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Identification of tropical shrimp species by RFLP and SSCP analysis of mitochondrial genes

Identifizierung tropischer Garnelenarten durch RFLP- und SSCP-Analyse mitochondrialer Gene

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Summary

Wild and farmed tropical shrimps form an important market share of seafood trade. Many of these species are differing in sensory quality, size and price. PCR-based methods have been developed for differentiation of nine penaeid shrimp species. Restriction fragment length polymorphism (RFLP) analysis of two amplicons (312 bp, 16S rRNA gene; 558 bp, cytochrome oxidase subunit I gene) was used to identify two commercially important species, *Penaeus monodon* and *Litopenaeus vannamei*. Differentiation between these two species and seven other species was achieved by single strand conformation polymorphism (SSCP) analysis of PCR products (134 bp and 312 bp) obtained from the 16S rRNA gene. Plasmids containing the 312 bp amplicon were constructed to serve as reference material in SSCP analysis. Whereas the SSCP patterns of the 312 bp amplicon expressed some intra-species variability, the patterns obtained for the short amplicon showed no variation between different specimens of a given species.

Keywords: shrimp, PCR, RFLP, SSCP, cytochrome oxidase I, 16S rRNA

Zusammenfassung

Wilde und gefarmte tropische Garnelen haben einen bedeutenden Anteil am internationalen Handel mit Meeresfrüchten. Dabei können zwischen den einzelnen Garnelenarten beträchtliche Unterschiede in Qualität und Preis auftreten. Zur Differenzierung zwischen neun Garnelenarten aus der Familie der Penaeidae (Geißelgarnelen) wurden folgende PCR-Methoden eingesetzt: (i) RFLP-Analyse (Restriktionsfragment-Längenpolymorphismus-Analyse) von zwei Amplikons (16S rRNA-Gen, 312 bp; Cytochromoxidase I-Gen, 558 bp) zur Identifizierung der beiden bedeutenden Handelsarten *Penaeus (P.) monodon* und *Litopenaeus (L.) vannamei*. (ii) Eine Differenzierung zwischen diesen beiden und sieben weiteren kommerziellen Garnelenarten gelang durch SSCP-Analyse (Einzelstrang-Konformationspolymorphismus-Analyse) von Amplikons aus dem 16S rRNA-Gen (134 bzw. 312 bp). Als Referenzmaterial für die SSCP-Analyse wurden Plasmide hergestellt, die das 312 bp-Amplikon von *P. monodon* oder *L. vannamei* enthielten. Während die SSCP-Muster des größeren (312 bp) Amplikons häufig eine intraspezifische Variabilität aufwiesen, waren die Muster bei dem kürzeren Amplikon innerhalb einer Spezies konstant.

Schlüsselwörter: Garnelen, PCR, RFLP, SSCP, Cytochromoxidase I, 16S rRNA

Introduction

Shrimps and prawns are an important segment of aquatic food with an annual production of 3 460 000 t in 2006 (FAO, 2006). Several hundred shrimp species are used for human consumption worldwide differing by origin (warm- or cold-water shrimps; marine shrimps, brackish water or freshwater shrimps) and production method (farmed versus wild catch) (Holthuis, 1980).

Many species are differing in sensory quality and price. After removal of external characteristics by peeling the shrimps it is very difficult to identify the species. The consumer can hardly recognize which species is offered in shops or restaurants; many consumers are also unable to distinguish between shrimp meat and surimi-based imitation crab meat, which are esteemed components of seafood salad or other dishes.

Against this background it is not very surprising that cases of mislabelling have been reported (Jacquet and Pauly, 2008; Pascoal et al., 2008a). Recently, a number of PCR-based methods have been developed for identification of shrimp meat. Rehbein (2001) made a comparative study of different methods of protein and DNA analysis to identify shrimp. Protein separation by isoelectric focusing (IEF) was of limited success in differentiation of shrimps, whereas PCR-based techniques (restriction fragment length polymorphism (RFLP) and single strand conformation polymorphism (SSCP)) applied to mitochondrial genes allowed to distinguish between a number of species. Khamnamtong et al. (2005) also used SSCP and RFLP analysis to differentiate between five penaeid shrimp species, whereas Brzezinski (2005) identified eleven crustacean species by RFLP analysis of a 205 bp fragment of the 16S rRNA gene. Later, Pascoal et al. (2008b) developed a PCR-RFLP protocol for the identification of penaeid shrimps in raw and processed products. A ~525 bp fragment of the 16S rRNA/tRNA^{Val} mitochondrial gene was amplified and cut by three endonucleases.

Phylogenetic analysis had been performed to clarify genetic relationship between penaeid shrimps (Tong et al., 2000; Quan et al., 2004), a topic which still is under discussion (Dall, 2007; Flegel, 2007).

The aim of our study was to test the suitability of RFLP and SSCP for differentiation of shrimp species relevant to the German market, to prepare a plasmid reference material to be used in SSCP analysis and to develop a PCR system with a short amplicon for analysis of highly processed shrimp.

Material and Methods

Collected samples

Samples of 15 tropical shrimp species, which are of commercial interest in Germany and other countries, were collected. Raw eviscerated shrimps, either fresh or deep-frozen, were purchased from fish farmers, at the fish market, from wholesale traders or in retail shops. A number of species could be identified by taxonomic analysis (Dall et al., 1991), but in case of samples without sufficient morphological characteristics for identification, species assignment was checked by sequencing a 134 bp fragment of the 16S rRNA gene and comparison to sequences deposited in GenBank by performing BLAST (Tab. 1A or authenticated species).

Six samples (listed in Tab. 1B), which could not be completely identified by this procedure, were nevertheless included in the further analysis to get information on the possible types of RFLP and SSCP patterns of traded shrimps.

DNA isolation

DNA was isolated from 70 to 100 mg of the tail muscle of shrimps using the cationic detergent cetyltrimethylammonium bromide (CTAB) (Rehbein, 2005). The DNA content of extracts was estimated by measuring the DNA-dependent enhancement of fluorescence intensity of Hoechst

TABLE 1A: *List of authentic samples*

Code	Origin	Product	Latin name	English name	Authentication
1	Fish market	cp/rp/r*	*Penaeus monodon*	Black tiger shrimp (prawn)	BLAST: 100 % id to FJ435645.1
2	Fish market/wholesale trader/fish farmer	cp/rp/r	*(Lito)penaeus vannamei*	Pacific white shrimp	vi**, BLAST: 100 % id to DQ534543.1
3	Wholesale trader	r	*(Fennero)penaeus*	indicus Indian white prawn	vi, BLAST: 100 % id to FJ002574.1
4	Fish farmer	r	*(Marsu)penaeus japonicus*	Kuruma prawn	vi
5	Wholesale trader	r	*Penaeus semisulcatus*	Green tiger prawn	vi
6	Research vessel	rp	*Parapenaeus longirostris*	Deep-water rose shrimp	vi
7	Wholesale trader	r	*Parapenaeopsis sculptilis*	Rainbow shrimp	vi
8	Wholesale trader/supermarket	cp/r	*Metapenaeus monoceros*	Speckled shrimp	vi
9	Retail trader	r	*Macrobrachium rosenbergii*	Giant river prawn	BLAST: 100 % id to FJ595481.1

*r = raw, c = cooked, p = peeled, ** vi = visual inspection of a whole animal.

TABLE 1B: *List of other commercial samples (species not identified) from the German market*

Code	Origin	Product	Labelling (Latin name)	English name	Result of BLAST
10	Retail trader	r*	*Penaeus notialis*	Southern pink shrimp	100 % id to AF192056.1 *(Penaeus duorarum)*
11	Wholesale trader	r	*Hymenopenaeus muelleri*	Argentine red shrimp	95 % id to AF335280.2 *(Fenneropenaeus merguiensis)*
12	Wholesale trader	cp	*Heterocarpus reedi*	Nylon shrimp	95 % id to AY612879.1 *(Heterocarpus laevigatus)*
13	Wholesale trader	cp	*Solenocera agassizii*	Kolibri shrimp	95 % id to FJ435644.1 *(Marsupenaeus japonicus)*
14	Wholesale trader	rp	*Solenocera melantho*	Razor mud shrimp	97 % id to AF279828.1 *(Penaeus esculentus)*
15	Wholesale trader	r	*Parapenaeopsis hardwickii*	Spear shrimp	97 % id to FJ435640.1 *(Parapenaeopsis hardwickii)*

*r = raw, c = cooked, p = peeled.

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33258 against a calf thymus DNA standard (Downs and Wilfinger, 1983).

PCR conditions

Three primer pairs were used to amplify different segments of the mitochondrial 16S rRNA and cytochrome oxidase subunit I (COX I) genes.

For all amplifications, DNA concentration in the PCR assay was adjusted to 1 ng DNA/?l; the primer concentration was 0.5 µM. PCR was performed with reagents from Solis BioDyne (Tartu, Estonia) using HotFirePol DNA polymerase I (final activity 2.5 units), BD Buffer (5 µl/assay), dNTP mix (200 µM final concentration), $MgCl_2$ (2.5 mM final concentration); the assay volume was 50 µl.

Amplification of a 312 bp fragment of the 16S rRNA gene (Khamnamtong et al., 2005): primer 16S 312F: 5' – GRA GGC TTG TAT GAA TGG TTG – 3', primer 16S 312R: 5' – AAG AAG ATT ACG CTG TTA – 3'; PCR conditions: preheating 15 min/95 °C, 35 cycles of 1 min/94 °C, 1 min/53 °C, 1 min/72 °C, final heating 7 min/72 °C.

Amplification of a 558 bp fragment of the mitochondrial COX I gene (Rehbein, 2001): primer IFF15: 5' – TTC GGT CAY CCA GAA GTM TAT – 3', primer IFF 16: 5' – TAA GCG TCT GGG TAG TCT GAR TAK CG – 3'; PCR conditions: preheating 15 min/95 °C, 35 cycles of 40 s/94 °C, 80 s/47 °C, 80 s/72 °C, final heating 10 min/72 °C.

Amplification of a 134 bp fragment of the 16S rRNA gene: primer BFEL28: 5' – AGT TAC TTT AGG GAT AAC AGC – 3', primer BFEL29: 5' – RRW TTT WAR RGT CGA ACA GAC – 3'; PCR conditions: preheating 15 min/95 °C, 35 cycles of 1 min/94 °C, 1 min/54 °C, 1 min/72 °C, final heating 10 min/72 °C.

Agarose gel electrophoresis

Prior to RFLP and SSCP analysis PCR results were checked by agarose gel electrophoresis; the gels contained 2 % agarose, PCR products were visualised by staining with ethidiumbromide (0.1 µg/ml final concentration).

Restriction fragment length polymorphism (RFLP) analysis

Without further purification, PCR products were digested with different commercially available enzymes: *Alu*I, *Vsp*I, *Sscp*I, *Apo*I, *Dde*I and *Rsa*I. Digestion always took place at 37 °C over night, except for *Apo*I (50 °C), then enzymes were inactivated by heating for 20 min at 65 °C. DNA fragments were run on 2 % agarose gels and stained with ethidiumbromide; fragment lengths were calculated by comparison of the position of bands with the DNA marker "100 bp ladder equalized" (Carl Roth, Karlsruhe, Germany) using the GELDOC 2000 System with the Quantity-One-Software (Bio-Rad, München, Germany).

Single strand conformation polymorphism (SSCP) analysis

Preparation of single-stranded DNA

For denaturising amplicons to single-stranded DNA (ssDNA), 5 µl of PCR assay were diluted with 15 µl distilled water, heated for 5 min at 95 °C, and cooled immediately in iced water.

Native polyacrylamide gel electrophoresis (PAGE) using CleanGel

For separation of ssDNA by native PAGE, 6 µl of the cooled ssDNA solution were applied without delay to the sample wells of a CleanGel 15 % (ETC, Kirchentellinsfurt, Germany), which had been rehydrated with DNA SSCP Gel Buffer (ETC). Rehydration of the gel (with 40 ml of buffer) and soaking of electrode strips with DNA SSCP Electrode Buffer were performed according to the instructions given by ETC (www.etcelpho.com).

Conditions of electrophoresis using Multiphor II (GE Healthcare, Freiburg, Germany)

Sets for running a full-size gel: Starting with 250 V, 22 mA and 10 W for 10 min, followed by 425 V, 30 mA and 26 W for 50 min (total electrophoresis time: 60 min), then 525 V, 30 mA, 26 W, for 60 min (total electrophoresis time: 120 min).

Temperature of the thermostating plate: 15 °C.

Silver staining

The following silver staining protocol (ETC) was used.

Stock solutions:
Fixing concentrate (5x): 15 g benzene sulfonic acid + 120 ml ethanol, filled up to 500 ml with distilled water.
Fixing diluter: 120 ml ethanol, filled up to 500 ml with distilled water.
Washing concentrate (6x): 1.75 g benzene sulfonic acid/500 ml of distilled water.
Silvering concentrate (5x): 5 g $AgNO_3$ + 1.75 g benzene sulfonic acid/500 ml distilled water.
Developing concentrate (5x): 62.5 g Na_2CO_3, filled up to 500 ml with distilled water.
Thiosulfate concentrate: 500 mg sodium thiosulfate pentahydrate/25 ml of distilled water.

Final solutions:
Fixing solution: 40 ml fixing concentrate + 160 ml fixing diluter.
Washing solution: 100 ml washing concentrate + 500 ml distilled water.
Silvering solution: 40 ml silvering concentrate + 160 ml distilled water + 260 ml formaldehyde (37 %, w/v).
Developing solution: 40 ml developing concentrate + 160 ml distilled water + 260 ml formaldehyde (37 %, w/v) + 200 ml thiosulfate concentrate.

Protocol:
In each step the gel was shaken gently in a volume of 200 ml solution. Fixing: 40 min, washing: 3 x 10 min, silvering: 40 min, washing in distilled water: 2 min, developing: 7 min, stopping and preserving: 3 x 10 min. Finally, the gel was dried overnight at ambient temperature and covered with a plastic film.

Sequencing and cloning of amplicon (312 bp) from 16S rDNA gene

DNA sequencing

After purification of PCR products with a Centricon 30 Filter device (Millipore, Schwalbach, Germany), sequencing was done in both directions using the ABI Prism® BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) with the same primer pairs as taken for PCR. After removing surplus dye-labelled dideoxynucleotides with DyeEx 2.0 Spin Kit (Qiagen, Hilden, Germany), the sequencing assays were dried in a vacuum centrifuge. The nucleotide sequences were determined by the University Medical Centre

Hamburg-Eppendorf (Hamburg, Germany) using the ABI Prism® 3100 DNA Sequencer.

Cloning of PCR products:

Two samples of *Penaeus (P.) monodon* with different SSCP patterns and one of *Litopenaeus (L.) vannamei* were selected for cloning. The same purified PCR products as used for sequencing were ligated into pDrive Cloning Vector (Qiagen) and transformed into Qiagen EZ Competent Cells regarding the instructions of the Qiagen PCR Cloning Kit Protocol. Aliquots of those mixtures were plated directly on LB (Luria Bertoni) agar plates containing 1 % (w/v) Trypton, 0.5 % yeast extract, 1 % NaCl and 1.5 % BactoAgar, 0.1 mg/ml ampicillin, 0.08 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl ß-Dgalactopyranoside) and 0.012 mg/ml IPTG (ß-D-thiogalactopyranoside). Inverted plates were incubated at 37°C overnight. White colonies were picked and grown in LB medium. Plasmid DNA was purified following the steps of the Plasmid Purification Kit (Qiagen) and checked by PCR for the presence of the integrated amplicon.

Results and Discussion

Reactivity of PCR primers

Three PCR systems yielding amplicons of different sizes (134, 312 and 558 bp) were tested for their reactivity against nine authenticated tropical shrimp species, as well as six other samples from the German market (Tab. 2). Amplification of the 134 bp fragment (primer pair BFEL 28/29) of the 16S rRNA gene was successful for all species of shrimps analysed. The primer pair 16S 312F/R also reacted with nearly all of the samples, with the exception of a sample tentatively assigned to Heterocarpus reedi, whereas primers IFF 15/16 did not show such a widespread reactivity as shown in Table 2.

TABLE 2: *Reactivity of PCR primers*

Code	Latin name	16S 312 F/R	BFEL 28/29	IFF 15/16
Authentic samples				
1	*Penaeus monodon*	+	+	+
2	*(Lito)penaeus vannamei*	+	+	+
3	*(Fennero)penaeus indicus*	+	+	+
4	*(Marsu)penaeus japonicus*	+	+	–
5	*Penaeus semisulcatus*	+	+	+
6	*Parapenaeus longirostris*	+	+	–
7	*Parapenaeopsis sculptilis*	+	+	–
8	*Metapenaeus monoceros*	+	+	+
9	*Macrobrachium rosenbergii*	+	+	–
Other samples, labelled as*				
10	*Penaeus notialis*	+	+	–
11	*Hymenopenaeus muelleri*	+	+	+
12	*Heterocarpus reedi*	–	+	–
13	*Solenocera agassizii*	+	+	–
14	*Solenocera melantho*	+	+	+
15	*Parapenaeopsis hardwickii*	+	+	–

*species not identified, Table 1B.

The results obtained for the primer pair 16S 312 F/R are in accordance with the findings of Khamnamtong et al. (2005), who received PCR products for five penaeid shrimp species (*P. monodon, P. semisulcatus, Feneropenaeus (F.) merguiensis, L. vannamei* and *Marsupenaeus (M.) japonicus*). In the present study, amplicons were also obtained for five other species *(F. indicus, P. notialis, P. longirostris, P. hardwickii, P. sculptilis)* of the genus *Penaeus* (Flegel, 2007), as well as for products tentatively assigned to the species *Solenocera (S.) agassizii, S. melantho* and *M. rosenbergii.*

The PCR system (primers IFF 15/16) for amplification of a 558 bp sequence of the cytochrome

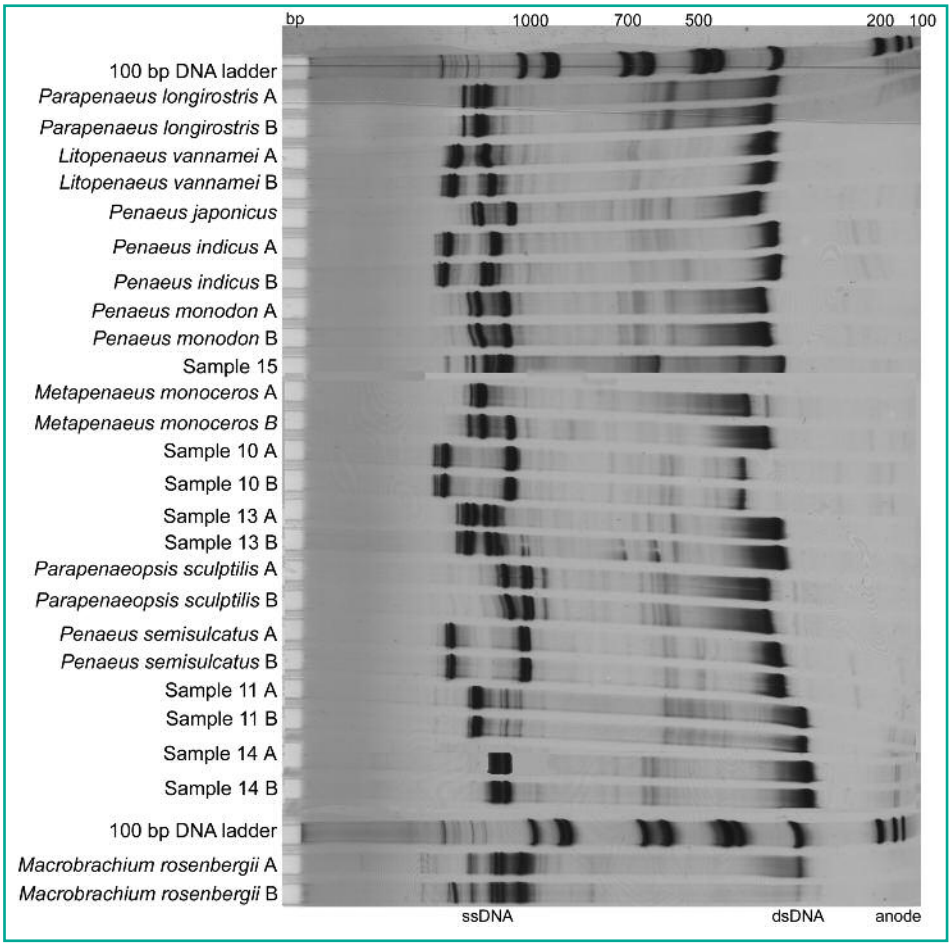

FIGURE 1: *Differentiation of shrimps by SSCP analysis of a 31 2bp sequence of the 16S rDNA gene. "A" and "B" denote different specimens; sample numbers refer to Table 1.*

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oxidase I gene gave products with some shrimp species possessing a high market share (*P. monodon* and *L. vannamei*), but generally turned out to be of a more limited applicability for differentiation of shrimps. Primers IFF 15/16 have the sequence of primers CO9/CO10 constructed by Baldwin et al. (1998), who found amplicons with 13 species of the genus *Penaeus.*

RFLP analysis

For identification of two important tropical shrimps offered on the German market, *P. monodon* and *L. vannamei,* the 312 bp and 558 bp amplicons were subjected to RFLP analysis. The fragment patterns were compared to those of some other shrimp species (Tab. 3). Differentiation between *P. monodon* and *L. vannamei* was easily achieved applying one restriction endonuclease (*Alu*I) for cutting the 312 bp amplicon. Results may be secured by using a second enzyme (*Sscp*I or *Vsp*I); in case of *P. monodon* intra-species variability of fragment patterns was observed. Recently, a survey of authenticity of shrimp products of the Spanish market was performed using RFLP analysis of a 16S rRNA/tRNA^{Val} sequence (Pascoal et al., 2008a). An amplicon of 530 bp in size was cut by three enzymes giving a set of fragments which allowed differentiation between 19 shrimp species. A considerable number of cases of mislabelling, namely for ten out of 41 products, had been detected.

SSCP analysis using the 312 bp amplicon

One of the most sensitive and rapid methods to characterize amplicons is SSCP analysis (Sunnucks et al., 2000). In the present study, the patterns of ssDNA were clearly different between 14 shrimp species analysed (Fig. 1). Polymorphism was observed for several species (*P. longirostris, L. vannamei, P. indicus, M. monoceros* and *M. rosenbergii*). Samples of black tiger shrimp *(P. monodon)* gave three different SSCP patterns (Fig. 2), as reported previously by Khamnamtong et al. (2005). The number of samples analysed in this study may be too small to detect all of the variants existing for the different shrimps found on the market. It has to be expected that application of this method by food control laboratories will result in a collection of various SSCP patterns for the different shrimp species.

The reliability of identification of unknown samples by SSCP analysis is greatly enhanced by running sample and

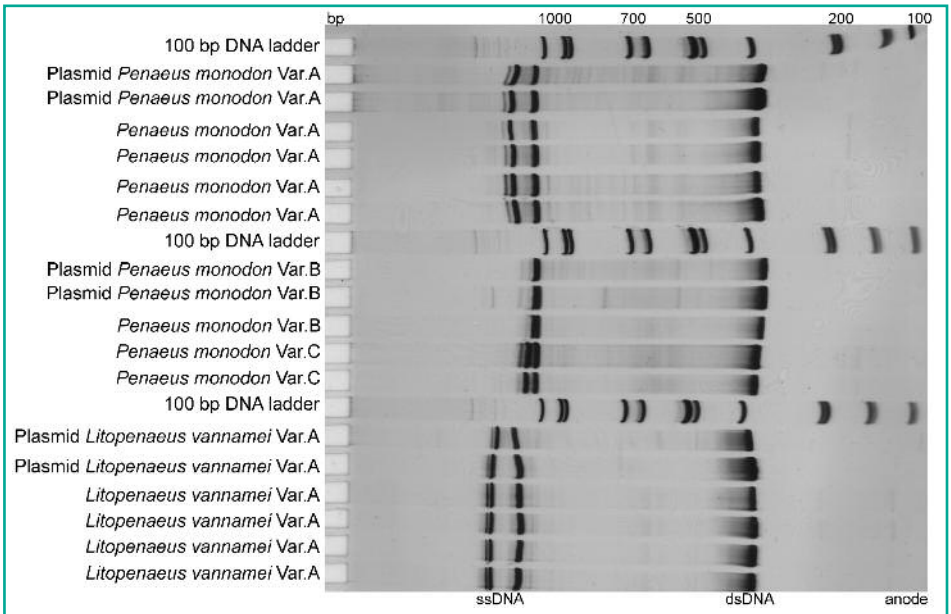

FIGURE 2: *Comparison of ssDNA patterns of shrimps and plasmid standards. Var: variation of SSCP pattern. In case of P. monodon seven specimens had been analysed, and for L. vannamei four samples were compared to the plasmid.*

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                                                                      60
Var-A TCCCTAAAGT AACTTAATCT TTTAATCTTT AATAAAGGAT CAAATATTCT TCAATTATAC
Var-B TCCCTAAAGT AACTTAATCT TTTAATCTTT AATAAAGGAT CAATTATTTT TCAATTATAC
Var-A TCCCTAAAGT AACTTAATCT TATGATCTCT AGTAGAGGAT CATTAATTTT CCAGAAATTT

                                                                     120
Var-A TTGTTAATAA AATATTTAAG AACAGTTAC TAATTATATTC CCGTCGCCCC AACGCAACAA
Var-A TTGTTAATAA G--TATTTAAG AACAGTTAC TAATTATATTC CCGTCGCCCC AACGCAACAA
Var-A CTGTCATTAA ATAACTTAAG AACAGTTAC TTATTATATTC TCGTCGCCCC AACGCAACAA

                                                                     180
Var-A ACATTAATTA AAATCAAGTT ATACTAACAA TTTATAATTT AATCAAATTA TTGTTAAGTT
Var-A ACATTAACTA AAATCAAGTT ATACTAACAA TTTATAATTT AACTAATTTA TTGTCAAGTT
Var-A ACGTTACCTA AACTCAAGTT ATACTAACAA TTTATAATAT AGGTAACTTA TTGTAAAGCT

                                                                     240
Var-A TTATAGGGTC TTATCGTCCC CTTAAAGTAT TTAAGCCTTT TCACTTAAAA GTTAAGTTCA
Var-A TTATAGGGTC TTATCGTCCC CTTAAAGTAT TTAAGCCTTT TCACTTAAAA GTTAAGTTCA
Var-A TTATAGGGTC TTATCGTCCC CTTAATTTAT TTAAGCCTTT TCACTTAAAA GTTAAATTCA

                                                  272
Var-A ACTATTATAAC TGAGACAGAT TACTTTTTG TC          Penaeus monodon
Var-A ACTATTACAAT TGAGACAGAT TACTTTTTG TC          Penaeus monodon
Var-A ATAAATATAAT TGAGACAGTT TGCTTTTTG TC          Litopenaeus vannamei
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FIGURE 3: *Comparison of sequences (without primers) of the 312 bp amplicon obtained from the 16S rDNA gene of P. monodon (variation A, Gen-Bank accession no. FJ976658; variation B, GenBank accession no. FJ976659) and L. vannamei (GenBank accession no. FJ976660). Sequences as obtained with primer 16S 312R.*

TABLE 3: *Fragments obtained by cutting amplicons of the 16S rDNA and the COX I gene with various endonucleases*

Code	Latin name	16S rRNA gene			COX I gene		
		AluI	*SscpI*	*VspI*	*ApoI*	*DdeI*	*RsaI*
Authentic samples							
1	Penaeus monodon	U	208,85	U or 150,125	U	U	340,260 or 275,230
2	(Lito)penaeus vannamei	180,105	U	240,70	U	270,269	260,259
3	(Fennero)penaeus indicus	180,74	ND	ND	U	270,160	345,145
4	(Marsu)penaeus japonicus	180,74	U	U	ND	ND	ND
5	Penaeus semisulcatus	180	U	U	U	315,270	345,260
6	Parapenaeus longirostris	178,100	U	U	ND	ND	ND
7	Parapenaeopsis sculptilis	170,74	U	ND	ND	ND	ND
8	Metapenaeus monoceros	123,74,60	U	U	ND	ND	ND
9	Macrobrachium rosenbergii	ND	ND	ND	ND	ND	ND
Labelling of other samples*							
10	Penaeus notialis	170	ND	235	ND	ND	ND
11	Hymenopenaeus muelleri	ND	ND	ND	U	315,270	ND
12	Heterocarpus reedi	ND	ND	ND	ND	ND	ND
13	Solenocera agassizii	175,105	ND	240	ND	ND	ND
14	Solenocera melantho	ND	ND	ND	U	330,215	U
15	Parapenaeopsis hardwickii	180,74	U	ND	ND	ND	ND

Mean values (n = 2–3) for fragment lengths, as determined by agarose gel electrophoresis, are given in base pairs; relative standard deviation was less than 10 %. ND: not determined, U: uncut amplicon, *species not identified, Table 1B.

reference side-by-side on the same electrophoresis gel. Therefore, plasmids were constructed containing amplicons of the most important species, black tiger shrimp *(P. monodon)* and Pacific white shrimp *(L. vannamei)* to be used as reference material in further studies. Two amplicons (variant A and B) of black tiger shrimp and one amplicon of Pacific white shrimp were cloned into the pDrive Cloning Vector (3.85 kb) and compared to the original nuclear DNA by SSCP. Identical SSCP patterns were obtained for shrimp and accompanying plasmid (Fig. 2), demonstrating the suitability of the plasmids as references.

The nucleotide sequences of the three amplicons are shown in Figure 3. Sequences of variant A and B of *P. monodon* are varying at four positions, whereas the sequence of *L. vannamei* differed in 38 positions from the sequence of *P. monodon* variant A. Comparison of the three sequences with sequences deposited in GenBank by BLAST (3. 7. 2008) gave for *P. monodon* variant A a 99 % sequence identity with >15 sequences of this species; for *P. monodon* variant B a 100 % identity with two deposited sequences was found. The sequence of *L. vannamei* corresponded to 100 % with >15 sequences of this species.

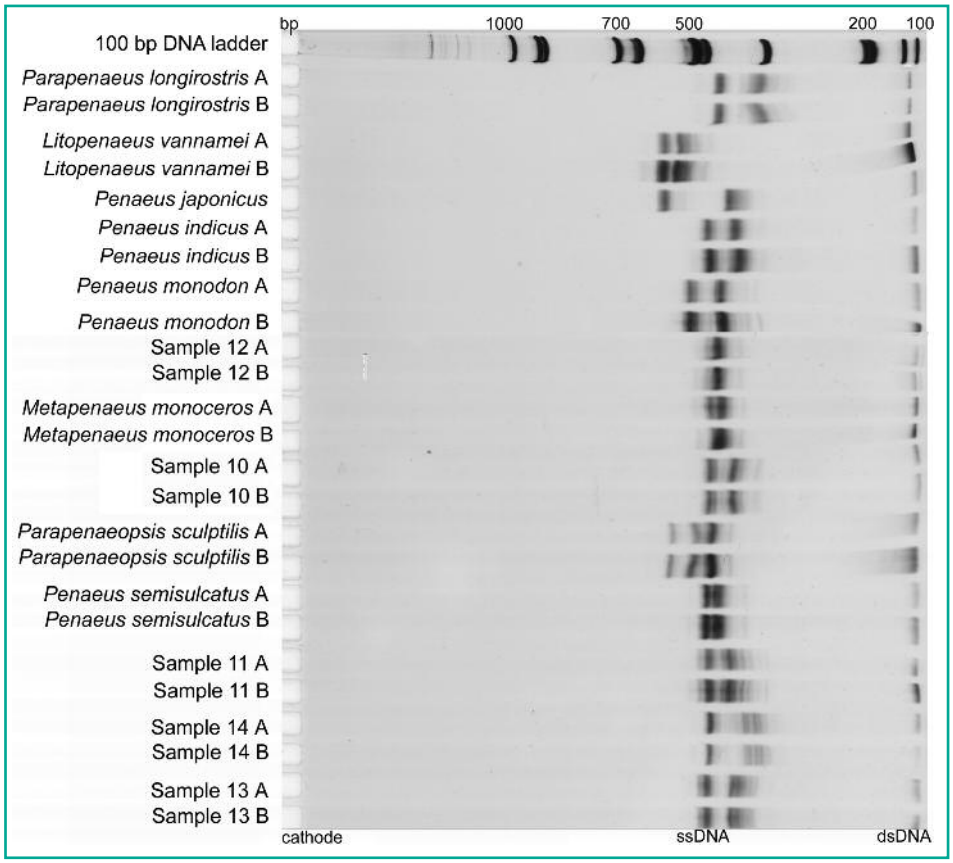

FIGURE 4: *Differentiation of shrimps by SSCP analysis of a 134 bp sequence of the 16S rRNA gene. "A" and "B" denote different specimens, sample numbers refer to Table 1.*

SSCP analysis using the 134 bp amplicon

In order to reduce intraspecies variability of amplicons and to establish a

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PCR system for heavily processed shrimp products like canned shrimp or shrimp meal, universal crustacean primers were constructed to amplify a 134 bp segment of the 16S rRNA gene. It is to be seen from Figure 4 that these PCR worked well with all the shrimp species analysed in this study; furthermore, patterns of ssDNA allowed differentiation between the following species: *F. longirostris, L. vannamei, P. japonicus, P. indicus, P. monodon, P. notialis, P. sculptilis, P. semisulcatus, Hymenopenaeus muelleri, S. melantho, S. agassizii.* Comparison of the pattern shown in Figure 1 and Figure 4 is demonstrating the lower variability of patterns within species.

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