# Short Communication

# A simple genetic identification method for Northeast Atlantic smoothhound sharks (*Mustelus* spp.)

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Considerable ambiguity exists in the identification of the commercially valuable smoothhound sharks (*Mustelus* spp.) in the Northeast (NE) Atlantic. The lack of a clear and accurate method of identification prevents the collation of reliable species-specific landings and survey data for these fish and hinders the accurate delineation of the distribution ranges of species and stock boundaries, making it impossible to apply sound species-specific conservation and management strategies. This paper reports on the development of a multiplex PCR reaction that utilizes a set of mtDNA primers for the identification of *Mustelus asterias*, *Mustelus mustelus*, and *Galeorhinus galeus*. The high throughput method allows for the rapid and cost-effective identification of large numbers of samples; its application to 431 fish collected between 2006 and 2008 also raises important questions regarding the biogeography of the genus *Mustelus* in the NE Atlantic.

**Keywords:** conservation, fisheries management, genetic identification method, mitochondrial DNA, multiplex PCR, *Mustelus*, ND2, sharks.

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## Introduction

Two species of smoothhound sharks are known in the Northeast (NE) Atlantic: the starry smoothhound (*Mustelus asterias*) and the common smoothhound (*Mustelus mustelus*). One other triakid is known in the area, the tope (*Galeorhinus galeus*). *Mustelus asterias* and *M. mustelus* are relatively small demersal sharks that occur in inshore temperate waters of the NE Atlantic, Mediterranean, and Southeast Atlantic (*M. mustelus* only; Compagno, 1984). They feed primarily on crustaceans and have specialized crushing dentition. *Galeorhinus galeus* has a more cosmopolitan distribution and is found widely throughout much of the Atlantic and Pacific Oceans, except in the northwestern waters of both (Compagno, 1984).

There is longstanding confusion regarding the identification and nomenclature of *Mustelus* species (Hubbs, 1938; Wheeler, 1969; Lopez *et al.*, 2006). Based on DNA sequences and reproductive modes, the genus is not monophyletic and can be divided into two clades: the unspotted placental viviparous species including *M. mustelus* and the spotted aplacental viviparous species including *M. asterias* (Lopez *et al.*, 2006). Based on these differences, the distinction between the target species may appear straightforward, but their external morphology is similar. The apparently diagnostic white spots of *M. asterias*, which are generally used as the principal method of distinguishing between the species, are highly variable and may be vivid and well defined, faint, or even absent in some cases (Heemstra, 1973). This scenario is also evident in the closely related starspotted smoothhound (*Mustelus manazo*) in Japanese waters (Teshima and Koga, 1973). As a result, the spots are a poor taxonomic indicator, and other discriminatory methods need to be employed. *Mustelus asterias* and *M. mustelus* may be distinguished based on a number of potentially ambiguous morphological characteristics including the position of fins, the internarial distance, the pattern of buccopharyngeal denticles, and the length of the ridges on the dermal denticles (Quignard and Capape, 1972; Heemstra, 1973). The only definitive method of discrimination is in the uterine connections between embryo and mother, but this is not practical for large fisheries samples, cannot be used for males, and is virtually inapplicable for quick diagnostics on live fish or body parts. *Galeorhinus galeus* are generally easy to distinguish from *Mustelus* spp. in both body shape and dentition. However, early juvenile *G. galeus* (<40 cm total length) can sometimes be confused with *Mustelus* spp. (EDF, pers. obs.), so it is necessary that they too be taken into consideration when developing a means of identifying tissue samples from the NE Atlantic. This becomes paramount when identifying dressed carcasses during market sampling, because *G. galeus* and *Mustelus* spp. are sometimes marketed together under the same local name (EDF, pers. obs.).

The lack of clear and accurate phenotypic-based identification methods prevents the collation of reliable species-specific landings and survey data for *Mustelus* species. Therefore, assessments of these species in the NE Atlantic currently use aggregated data

under the generic heading *Mustelus* spp. (ICES, 2007). This makes it difficult to delineate the distribution of species or stock boundaries accurately and prevents the application of sound conservation and management strategies. Therefore, a simple, robust, but reliable method for the non-destructive identification of large numbers of *Mustelus* spp. sampled either during fishery surveys, market sampling, or by recreational anglers is needed to rectify the longstanding confusion.

Here, we report on the development of four new primers for the mitochondrial gene, NADH dehydrogenase subunit 2 (ND2), which can be used in a multiplex PCR reaction to differentiate the three target species reliably and quickly by simple agarose gel running. We applied the method to 431 *Mustelus* specimens from the NE Atlantic, the Irish Sea, the Celtic Sea, the Bristol Channel, and the North Sea, the results of which pose some intriguing questions on the status and biogeography of *Mustelus* spp. in the region.

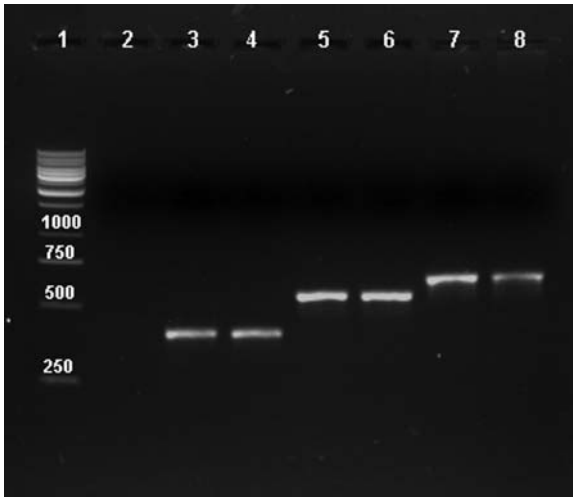


**Figure 1.** The result of the four primer multiplex PCR amplification of the ND2 gene for *M. mustelus*, *M. asterias*, and *G. galeus*. Lanes 1 and 2 are the 1-kb molecular ladder and the negative control, respectively. Lanes 3 and 4 are *M. mustelus* from South Africa and the northern Adriatic, respectively. Lanes 5 and 6 are *M. asterias* from the Irish Sea and the North Sea, respectively. Lanes 7 and 8 are *G. galeus* from the Celtic Sea.

## Material and methods

### Primer design and testing

Mitochondrial DNA primers were designed based on sequences of the NADH dehydrogenase subunit 2 (ND2) gene (Lopez *et al.*, 2006). GenBank accession numbers: *M. asterias*, DQ422123; *M. mustelus*, DQ422128; and *G. galeus*, DQ422118. A universal forward primer MUND2F1 (5′-TGTGAATAGGCCTCGAAATC A-3′) was designed to anneal in all three of the species in question. Three target species reverse primers were designed to amplify a fragment of different length for each species: *M. asterias*, 564 bp, MAND2R (5′-GGAAGGTTGTAAGTGTTATGATGA-3′); *M. mustelus*, 392 bp, MMND2R (5′- AATGCCAAGGAATAGTA GGAGGT-3′); *G. galeus*, 671 bp, GGND2R (5′-TCCTAAGGAAA GGAGAGTCAGTAA-3′).

To test for the specificity of these primers, reference tissue samples (as confirmed through detailed anatomical examination) of four *M. asterias*, four *M. mustelus*, and two *G. galeus* were collected. The *M. asterias* samples were from the Irish Sea, Celtic Sea, and North Sea (ICES Areas VIIa, VIIg, and IVc) and included specimens with vivid, faint, and no spots. The *G. galeus* samples were from the Celtic Sea (ICES Area VIIg), and the *M. mustelus* samples were from South Africa and the northern Adriatic. All samples consisted of a 1-$cm^2$ piece of tissue from the second dorsal fin. Samples were stored at 4°C in 95% ethanol. Total genomic DNA was extracted using a modified chloroform/isoamyl alcohol protocol (Petit *et al.*, 1999). The primer sequences were also analysed for potential cross-amplifications using BLAST (Basic Logical Alignment Search Tool; Zhang *et al.*, 2000). An additional specificity test was conducted, following the outlined protocol, with tissue samples of the spotted estuary smoothhound (*Mustelus lenticulatus*) from New Zealand, and the dusky smoothhound (*Mustelus canis*) from the NW Atlantic. These species are spotted aplacental viviparous and unspotted placental viviparous, respectively, and are both geographically isolated from the target species (Compagno, 1984).

### PCR conditions

Total amplification reaction volumes were 12.5 μl and contained 1.5 μl of the extracted DNA (25 ng $\mu l^{-1}$), 0.5 μl of universal forward primer MUND2F1 (10 μM), and 0.5 μl each of the three species-specific reverse primers MAND2R, MMND2R, and GGND2R (10 μM), as well as 1.25 μl of the 10× PCR Rxn buffer, 1.25 μl of dNTPs (2 mM), 0.5 μl of $MgCl_2$ (50 mM), 0.1 μl of Taq polymerase, and 5.9 μl of $ddH_2O$. A negative control containing no template DNA was included in all PCR reactions. Amplifications were performed in a Biometra T3000 Thermocycler using a thermal cycling profile of initial heating of 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, followed by a final extension step of 72°C for 7 min. Completed reactions were kept at 4°C before running gel electrophoresis on a 1% agarose gel. The gels were viewed under ultraviolet light. Species was assigned based on the fragment length visualized on the gel (Figure 1).

### Large-scale application

A further 431 *Mustelus* spp. samples were collected between 2006 and 2008 from fishery surveys, commercial fishing boats, and recreational anglers. The extensive sampling covered all seasons and a large geographic area including the NE Atlantic, the Irish Sea, the Celtic Sea, the Bristol Channel, and the North Sea (Figure 2). Samples were preserved dried, frozen, or in 95% ethanol. In all, 43 of the 431 samples were initially identified by survey scientists, based on putative morphological differences, as *M. mustelus*, and the remaining 388 as *M. asterias*. DNA was extracted from all samples and PCR reactions were run as outlined above. In addition to the negative control, a positive control containing a mixture of the DNA of all three species was included in the tests (Figure 3). This allowed easy identification of the test samples on an agarose gel.

## Results

Each of the three specific primers amplified the expected length fragment for their respective species (Figure 1). The fragments were clear, defined, and well separated on a 1% agarose gel, so allowing for easy and unambiguous identification of the three target species (Figures 1 and 3).

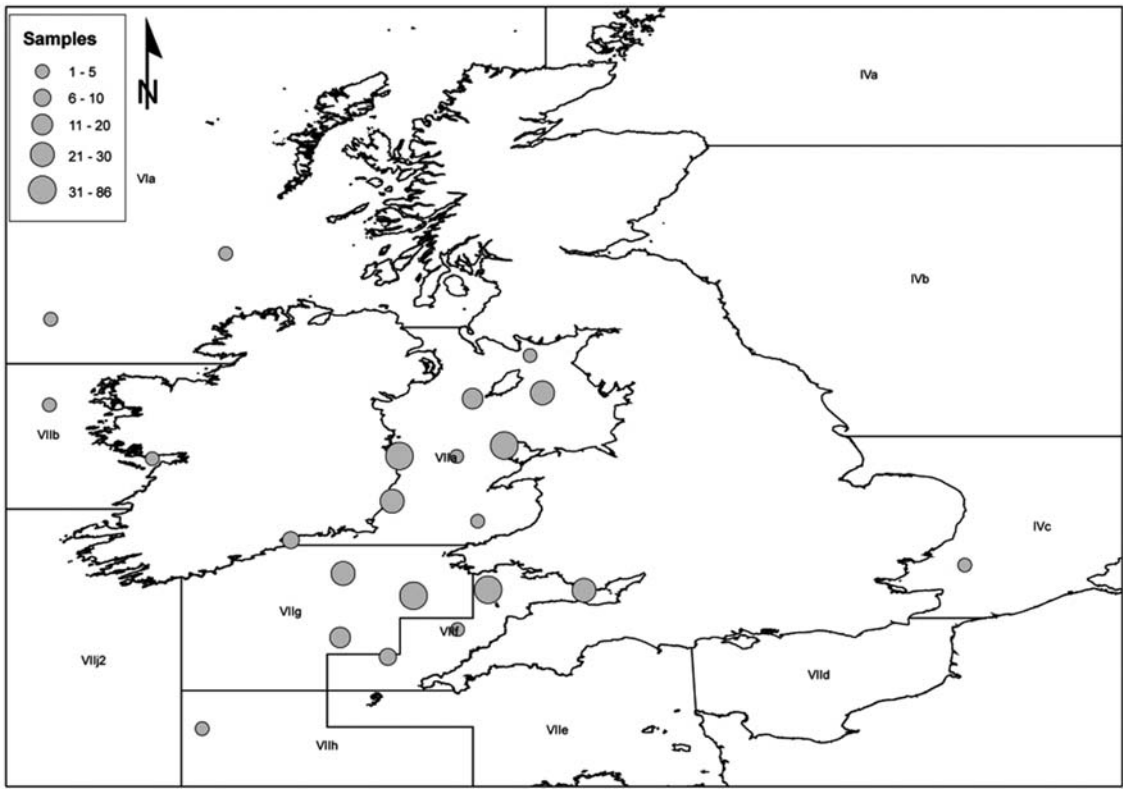


**Figure 2.** Distribution of *Mustelus* spp. samples collected in the NE Atlantic, the Irish Sea, the Celtic Sea, the Bristol Channel, and the North Sea. Roman numerals indicate ICES assessment areas. All 431 samples were positively identified as *M. asterias*.

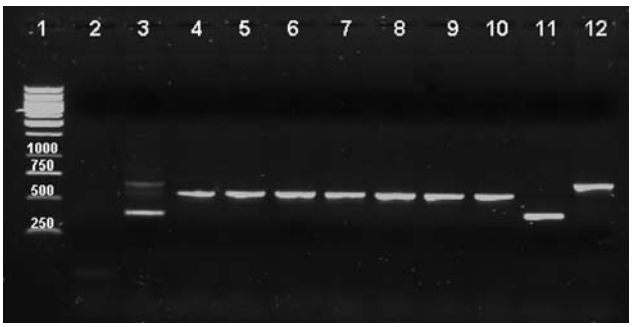


**Figure 3.** The result of four primer multiplex PCR amplification of the ND2 gene for the identification of triakid sharks in the NE Atlantic. Lanes 1 and 2 are the 1-kb molecular ladder and the negative control, respectively. Lane 3 is the positive control, which includes DNA from *M. mustelus*, *M. asterias*, and *G. galeus*; the three diagnostic bands are visible in this lane. Lanes 4–10 are positively identified as *M. asterias*. Lane 11 is *M. mustelus*, and Lane 12 is *G. galeus*.

Analyses of the annealing sites and primers with BLAST revealed that their specificity appeared to be limited to distinguishing the three target NE Atlantic species from each other (Zhang *et al.*, 2000). The annealing site in *M. asterias* is identical in other spotted aplacental *Mustelus* spp., such as *Mustelus manazo* and *Mustelus schmitti* (Zhang *et al.*, 2000). Similarly the annealing site in *M. mustelus* is identical in other unspotted placental *Mustelus* spp., such as *M. canis* and *Mustelus californicus* (Zhang *et al.*, 2000). The additional diagnostic tests on samples of *M. lenticulatus* and *M. canis* confirmed this, with clear bands evident with the *M. asterias* and *M. mustelus* primers, respectively.

Large-scale application of the genetic identification method to the 431 samples of NE Atlantic *Mustelus* spp. revealed that all samples belonged to *M. asterias*. Therefore, there was a 10% error in the morphological identification of *Mustelus* spp. from the area.

## Discussion and conclusions

Genetic identification of elasmobranchs is increasingly being accepted as a tool for the implementation of effective conservation strategies and the monitoring of trade in endangered species (Pank *et al.*, 2001; Shivji *et al.*, 2002). Methods such as bar-coding (Ward *et al.*, 2005) can prove costly if large numbers of samples need to be identified, so the development of more cost-effective methods suitable for large-scale screening is necessary (Pank *et al.*, 2001; Shivji *et al.*, 2002; Castilho *et al.*, 2007). The method described here fulfils this requirement and can ensure quick and reliable results, which are simple to interpret (Figures 1 and 3).

The annealing sites of the primers were chosen because they were the most variable between the target species, so preventing cross-amplification and ensuring that well-separated and easily distinguishable bands were produced during gel electrophoresis. However, the low levels of interspecific divergence in the ND2 gene within the two clades of *Mustelus* (Lopez *et al.*, 2006) and the resulting cross-amplification tests outlined above means that the method developed cannot be applied safely on a global scale. However, as *M. asterias* is the only spotted aplacental and *M. mustelus* the only unspotted placental species in the NE

Atlantic, it is arguable that the test can be a valuable and powerful diagnostic within the region. Moreover, the lack of interspecific variation at the primer sites within each one of the two clades, and the neat identification of *M. mustelus* reference specimens from locations as far apart as South Africa and the Adriatic Sea, confirms that potential intraspecific variation at the primer annealing sites does not represent a problem for the amplification of specific bands.

There is no evidence to suggest another *Mustelus* species in the NE Atlantic. One additional species is present in the Mediterranean Sea, the blackspotted smoothhound (*Mustelus punctulatus*). However, mtDNA analyses have shown that species to be more divergent than the target species, so cross-amplification would be unlikely (Cigala Fulgosi *et al.*, 2000). Despite the limitations discussed above, the usefulness and ease with which *M. mustelus*, *M. asterias*, and *G. galeus* samples can be distinguished makes this method an important tool for shark management in the NE Atlantic. Moreover, the high rate of misidentification observed in the 431 samples is indicative of the confusion that currently exists and demonstrates the need for large-scale application of this genetic identification method.

*Mustelus* spp. in the NE Atlantic have historically been considered to be of little commercial value. In the Mediterranean, however, their flesh is considered superior and more valuable than that of many other commercially important shark species, including the blue shark (*Prionace glauca*) and the porbeagle (*Lamna nasus*) (Renon *et al.*, 2001). Fisheries for *Mustelus* spp. in the Mediterranean peaked in 1994 when Italy landed 9999 t (FAO, 2000). The fishery subsequently declined, and landings were ~462 t by the start of the 21st century (FAO, 2000). *Mustelus* spp. have since been reported to be locally extirpated in many areas where they were previously considered abundant, including the Gulf of Lions, the upper Tyrrhenian Sea, and the Adriatic Sea (Aldebert, 1997; Jukic-Peladic *et al.*, 2001; Ferretti *et al.*, 2005). Similar decreases in landings and local extirpations are also evident in Portuguese waters and in the south of the Bay of Biscay (Quero, 1998; Correia and Smith, 2003). These examples highlight the vulnerability of *Mustelus* spp. to overexploitation and illustrate the need for conservation measures to be introduced into the NE Atlantic.

Survey data suggest that the abundance of *Mustelus* spp. has increased in recent years in the Bristol Channel, the Celtic Sea, and the North Sea and that it has remained stable in the Irish Sea (ICES, 2007). However, French landings in the NE Atlantic have concurrently been increasing steadily since the late 20th century, up to some 2416 t year$^{-1}$ (FAO, 2000). Therefore, the introduction of sound management actions for this and similar fisheries is required before unsustainable levels are reached. A first and critical step in this process is the reliable identification of the species in question.

In this light, the lack of *M. mustelus* in the samples collected is of particular interest. Given the geographical and temporal extent of the sampling undertaken, it would have been expected that some *M. mustelus* would be encountered if they were present in the sampling area (Figure 2). Their rarity in catches may be an indication of the scarcity of *M. mustelus* in the NE Atlantic or perhaps that the species has been locally or even generally extirpated in the area, as has happened elsewhere. Our findings may also indicate that only *M. asterias* is present in the study area and that the natural distribution limit of *M. mustelus* lies farther to the south. The unreliability of previously used methods of phenotypic identification may have historically confounded the delineation of the distribution of these species. The genetic identification method outlined here, therefore, offers a rapid, convenient, and reliable method to investigate these important questions.

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## References

Aldebert, Y. 1997. Demersal resources of the Gulf of Lions (NW Mediterranean) impact of exploitation on fish diversity. Vie et Milieu, 47: 275–284.

Castilho, R., Freitas, M., Silva, G., Fernandez-Carvalho, J., and Coelho, R. 2007. Morphological and mitochondrial DNA divergence validates blackmouth, *Galeus melastomus*, and Atlantic sawtail catsharks, *Galeus atlanticus*, as separate species. Journal of Fish Biology, 70: 346–358.

Cigala Fulgosi, F., Nonnis Marzano, F., Gandolfi, G., and Tagliavini, J. 2000. Evidence of interspecific low mitochondrial divergence between *Mustelus asterias* and *M. manazo* (Chondrichthyes, Triakidae). Biologia Marina Mediterranea, 7: 440–443.

Compagno, L. J. V. 1984. FAO species catalogue. 4. Sharks of the world. An annotated and illustrated catalogue of shark species known to date. Part 2. Carcharhiniformes. FAO Fisheries Synopsis, 125: 251–655.

Correia, J. P. S., and Smith, M. F. L. 2003. Elasmobranch landings for the Portuguese commercial fishery from 1986–2001. Marine Fisheries Review, 65: 32–40.

FAO. 2000. FAO Fisheries Department, Fishery Information, Data and Statistics Unit. FISHSTAT Plus: Universal software for fishery statistical time series.

Ferretti, F., Myers, R. A., Sartor, P., and Serena, F. 2005. Long term dynamics of the chondrichthyan fish community in the upper Tyrrhenian Sea. ICES Document CM 2005/N: 25. 34 pp.

Heemstra, P. C. 1973. A revision of the shark genus *Mustelus* (Squaliformes: Carcharhinidae). PhD thesis, University of Miami, Coral Gable, FA.

Hubbs, C. L. 1938. Scientific names of the American "smooth dogfish", *Mustelus canis* (Mitchell), and of related European species. Occasional Papers of the Museum of Zoology, University of Michigan, 374. 19 pp.

ICES. 2007. Report of the Working Group on Elasmobranch Fishes (WGEF). ICES Document CM 2007/ACFM: 27. 318 pp.

Jukic-Peladic, S., Vrgoc, N., Krstulovic-Sifner, S., Piccinetti, C., Piccinetti-Manfrin, G., Marano, G., and Ungaro, N. 2001. Long-term changes in demersal resources of the Adriatic Sea: comparison between trawl surveys carried out in 1948 and 1998. Fisheries Research, 53: 95–104.

Lopez, J. A., Ryburn, J. A., Fedrigo, O., and Naylor, G. J. P. 2006. Phylogeny of sharks of the family Triakidae (Carcharhiniformes) and its implications for the evolution of carcharhiniform placental viviparity. Molecular Phylogenetics and Evolution, 40: 50–60.

Pank, M., Stanhope, M., Natanson, L., Kohler, N., and Shivji, M. 2001. Rapid and simultaneous identification of body parts from the morphologically similar sharks *Carcharhinus obscurus* and *Carcharhinus plumbeus* (Carcharhinidae) using multiplex PCR. Marine Biotechnology, 3: 231–240.

Petit, E., Excoffier, L., and Mayer, F. 1999. No evidence of bottleneck in the postglacial recolonization of Europe by the noctule bat (*Nyctalus noctula*). Evolution, 53: 1247–1258.

Quero, J. C. 1998. Changes in the Euro-Atlantic fish species composition resulting from fishing and ocean warming. Italian Journal of Zoology, 65: 493–499.

Quignard, J. P., and Capape, C. 1972. Note sur les espèces méditerranéennes du genre *Mustelus* (Selachii, Galeoidea, Triakidae). Revue des Travaux de l'Institut des Pêches Maritimes, 36: 15–29.

Renon, P., Colombo, M. M., Colombo, F., Malandra, R., and Biondi, P. A. 2001. Computer-assisted evaluation of isoelectric focusing patterns in electrophoretic gels: identification of smoothhounds (*Mustelus mustelus, Mustelus asterias*) and comparison with lower value shark species. Electrophoresis, 22: 1534–1538.

Shivji, M., Clarke, S., Pank, M., Natanson, L., Kohler, N., and Stanhope, M. 2002. Genetic identification of pelagic shark body parts for conservation and trade monitoring. Conservation Biology, 16: 1036–1047.

Teshima, K., and Koga, S. 1973. Studies on sharks. 5. Taxonomic characteristics of reproductive organs in Japanese *Mustelus*. Marine Biology, 23: 337–341.

Ward, R. D., Zemlak, T. S., Innes, B. H., Last, P. R., and Hebert, P. D. N. 2005. DNA barcoding Australia's fish species. Philosophical Transactions of the Royal Society, B: Biological Sciences, 360: 1847–1857.

Wheeler, A. 1969. The Fishes of the British Isles and North West Europe. Macmillan, London. 613 pp.

Zhang, Z., Schwartz, S., Wagner, L., and Miller, W. 2000. A greedy algorithm for aligning DNA sequences. Journal of Computational Biology, 7: 203–214.

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