 2 (Continued)

			TLA of aquafeed products					
			Meals				Feeds	
a/a	Species/Taxon	TLN	M1	M2	M3	M4	F1	F2
43	*Tetragonurus cuvieris*	3.80		0.04				
44	*Thunus* sp	4.30						0.04
45	*Trachurus longimanus*	3.40		0.07				
46	*Trachurus mediterraneus*	3.60		0.48			0.07	
47	*Trichiurus lepturus*	4.50	0.09	0.92			0.08	
48	*Trisopterus esmarkii*	3.20					0.06	
49	*Triticum* sp. *or T. aestivum or T. urartu*	1.00					0.03	0.03
	Total number of species/taxon detected		8	21	12	8	21	8
	Sum of TL_N x proportional number of clones for a given species		**3.15**	**3.63**	**2.90**	**2.92**	**3.19**	**2.98**

TABLE 3 Type and formulation of the diets of five farmed fish species of Greek mariculture, trophic level of each species (TL_Fs) feeding on aquafeeds formulated by fishmeals (and oils) having TL_Ms values from 2.90 to 3.63 (M3, M4, M1, M2), mean trophic level of farmed species (TL_F (SE), trophic level of the species in their natural environment (TL_N) and percentage of difference of TL_F from TL_N values (100*(TL_Fs–TL_N)/TL_N)

	Ingredients (%)				TL_Fs						
Species	FM	PM	FO	W	M3 (2.90)	M4 (2.92)	M1 (3.15)	M2 (3.63)	Mean TL_F (SE)	TL_N	100*[(TL_Fs–TL_N)/TL_N] (%)
Sparus aurata	50.1	23.5	14.0	11.7	4.20	4.22	4.45	4.92	4.45 (0.2)	3.26	36.4
Dicentrarchus labrax	56.1	16.1	7.1	18.2	3.68	3.69	3.88	4.27	3.88 (0.1)	3.79	2.4
Pagellus erythrinus	69.9	0.0	3.1	26.5	3.60	3.62	3.80	4.19	3.80 (0.1)	3.40	11.8
Dentex dentex	71.3	0.0	15.3	8.2	4.68	4.70	4.99	5.58	4.99 (0.2)	4.50	10.8
Pagrus pagrus	66.7	22.3	6.5	0.0	3.81	3.82	4.03	4.46	4.03 (0.2)	3.65	10.4

FM: fish meal; FO: fish oil; PM: plant material; W: wheat.

low number of species used to produce each feed could explain the difference in the total number of species identified.

The results clearly show that fish are the main animal constituents of fish feeds employed in Greek aquaculture. Even though the small pelagic fish (i.e., *E. ringens*, *E. encrasicolus*, *C. mysticetus*, *M. villosus*, *S. aurita*, *T. mediterraneus*, *etc)* derived from marine fisheries were the main fraction of fish constituents, a great number of non pelagic fish species (i.e., *Trichiurus lepturus*, *Salmon salar*, *Merluccius* sp., *Coryphaena* sp.) with high or low proportional frequencies constitute remarkable portion of the meals. These results are in accordance with FAO and IFFO trade and production databases, that fish meals contain a wide range of fish species (Péron, François Mittaine, & Le Gallic, 2010). Those species are probably derived as bycatches of fisheries or wastes of the fish processing industry in such a case, their use would not necessarily be a cause of increasing fishing pressure (Ardura et al., 2012). The limited number of common species as well as the big differences in the representation of each common species among the four fish meals and among the two fish feed (seven and three, respectively) reflects probable differences in the producing countries related to annual catches per species used and thus to the availability of fish resources.

Most of the species identified belong to high trophic levels of the marine fish community, being predators or high predators. Catches of those predators are increasing during last decade as a result of an increasing fisheries pressure. The intensive exploitation of fish communities often leads to reductions in the abundance of target species to drastically altering biodiversity (Tisdell, 2003). The overexploitation of high trophic level predators for feeding farmed species decreases the mean trophic level of fish worldwide (the fishing down phenomenon, (Pauly & Watson, 2005; Pinnegar, Jennings, O'Brien, & Polunin, 2002)). In a similar way the overexploitation of low trophic levels (like *Engraulis* sp. that reached 91.6% in M1) fish could have significant impacts on other parts of the ecosystem i.e., on the larger commercially harvested species, on other fish and marine mammals particularly when they constitute a high proportion of the biomass in the ecosystem or are highly connected in the food web (Smith et al., 2011). The overexploitation of fish species for feeding farmed fish raises concerns about the sustainability of fish meal production practices. Similarly to (Ardura et al., 2012) the fish meals used in Greek mariculture contribute at least to some extend to increase fisheries effort and their concomitant risk of fish overexploitation.

Using the TL_F to examine potential farming up trends caused by the fish meal (and corresponded oil) portions in fish feeds (Tsikliras et al., 2014) we demonstrated here that feed products employed in Greek mariculture still contain large portions of fish meals and cause

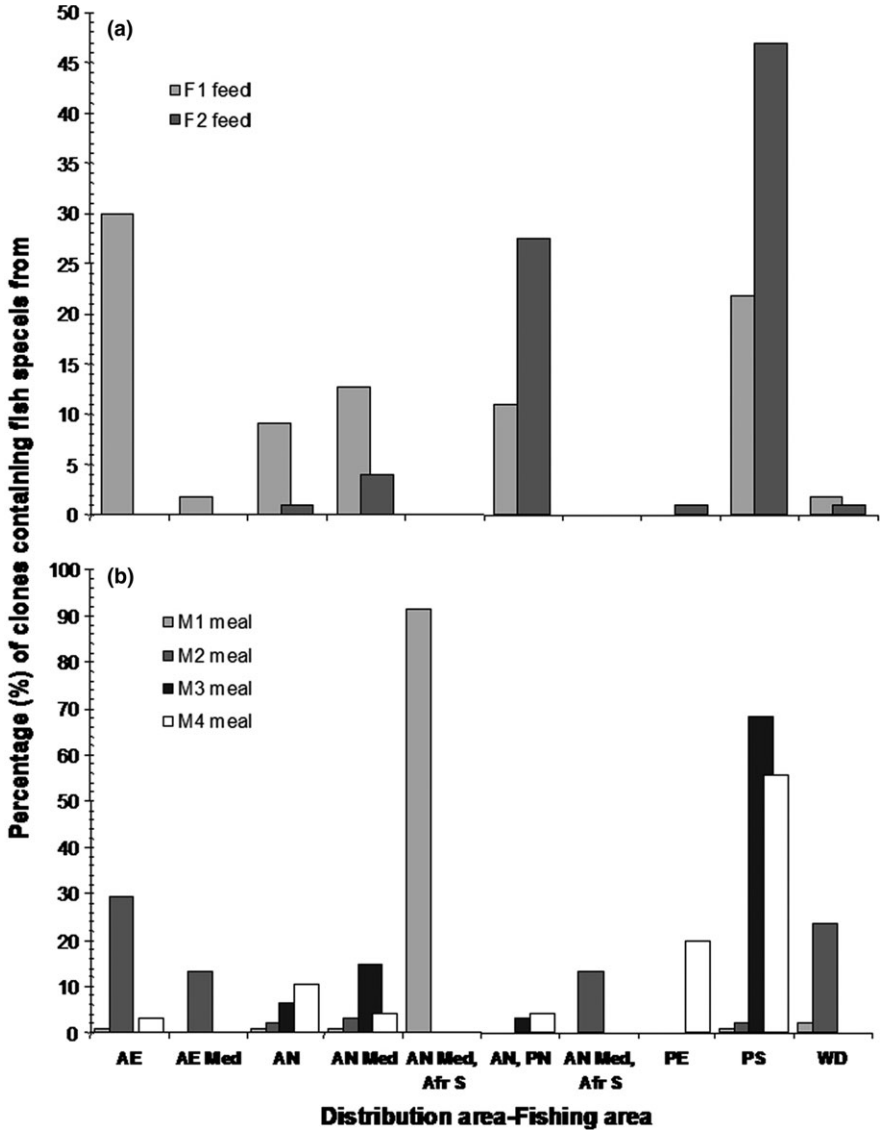

FIGURE 3 Geographic distribution (%) of the main fish species detected in fish feeds (a) and fish meals (b) based on the proportional number of clones containing given fish species originated from a given geographic-fishing area. AE: Atlantic East; AE Med: Atlantic east, Mediterranean; AN: Atlantic north; AN Med: Atlantic north, Mediterranean; Afr S: Atlantic north, Mediterranean, Africa south; AN, PN: Atlantic north, Pacific north, AN Med; Afr S: Atlantic north east, Africa south west, Mediterranean; PE: Pacific east; PS: Pacific south; WD: World distribution

a farming up trend (Pauly, Tyedmers, & Froese, 2001), a common trend already reported in many parts including the Mediterranean (Ardura et al., 2012; Tsikliras et al., 2014). Further reduction in fish meal (and oil) or replacement of fish meal portion by plant proteins but also the promotion of more efficient use of living aquatic resources (Tsikliras et al., 2014) are necessary to generate viable strategies to decrease the risk of aquaculture industry on the marine environment (Mente, Karalazos, & Karapanagiotidis, 2011).

Since aquaculture relies mainly on world fisheries production for feeding cultured species, identifying the origin of fish meal is crucial for understanding the effects of this new pressure on the marine ecosystem. Based on the geographical distribution of fish species we assumed that SE Pacific, NE Atlantic and Mediterranean Sea are the fishing areas more exploited for the production of the analysed feed products. These results are in accordance with landings data for the main small pelagic fish species targeted in industrial fisheries in major producing countries of fish meal and oil products like Peru, Chile, South Africa, Morocco, etc (Péron et al., 2010).

4.2 | Reliability of the PCR-cloning methodology and quantification of species composition

PCR-cloning is a sensitive methodology and has proven capable to identify 89% of sequences to species and only 11% to genus/family level. Moreover, both the plant and animal species were detected as alternative protein sources. The use of universal primers that can successfully amplify 16s rRNA mtDNA gene even from the highly processed fish meals in combination with the high number of clones analyzed overcame limited drawbacks of the method (loss of some species present in the sample during the DNA isolation, PCR amplification and cloning of PCR products), and provided an accurate methodology for the quantification of species composition. However, since the number of mitochondrial targets available is largely tissue-dependent, more research is needed to be able to provide an accurate quantification of fish meal composition.

PCR-cloning methodology was proven capable to detect traces of pork (meal 3, 2.11%) that is totally forbidden. This could be due

either to intentional use for economic reasons or due to contamination in the production process (i.e., M3 meal). Animals such as pork and chicken were also detected in feed (around 3.5%) but their use is allowed in aquafeed production (EU legislation 58/2013). Although EU banned the use of non-ruminant species (i.e., cow and sheep), they have been observed in some fish meals analyzed (Doosti et al., 2014). This was not our case since all the species detected in feed of this study where in the frame of EU legislation. However, we should mention that although the use of gastropod (sea snails; *Bulla* sp., constituent of M4 meal) is not forbidden, various gastropods are known to accumulate toxins harmful for human health (e.g., cone snail; (Nelson, 2004)) and thus their use in aquafeeds production raises concerns about risks of feeding farmed fish.

Plants such as wheat and soya were also identified in the fish feeds analyzed. The replacement of fish with plant protein is a common practice in an attempt to move towards the use of more sustainable aquafeeds that will contain less fish meal portion thus decreasing feed cost and overexploitation of fishery resources. A number of plant species (i.e., soya, barley, pea and canola products) have been tested in an attempt to maintain high fish growth performance at the same time (Barrows, Gaylord, Stone, & Smith, 2007; Forster, Higgs, Dosanjh, Rowshandeli, & Parr, 1999; Gaylord & Barrows, 2009; Thiessen, Campbell, & Adelizi, 2003). A reliable DNA methodology is needed to detect and quantify DNA plant protein sources in aquafeeds aiming to examine their nutritional value in the diet of large sized farmed fish. The PCR-cloning methodology used in this study has the disadvantage that the 16s rRNA gene is not the suitable DNA barcoding marker for the identification of plant species. Moreover, the primers used could amplify the chloroplastic 16S rRNA of only some higher plant species (Bendich & McCarthy, 1970). Thus, we were unable for an accurate identification of plant species in fish feeds. For this reason, specific primers for the amplification and detection and a QC-PCR methodology for a more accurate quantification of plant species in fish feeds are needed. Additionally, advanced technologies like NGS sequencing can increase the resolution of fish meal analysis since they are faster and even more informative than cloning, since they can detect also low-represented species in mixtures as well as plants and they can lead to relative quantification of detected species (Giusti, Armani, & Sotelo, 2017; Ribani et al., 2018). However, they are still costly and they cannot yet be considered enough mature to be applied as routine method. More studies aimed at improving its accuracy as well as correcting their error sources are needed (Zaiko et al., 2015). For these reasons, classical DNA barcoding methodologies as the one used in the present study are still of high preference by the researchers (e.g. Hu, Huang, Hanner, Levin, & Lu, 2018).

5 | CONCLUSIONS

Although fish meal production is expected to remain around current levels and alternative protein sources are increasingly used, fishmeal will continue to be a strategic ingredient for farmed species, when optimum performance is required. Although quality and price were the main determinants for fishmeal purchasers in the aquafeeds industry since now, there is a growing need for fish feed producers and farmers to demonstrate that raw materials in aquafeeds are being responsibly sourced. Methodologies such as the PCR-cloning methodology presented here, or new ones based on next generation sequencing analyses allow for a robust description of aquafeeds composition providing information about species presence in aquafeeds, geographical origin of raw materials, legal or illegal species adulteration. Such methodologies are needed to certify aquafeeds now entering the market, allowing fish farmers to demonstrate their commitment to sustainable aquaculture.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

ORCID


Nikoleta Karaiskou http://orcid.org/0000-0003-0464-7154

REFERENCES

Allan, G. L., Parkinson, S., Booth, M. A., Stone, D. A. J., Rowland, S. J., Frances, J., & Warner-Smith, R. (2000). Replacement of fish meal in diets for Australian silver perch, Bidyanus bidyanus: I. Digestibility of alternative ingredients. *Aquaculture*, *186*(3–4), 293–310. https://doi.org/10.1016/S0044-8486(99)00380-4

Ardura, A., Horreo, J. L., Hernandez, E., Jardon, A., Pola, I. G., Martinez, J. L., & Garcia-Vazquez, E. (2012). Forensic DNA analysis reveals use of high trophic level marine fish in commercial aquaculture fish meals. *Fisheries Research*, *115–116*, 115–120. https://doi.org/10.1016/j.fishres.2011.08.011

Barrows, F. T., Gaylord, T. G., Stone, D. A. J., & Smith, C. E. (2007). Effect of protein source and nutrient density on growth efficiency, histology and plasma amino acid concentration of rainbow trout (Oncorhynchus mykiss Walbaum). *Aquaculture Research*, *38*(16), 1747–1758. https://doi.org/10.1111/j.1365-2109.2007.01854.x

Bendich, A. J., & McCarthy, B. J. (1970). Ribosomal RNA homologies among distantly related organisms. *Proceedings of the National Academy of Sciences of the United States of America*, *65*(2), 349–356. https://doi.org/10.1073/pnas.65.2.349

Doosti, A., Ghasemi Dehkordi, P., & Rahimi, E. (2014). Molecular assay to fraud identification of meat products. *Journal of Food Science and Technology*, *51*(1), 148–152. https://doi.org/10.1007/s13197-011-0456-3

Farajollahi, H., Aslaminejad, A. A., Nassiry, M. R., Sekhavati, M. H., Mahdavi, M., & Javadmanesh, A. (2009). Development and use of quantitative competitive PCR assay for detection of poultry DNA in fish meal. *Journal of Animal and Feed Sciences*, *18*, 733–742. https://doi.org/10.22358/jafs/66447/2009

Forster, I., Higgs, D. A., Dosanjh, B. S., Rowshandeli, M., & Parr, J. (1999). Potential for dietary phytase to improve the nutritive value of canola protein concentrate and decrease phosphorus output in rainbow trout (Oncorhynchus mykiss) held in 11 C fresh water. *Aquaculture*, *179*(1–4), 109–125. https://doi.org/10.1016/S0044-8486(99)00156-8

Froese R. & Pauly D. (Eds). (2018). FishBase (version Feb 2018). In: Y. Roskov, G. Ower, T. Orrell, D. Nicolson, N. Bailly, P. M. Kirk, T. Bourgoin, R. E. DeWalt, W. Decock, E. van Nieukerken, J. Zarucchi & L. Penev (Eds.), *Species 2000 & ITIS Catalogue of Life, 30th October*

2018. Digital resource at www.catalogueoflife.org/col. Species 2000. Leiden, the Netherlands: Naturalis.

Gaylord, T. G., & Barrows, F. T. (2009). Multiple amino acid supplementations to reduce dietary protein in plant-based rainbow trout, Oncorhynchus mykiss, feeds. *Aquaculture*, *287*(1–2), 180–184. https://doi.org/10.1016/j.aquaculture.2008.10.037

Giusti, A., Armani, A., & Sotelo, C. G. (2017). Advances in the analysis of complex food matrices: Species identification in surimi-based products using Next Generation Sequencing technologies. *PLoS ONE*, *12*(10), e0185586. https://doi.org/10.1371/journal.pone.0185586

Gomes, E. F., Rema, P., & Kaushik, S. J. (1995). Replacement of fish meal by plant proteins in the diet of rainbow trout (Oncorhynchus mykiss): Digestibility and growth performance. *Aquaculture*, *130*(2–3), 177–186. https://doi.org/10.1016/0044-8486(94)00211-6

Horreo, J. L., Ardura, A., Pola, I. G., Martinez, J. L., & Garcia-Vazquez, E. (2013). Universal primers for species authentication of animal foodstuff in a single polymerase chain reaction. *Journal of the Science of Food and Agriculture*, *93*(2), 354–361. https://doi.org/10.1002/jsfa.5766

Hu, Y., Huang, S. Y., Hanner, R., Levin, J., & Lu, X. (2018). Study of fish products in Metro Vancouver using DNA barcoding methods reveals fraudulent labeling. *Food Control*, *94*, 38–47. https://doi.org/10.1016/J.FOODCONT.2018.06.023

Huson, D. H., Auch, A. F., Qi, J., & Schuster, S. C. (2007). MEGAN analysis of metagenomic data. *Genome Research*, *17*(3), 377–386. https://doi.org/10.1101/gr.5969107

Luo, J. Q., Wang, J. Q., Bu, D. P., Dan, L. I., Li, W. A. N. G., Wei, H. Y., & Zhou, L. Y. (2008). Development and application of a PCR approach for detection of bovis, sheep, pig, and chicken derived materials in feedstuff. *Agricultural Sciences in China*, *7*(10), 1260–1266. https://doi.org/10.1016/S1671-2927(08)60173-X

Mente, E., Karalazos, V., Karapanagiotidis, I. T., & Pita, C. (2011). Nutrition in organic aquaculture: An inquiry and a discourse. *Aquaculture Nutrition*, *17*, 798–817. https://doi.org/10.1111/j.1365-2095.2010.00846.x

Nagase, M., Maeta, K., Aimi, T., Suginaka, K., & Morinaga, T. (2009). Authentication of flying-fish-meal content of processed food using PCR-RFLP. *Fisheries Science*, *75*(3), 811–816. https://doi.org/10.1007/s12562-009-0097-x

Naylor, R. L., Goldburg, R. J., Primavera, J. H., Kautsky, N., Beveridge, M. C., Clay, J., … Troell, M. (2000). Effect of aquaculture on world fish supplies. *Nature*, *405*(6790), 1017–1024. https://doi.org/10.1038/35016500

Nelson, L. (2004). Venomous snails: One slip, and you're dead. *Nature*, *429*(6994), 798–799. https://doi.org/10.1038/429798a

Pauly, D., Christensen, V., Dalsgaard, J., Froese, R., & Torres, F. (2001). Fishing down and farming up the food web. *Conservation Biology in Practice*, *2*(4), 25.

Pauly, D., & Watson, R. (2005). Background and interpretation of the "Marine Trophic Index" as a measure of biodiversity. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *360*(1454), 415–423. https://doi.org/10.1098/rstb.2004.1597

Péron, G., François Mittaine, J., & Le Gallic, B. (2010). Where do fishmeal and fish oil products come from? An analysis of the conversion ratios in the global fishmeal industry. *Marine Policy*, *34*(4), 815–820. https://doi.org/10.1016/j.marpol.2010.01.027

Pinnegar, J. K., Jennings, S., O'Brien, C. M., & Polunin, N. V. C. (2002). Long-term changes in the trophic level of the Celtic Sea fish community and fish market price distribution. *Journal of Applied Ecology*, *39*(3), 377–390. https://doi.org/10.1046/j.1365-2664.2002.00723.x

Ribani, A., Schiavo, G., Utzeri, V. J., Bertolini, F., Geraci, C., Bovo, S., & Fontanesi, L. (2018). Application of next generation semiconductor based sequencing for species identification in dairy products. *Food Chemistry*, *246*, 90–98. https://doi.org/10.1016/j.foodchem.2017.11.006

Santaclara, F. J., Espiñeira, M., Cabado, A. G., & Vieites, J. M. (2007). Detection of land animal remains in fish meals by the polymerase chain reaction-restriction fragment length polymorphism technique. *Journal of Agricultural and Food Chemistry*, *55*(2), 305–310. https://doi.org/10.1021/jf061840l

Shepherd, C. J., & Jackson, A. J. (2013). Global fishmeal and fish-oil supply: Inputs, outputs and marketsa. *Journal of Fish Biology*, *83*(4), 1046–1066. https://doi.org/10.1111/jfb.12224

Sitja-Bobadilla, A., Pena-Llopis, S., Gomez-Requeni, P., Medale, F., Kaushik, S., & Perez-Sanchez, J. (2005). Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (Sparus aurata). *Aquaculture*, *249*, 387–400. https://doi.org/10.1016/j.aquaculture.2005.03.031

Smith, A. D. M., Brown, C. J., Bulman, C. M., Fulton, E. A., Johnson, P., Kaplan, I. C., … Tam, J. (2011). Impacts of fishing low-trophic level species on marine ecosystems. *Science*, *333*(6046), 1147–1150. https://doi.org/10.1126/science.1209395

Stergiou, K. I., Tsikliras, A. C., & Pauly, D. (2009). Farming up mediterranean food webs. *Conservation Biology*, *23*(1), 230–232. https://doi.org/10.1111/j.1523-1739.2008.01077.x

Tacon, A. G. J., & Metian, M. (2008). Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. *Aquaculture*, *285*(1–4), 146–158. https://doi.org/10.1016/j.aquaculture.2008.08.015

Teletchea, F. (2009). Molecular identification methods of fish species: Reassessment and possible applications. *Reviews in Fish Biology and Fisheries*, *19*(3), 265–293. https://doi.org/10.1007/s11160-009-9107-4

Thiessen, D. L., Campbell, G. L., & Adelizi, P. D. (2003). Digestibility and growth performance of juvenile rainbow trout (Oncorhynchus mykiss) fed with pea and canola products. *Aquaculture Nutrition*, *9*(2), 67–75. https://doi.org/10.1046/j.1365-2095.2003.00203.x

Tisdell, C. (2003). Socioeconomic causes of loss of animal genetic diversity: Analysis and assessment. *Ecological Economics*, *45*(3), 365–376. https://doi.org/10.1016/S0921-8009(03)00091-0

Trujillo, P., Piroddi, C., & Jacquet, J. (2012). Fish farms at Sea: The ground truth from Google Earth. *PLoS ONE*, *7*(2), e30546. https://doi.org/10.1371/journal.pone.0030546

Tsikliras, A. C., Stergiou, K. I., Adamopoulos, N., Pauly, D., & Mente, E. (2014). Shift in trophic level of mediterranean mariculture species. *Conservation Biology*, *28*(4), 1124–1128. https://doi.org/10.1111/cobi.12276

von Holst, C., Baeten, V., Boix, A., Slowikowski, B., Fernández Pierna, J. A., Tirendi, S., ... Dardenne, P. (2008). Transferability study of a near-infrared microscopic method for the detection of banned meat and bone meal in feedingstuffs. *Analytical and Bioanalytical Chemistry*, *392*(1-2), 313–317. https://doi.org/10.1007/s00216-008-2232-4

Zaiko, A., Martinez, J. L., Ardura, A., Clusa, L., Borrell, Y. J., Samuiloviene, A., … Garcia-Vazquez, E. (2015). Detecting nuisance species using NGST: Methodology shortcomings and possible application in ballast water monitoring. *Marine Environmental Research*, *112*, 64–72. https://doi.org/10.1016/J.MARENVRES.2015.07.002

How to cite this article: Vlachavas A, Karaiskou N, Kokokiris L, Zampeta F-I, Drosopoulou E, Triantafyllidis A. Using genetic methods for analysis of fish meals and feeds employed in Greek mariculture. *Aquac Res*. 2019;50:312–322. https://doi.org/10.1111/are.13900